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EDITORIAL

Practical guidelines for the treatment of inflammatory bowel disease

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Abstract

In recent years, great progress has been made regarding the treatment of inflammatory bowel disease (IBD), particularly in the field of biological therapies. Nevertheless, the ultimate treatment is not in sight. With the development of new medication, it has become clear that we need a new understanding of IBD. Therapy needs to fit the different subtypes of IBD; e.g. mild disease in comparison to severe chronic active disease or Crohn's disease with or without fistulation or stenosis. The following article gives a practical overview of actual treatments for IBD. The intention of this article is not to provide a complete review of all new scientific developments, but to give a practical guideline for therapy of IBD.

Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Immunomodulators; Anti-tumor necrosis factor

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INTRODUCTION

Crohn's disease and ulcerative colitis are both defined as inflammatory bowel diseases (IBD), characterized by a chronic inflammation of the gut mucosa. The clinical course of both diseases can differ from a mild form, in which the patient reaches long-term remission without taking permanent medication, to a chronic active form, in which remission is only reached by permanently taking immunosuppressives and/or biologics or by taking them for a long period of time. Patients are not only burdened by the most common symptoms of IBDs; e.g. diarrhea, bowel pain, fever and complications such as fistulation, stenosis and abscesses in Crohn's disease and megacolon in ulcerative colitis, but are also burdened by the side-effects of the therapeutics, which the patients take to achieve a normal quality of life. Therefore, it is a great responsibility for a physician to consider treatment for an individual patient.

Recent discussions in the field of IBD are concerned with recommendations for a step-down or step-up therapy. A step-down therapy means using the most effective biologic or immunosuppressive treatment on the market, even without prior use of therapeutics such as steroids, in order to reach an effective remission as soon as possible. A step-up therapy means using a "classical" treatment by, for example, starting with aminosalicylates and ending up with an immunosuppressive and/or biological. Even if using a common step-up therapy treatment, the decision about which drug to use should be based on the individual patient, considering the clinical course and diagnosed complications according to current treatment guidelines.

This article provides a short overview of IBD and practical guidelines for the actual treatment of IBD (Figures 1 and 2). It is recognized that there is still an urgent medical need for improvement in the treatment in IBD and that treatments may not be sufficient for all patients, although great progress has been made in therapeutic approaches in the last decades (especially regarding biological therapeutics). The current guidelines for IBD therapy are distinguished by the course, place and severity of the clinical disease[1-7]. In Crohn's disease, a mild active form, a moderate or severe active form or a severe chronically active form can be differentiated. Patients can also be defined as steroid-dependent or steroid-refractory. The endoscopic and clinical pattern, the segments of the body that are involved (e.g. only small colon or small and large colon and/or stomach), the complications (such as fistulation and/or stenosis) as well as the duration of the inflammation and the response (or loss of response) to steroids are taken into account in the evaluation. In ulcerative colitis, differentiation is easier because inflammation only involves the large colon, starting from the rectum to the coecum. Therefore, treatment is determined by the clinical disease course and the involved segments of the colon, such as left-sided disease or pancolitis, as well as the response to steroids

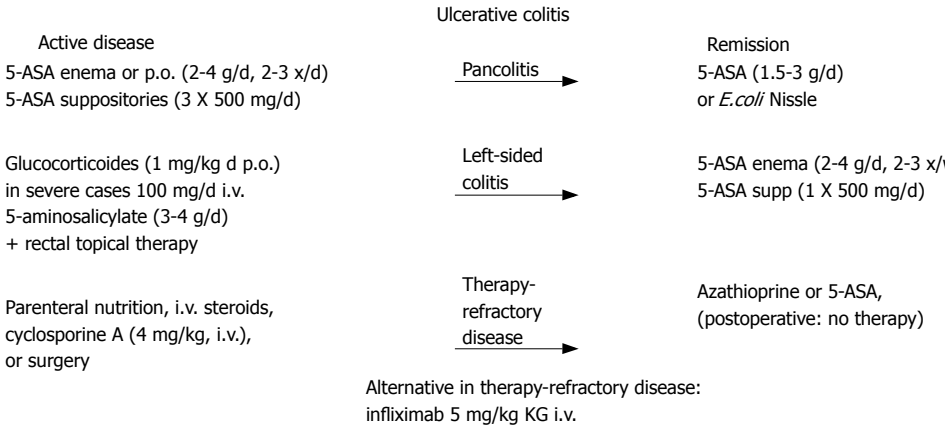

Figure 1 Short overview of the treatment regime of ulcerative colitis.

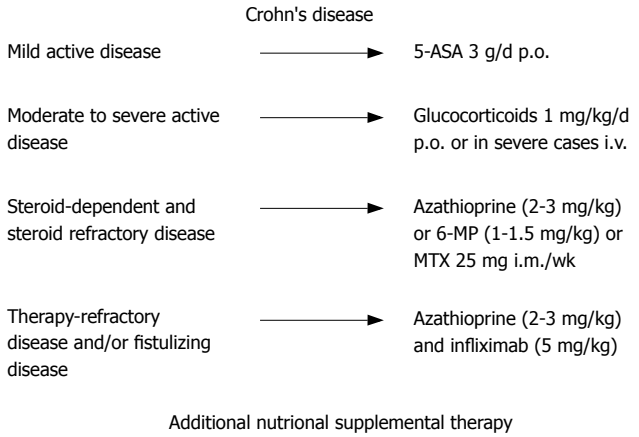

Figure 2 Short overview of treatment regimen in Crohn´s disease.

(steroid-dependent or steroid-refractory). In the following paragraphs the treatment of IBD will be discussed according to use of different therapeutic drugs.

5-Aminosalicylates (5-ASA)

5-ASA are bowel-specific drugs that are metabolized in the gut where the predominant actions occur. As a derivative of salicylic acid, 5-ASA is also an antioxidant that traps free radicals, which are potentially damaging by-products of metabolism. In the radical induction theory of ulcerative colitis, 5-ASA functions as a free radical trap as well as an anti-inflammatory drug. 5-ASA is considered to be the active moiety of sulphasalazine, which metabolizes to it. Oral and/or topical 5-ASA is recommended for mild to moderate ulcerative colitis to induce and maintain remission. The dosage of 5-ASA should be no less than 3 g/d. In practice, most patients do not like a topical treatment, but in left-sided disease, an enema with 4 g of 5-ASA 2 times per day or suppositories with 500 mg 5-ASA 3 times per day are most effective. Sometimes it is helpful for the patient to use the enema only in the evening and the suppositories in the morning. In Crohn's disease, aminosalicylates should also be used in initial therapy for mild disease, although discussion about efficacy has increased recently. In contrast to ulcerative colitis, the use of aminosalicylates for the maintenance of remission is not recommended for Crohn's disease because clinical studies have not shown success in remission maintenance[8,9]. 5-ASA is a standard treatment for ulcerative colitis, but not for Crohn's disease. In the last few years, new formulations have been on the market with varying clinical value. In some special cases; e.g. patients with an ileostoma, a formulation that sets metabolites free prior to the large bowel may be helpful. In patients with IBD and arthritis, sulphasalazine rather than 5-ASA formulations seems to be more effective regarding arthritis. In most patients, the side-effects are not very severe with headache as one of the most common side-effects. However, in some cases nephritis, pancreatitis and hair loss have been reported, which suggests regular monitoring of renal and liver enzymes at least once in three months.

Antibiotics

Antibiotics (e.g. metronidazole) play only a minor role in the additional treatment of fistulizing disease[10]. Although metronidazole (for up to 3 mo in a dosage of 400 mg 2 times per day) is often used in post-operative management after an ileocecal resection or fistula/abscess operation for Crohn's disease, this therapy is not based on study evidence. A side-effect of long-term treatment with metronidazole is polyneuropathy and monitoring is, therefore, required. Antibiotics are also used in the conservative treatment of small abscesses. Understanding the role of microbiota and antibiotics in IBD may become important in the future, but currently clinical studies have not provided support for this concern.

Corticosteroids

In patients with moderate to severe Crohn's disease or ulcerative colitis, corticosteroids are effective for the induction of clinical response and remission[11-16]. Dosages from 40-60 mg/d or 1 mg/kg per day orally are effective for the induction of remission. After the induction of remission, the steroid dose should be tapered (10 mg/wk until 40 mg; 5 mg/wk until 20 mg, followed by a tapering of 2.5 mg/wk). In severe disease, the application of parenteral glucocorticoids as soon as possible is useful for an anti-inflammatory response. Before the initiation of steroid treatment, the presence of an abscess should be excluded. In patients who have been on glucocorticoids

for more than one month, an ACTH (Adrenocorticotropic hormone)-Test should be performed before beginning tapering of the steroid. The ACTH-test can detect deficient cortisol production in the body. If there is a deficiency, hydrocortisone should be used as a substitute.

Budesonide

The benefits of glucocorticoid therapy should be carefully balanced against possible side-effects. Budesonide can reduce typical steroid side effects by a 90% first-pass metabolism in the liver and erythrocytes. Due to a special structural formulation, budesonide achieves the best anti-inflammatory effect in ileocecal inflammation[17-20]. Therefore, it is useful in therapy for Crohn's disease with ileocecal inflammation only. However, neither budesonide nor any other glucocorticosteroid should be used for a maintenance therapy due to the side-effects (e.g. Cushing-syndrome, osteoporosis or cardiomyopathy[21,22]). All patients treated with corticosteroids should additionally receive vitamin D and calcium substitution to avoid bone loss.

Immunosuppressives

The clinical course of 36% of IBD patients is defined as steroid-refractory and in 20% as steroid-dependent[23]. Immunomodulators are, therefore, recommended for the treatment of chronic active IBD. Studies have shown an efficacy for immunosuppressives that is similar to azathioprine and its metabolite, 6-mercaptopurine (6-MP; 2-3 mg/kg per day, resp. 1-1.5 mg/kg per day), in the long-term use of chronic active disease. Immunomodulators have been shown to be efficient for the control of inflammation and remission maintenance. Only limited data exists on the efficacy of immunosuppressants in fistulizing Crohn's disease and the prevention of post-operative recurrence[24-30]. Evidence-based data is missing on the post-operative use of azathioprine and many IBD referral centers are using azathioprine for the prevention of post-operative recurrence. Prior to an initiation of treatment with azathioprine or 6-MP, patients should be thiopurine methyltransferase (TPMT) genotype assessed in order to detect for a homozygous deficiency in TPMT in an effort to avert AZA or 6-MP-induced potential adverse events. All patients on azathioprine or 6-MP should be monitored weekly in the first month and after that once a month regarding their white blood count and liver enzymes because a myelosuppression or an elevation of liver enzymes subsequent to the use of azathioprine or 6-MP can occur. In such cases, the dosage of azathioprine or 6-MP should be reduced or paused until lab values are normal. In patients with gastrointestinal side-effects after the intake of azathioprine, a change to 6-MP should be considered.

Methotrexate and cyclosporine

Methotrexate is another immunomodulatory agent that is used in long-term treatment of IBD. The dosage for induction of remission in chronic active disease is 25 mg i.m. per week for 16 wk, followed by a maintenance treatment of 15 mg i.m. per week. In contrast to azathioprine and 6-MP, the data on use of methotrexate is rather scarce[31-33]. Cyclosporine is reserved for the treatment of severe steroid-refractory ulcerative colitis only. Intravenous cyclosporine (2-4 mg/kg) was able to prevent a decidered colectomy in two of three patients with severe ulcerative colitis. Due to its toxicity, use should be considered carefully; i.e. it should be used only in very severe active disease cases to avoid a colectomy. In Crohn' s disease, cyclosporine has been shown to be effective only in fistulizing, but not luminal disease[34-37].

Tacrolimus

Only very inadequate data is available for tacrolimus. Improvement of fistula drainage, but not closure was demonstrated in a randomized, placebo-controlled trial with tacrolimus[38,39].

Infliximab

Biological therapies, especially anti-TNF agents, play a pivotal role in the treatment of chronic active IBD and fistulizing disease[40-43]. The first anti-TNF agent on the market, infliximab, is a chimeric IgG1 mouse/human monoclonal antibody. Randomised, placebo-controlled trials (ACCENT I and Ⅱ) demonstrated the efficacy of infliximab (5 mg/kg, i.v.) in the induction of clinical response and remission in patients with active Crohn´ s disease. In fistulizing disease, complete fistula closure of at least 50% of the fistulas could be seen in 55% of the patients after three infusions of infliximab at wk 0, 2 and 6 (ACCENT Ⅱ). Given on a regular basis in intervals of 8-12 wk (5 mg/kg i.v.), infliximab is able to maintain remission[44-50].

In ulcerative colitis, the recently completed ACT I and ACT Ⅱ randomised, placebo-controlled trials demonstrated the efficacy of infliximab treatment in induction of remission and mucosal healing in 61.2% of the infliximab treated patients *versus* 32.4% of placebo treated patients[51,52].

Contraindications and side-effects should be taken into consideration carefully prior to infliximab therapy[53]. Due to immunogenicity, infliximab can lead to the formation of human anti-chimeric antibodies (HACA) in 30% to 75% of the patients. Additional administration of immunosuppressants; e.g. azathioprine and/or pretreatment with intravenous prednisolone, can reduce the risks of HACA formation. The main reported side-effect is an infusion reaction, which can occur as an acute allergic/anaphylactic reaction or a delayed hypersensitivity reaction. In clinical trials, observations have included infections, drug-induced lupus, cardiac failure, non-Hodgkin's lymphoma and, in post-marketing surveillance, tuberculosis, pneumonia, histoplasmosis, listeriosis and aspergillosis. To avoid a potential tuberculosis reactivation, a purified protein derivative (PPD) skin test and a chest-X-ray should be performed prior to infliximab treatment[54-67]. Patients with perianal or enterocutaneous fistulizing Crohn's disease should be treated first with infliximab. The effect of infliximab is not as effective on entero-enteral or recto-vaginal fistulas. Patients with steroid-refractory or chronic active Crohn's disease or ulcerative colitis who do not respond to immunosuppressive therapy alone should also be treated with infliximab. The recommended

treatment regimen is an induction scheme with three infusions (5 mg/kg i.v.) at 0, 2 and 6 wk, followed by a maintenance treatment of infliximab every 8 wk (5 mg/kg i.v.). Additionally, immunosuppressive therapy with azathioprine, for example, is recommended. HACA testing is not recommended routinely for every patient on infliximab, but it is recommended if there is a delayed hypersensitivity reaction or if the last infliximab infusion was more than 12 wk previous.

Adalimumab

Other TNF agents also showed efficacy in Crohn's disease. The human IgG1 antibody adalimumab, which is a therapeutic agent used for rheumatoid arthritis, was effective in open-label experience. A placebo-controlled, randomised trial was also conducted. One advantage, in comparison to infliximab, might be the completely human structure of the antibody, which leads to better tolerance and a subcutaneous route of administration. Data on adverse reactions in Crohn's disease patients are still not available, but adalimumab is well-tolerated in patients with rheumatoid arthritis[68-71].

CDP-870

Certolizumab pegol (CDP-870), which is a polyethylene-glycolated Fab-fragment of the anti-tumour necrosis factor, has been shown to be effective in the treatment of Crohn's disease in a recent published, randomised, placebo-controlled trial. At week ten, 52.8% of the certolizumab (400 mg) treated patients showed a clinical response versus 30.1% in the placebo treated group (the high placebo response was seen in a large patient subgroup with low C-reactive protein levels; this might have been due to statistical separation between treatment and placebo group[72]). The antibody was well tolerated. Ongoing trials, however, are necessary to establish efficacy in Crohn's disease.

CDP-571

CDP-571, which is a humanized IgG4 monoclonal antibody against tumour necrosis factor alpha, initially showed an induction of clinical response in controlled trials, but failed in a phase Ⅲ trial which was discontinued[73].

Onercept and eternacept

Onercept, which is a recombinant human p55 soluble receptor to TNF, and also eternacept, which is a recombinant human p75 soluble receptor to TNF, failed in a phase Ⅱ trial with Crohn's disease and both trials were discontinued[74-76].

Natalizumab

Adhesion molecule inhibiting agents, such as natalizumab, which is a humanized IgG4 antibody, demonstrated a clinical response in a clinical trial in Crohn's disease, but all trials had to be stopped immediately after cases of progressive multifocal leucencephalopathy in patients receiving natalizumab for multiple sclerosis were reported[77-79].

The antisense oligonucleotide of the adhesion molecule ICAM-1 (anti-ICAM-1) was ineffective in Crohn's disease[80].

A hopeful, novel approach for the treatment of Crohn's disease is an anti-IL-12/IL-23p40 antibody that proved effective for induction of response and remission in a phase Ⅱ study[81].

β-Interferon

The use of β-Interferon, which has been investigated in a small pilot study in ulcerative colitis with a subcutaneous administration, seems to be effective, but larger, randomised, placebo-controlled studies need to be performed to clarify the clinical efficacy[82].

In conclusion, the only biological therapeutic today, which has been proven effective in IBD and is available on the market is infliximab. The market release of new TNF agents might happen in the near future.

Probiotics

A different group of therapeutic agents for therapy of IBD are probiotics. The use of probiotics has been advocated in colonic inflammatory disease for a long time. Only recently, two controlled trials demonstrated that E. coli nissle is as effective as 5-ASA for remission maintenance in ulcerative colitis[83,84]. For remission maintenance and pouchitis, studies demonstrated the benefit of probiotics[85,86]. Due to a better understanding of the molecular events and the pathophysiological processes of this disease, it is hoped that more probiotic agents will be developed in the near future.

5-ASA

A short, practical guideline would be incomplete without discussing IBD and pregnancy. 5-ASA is not harmful during pregnancy and there is very little placental transport. 5-ASA should be avoided during breast feeding because there are no studies on 5-ASA use during breast feeding[87]. Acute disease or a flare up of IBD can be treated throughout an entire pregnancy with steroids. Glucocorticoids pass the placental barrier, but there has been no significant evidence of teratogenesis. There have been some observations of cleft lip and palate associated with the intake of steroids. In general, no increase in fetal complications have been found with use of 5-ASA compared to the general population[88].

Azathioprine or 6-mercaptupurine

The use of azathioprine or 6-mercaptupurine should not be completed during pregnancy. Extensive experience with use of these substances during pregnancy exists with other autoimmune diseases and patients who received renal transplants. No teratogenic effects in humans have been reported so far. However, it is very important to discuss all the data and possible complications with the patient and it is essential that the decision to take the drug should be made by the patient[89].

Methotrexate is contraindicated during pregnancy as it is mutagenic and teratogenic[90]. Cyclosporine is not teratogenic, but due to its side-effects, it needs to be considered very carefully and should only be used to avoid a colectomy[91]. Infliximab should not be given as

maintenance therapy during pregnancy. In very severe disease, it can be considered as an emergency therapy[92]. Nutrition has not been discussed in this article so far. However, the balance of trace elements and vitamins is essential for successful therapy. Vitamin B12 and folinic acid, for example, should be monitored in Crohn's disease patients with ileal inflammation to avoid a deficiency syndrome, which can lead to severe anemia. Also ferritin should be monitored and, if necessary, substituted orally or intravenously to avoid severe iron deficiency anemia. In severe cases of iron deficiency anemia, erythropoietin (10 000IE s.c. 3 times per week) plus iron i.v. (62.5 mg in 250 mL NaCl) is effective[93]. In severe ulcerative colitis or Crohn's disease, patients profit from short term parenteral or additional high calorie nutrition[94].

All above treatment regimens attempt to avoid complications and inflammation in IBD. However, all conservative therapy sometimes fails or is not effective enough. Examples might be therapy for refractory ulcerative colitis, which can only be successfully treated by an operation (e.g. a colectomy with an ileoanal pouch anastomosis), non-inflammatory stricture in Crohn's disease or severe fistulizing disease, in which a protective ileostoma can be very useful for supporting conservative treatment. In such cases, close collaboration with an experienced IBD surgeon is essential.

It can be summarized that although a number of new biological agents have been developed in the last decades for the treatment of IBD, there is still a great need for new pharmcotherapeutics, particularly for chronic refractory disease patients with multiple complications.

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EDITORIAL

New aspects in celiac disease

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Abstract

Celiac disease (CD) is a common autoimmune disorder characterized by an immune response to ingested gluten and has a strong HLA association with HLA-DQ2 and HLA-DQ8 molecules, but human HLA-DQ risk factors do not explain the entire genetic susceptibility to gluten intolerance. CD is caused by the lack of immune tolerance (oral tolerance) to wheat gluten. In this sense, the expression of soluble HLA-G in CD is of special interest because the molecule plays an important role in the induction of immune tolerance. The enhanced expression of soluble HLA-G found in CD may be part of a mechanism to restore the gluten intolerance. In this editorial, we review recent progress in understanding CD in relation to its prevalence, diagnosis and possible mechanisms of pathogenesis.

Key words: Celiac disease; Oral tolerance; IL-10; TGF-beta; Gluten intolerance

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INTRODUCTION

Celiac disease (CD) is a chronic inflammatory disease which develops in genetically predisposed individuals. CD is a T cell-mediated inflammatory disorder with autoimmune features and it has environmental and immunologic components[1,2]. It is characterised by an immune response to ingested wheat gluten and related proteins of rye and barley that leads to inflammation, villous atrophy and crypt hyperplasia in the proximal part of the small intestine[2,3]. The common presentation symptoms and signs of CD include diarrhea, abdominal distention, abdominal pain, weight loss, fatigue, and malnutrition.

The clinical classification of CD is based on the presence or lack of gastrointestinal symptoms: "classical" or "symptomatic" CD refers to presentations with diarrhea and with malabsorption syndrome, whereas "atypical" or "asymptomatic" form has no gastrointestinal manifestations[4]. CD is defined as silent when the typical enteropathy is found in patients who apparently are healthy. Potential CD refers to a risk for developing a typical CD later in life; the patients have Endomysial antibody (EMA) and tissue transglutaminase (tTG) antibodies with HLA DQ2 or DQ8 predisposing genotype[6,7], and normal or minimal abnormal mucosa. Finally, a specific manifestation of CD is refractory sprue defined as symptomatic, severe villous atrophy with increased intraepithelial lymphocytes (IELs) despite maintenance of a strict gluten-free diet[5].

Although there have been major advances in our knowledge of the disease, there are few advances in the therapy. The only accepted treatment for CD is a nutritional therapy with a gluten-free diet for lifelong[8]. Here, we review recent advances in our understanding of CD with respect to its prevalence and diagnosis, and possible mechanisms underlying the gluten intolerance.

PREVALENCE

CD is the most common food-sensitive enteropathy in humans. The prevalence is estimated in the range of 1:100 to 1:300 in North America and Europe[9,10]. Recently, it has become clear that CD is much more prevalent than previously thought. We observed in our pediatric population (south-east of Spain) a 1:75 prevalence, which is in good agreement with the studies by Fasano et al [9] and Ciclitira *et al*[10] showing a prevalence of 1:133 in the United States and 1:100 in Europe, respectively.

At the present time, the form of clinical presentation of CD is changing. In our experience, CD is a very common disorder, and most affected individuals have the atypical and silent forms of the disease, which are also the prevalent forms of disease presentation in our population. Indeed, we observed a more elevated percentage of patients associated with HLA-DQ8 molecule in CD (around 20%-25%) than have been described previously by other studies, which estimated the percentage of HLA-DQ8 patients to be about 10%[11]. Furthermore, the HLA-DQ8 CD patients are commonly associated with extraintestinal symptoms presentations, and are frequently

Table 1 Clinical manifestations of untreated celiac disease

Manifestations	Associated diseases	Genetic associated diseases
Classic symptoms:	Autoimmune diseases:	Down syndrome
		Turner syndrome
Abdominal pain	Type 1 diabetes	William syndrome
Anorexia	Thyroiditis	IgA deficiency
Diarrhea	Sjogren's syndrome	
Weight loss	IgA nephropathy	
Short stature		
Irritability	Neurologic disturbances:	
Nonclassic symptoms:	Autism	
	Depression	
Dermatitis hepertiformis	Epilepsy	
Hepatitis	Cerebellar ataxia	
Anemia		
Arthritis	Other diseases:	
Constipation		
Alopecia	Osteopenia/osteoporosis	
Pubertal delay	Infertility	
Vomiting	Intestinal adenocarcinoma	
Inflammatory bowel disease	Non-Hodgkin lymphoma	
Migraine headaches		

associated with others autoimmune diseases (unpublished results).

DIAGNOSIS

We believe that the current diagnostic criteria for CD need a revision. CD is often missed during diagnosis because many individuals do not present the disease with the classical gastrointestinal symptoms. In fact, the percentage of undiagnosed population has markedly increased, and significant risks and complications are associated with untreated CD (Table 1).

As a multi-factorial disorder, CD may present highly diverse clinical manifestations[12]. CD patients typically display extraintestinal (non-classical) symptoms or lack classical gastrointestinal symptoms such as diarrhea, abdominal pain, distention, or weight loss. In our pediatric population, the non-classical symptoms are often the most common presentation in newly diagnosed CD cases. These include autoimmune alopecia, iron deficiency, or elevated levels of liver transaminases, and they often may be the only manifestation of CD in an affected individual (Table 1).

In our experience, small bowel biopsy is critical for diagnosing symptomatic patients with negative serology for CD and with HLA compatible with the disease. Although we consider intestinal biopsy as the gold standard for CD diagnosis, in many occasions the permission for intestinal biopsy is refused. Both serologic tests and genetic study are necessary to select subjects who need intestinal biopsy (Table 2).

CD is more common in certain risk groups. Family members of known celiac patients represent the most important group of study patients. The determination of HLA typing as a first step is useful as it would exclude approximately one third of first degree relatives in a CD family. We found a high prevalence of CD between relatives, and the highest percentage was observed among daughters. The CD relatives are a population at high risk of developing gluten intolerance and we propose that an extensive study of such group should be undertaken. It is noteworthy that after the first diagnosis of CD, the family will become more self-aware in recognizing early symptoms related to the disease.

PATHOGENESIS

A defect in antigen processing by epithelial cells, together with the intrinsic properties of the gliadins, as well as the HLA-DQ haplotype of the individual are considered the principal factors involved in the pathogenesis of CD[3]. CD is strongly associated with HLA class II genes that map to the DQ locus. It has been shown that CD is associated with the expression of HLA-DQ2 and HLA-DQ8[6,7]. Several studies found that the majority of celiac patients carry DQ2 (DQA1*05/DQB1*02), with the remaining patients displaying an association with DQ8 (DQA1*0301/DQB1*0302). Collectively, these HLA genes confer up to 40% of the genetic risk for CD development.

Gliadin peptide presentation and T-cell activation are critical events in the pathogenesis of CD. Gluten peptides are not fully digested by the action of gastric, intestinal and pancreatic enzymes in CD patients. A 33-mer peptide was isolated and identified as the primary initiator of the inflammatory response to gluten in celiac patients[16]. This peptide reacted with tissue transglutaminase (tTG), the major autoantigen in CD that deamidates certain glutamine residues of gluten to glutamic acid. This in turn produces a negative charge that favours binding and presentation by HLA-DQ2 and DQ8 molecules, which are responsible for T-cell activation and subsequent production of cytokines, leading to tissue damage[17,18]. The inappropriate CD4+ T-cell activation in the lamina propia commonly observed in CD is triggered by specific gluten peptides bound to DQ2 and DQ8 heterodimers on the surface of antigen presentation cells[2,6]. The mucosal intestinal lesion is believed to be mainly induced by the production of IFN-gamma from these gluten specific T cells[1, 2]. Moreover, changes in intestinal permeability, secondary to alterations in intercellular tight junctions or in the processing of the food antigen, have also been recently implicated in the loss of tolerance to gluten[19].

Immune (oral) tolerance

The gut immune system is exposed to a wide variety of antigens derived from foods, resident bacteria and invading microorganisms. Oral tolerance is a physiological condition characterized by induction of immune unresponsiveness toward intestinal alimentary and bacterial antigens of the intestinal flora. Multiple cellular and molecular mechanisms are involved in the regulation of this fundamental property of the gut immune system[20].

CD is the most common food-sensitive enteropathy in humans and is caused by the lack of immune tolerance (oral tolerance) to wheat gluten and the prolamin fractions of rye and barley. Many different gluten peptides recognized by intestinal T cells have been identified[21]. The activation

Table 2 Diagnosis in celiac disease

Diagnostic criteria used in celiac disease	Diagnostic propose for celiac disease	Comments
Serological test: Tissue transglutaminase antibody (tTGA) Endomysial antibody (EMA) Gliadin antibodies (AGA) Total IgA	With classical symptoms: Serological test: Tissue transglutaminase antibody (tTGA) Endomysial antibody (EMA) Gliadin antibodies (AGA)	Positive serology supports a diagnosis of CD, but they are not essential. Compatible with HLA-DQ2/D8 testing and identify individuals for further biopsy evaluation. Small bowel biopsy is critical in symptomatic patients with negative serology for CD and with HLA compatible with the disease
Endoscopy	HLA-DQ2/DQ8 testing	Determination of HLA typing as a first step in diagnosis in CD family
	Capsule endoscopy (with adequate pathological interpretation)	Adequate number of biopsies and well oriented. Estimate lymphocyte infiltration and partial or total villus atrophy
	Without classical symptoms HLA-DQ2/DQ8 testing	Primordial role of HLA-typing if serology is negative and with biopsy refused or equivocal to identify individuals
	Serologic test: Tissue transglutaminase antibody (tTGA) Endomysial antibody (EMA) Gliadin antibodies (AGA)	Adequate number of biopsies and well oriented. Estimate lymphocyte infiltration and partial or total villous atrophy
	Capsule endoscopy (with adequate pathological interpretation)	

of these gluten-reactive T cells represents a key event in the pathogenesis of CD[1,2]. The mucosa of CD patients is characterized by a high proportion of intraepithelial T cells bearing gamma-delta chain of the antigenic T cell receptors (γδ IEL)[22].

Ingested gliadin, the triggering agent of the disease, can cross the epithelial barrier and elicit a harmful T cell-mediated immune response. Dendritic cells are supposed to play a pivotal role in shaping the immune response[23]. Immature dendritic cells are characterised by low levels of MHC class II expression and co-stimulatory molecules and can mediate tolerance presumably by induction of T regulatory cells (Treg cells)[24]. Moreover, IL-10 released by Treg cells can modulate the function of immature dendritic cells and inhibit their differentiation, amplifying the local presence of "tolerizing dendritic cells"[25]. IL-10-modulated dendritic cells induce anergy of effector T cells through still undefined mechanisms requiring cell-cell contact.

Therefore, the direction of the immune response toward immunity or tolerance depends on the stage of maturation and the functional properties of the dendritic cells. Gliadin peptides can contribute to overcoming the stage of unresponsiveness of immature dendritic cells by inducing phenotypic and functional dendritic cell maturation, resulting in more efficient processing and presentation of gliadin peptides to specific T lymphocytes[26].

HLA-G: molecule of immune tolerance

HLA-G is a non-classical major histocompatibility complex class I molecule selectively expressed at the maternal-foetal interface on cytotrophoblast cells, protecting the fetus from the maternal immune rejection, and creating a general state of tolerance[27]. HLA-G exhibit tolerogenic properties via interaction with inhibitory receptors presented in natural killer (NK) cells, T cells and antigen-presenting cells (APC)[28].

The presence of soluble HLA-G (sHLA-G) in the cerebrospinal fluid of multiple sclerosis[29] and allograft acceptance after transplantation[30] suggests a tolerogenic function for this molecule against innate and adaptive cellular immune responses. Interestingly, work from our lab and others have suggested that HLA-G antigens may play a protective role in inflammation[31,32]. Further analysis revealed that, sHLA-G molecules inhibit lytic activity of NK cells, induce apoptosis of CD8+ CTLs and affect CD4+ alloproliferation[33]. Thus, the immune modulatory properties of sHLA-G may suggest a potentia role in CD.

We have demonstrated an association of CD with sHLA-G expression (Figure 1A) and we found a correlation between increased levels of sHLA-G expression and CD associated with other autoimmune diseases, being dependent on a genetic link of these diseases through HLA genes[34].

The expression of soluble HLA-G in CD is of special interest because its molecule plays an important role in the induction of immune tolerance[35]. We propose that the enhanced expression of sHLA-G found in CD could be part of a mechanism to restore the gluten intolerance. HLA-G may act through inhibitory receptor (ILT) interactions that lead to development of tolerogenic dendritic cells with the induction of anergic and immunosuppressive T cells, and an arrest of maturation/activation of dendritic cells. The expression of these ILT receptors on dendritic cells is tightly controlled by inflammatory stimuli, and by cytokines[36].

Thus, a powerful anti-inflammatory response to gliadin might occur during the development of the disease with uncontrolled production of HLA-G and anti-inflammatory cytokines, such as IL-10 and TGF-beta, which counteract the inflammation and/or may cause recruitment of intra-epithelial lymphocytes, maintaining the intestinal lesions presented in CD[34].

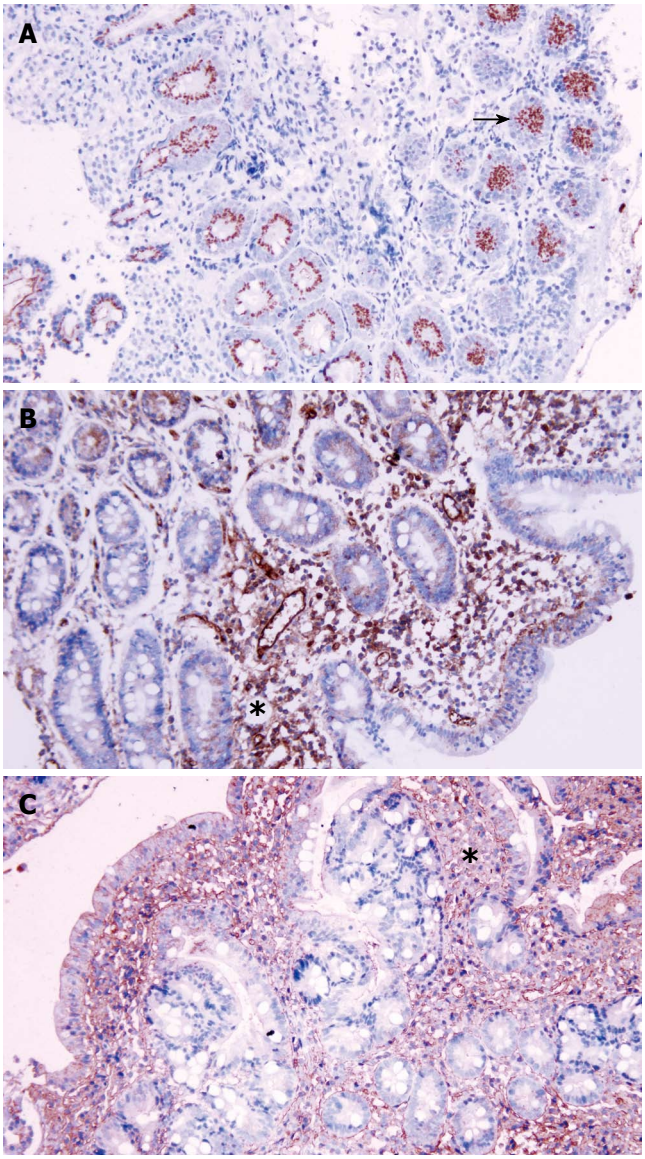

Figure 1 Immunohistochemistry analysis in celiac patients. **A**: Immunohistochemical staining for HLA-G in the Lieberkühn crypts; **B**: Immunohistochemical staining for IL-10 at the lamina propria level; **C**: Immunoreaction for TGF-beta in areas of infiltrated inflammatory cells. Arrows and asterisks: cells showing immunoreactivity (x 200).

Cytokines

The characteristics of an intestinal inflammatory response depend on the cytokines produced during this response. The production of cytokines from T cells and macrophages is of potential importance for the histological lesions that appear in CD. The CD lesions are associated with a marked infiltration of Th1 cells dominated by the synthesis of the pro-inflammatory cytokines, IFN-gamma and TNF-alpha[37]. IL-15 also has an important role in CD, because it orchestrates intraepithelial lymphocytes changes in the disease. Certain parts of gluten may stimulate the innate part of the immune system, and IL-15 is a central player in this immune response-induced by gluten, inducing the activation of IEL in CD[38]. Engineered IL-15 over-expression by epithelial cells in the intestine leads to a massive expansion of CD8+ intestinal T cells accompanied by a CD-like enteropathy[39]. Furthermore, IL-15 induces the expression of MICA, the epithelial ligand of NKG2D[40].

We found in the celiac patients increased levels of anti-inflammatory cytokines IL-10 and TGF-beta at the lamina propria level in biopsy samples of patients with villous atrophy and elevated lymphocyte infiltration (Figure 1B and C). IL-10 predominantly acts as a potent anti-inflammatory factor and a suppressive agent for Th1 responses[41]. TGF-beta is a major factor for the production of immunoglobulin A (IgA) and acts as a potent mediator of the tissue repair[42]. Interestingly, tTG, described as the major endomysial autoantigen in CD, is necessary for the activation of TGF-beta[43]. The elevated levels of IL-10 and TGF-beta are insufficient for the inhibition of the autoimmune reaction, and would modulate the immune response and induce the activation of B lymphocytes. These cytokines may play important roles in inducing HLA-G. In this regard, the possible role of IL-10 in inducing HLA-G protein expression has been already previously described in CD[34].

Table 3 Future directions in celiac disease

Unanswered questions	Future directions
Immunopathogenesis	
How is oral tolerance broken?	Restoring immunological tolerance to gluten would represent the ideal cure for CD
Gluten-specific T cells	Gluten-specific T cells could be inactivated or deleted, tolerance to gluten should be restored
Diagnosis	
What is the significance of the vast number of currently undiagnosed people with the disease?	The rate of the diagnosis will continue to increase with better diagnosis.
What is the significance of the increase number of people with extraintestinal symptoms or without classical symptoms in CD?	"the suspicion of the disease"
Genetics	
Identify the genetic risk factors that predispose to CD	Polymorphic genes located in the MHC region and CD Genetic polymorphism of cytokine genes may influence the risk of CD Associated polymorphism with serological markers HLA-G polymorphism?

FUTURE DIRECTIONS

There are many unanswered questions about CD. What is responsible for the collapse of CD oral tolerance? Restoring immunological tolerance to gluten would represent the ideal way to treat or cure this disease. However, if gluten-specific T cells could be inactivated or deleted, tolerance to gluten should be restored. What is the significance of the vast number of currently undiagnosed people with the disease? What is the significance of the increased number of people with extraintestinal symptoms

or without classical symptoms in CD? As we anticipate that the rate of the diagnosis will continue to increase, it will be important to further our understanding of the pathogenesis of CD and improve its diagnosis (Table 3). The diversity of the clinical presentation in CD can complicate the diagnosis and delay the treatment initiation. In our experience, the most important diagnostic test in CD is "the suspicion of the disease". The screening of CD should start with a serologic test (for IgA and IgG anti-endomysial and anti-tTG antibodies), and HLA typing. If the patient is genetically compatible with the disease (even with negative serology), a confirmatory small biopsy by capsule endoscopy should be followed.

Recently, many efforts have been made to identify the genetic risk factors that predispose to CD. The lack of immune tolerance in CD could occur due to genetic polymorphisms in any of the non-MHC molecules involved in the induction, regulation or expression of mucosal immune responses, such as IL-10, TGF-beta, IFN-gamma. Most of these polymorphisms affect gene transcription influencing the individual susceptibility to CD[44]. In addition, several associations between different polymorphic genes located in the MHC region and CD have been reported[45]. Furthermore, we found evidence that HLA-G is an interesting candidate as susceptibility gene in CD. These findings should open new perspectives for the identification of genetic susceptibility to CD.

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GASTRIC CANCER

(-)-Epigallocatechin-3-gallate inhibits growth of gastric cancer by reducing VEGF production and angiogenesis

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Abstract

AIM: To investigate the effect of (-)-epigallocatechin-3-gallate (EGCG) on growth of gastric cancer and its possible mechanism.

METHODS: Heterotopic tumors were induced by subcutaneously injection of SGC-7901 cells in nude mice. Tumor growth was measured by calipers in two dimensions. Tumor angiogenesis was determined with tumor microvessel density (MVD) by immunohistology. Vascular endothelial growth factor (VEGF) protein level and activation of signal transducer and activator of transcription 3 (Stat3) were examined by Western blotting. VEGF mRNA expression was determined by RT-PCR and VEGF release in tumor culture medium by ELISA. VEGF-induced cell proliferation was studied by MTT assay, cell migration by gelatin modified Boyden chamber (Transwell) and *in vitro* angiogenesis by endothelial tube formation in Matrigel.

RESULTS: Intraperitoneal injection of EGCG inhibited the growth of gastric cancer by 60.4%. MVD in tumor tissues treated with EGCG was markedly reduced. EGCG treatment reduced VEGF protein level *in vitro* and *in vivo*. Secretion and mRNA expression of VEGF in tumor cells were also suppressed by EGCG in a dose-dependent manner. This inhibitory effect was associated with reduced activation of Stat3, but EGCG treatment did not change the total Stat3 expression. EGCG also inhibited VEGF-induced endothelial cell proliferation, migration and tube formation.

CONCLUSION: EGCG inhibits the growth of gastric cancer by reducing VEGF production and angiogenesis, and is a promising candidate for anti-angiogenic treatment of gastric cancer.

Key words: Epigallocatechin-3-gallate; Angiogenesis; Migration; Tube formation; Vascular endothelial growth factor; Signal transducer and activator of transcription 3; Gastric cancer

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INTRODUCTION

Gastric cancer is the leading cause of cancer-related death in most countries, but the incidence has steadily decreased throughout the world in recent decades[1]. Although the exact reason for the decline is uncertain, it was reported that dietary factors play a role[2]. Epidemiological studies showed that regular drinking of green tea significantly reduces the occurrence and mortality of many types of cancer[3]. Case-control studies have provided evidence for protective effect of green tea against gastric cancer[4]. Experiment and animal model studies also demonstrated that green tea and its components have anti-cancer effect against gastric cancer[5,6], but conclusive results are not available at present[7].

(-)-Epigallocatechin-3-gallate (EGCG) is the most abundant and active component of green tea, and the beneficial properties of green tea appear to be ascribed to EGCG. The effect of EGCG on cell proliferation and apoptosis has been well established. EGCG suppresses tumor growth by inhibiting proliferation and inducing apoptosis through a variety of mechanisms[8]. Recent studies showed that EGCG has anti-angiogenic property and reduces tumor growth in mouse model by inhibiting angiogenesis[9,10]. Angiogenesis, the growth of new blood vessels from preexisting capillaries, is necessary for solid tumor growth and metastasis. Angiogenesis is initiated by the release of certain angiogenic factors from tumor cells. Vascular endothelial growth factor (VEGF), which has

been shown to be the most potent angiogenic factor, is associated with tumor-induced angiogenesis. Angiogenesis is closely associated with progression and prognosis of gastric cancer, and VEGF expression is a predictive and prognostic factor of gastric cancer[11]. Anti-angiogenic therapy targeting VEGF can inhibit growth and metastasis of gastric cancer[12]. However, whether EGCG suppresses growth of gastric cancer by anti-angiogenesis remains to be elucidated.

In this study, we investigated the effect of EGCG on angiogenesis and growth of gastric cancer with an *in vitro* and *in vivo* model. Data reported here show that EGCG suppresses growth of gastric cancer by reducing VEGF production and VEGF-induced angiogenesis.

MATERIALS AND METHODS

Cell culture

Human gastric cancer cells (SGC-7901 cells, Cell Bank of Sun Yat-Sen University, Guangzhou, China) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD) and incubated at 37℃ in a humidified incubator containing 50 mL/L CO_2. Human umbilical vein endothelial cells (HUVECs) were prepared from fresh human umbilical cord and grown in human endothelial-serum free medium (endothelial-SFM, Gibco BRL, Gaithersburg, MD) supplemented with 10% FBS, 100 U penicillin, streptomycin and fungizone, and incubated at 37℃ in a humidified incubator containing 50 mL/L CO_2. To maintain uniform conditions, all experiments were carried out between cell passages 4-6.

Tumor growth assay

SGC-7901 cells (5×10^6 cells/0.2 mL) in SFM were inoculated subcutaneously into the dorsal area of 6-8-wk female BALB/c nude mice, weighing 18-22 g (Experimental Animal Center of Sun Yet-Sen University, Guangzhou, China). When tumors reached a volume of 50 mm^3, intraperitoneal injection of EGCG was carried out at the dosage of 1.5 mg/d per mouse ($n = 6$). The control group ($n = 6$) was treated with the same volume of PBS. Tumor growth was monitored by external measurement in two dimensions every other day with calipers. Tumor volume was determined according to the equation: $V = (L \times W^2)/2$, where V is volume, L is length and W is width. Twenty-eight days after EGCG injection, all animals were sacrificed, tumors were excised and weighed. The tumor inhibition ratio was calculated as follows: inhibition ratio (%) = $[(C-T)/C] \times 100\%$, where C is the average tumor weight of the control group and T is the average tumor weight of the EGCG-treated group. Some tumor tissues were frozen at -80℃ for Western blot analysis and others fixed in 4% formaldehyde for immunohistochemistry analysis.

All animal studies were performed according to the guides for the Care and Use of Laboratory Animals approved by Sun Yat-Sen University.

Determination of tumor microvessel density

Histologic 4-μm thick sections prepared from formaldehyde fixed and paraffin embedded tumor tissues were used for immunohistochemical analysis. After deparaffinization, rehydration, antigen retrieval and blocking of endogenous peroxidase, the specimens were incubated overnight at 4℃ with goat polyclonal anti-CD34 antibodies diluted at 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were rinsed and incubated with peroxidase-conjugated second antibody followed by enzyme conjugate (HRP-streptavidin). Microvessels were revealed with diaminobenzidine (DAB) and the sections were counterstained with Mayer's hematoxylin. In negative-control staining, the primary antibodies were omitted. Tumor vasculature was quantified by the Weidner's method[13]. The area of tumor containing the maximum number of microvessels to be counted was identified by scanning the entire tumor at low power (100 ×). The number of highlighted vessels from 3 fields was then counted in this area at high power (200 ×).

Western blot analysis

SGC-7901 cells were seeded in 90 mm plates and cultured in growth medium until 70%-80% confluence. The culture medium was replaced with SFM supplemented with different concentrations of EGCG (0, 5, 10, 20, and 40 μmol/L). The cells were incubated for another 24 h. Total protein from cell lysates and tumor tissue homogenizations was extracted with mammalian cell lysis kit (Bio Basic Inc., Ontario, Canada). Protein levels were quantified with Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA). Total protein (100 μg) was separated in 12% SDS–PAGE, and transferred onto PVDF membrane (Invitrogen, Carlsbad, CA, USA). The membrane was blocked with 5% skim milk and incubated at 4℃ overnight with rabbit polyclonal anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-Stat3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or goat polyclonal anti-p-Stat3 antibody (Phospho-Stat3 [tyr-705], Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing three times with 0.1% Tween 20 in Tris-saline, the membranes were incubated with biotin-labeled anti-rabbit or anti-goat IgG for 1 h at room temperature with agitation. The probe proteins were detected using enhanced chemiluminescence system (Amersham International, Piscataway, N.J., USA).

Measurement of VEGF in supernatant by ELISA

The effect of EGCG on VEGF release in tumor cells was measured by ELISA. SGC-7901 cells seeding in 90 mm plates were cultured until 70%-80% confluence. The cells were washed three times with PBS and the culture medium was replaced with SFM. EGCG was added to the medium to various concentrations (0, 5, 10, 20 and 40 μmol/L) and incubated with the cells for 24 h. The conditioned medium was harvested and centrifuged. VEGF concentration in the supernatant was measured using a VEGF ELISA kit (R & D systems, Minneapolis, Minn., USA).

RT-PCR analysis

SGC-7901 cells were seeded in 90 mm plates and cultured in growth medium until 90% confluence. The culture medium was replaced with SFM supplemented

with EGCG at different concentrations (0, 5, 10, 20 and 40 μmol/L). The cells were incubated for another 24 h. Total RNA was extracted from the cell lysates. The levels of human VEGF cDNAs were evaluated by RT-PCR and normalized by the levels of human cytoplasmic β-actin. Reverse transcription was performed with 1 μg total RNA and 100 pmol random hexamers in a total volume of 20 μL to produce first-strand cDNA. PCR experiments were performed with 1 μL of the first-strand cDNA in a 50 μL reaction mixture. Human VEGF cDNA was amplified with specific primers (sense primer: 5'TGC ATT CAC ATT TGT TGT GC 3'; antisense primer: 5'AGA CCC TGG TGG ACA TCT TC-3', a 200 bp product) and β-actin specific primers (sense primer: 5'-TCA TCA CCA TTG GCA ATG AG-3'; antisense primer: 5'-CAC TGT GTT GGC GTA CAG GT-3'; a 150 bp product). Amplification conditions were as follows: denaturation at 94℃ for 1 min, annealing at 60℃ (for β-actin at 55℃) for 1 min, and extension at 72℃ for 1 min. All PCRs were linear up to 30 cycles.

Cell proliferation assay by MTT assay

The effect of EGCG (Sigma, St. Louis, MO., USA) on endothelial cell proliferation induced by VEGF was determined by MTT assay (Sigma, St. Louis, MO., USA). Briefly, 2.5 × 10^4 HUVECs were plated in 24-well plates pre-coated with 2.0% gelatin in triplicate and cultured overnight in growth medium. Then the culture medium was changed with endothelial-SFM supplemented with 2% FBS and 20 ng/mL recombinant human vascular endothelial growth factor (hrVEGF; R & D Systems, Minneapolis, Minn., USA) in the presence of EGCG at various concentrations (0, 5, 10, 20 and 40 μmol/L). The viable cells were quantified by MTT assay at indicated time point following the manufacturer's instructions.

Cell migration assay

Endothelial cell migration induced by VEGF was assessed using a modified Boyden chamber (8 μmol/L pores, Transwell®; Corning Costar Corp., Cambridge, MA). The transwell inserts were coated with 2.0% gelatin. HUVECs were treated with EGCG at various concentrations (0, 5, 10, 20 and 40 μmol/L) for 24 h. Cells were harvested by trypsinization, washed twice with PBS, and resuspended in endothelial-SFM containing 2% FBS and EGCG at the indicated concentrations. Then the treated cells were added to the upper chamber at 2 × 10^5 cells per well and 0.6 mL endothelial-SFM containing 2% FBS and 20 ng/mL VEGF was added to the bottom chamber. After 4-h incubation at 37℃, cells on the upper surface of the membrane were mechanically removed, and the migrated cells on the lower surface of the membrane were fixed and stained with hematoxylin. The average number of migrated cells from 5 randomly chosen fields (× 200) on the lower surface of the membrane was counted. Each experiment was performed in triplicate.

Tube formation assay

Matrigel was thawed at 4℃ in an ice-water bath, and 300 μL/well was carefully added to a pre-chilled 24-well plate using a cold pipette. Matrigel was allowed to polymerize for 1 h at 37℃. After polymerization, 5 × 10^4 cells/well in endothelial-SFM with 2% serum, 20 ng/mL VEGF and EGCG at different concentrations (0, 5, 10, 20 and 40 μmol/L) was layered on top of polymerized gel. Cells were incubated for 48 h and photographed. For quantification of tube formation, the total length of tubes formed in a unit area was measured using an NIH Image program.

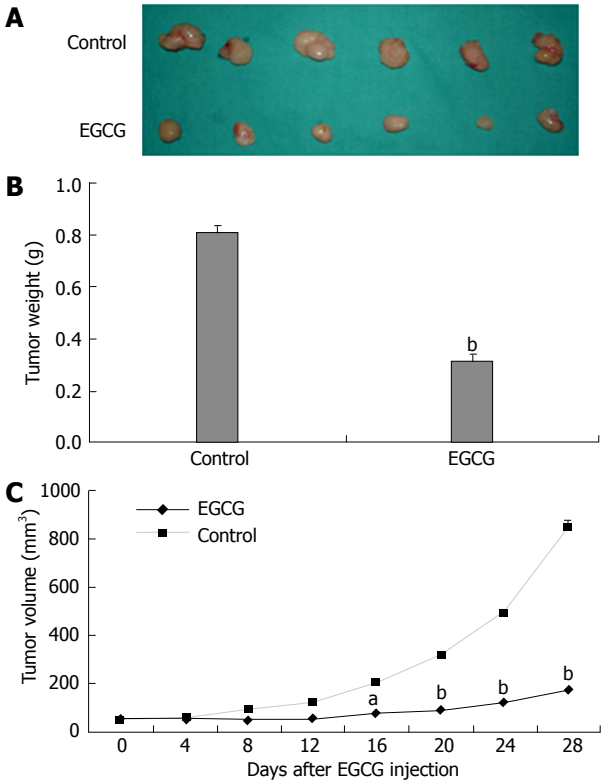

Figure 1 Inhibition of growth of human gastric cancer xenografts in an athymic mouce model. **A**: tumor tissues treated with EGCG or PBS on d 28 after treatment; **B**: an average of 60.4% suppression of primary tumor growth in the EGCG treatment group compared with control group; **C**: tumor growth suppression curve: volumes of EGCG treatment group versus PBS treatment group on indicated days. Data are presented as mean ± SE (n = 6, $^aP < 0.05$, $^bP < 0.01$).

Statistical analysis

Student's t test was used in all statistical analyses. $P < 0.05$ was considered statistically significant.

RESULTS

EGCG suppressed growth of human gastric carcer xenografts in athymic mice

To evaluate the effect of EGCG on tumor growth, tumor xenografts were created by implanting SGC-7901 cells into the dorsal area of athymic mice. Nine days after implantation, tumors reached a size of 50 mm^3. The mice were randomized into two groups and received intraperitoneal injection of EGCG or PBS, respectively. EGCG treatment markedly inhibited tumor growth when compared to control group (Figure 1A). The mean weight of tumors of EGCG treatment was significantly lower than that of control group, and an average of 60.4% suppression of primary tumor growth was observed ($P < 0.01$, Figure 1B). From d 16 after EGCG

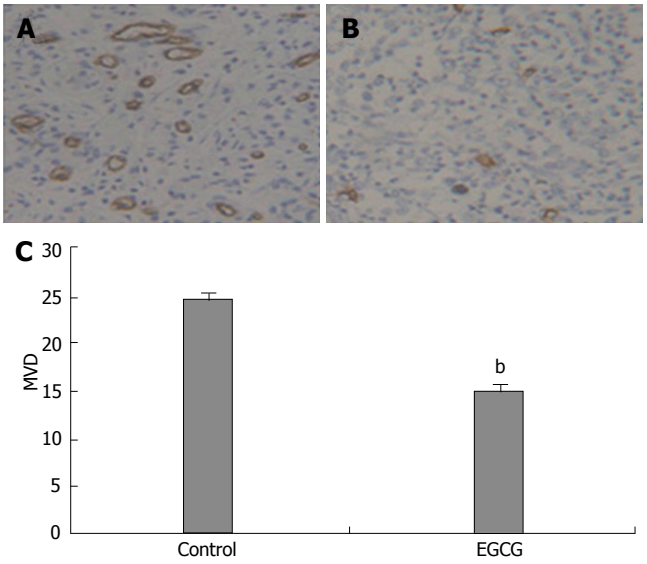

Figure 2 MVD in tumor tissues determined by a specific antibody against mouse endothelial CD34 in PBS group (**A**) and EGCG group (**B**) (200 ×) while quantitative analysis showing significant reduction of tumor MVD after EGCG treatment (**C**). Data are presented as mean ± SE (n = 6, $^bP < 0.01$).

injection, the average volume of tumors was significantly lower in EGCG treatment group than in control group (Figure 1C).

EGCG inhibited tumor neovascularization

To elucidate the anti-angiogenic activity of EGCG *in vivo*, the effect of EGCG on tumor angiogenesis was evaluated by CD34 immunostaining for capillaries in tumor tissues. The density of CD34-stained capillaries was significantly lower in EGCG treatment group (Figure 2A) than in control group (Figure 2B). Microvessel density in tumors receiving EGCG treatment was markedly reduced ($P < 0.01$, Figure 2C), and an average of 38.2% suppression was observed.

EGCG inhibited VEGF expression and Stat3 activation in vitro and in vivo

Gastric cancer had a high expression level of VEGF and Stat3, and activation of Stat3. To determine the effect of EGCG on Stat3 activation and expression of VEGF and total Stat3, the protein level of VEGF, total Stat3 and phosphorylated Stat3 in tumor cells and tissues was examined by Western blot. As shown in Figure 3, EGCG treatment down-regulated VEGF expression in cultured tumor cells in a dose-dependent manner. VEGF expression in tumor tissues treated with EGCG was also markedly reduced. Consistent with the results of VEGF expression, activation of Stat3 in cultured tumor cells was decreased in a concentration-dependent manner, and also apparently reduced in EGCG treated tumor tissues. However, EGCG treatment did not change the expression of total Stat3 either in cultured cells or in tumor tissues.

EGCG decreased VEGF secretion from tumor cells

Angiogenesis is initiated by the release of angiogenic factors from tumor cells. VEGF is the most potent angiogenic factor and associated with tumor-induced

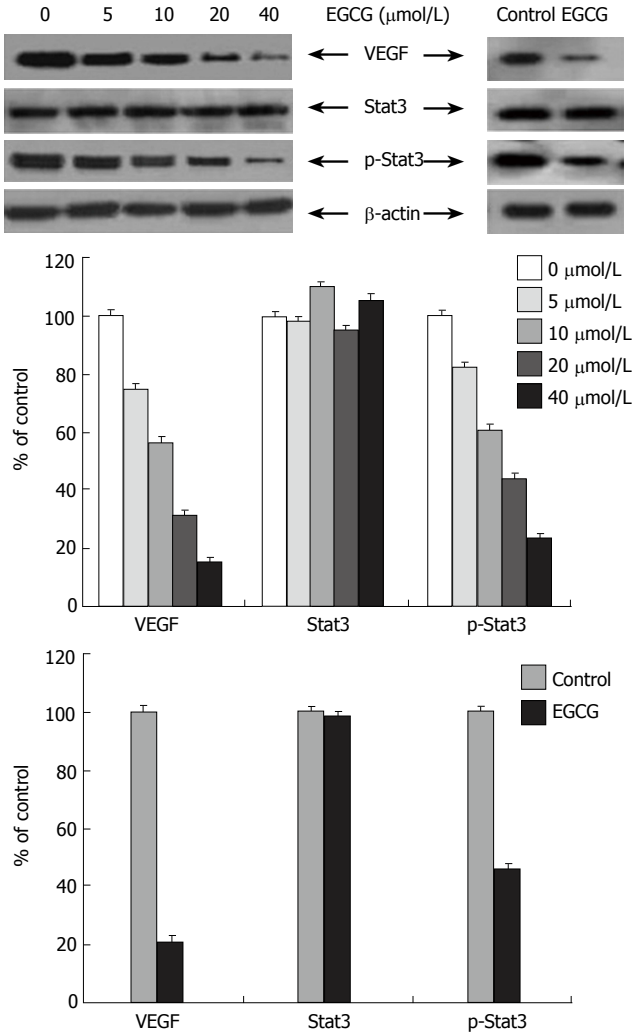

Figure 3 Suppression of VEGF expression and Stat3 activation in tumor cells and tissues by EGCG. Protein level of VEGF and phosphorylated Stat3 was dose-dependently reduced in tumor cells treated with EGCG and also markedly in EGCG treated tumor tissues. EGCG did not change expression of Stat3 in tumor cells or tumor tissues.

angiogenesis. To examine the effect of EGCG on VEGF secretion, VEGF protein level in the conditioned medium was measured by ELISA. EGCG treatment reduced VEGF secretion in the culture medium in a dose-dependent manner (Figure 4A). EGCG at the concentration of 5 μmol/L significantly reduced VEGF secretion by 16.0% ($P < 0.05$).

EGCG inhibited VEGF expression at transcription level

To determine whether the inhibitory effect of EGCG on VEGF expression is at the transcriptional level, we examined VEGF mRNA expression in tumor cells by RT-PCR, showing that VEGF mRNA expression in EGCG treated cells was reduced in a dose-dependent manner (Figure 4B). These results suggested that EGCG reduced VEGF expression by inhibiting VEGF gene transcription and decreasing VEGF mRNA expression.

EGCG inhibited endothelial cell proliferation induced by VEGF

Tumor angiogenesis is induced by VEGF mainly produced by tumor cells. VEGF-induced angiogenesis is very important

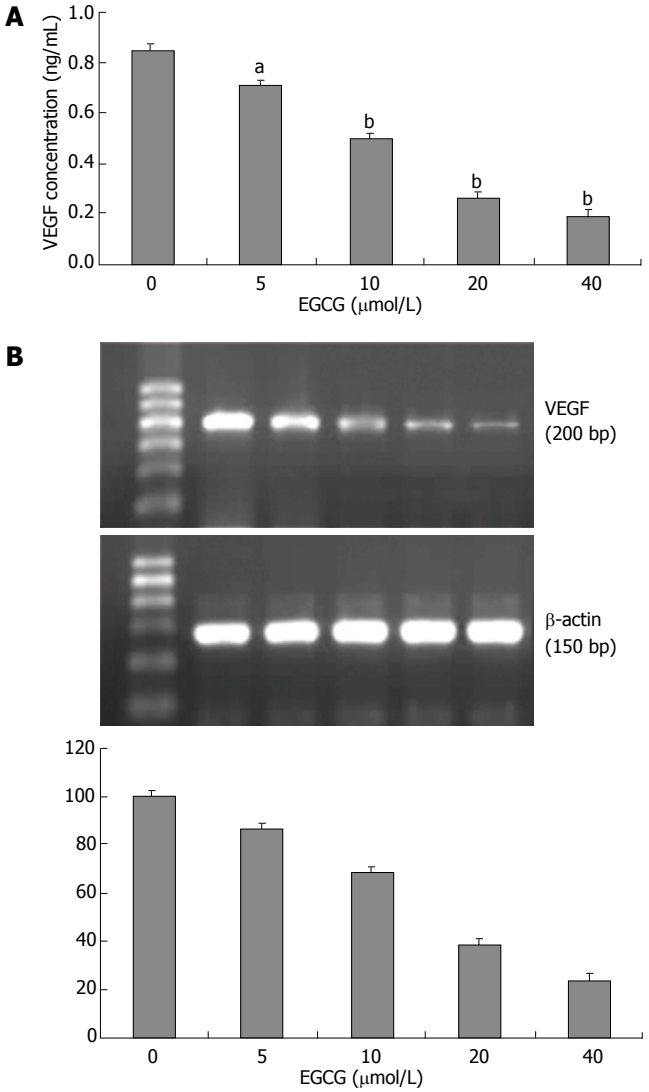

Figure 4 Decreased VEGF secretion (**A**) and inhibition of of VEGF mRNA expression (**B**) in tumor cells. SGC-7901 cells were treated with various concentrations of EGCG for 24 h and VEGF protein level in the supernatant was measured by ELISA and mRNA expression in tumor cells was examined by RT-PCR. Data are presented as mean ± SE (n = 3, $^aP < 0.05$, $^bP < 0.01$).

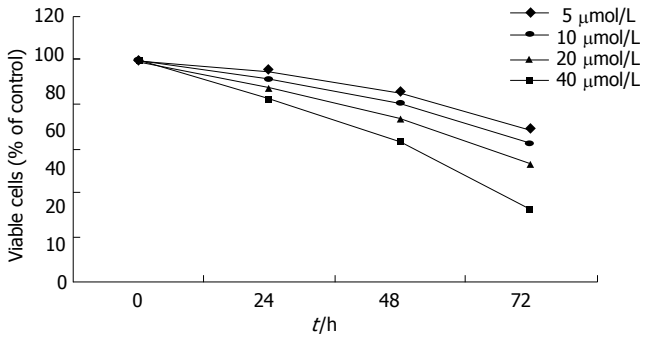

Figure 5 Inhibitory effects of EGCG on VEGF-induced proliferation of endothelial cells. HUVECs were treated with 20 ng/mL VEGF and EGCG at the indicated concentration and time. EGCG time- and dose-dependently inhibited HUVEC proliferation induced by VEGF. Values are expressed as percent of control (means ± SE, n = 3).

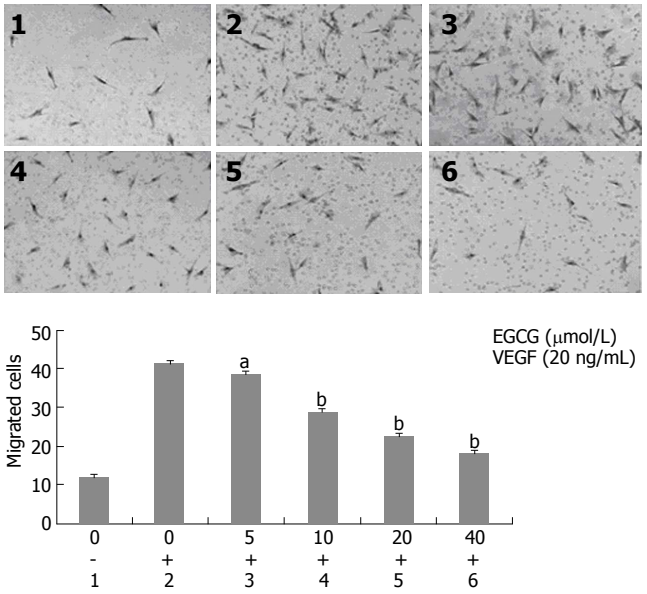

Figure 6 EGCG inhibits endothelial cell migration induced by VEGF. EGCG inhibited migration of HUVECs induced by VEGF in a dose-dependent manner. Data are presented as mean ± SE (n = 3). VEGF-induced migration without EGCG treatment was used as a control. Values significantly lower than control are indicated ($^aP < 0.05$, $^bP < 0.01$).

for tumor growth and metastasis. To further investigate whether EGCG inhibits VEGF-induced angiogenesis, we examined the effect of EGCG on VEGF-induced endothelial proliferation, migration and tuber formation. The effect of EGCG on VEGF-induced proliferation of HUVECs was determined by MTT assay. HUVECs were treated with 20 ng/mL VEGF and EGCG at the indicated concentrations for 24, 48 and 72 h. As shown in Figure 5, EGCG treatment abrogated VEGF-induced proliferation of HUVECs in a time- and dose-dependent manner.

Inhibitory effect of EGCG on endothelial cell migration induced by VEGF

Endothelial cell migration is one of the key steps in angiogenesis. We examined the inhibitory effect of EGCG on endothelial cell migration induced by VEGF. As shown in Figure 6, EGCG treatment significantly and dose-dependently inhibited migration of HUVECs induced by VEGF. EGCG at the concentration of 5 μmol/L could effectively reduce VEGF-induced endothelial cell migration ($P < 0.05$).

Inhibition of VEGF-induced tube formation by EGCG

We determined the effect of EGCG on tube formation induced by VEGF. When treated with EGCG, tube formation induced by VEGF was inhibited in a concentration-dependent manner. EGCG reduced the length of endothelial tubes even at the concentration of 5 μmol/L, and an average of 24.2% inhibition was observed ($P < 0.01$). At the concentration of 40 μmol/L, EGCG abrogated almost all tube formations induced by VEGF (Figure 7).

DISCUSSION

Despite significant advance in treatment, gastric cancer remains one of the leading causes of cancer-related death in many countries such as China[14]. Tremendous efforts

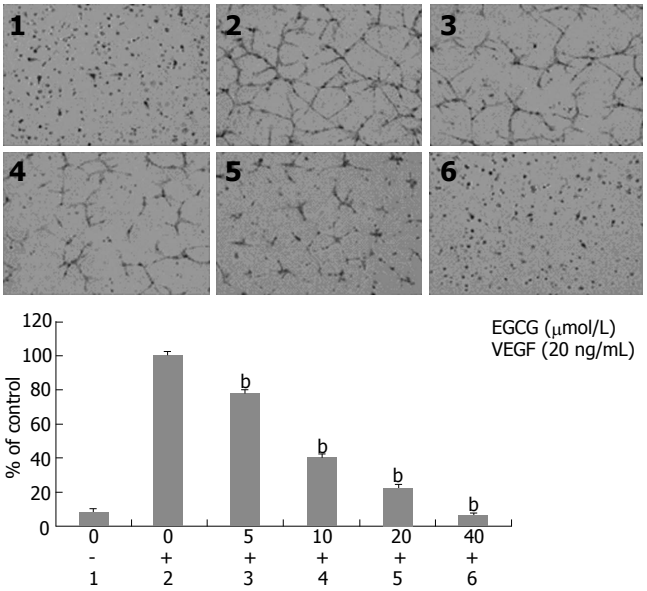

Figure 7 Inhibition of EGCG on VEGF-induced tube formation of endothelial cells. EGCG dose-dependently inhibited tube formation induced by VEGF on Matrigel. Data are expressed as percent of control. VEGF-induced tube formation without EGCG treatment was used as a control. Values significantly lower than control are indicated ($^bP < 0.01$).

have been made to identify new chemopreventive and chemotherapeutic agents and strategies. Anti-angiogenic therapy is one of the most promising novel strategies and many attempts have been made to prevent or delay tumor growth by anti-angiogenesis. As the most potent angiogenic factor, VEGF has become one of the most common targets in anti-angiogenic therapy. In this study, we demonstrated that EGCG suppressed growth of gastric cancer by reducing VEGF production and VEGF-induced angiogenesis.

Green tea has been shown to have anti-angiogenic activity and drinking tea can significantly prevent VEGF-induced corneal neovascularization[15]. As the most abundant and active component of green tea, EGCG has also been shown to have anti-angiogenic property[9]. Treatment with EGCG can inhibit tumor growth[10]. In this study, intraperitoneal injection of EGCG markedly suppressed growth of gastric cancer, and an average of 60.4% inhibition was observed. MVD in tumor tissues treated with EGCG were significantly decreased. These results suggest that EGCG inhibits growth of gastric cancer.

Previous studies indicated that EGCG inhibits VEGF expression in tumor cells[10,16]. Treatment with EGCG also dose-dependently suppresses the secretion of VEGF both in endothelial cells and in tumor cells[17]. To evaluate the effect of EGCG on expression of VEGF in gastric cancer, we determined the expression of VEGF in cultured cells and tumor tissues. As shown in Figure 3, EGCG treatment dose-dependently decreased the expression of VEGF in cultured tumor cells. In tumor tissues treated with EGCG, the expression of VEGF was markedly reduced. Treatment with EGCG also reduced VEGF secretion in tumor cells in a dose-dependent manner. EGCG dose-dependently suppressed VEGF mRNA expression (Figure 4B). These findings suggest that EGCG inhibits angiogenesis in gastric cancer by reducing production of VEGF at transcriptional level.

VEGF expression is associated with a variety of transcription factors, genes and modulators[18]. Several mechanisms have been proposed for the inhibitory effect of EGCG on VEGF expression. EGCG strongly inhibits the transcriptional activity of transcription factors, such as NF-kappa B[16,19] and activator protein-1[20]. Treatment with EGCG also decreases the constitutive activation of EGFR[16] and the expression of protein kinase C[19], transcription modulators of VEGF, suggesting that these changes are associated with inhibition of VEGF promoter activity and cellular production of VEGF. Recent studies showed that EGCG strongly inhibits the constitutive activation of Stat3 in tumor cells[16]. Stat3 is one of the key transcription factors in regulation of VEGF expression[18]. Stat3 activation directly promotes VEGF expression and stimulates tumor angiogenesis[21]. In this study, high activation level of Stat3 was observed in gastric cancer, showing that abnormally activated Stat3 expression significantly correlates with VEGF expression and microvessel density (MVD), and is an independently prognostic factor of poor survival[22]. EGCG can reduce VEGF expression by inhibiting activation of Stat3 in human head, neck and breast cancer[16]. In this study, EGCG dose-dependently inhibited activation of Stat3. In tumor cells and tissues treated with EGCG, the protein level of phosphorylated Stat3 was markedly reduced, but the total Stat3 level remained unchanged, suggesting that EGCG down-regulates VEGF expression at least in part by inhibiting Stat3 activation in gastric cancer.

It was reported that EGCG is most effective in inhibiting angiogenesis among the four main catechins of green tea[23]. Angiogenesis is initiated by the release of angiogenic factors from tumor cells, and VEGF is the most potent angiogenic factor. In this study, EGCG directly inhibited VEGF-induced angiogenesis. As shown in Figure 5, treatment with EGCG inhibited VEGF-induced HUVEC proliferation in a time- and dose-dependent manner and endothelial cell migration and tuber formation were also abrogated by EGCG, suggesting that EGCG inhibits angiogenesis in gastric cancer by inhibiting VEGF-induced angiogenesis and reducing production of VEGF.

VEGF induces angiogenesis by binding to its receptors on endothelial cell surface. It was reported that EGCG dose-dependently inhibits VEGF binding to its receptors on endothelial cell surface, and EGCG is the only catechin of green tea that could disrupt VEGF receptor binding[23]. Importantly, EGCG at low doses (0.5-10 μmol/L) markedly inhibits formation of VEGFR-2 complex[19]. The exact mechanism underlying the inhibition of VEGF receptor binding is unclear. A recent report suggested that green tea extract dose-dependently inhibits expression of VEGFR-1 and -2 on HUVECs[24]. But in our study, EGCG could not modulate VEGFR-1 and VEGFR-2 expression (data not shown). Disruption of VEGF receptor binding would block receptor tyrosine phosphorylation and VEGF-induced growth and survival signaling. Treatment with EGCG inhibits VEGF-induced VEGFR-1 and VEGFR-2

phosphorylation on endothelial cells in a time- and dose-dependent manner[25]. This disruption decreases PI3-kinase activity in a dose-dependent manner[19], suppresses VE-cadherin tyrosine phosphorylation and Akt activation[26], and also decreases the PI3 kinase-dependent activation and DNA-binding ability of NF-kappaB[19], suggesting that EGCG inhibits VEGF-induced angiogenesis by disrupting VEGF signaling pathway.

In the present study, EGCG suppressed growth of gastric cancer by targeting multiple steps of angiogenesis. EGCG at the concentration 5 μmol/L effectively inhibited VEGF production and VEGF-induced angiogenesis. EGCG at 40 μmol/L almost totally abolished tube formation induced by VEGF. Previous studies also showed that concentrations of EGCG used in anti-angiogenesis experiments are usually lower than those in anti-cancer experiments, thus strongly supporting the concept that EGCG represents a potent angiogenic inhibitor and EGCG has dual anti-angiogenesis and anti-cancer activities. EGCG might mainly act as an angiogenic inhibitor *in vivo*, and this might in part explain the discrepancy between cell line and animal model studies. The concentration of EGCG tested in cell line system usually is 10-100 μmol/L, or higher. It is much higher than that observed in plasma or tissues of animals or human beings after ingestion of tea or related tea preparations for the poor bioavailability of EGCG[27].

As an angiogenic inhibitor targeting tumor-induced angiogenesis, EGCG might have some advantages over conventional cytotoxic chemotherapy for genetic stability of endothelial cells and abnormality of tumor vasculature. EGCG is less likely to induce drug resistance and has little or no toxicity. During the treatment, EGCG treated mice did not show weight loss or unusual behavior, histopathological examination also did not reveal any detectable toxicity to liver or kidney (data not shown), suggesting that EGCG at the concentrations used does not cause any detectable toxicity. Previous studies also showed that EGCG induces apoptosis and cell cycle arrest in many cancer cells without affecting normal cells[28]. It was reported that EGCG has anti-cancer effect without any severe side effects[29]. Taken together, as a natural and non-toxic product, EGCG might be a promising candidate for anti-angiogenic treatment of gastric cancer.

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GASTRIC CANCER

Downregulation of survivin by RNAi inhibits growth of human gastric carcinoma cells

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Abstract

AIM: To investigate the inhibitory effect of a specific small survivin interfering RNA (siRNA) on cell proliferation and the expression of survivin in human gastric carcinoma cell line SGC-7901.

METHODS: To knockdown survivin expression, a small interfering RNA targeting against survivin was synthesized and transfected into SGC-7901 cells with lipofectamine™2000. The downregulation of survivin expression at both mRNA and protein levels were detected by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. Cell proliferation inhibition rates were determined by methyl thiazolyl tetrazolium (MTT) assay. The effect of survivin siRNA on cell cycle distribution and cell apoptosis was determined by flow cytometry (FCM).

RESULTS: RNA interference could efficiently suppress the survivin expression in SGC-7901 cells. At 48 h after transfection, the expression inhibition rate was 44.52% at mRNA level detected by RT-PCR and 40.17% at protein level by Western blot analysis. Downregulation of survivin resulted in significant inhibition of tumor cell growth *in vitro*. The cell proliferation inhibition rates at 24, 48 and 72 h after survivin siRNA and non-siliencing siRNA transfection, were 34.06%, 47.61% and 40.36%, respectively. The apoptosis rate was 3.56% and the number of cells was increased in G_0/G_1 phase from 38.2% to 88.6%, and decreased in S and G_2/M phase at 48 h after transfection.

CONCLUSION: Downregulation of survivin results in significant inhibition of tumor growth *in vitro*. The inhibition of survivin expression can induce apoptosis of SGC-7901 cells. The use of survivin siRNA deserves further investigation as a novel approach to cancer therapy.

INTRODUCTION

Survivin was initially identified as a member of the inhibitor of apoptosis protein (IAP) family. Up to the present, overexpression of survivin has not been reported in differentiated normal tissues with exception of thymus, basal colonic epithelium endothelial cells and neural stem cells during angiogenesis[1]. However, the highly specific expression of survivin was found in many human cancers, including gastric cancer. By inhibiting apoptosis and promoting mitosis, survivin facilitates cancer cell survival and growth[2-6]. Several studies have revealed that survivin as an indispensable factor can regulate and assist completion of cytokinesis[7-10]. Recently, it has been also proven that overexpression of survivin can protect cells from apoptosis by inhibiting pro-apoptotic caspases-3 and caspases-7; when acting as a microtubule stabilizer during mitosis, it promotes cell cycle progression as well[11-14]. In addition, Asanuma K *et al*[15] reported that survivin enhanced the expression of the Fas ligand in cancer cells through up-regulation of specific protein-1-mediated gene transcription, and enabled cancer cells to counter-attack immune cells by inducing FasL-triggered apoptosis of cells in the immune surveillance system.

RNAi is a genetic interference phenomenon that is effective for suppressing gene expression. It involves post-transcriptional gene silencing *via* a process in which double-stranded RNA (dsRNA) inhibits gene expression in a sequence-dependent manner through degradation of the corresponding mRNA. Its blocking action on gene expression has been successfully observed in rat and human cells cultured *in vitro*, and the knockdown of genes in cells has been achieved[16,17].

In the present study, siRNA targeting to the survivin gene was introduced into gastric carcinoma cell line SGC-7901, which overexpresses survivin. Its effect on cancer cell growth was investigated.

MATERIALS AND METHODS

Cell lines and culture

Human gastric carcinoma cell line SGC-7901 was a present from Dr. Wei Liu. The cells were cultured in RPMI 1640 medium supplemented with 100 mL/L fetal bovine serum (FBS), 8×10^5 U/L penicillin and 0.1 g/L streptomycin in humidified incubator containing 50 mL/L CO_2 at 37℃.

siRNA preparation and transfection of short interfering RNA

siRNA oligonucleotides with two thymidine residues (tt) at the 3' end of the sequence were designed for survivin (sense, 5'-GCAUUCGUCCGGUUGCGCUtt-3'antisense, 5'-AGCGCAACCGGACGAAUGCtt-3'). Cells were treated in parallel with a non-silencing-siRNA (sense, 5'-UUCUCCGAACGUGUCACGUtt-3'; antisense, 5'-ACGUGACACGUUCGGAGAAtt-3') as control oligonucleotides were synthesized by Shanghai Genechem Co. These cells were cultured in medium without antibiotics, and 24 h before transfection resulting in a confluence of the cell monolayer by 50%-70%. Specific survivin siRNA or non-silencing siRNA (70 nmol) were mixed with lipofectamine ^{TM}2000 (Invitrogen) according to manufacturer's recommendation and added to the cells. After 6 h at 37℃, the medium was changed, and the cells were cultivated in RPMI 1640 supplemented with 10% heat-inactivated FBS.

3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay

SGC-7901 cells (5×10^5) were placed onto 96-well plates in RPMI-1640 containing 10% FBS in a final volume of 0.1 mL. The next day, the cells were treated with siRNA. MTT was added (20 μL/well of 5 g/L solution in PBS) after culture for 24 h, 48 h and 72 h. When incubated at 37℃ for 4 h, the reaction was stopped by addition of 100 μL DMSO. The reaction product was quantified by measuring the absorbance at 490 nm using an ELISA reader (WALLAC 1420 VICTOR 2, Victor Co, Finland) and Software HT-Soft (Perkin-Elmer). All samples were assayed repeatedly in six wells.

Reverse transcription polymerase chain reaction

SGC-7901 cells (5×10^5) were seeded onto 6-well plates. Total RNA was extracted 48 h after transfection using trizol reagent. Reverse transcription was performed using one step RT-PCR kit. The primers of human survivin were 5'-GGACCGCCTAAGAGGGCGTGC-3' (forward primer) and 5'-AATGTAGAGATGCGGTGGTCCTT-3' (reverse primer). The primers of human β-actin were 5'-GTGGGCATGGGTCAGAAG-3' (forward primer) 5'-GAGGCGTACAGGGATAGCAC-3' (reverse primer). Thermal cycle conditions were as follows: 42℃ for 30 min, 94℃ for 2 min, followed by 28 cycles of 94℃ 15 s, 55℃ 30 s, 72℃ 1 min, with a final extension at 72℃ for 10 min. RT-PCR products were visualized by ethidium bromide-stained agarose gels.

Western immunoblot analysis

SGC-7901 cells (5×10^5) were seeded onto 6-well plates. Forty-eight hours after transfection, cells were collected and washed twice by cold PBS, and each well was treated with 50 μL lysis buffer (2 mmol/L Tris-HCl pH 7.4, 50 mmol/L NaCl, 25 mmol/L EDTA, 50 mmol/L NaF, 1.5 mmol/L Na_3VO_4, 1% Triton X-100, 0.1% SDS, supplemented with protease inhibitors 1 mmol/L phenylmethylsulfonylfluoride, 10 mg/L pepstatin, 10 mg/L aprotinin, and 5 mg/L leupeptin) (all from Sigma). Protein concentrations were determined using the bicinchoninic acid protein assay. Equal amounts of protein (40 μg) were separated on a 15% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond C, Amersham, Freiburg, Germany). Membranes were blocked in 5% nonfat dry milk in TBS for 1 h at room temperature and probed with rabbit antisurvivin antibodies (dilution, 1:500 Santa Cruz Biotechnology, USA) overnight at 4℃. After 3 times washing with TBS containing 0.1% Tween 20, membranes were incubated with anti-rabbit IgG-horseradish-peroxidase (1:5000, Santa Cruz Biotechnology, USA), and developed by luminol mediated chemiluminescence (Appylgen Technologies Inc, China). To confirm equal protein loading, membranes were reprobed with a 1:1000 dilution of an anti-actin antibody (Santa Cruz Biotechnology, USA). Densitometric analyses were performed using Scion Image software.

Flow cytometry

SGC-7901 (5×10^5) cell lines were seeded in triplicate onto 6 well-plates, and cultured in RPMI-1640 supplemented with 100 mL/L FBS. When transfected for 48 h, the cells were collected and washed with ice-cold PBS, and fixed in 70% ethanol overnight at 4℃. The fixed cells were pelleted, washed in PBS, resuspended in PBS containing 0.1 mg/mL of propidium iodide, and analysed by flow cytometry.

Statistical analysis

Data were expressed as mean ± SD. All statistical analyses were performed using the SPSS 10.0 software package for Windows (SPSS Inc., Chicago, IL). One-way ANOVA followed by Bonferroni correction was used to compare the data among three or more groups. A value of $P < 0.01$ was considered statistically significant.

RESULTS

Expression of survivin gene in SGC-7901 cells

To examine the specific effect of survivin siRNA treatment on survivin expression in SGC-7901 line, the survivin mRNA and protein expression levels were determined quantitatively with RT-PCR and Western blot analyses, respectively. Results are displayed in Figure 1 and Figure 2. Survivin mRNA and protein were strongly expressed in gastric cancer SGC-7901 cells as reflected by RT-PCR and Western blot. The inhibition rate of survivin mRNA after transfection with specific survivin siRNA was 44.52%. The inhibition rate of survivin protein after transfection with specific survivin siRNA was 40.17%. Survivin expression was decreased significantly at 48 h after transfection with specific survivin siRNA.

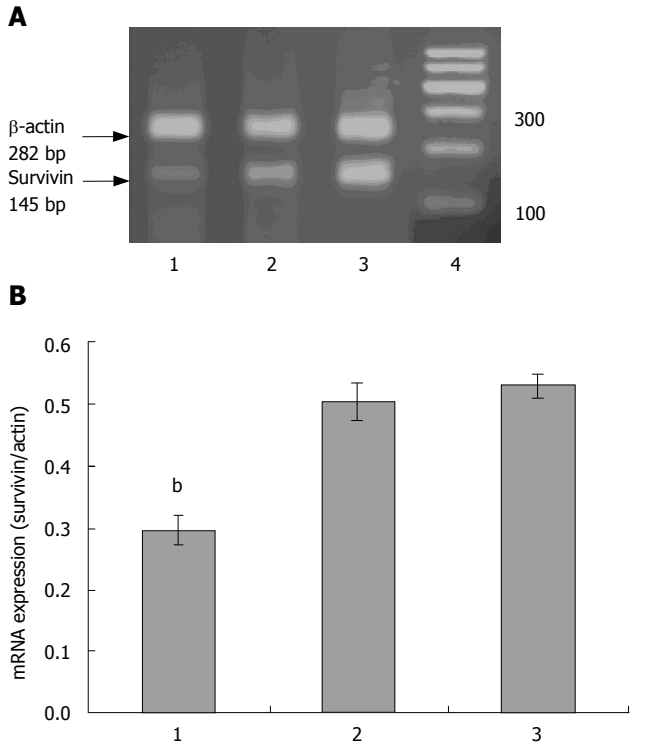

Figure 1 Expression of survivin mRNA in different groups of SGC-7901 cells. **A**: Survivin mRNA expression in RT-PCR assay; **B**: Survivin mRNA expression in SGC-7901. 1: treated with survivin siRNA; 2: treated with non-silencing siRNA; 3: untreated. Data are expressed as mean ± SD of three experiments, survivin siRNA group *vs* non-silencing siRNA group, $^bP < 0.01$; survivin siRNA group *vs* untreated group, $^bP < 0.01$.

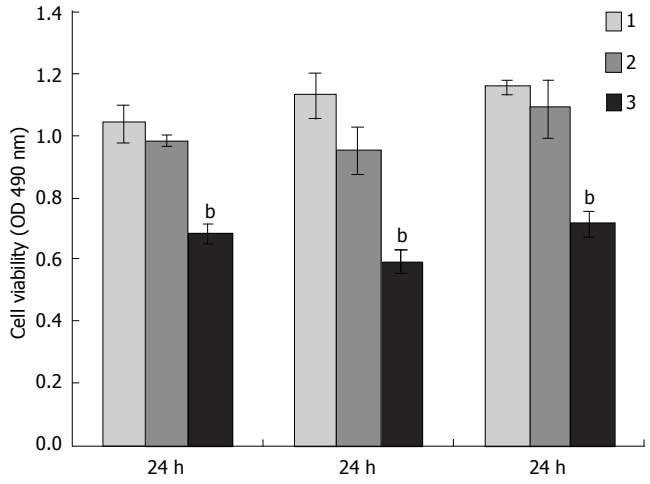

Figure 3 Effects on cell viability after non-silencing siRNA and survivin siRNA treatment. 1: untreated; 2: non-silencing siRNA; 3: survivin siRNA. Data are expressed as mean ± SD, survivin siRNA group *vs* non-silencing siRNA group, $^bP < 0.01$; survivin siRNA group *vs* untreated group, $^bP < 0.01$.

Effect of survivin siRNA on cell proliferation of gastric cancer cells

To determine whether inhibition of survivin affects cell proliferation in SGC-7901, metabolic activity at 24, 48, and 72 h was determined by the MTT assay. The cell viability was reduced significantly after treatment with specific survivin siRNA ($P < 0.01$) at 24, 48, and 72 h as compared with non-silencing siRNA treatment or untreated controls (Figure 3). The cell proliferation inhibition rates were

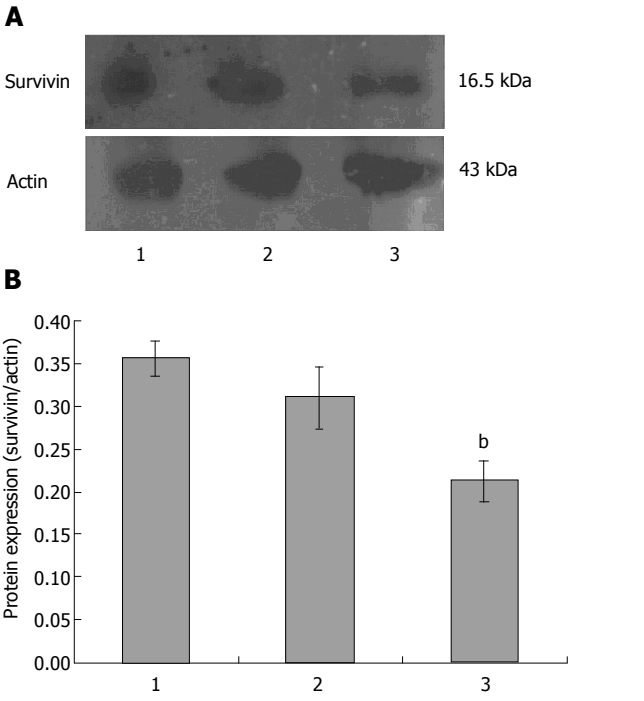

Figure 2 Western blot assay of survivin protein expression in SGC-7901 cells. **A**: Survivin protein expression in Western blot assay; **B**: Survivin protein expression in SGC-7901.1: untreated; 2: non-silencing siRNA; 3: survivin siRNA. Data are expressed as mean ± SD of three experiments, survivin siRNA group *vs* non-silencing siRNA group, $^bP < 0.01$; survivin siRNA group *vs* untreated group, $^bP < 0.01$.

Table 1 Effects of treatment with survivin-siRNA on cell cycle distribution and apoptosis 48 h after transfection

Groups	Cell cycle			Apoptosis rate
	G_0/G_1	S	G_2/M	
Untreated	38.21 ± 3.39	47.71 ± 2.73	14.00 ± 0.92	0.83 ± 0.34
Non-silencing	42.53 ± 2.78	43.28 ± 4.12	14.20 ± 2.90	1.08 ± 0.33
Sur-siRNA	88.60 ± 4.04^b	12.00 ± 3.42^b	0.10 ± 0.07^b	3.56 ± 0.91^b

Data are expressed as mean ± SD of the three experiments, sur-siRNA group *vs* non-silencing group, $^bP < 0.01$; sur-siRNA group *vs* untreated group, $^bP < 0.01$.

34.06%, 47.61% and 40.36%, respectively.

Effect of survivin siRNA on cell cycle distribution and apoptosis

Survivin has been reported to express in the G2/M phase of the cell cycle and to contribute to the aberrant progression of cancer cells through mitosis. In our study, in comparison of non-silencing siRNA group and untreated controls, specific survivin siRNA caused accumulation in the G_0/G_1 phase, and decreased the number of cells of G_2/M phase and increased hypodiploid DNA content ($P < 0.01$) at 48 h after transfection. Apoptosis rate was 3.56% in the group of specific survivin siRNA transfection (Table 1).

DISCUSSION

The balance between apoptosis and anti-apoptosis signal pathways plays a role in the pathogenesis of a variety of

cancers. It has been demonstrated that the inhibition of apoptosis promotes the mitotic progression in cancer cells[18]. Based on its specific overexpression in a vast majority of solid cancers and its anti-apoptotic function, survivin represents a suitable target for antitumor approaches. The inhibition of survivin would block the anti-apoptosis and mitotic progression in tumor cells[19], as a result, tumor development is suppressed. So, survivin has attracted considerable attention as a novel member of IAP family.

RNAi is an evolutionarily conserved phenomenon in which gene expression is suppressed by the introduction of homologous double-stranded RNAs (dsRNAs). Synthetic siRNA can trigger an RNA interference response in mammalian cells and induce strong inhibition of specific gene expression[20]. Therefore, it can be used as a powerful approach to silence mammalian gene expression for gene function studies[21-24].

Since survivin is important for the survival of various human tumors, survivin siRNA could become an effective therapeutic agent for tumors with overexpression of survivin. Survivin has been successfully down-regulated by RNAi in some previous studies[25-29]. However, the down-regulation of survivin expression by RNAi in gastric cancer SGC-7901 cells has not been performed until now.

In addition, it is reported that the effect of RNAi may be greatly different, targeting to different sequence of the gene[30-32]. It is therefore, very important to find the proper sequence. The sequence designed by Williams *et al*[26] has been confirmed effective in their research. So in our study, we synthesized this siRNA duplex with a thymidine overhang on 3' terminus *in vitro* to diminish the survivin expression of gastric carcinoma SGC-7901 cell line and to impair its growth potential. We observed that SGC-7901 cells transfected with survivin siRNA grew slowly as compared with the control groups. Survivin siRNA showed anti-proliferation function. RT-PCR and Western blot findings demonstrated that survivin mRNA and protein expression were reduced by over 40%, and survivin gene was blocked in SGC-7901 cells transfected with surviving siRNA at the level of transcription and protein expression. Flow cytometry revealed that the cell growth inhibition by survivin siRNA was a result of cell cycle arrest at G_0/G_1-phase and induction of apoptosis indicated by increased hypodiploid DNA content. The possible mechanism is that survivin is characterized by cell cycle dependent expression, i.e., it is expressed in G_2/M. Ito and co-workers transfected survivin into four human hepatocellular carcinoma cell lines and found that the number of cells in G_0/G_1 was remarkably reduced, and the number of cells in S or G2/M increased. In our study, after SGC-7901 cells were transfected with survivin siRNA, the number of cells in G_0/G_1 was remarkably increased, and the number of cells in S or G_2/M decreased. The effect showed the same mechanism. However, in the study by Kappler and co-workers, five human sarcoma cell lines transfected with specific survivin siRNA showed G_2/M arrest[26]. Ning *et al*[33] also observed a specific G_2/M arrest when using survivin-directed siRNA as a gene therapeutic approach to human bladder cancer cells. The reason remains unclear, and it may be due to different cell lines.

The above-mentioned findings confirm that chemically synthesized siRNAs can specifically block survivin gene expression, inducing cell apoptosis and inhibiting the growth of carcinoma cells.

In conclusion, our data suggest that survivin gene can be regarded as a very good target gene in genetic therapy for gastric carcinomas and the use of survivin siRNA deserves further investigations as a novel approach to cancer therapy.

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Selective COX-2 inhibitor, NS-398, suppresses cellular proliferation in human hepatocellular carcinoma cell lines *via* cell cycle arrest

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Abstract

AIM: To investigate the growth inhibitory mechanism of NS-398, a selective cyclooxygenase-2 (COX-2) inhibitor, in two hepatocellular carcinoma (HCC) cell lines (HepG2 and Huh7).

METHODS: HepG2 and Huh7 cells were treated with NS-398. Its effects on cell viability, cell proliferation, cell cycles, and gene expression were respectively evaluated by water-soluble tetrazolium salt (WST-1) assay, 4'-6-diamidino-2-phenylindole (DAPI) staining, flow cytometer analysis, and Western blotting, with dimethyl sulfoxide (DMSO) as positive control.

RESULTS: NS-398 showed dose- and time-dependent growth-inhibitory effects on the two cell lines. Proliferating cell nuclear antigen (PCNA) expressions in HepG2 and Huh7 cells, particularly in Huh7 cells were inhibited in a time- and dose-independent manner. NS-398 caused cell cycle arrest in the G1 phase with cell accumulation in the sub-G1 phase in HepG2 and Huh7 cell lines. No evidence of apoptosis was observed in two cell lines.

CONCLUSION: NS-398 reduces cell proliferation by inducing cell cycle arrest in HepG2 and Huh7 cell lines, and COX-2 inhibitors may have potent chemoprevention effects on human hepatocellular carcinoma.

Key words: Selective cyclooxygenase 2 inhibitor; Cell growth; Cell cycle; Hepatocellular carcinoma cells

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide[1]. Although HCC is significantly more prevalent in Asia and Africa, a rising incidence has been reported in Western countries. Most patients present at an advanced stage when successful surgical treatment is no longer feasible and current therapeutic options achieve clinical responses in only a small percentage of cases. As a consequence, effective approaches for prevention and treatment need to be established.

Epidemiological and experimental studies have demonstrated the effect of non-steroidal anti-inflammatory drugs (NSAIDs) in the prevention of human cancers, especially those in the gastroenterological tract[2-4]. Epidemiological studies have shown that the rate of mortality of colorectal cancer in individuals taking NSAIDs is 40%-50% lower than that in non-users[4]. NSAIDs inhibit endogenous prostaglandin synthesis. The key step in the enzymatic conversion of arachidonic acid to prostaglandins is catalyzed by cyclooxygenases (COX), and represents the specific target of NSAIDs.

Two forms of cyclooxygenase, COX-1 and COX-2 have been identified. COX-1 is expressed constitutively in most tissues and appears to be responsible for prostaglandin production in various physiological functions such as platelet aggregation and maintenance of gastric mucosa. In contrast, COX-2 is induced by several inflammatory cytokines and growth factors[5] and frequently over-expressed in various tumor cells. Selective COX-2 inhibitors do not affect platelet aggregation or bleeding time and cause far fewer acute gastric erosions than several nonselective inhibitors. Therefore, selective COX-2 inhibitors such as celecoxib and NS-398 are preferable to nonselective COX-2 inhibitors.

The role of COX-2 in hepatocellular carcinogenesis

is still unclear. Previous studies have shown increased expression of COX-2 in patients with various liver diseases, suggesting its possible role in chronic liver disease and during HCC progression[6-8]. In addition, some studies have described the possible benefits of treatment with COX-2 inhibitors as indicated by their effects on human HCC cell lines[8-10]. However, contrasting results have also been reported about the expression of COX-2 or the effects of COX-2 inhibitors on HCC cell lines[11]. COX-2 may be a logical therapeutic target in various human cancers including HCC. However, information regarding the mechanisms involved in these effects is scant and sometimes contradictory[12-15].

In this study, we examined the effects and possible mechanism of NS-398, a selective COX-2 inhibitor in two human hepatoma cell lines, HepG2, and Huh7.

MATERIALS AND METHODS

Materials

Human hepatoma cell lines HepG2 (showing wild-type p53) and Huh7 (showing mutant-type p53) were obtained from the American Type Culture Collection (Rockville, MD, USA). Each cell line was cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), essential amino acids, 100 units/mL penicillin (Invitrogen), and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA). All cells were maintained in a humidified atmosphere containing 950 mL/L air and 50 mL/L CO_2 at 37℃. NS-398 was purchased from Cayman Chemical (Ann Arbor, MI, USA) and dissolved in dimethyl sulfoxide (DMSO).

Cell viability assay

To evaluate the inhibitory growth effect of NS-398, we confirmed its cytotoxicity and proliferating activity by water-soluble tetrazolium salt (WST-1) assay (Roche Diagnostics, Mannheim, Germany). The WST-1 assay is a colorimetric method in which the dye intensity is proportional to the number of viable cells. Cells were seeded into 96-well microtiter plates at a concentration of 5×10^3 cells/well. After 12-h incubation, cells were treated with serum-free media containing various concentrations of NS-398 (0, 50, 100, and 200 μmol/L) for 24, 48, and 72 h. Because we used DMSO-dissolved NS-398, cells cultured in the serum-free media containing an equivalent volume of DMSO without NS-398 respectively were used as controls to ensure DMSO not to promote unwanted cellular effects. The final concentration of DMSO was < 0.3%. After incubation, the cells were washed with PBS and the cell proliferation reagent WST-1 was added with cell culture medium, and incubated for 4 h. Sample absorbence was analyzed using a bichromatic ELISA reader at 450 nm. All experiments were performed in triplicate.

Nuclei staining and FACS analysis

Characterization of apoptosis and/or necrosis was carried out after propidium iodide (PI) and Annexin V-FITC staining with apoptosis detection kit I (Pharmingen, San Diego, CA, USA) followed by flow cytometric analysis after 24, 48 and 72 h of 100 μmol/L NS-398 treatment in HepG2 and Huh7 according to the manufacturer's instructions. For histogram analysis after PI staining, the cells were fixed with 70% ethanol in PBS for 15 min at -20℃. The fixed cells were incubated with RNase A (100 μg/mL) for 30 min at 37℃ and then stained with PI (50 mg/mL in PBS). Fluorescence of individual nuclei and intact cells was determined using a FACScalibur flow cytometer (Beacton Dickinson, San Jose, CA).

For detection of apoptosis, cells were washed twice with ice cold PBS and stained simultaneously with FITC-conjugated Annexin V and PI for 20 min on ice in the dark with a binding buffer. Within the next hour, cells were analyzed for apoptosis. The total number of apoptotic cells was determined by calculation of Annexin V+ and PI-cells (reflecting early apoptosis) together with annexin V+ and PI+ cells (reflecting late apoptosis/secondary necrosis).

DAPI staining

4'-6-diamidino-2-phenylindole (DAPI), a DNA-binding fluorescent dye, was used to determine whether the mechanism of growth inhibition upon NS-398 treatment is apoptosis. After treatment with 100 μmol/L NS-398 for 0, 24, 48, and 72 h, the cells were washed three times with PBS, fixed in a 3.7% formaldehyde solution for 10 min, fixed once in 1 mL of methanol, and then stained with 4 μg/mL DAPI (Oncor, Gaithersburg, MD) for 10 min. Results were determined by visual observation of nuclear morphology *via* fluorescence microscopy.

Immunoblot analysis

HepG2 and Huh7 cells were seeded on 100-mm dishes at a density of 1.5×10^6 cells/dish. After seeding and overnight incubation, cells were serum-starved for 24 h and incubated in serum-free media with 100 μmol/L NS-398 for 0, 24, 48 and 72 h. Attached and floating cells were harvested together. Cells cultured in the same condition without NS-398 were used as controls. Proteins extracted from either treated or control cells were measured by Bradford assay, and subjected to SDS-PAGE. The proteins were then transferred electrophoretically to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) at 100 V for 1 h at 4℃. To stain the proteins and to validate that equal amounts of protein were loaded in each lane and transferred efficiently, the membrane was immersed in 0.5% Ponceau S (Sigma Chemical Co., St. Louis, MD) in 1% acetic acid. The membrane was washed three times with TBS containing 0.1% Tween 20 (TBS-T). After blocking with 5% skim milk in TBST buffer for 1 h, the membrane was incubated with primary antibodies (anti-mouse COX-2 antibody, 1:500; anti-rabbit caspase-9, caspase-3 and caspase-8, 1:1000; anti-mouse poly [ADP-ribose] -polymerase (PARP) antibody, 1:1000; anti-mouse proliferating cell nuclear antigen (PCNA) antibody, 1:1000 and anti-mouse β-actin antibody, 1:2500) overnight at 4℃. The membrane was incubated with secondary antibodies for 1 h and detected with ECL reagent (Amersham-Pharmacia, UK).

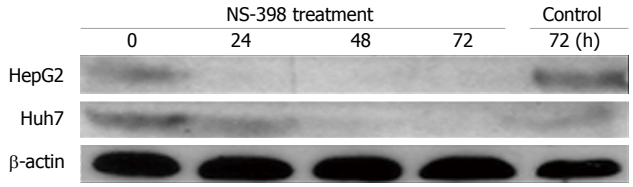

Figure 1 Immunoblot analysis for COX-2 protein levels in HepG2 and Huh7 cells. Cells were treated with 100 μmol/L NS-398 for 0, 24, 48 and 72 h. Control cells were treated with DMSO without NS-398. The upper bands represent 72 kDa COX-2-specific staining while the lower bands are β-actin controls. COX-2 expression was detectable but low in both cell lines. Decreased changes were observed in COX-2 expression with increasing time.

Statistical analysis

One-way analysis of variance (ANOVA) was performed to test the differences in cellular proliferation and apoptosis of the two cell lines treated with different NS-398 concentrations for varying times. $P < 0.05$ was considered statistically significant. Data were analyzed using the Statistical Package for Social Sciences (SPSS) (version 11.0).

RESULTS

Inhibition of COX-2 expression by NS-398

We first characterized the hepatoma cell lines by their expression of COX-2. Samples containing 50 μg of extracted protein were loaded onto the gel. Immunoblot analysis revealed that both cell lines expressed COX-2 protein. The expression of COX-2 was inhibited by NS-398. Immunoblot analysis also demonstrated different COX-2 expressions after treatment with 100 μmol/L NS-398 for different periods of time (Figure 1).

Inhibition of HepG2 and Huh7 cell viability by NS-398

To determine the effect of the selective COX-2 inhibitor NS-398 on HepG2 and Huh7 cells, cell viability was determined by WST-1 assay. Growth-inhibitory experiments were performed by treating cells with various doses of NS-398 for different periods of time. As shown in Figure 2, NS-398 reduced cell viability in a time- and dose-dependent manner on both cell lines. However, treatment with only DMSO as a control did not alter HepG2 and Huh7 cell proliferation ($P > 0.05$).

No evidence of apoptosis was observed in two cell lines

To confirm that NS-398 induces apoptosis of COX-2-expressing HepG2 and Huh7 cells, a double-staining method using FITC-conjugated Annexin V and PI, DAPI staining and Western blotting were done. In the Annexin and PI staining, the percentages of apoptotic and necrotic cells at 0, 24, 48 and 72 h after 100 μmol/L of NS-398 treatment were not significantly different in HepG2 and Huh7 cells (Figure 3). Moreover, no condensed and fragmented nuclei were found in DAPI staining (data not shown). Immunoblot analysis showed that there was no activation of caspase-3, caspase-8, caspase-9 and PARPs in HepG2 and Huh7 cells treated with 100 μmol/L of NS-398 (Figure 4). Taken together, NS-398 did not induce apoptosis in HepG2 and Huh7 cells.

NS-398 suppressed cell proliferation by inducing cell cycle arrest, especially in Huh7 cell lines

To test the effects of NS-398 on cellular proliferation, the two HCC cell lines were treated with 100 μmol/L of NS-398. We detected PCNA protein by IB analysis. As shown in Figure 5, both cell lines showed a down-regulation of PCNA expression after treatment with 100 μmol/L of NS-398 in a time dependent manner. In particular, NS-398 treatments significantly suppressed the cellular proliferation in Huh7 cells.

To determine whether suppressions of proliferation in Huh7 cells induced by NS-398 are associated with cell-cycle arrest, we measured DNA content by flow cytometry. We observed that the population of cells in sub-G1 phase increased after 100 μmol/L NS-398 treatment in both cell lines for different periods of time. A significant correlation was observed between the growth inhibition and accumulation of cells in sub-G1 phase for Huh7 at 48 h (Figure 6).

DISCUSSION

COX-2 is involved in several pathological processes such as inflammation and cancer, and inhibition of apoptosis is regarded as one mechanism by which COX-2 contributes to tumorigenesis. Recent studies demonstrated that selective COX-2 inhibitors (including NS-398) which can inhibit cell proliferation or induce apoptosis are found not only in HCC cell lines but also in colorectal[2,3], esophageal[14], bladder[15], prostatic[16], lung[17], cervix[18] carcinoma cells.

Over-expression of COX-2 has been demonstrated in patients with HCC and high COX-2 expression in non-tumor liver tissue is significantly associated with inflammatory activity[6,8,19]. These findings indicate that COX-2 expression plays an important role in hepatic inflammation and malignant transformation of hepatocytes[20]. However, the key mechanism by which COX-2 affects HCC cell growth remains unclear.

In this study, we detected the expression of COX-2 protein in HepG2 and Huh7 cells by Western blotting. Decreased changes in COX-2 protein expression were observed during the course of growth inhibition by NS-398 treatment (Figure 1). Our findings suggest that the growth-inhibitory effect on hepatocellular carcinoma cell lines is dependent on COX-2 expression[10]. However, several other studies have shown that there is no correlation between COX-2 expression and growth-inhibitory effects induced by NSAIDs[9,11,21].

NS-398-induced inhibition of cell growth is both dose- and time-dependent (Figure 2). The association of COX-2 inhibition with suppression of tumor cell proliferation remains unclear in HCC. Our findings demonstrate that NS-398 may inhibit cell growth in a dose- and time-dependent manner, due to the result of evident cell cycle arrest at G1 (Figures 2 and 6)[11]. Decreased PCNA expression supports the finding of a reduced number of cells in S phase by cell cycle analysis, which is an important event in cell cycle arrest of HepG2 (wild-type p53) and Huh-7 (p53-220Cys) induced by NS-398.

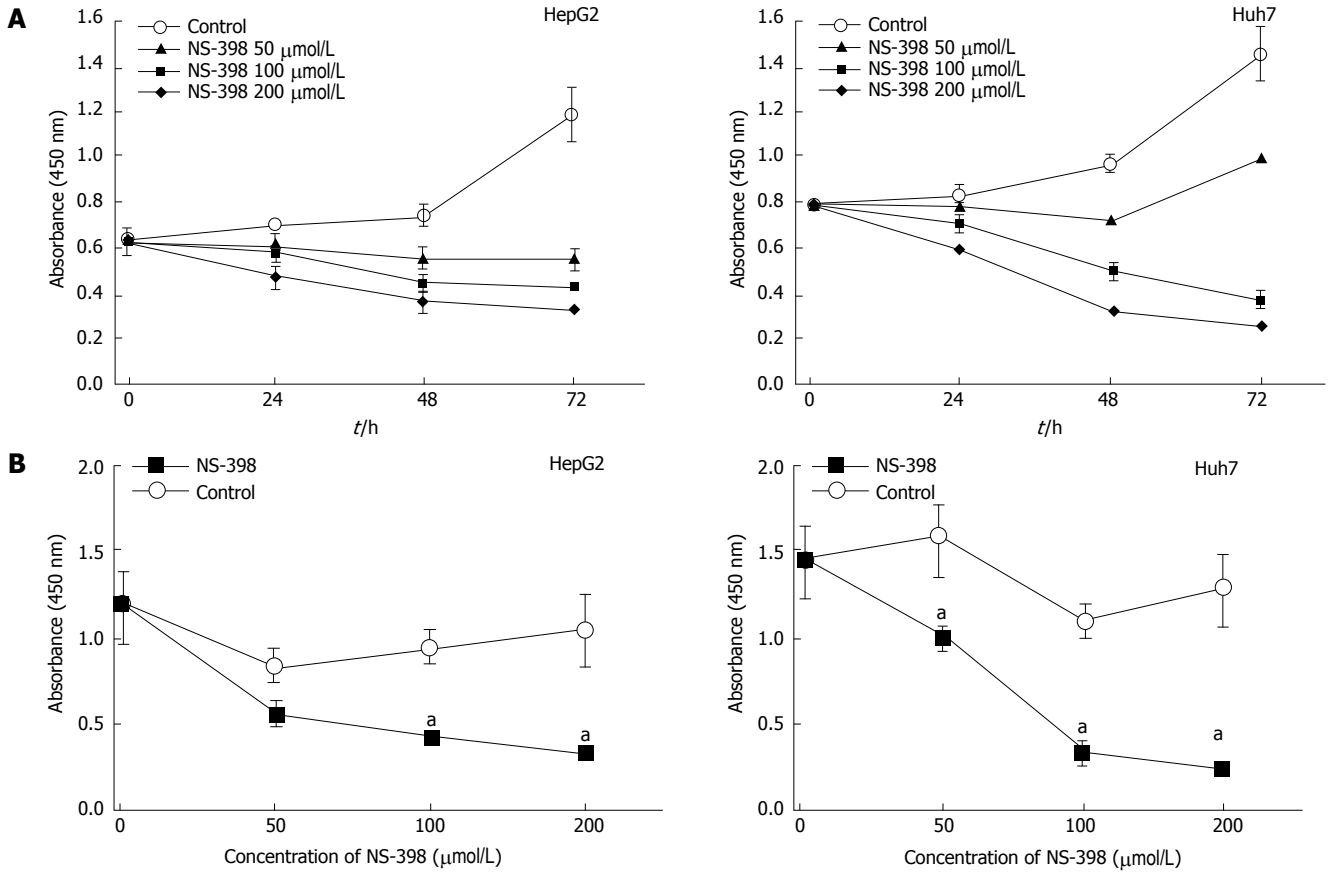

Figure 2 Proliferation of HepG2 and Huh7 cells inhibited by NS-398. **A**: Time-dependent growth-inhibitory effect of NS-398. One hundred μmol/L NS-398 was added to HepG2 and Huh7 cells, which were cultured for 24, 48 or 72 h, and subjected to WST-1 assay. Absorbance was detected at 450 nm with an ELISA plate reader, and the growth rates of both cell lines treated with NS-398 were reduced compared to controls. Control cells were treated with an equivalent volume of DMSO without NS-398, respectively; **B**: Dose-dependent growth-inhibitory effect of NS-398. Cells treated with various concentrations of NS-398 were cultured for 72 h, and quantified by WST-1 assay. Data from 3 independent experiments are shown as e mean ± SD ($^aP < 0.05$).

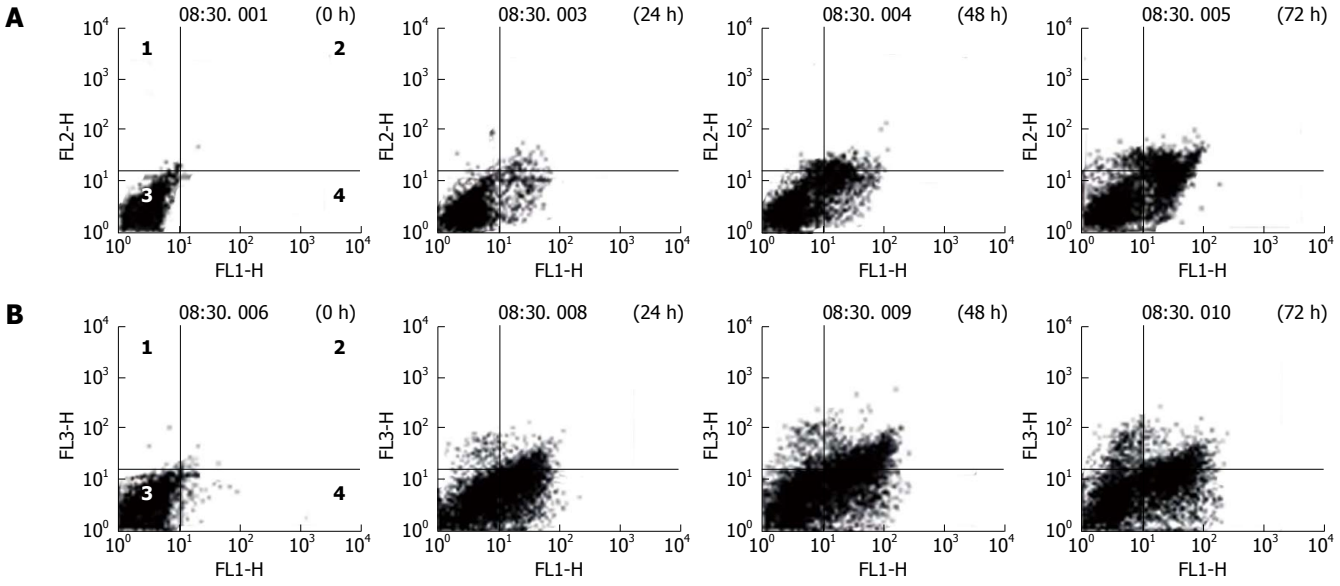

Figure 3 Flow cytometric analysis after exposure of HepG2 (**A**) and Huh7 (**B**) cells to NS-398. Cells were treated with 100 μmol/L NS-398 for 24, 48, and 72 h, stained with annexin V-FITC and propidium iodide and analyzed on a FACScalibur flow cytometer. Quadrants 2-4 represent secondary necrotic cells, viable cells, and apoptotic cells, respectively.

In particular, NS-398 treatment significantly suppressed Huh7 proliferation. Recent reports indicate a complex relationship between the tumor suppressor p53 and COX-2[22,23]. While wild-type p53 over-expression leads to increased COX-2 levels[24], opposite effects where p53 inhibits COX-2 transcription have been reported[25]. Thus,

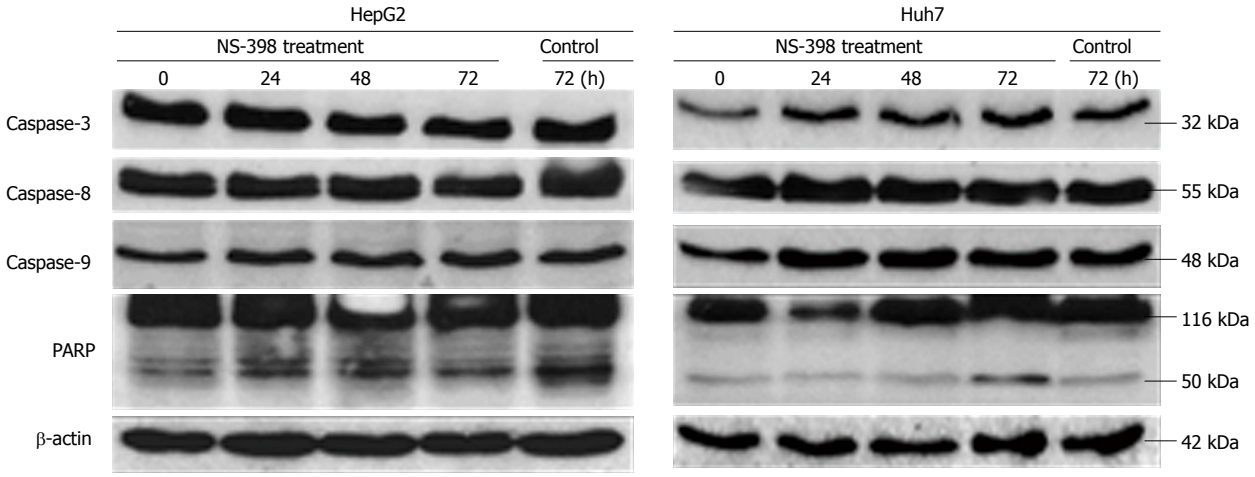

Figure 4 No evidence of activation of the caspase pathway and PARP by NS-398. No evidence of apoptosis in HepG2 and Huh7 cells was observed after NS-398 treatment. No caspase pathway activation or PARP cleavage was detected in both cell lines.

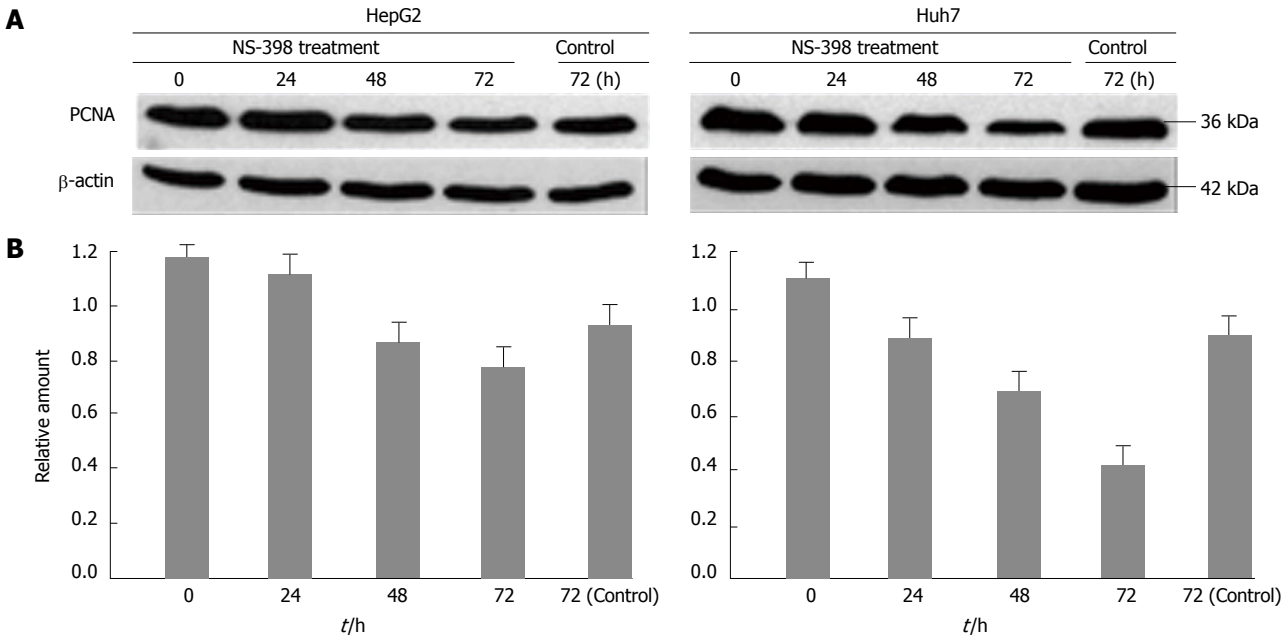

Figure 5 Inhibition of PCNA expression by NS-398. **A**: Down-regulation of PCNA expression in both cell lines after NS-398 treatment. Cells were treated for 24, 48, and 72 h with 100 μmol/L NS-398. Controls were treated with an equivalent volume of DMSO without NS-398. PCNA expressions in HepG2 and Huh7 cells were inhibited in a time-dependent (as shown above) and dose-independent (data not shown) manner; **B**: Quantification of the relative amount of each band by image analysis.

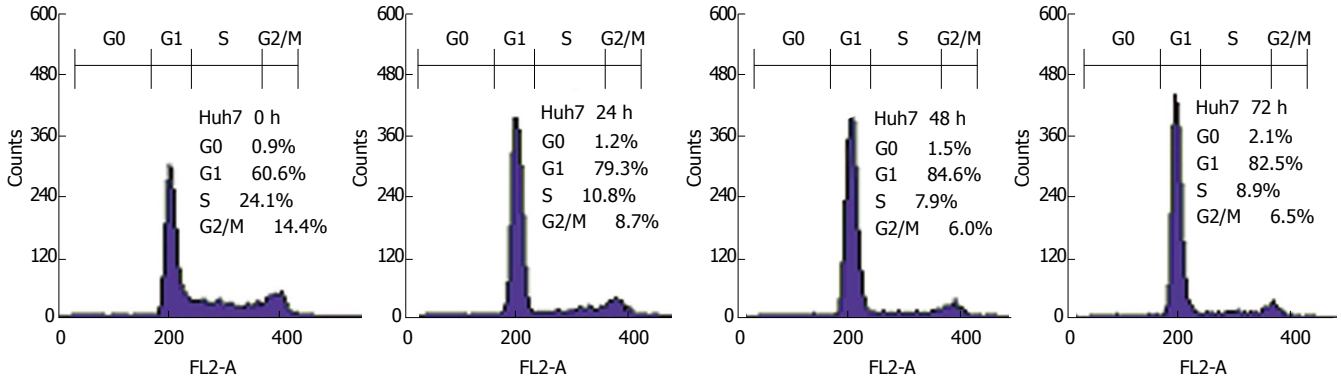

Figure 6 Inhibition of cell cycle arrest in Huh7 by NS-398. The cell cycle distribution was determined using a FACScan flow cytometer. Cells were harvested at 24, 48 and 72 h after treatment with 100 μmol/L NS-398 and collected. RNA was digested and DNA content was stained with propidium iodide (PI). Representative DNA histograms for Huh7 cells were shown.

different chemosensitivity to the selective COX-2 inhibitor NS-398 might be associated with different p53 status, including Huh-7 (mutated p53) and HepG2 (wild-type p53).

Several other studies, however, have shown that NS-398 inhibits cell proliferation and induces apoptosis *via* caspase-dependent or caspase-independent pathway in hepatoma cell lines[7,26,27]. But in our experiments, we obtained all negative data in Western blots and DAPI staining. This discrepancy may be due, at least in part, to sub-clone variation and different culture conditions[9,28].

Alteration of apoptosis is considered an important mechanism of carcinogenesis[21,26]. Caspases can be divided, based on their activity, into initiator caspases (caspase-8 and -9) and effector caspases (caspases-3, -6 and -7). In our study, no definite evidence of apoptosis was found after NS-398 treatment of both cell lines, suggesting that the major mechanism of growth inhibition by NS-398 may be anti-proliferation by cell cycle arrest rather than apoptosis.

In conclusion, selective COX-2 inhibitor, NS-398, inhibits the growth of HepG2 and Huh7 cells by inducing cell cycle arrest and is a potential candidate as an effective chemopreventive tool against human HCC.

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LIVER CANCER

Assessment of hemodynamics in precancerous lesion of hepatocellular carcinoma: Evaluation with MR perfusion

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Abstract

AIM: To investigate the hemodynamic changes in a precancerous lesion model of hepatocellular carcinoma (HCC).

METHODS: Hemodynamic changes in 18 Wistar rats were studied with non-invasive magnetic resonance (MR) perfusion. The changes induced by diethylnitrosamine (DEN) developed into liver nodular lesions due to hepatic cirrhosis during the progression of carcinogenesis. The MR perfusion data [positive enhancement integral (PEI)] were compared between the nodular lesions corresponding well with MR images and pathology and their surrounding hepatic parenchyma.

RESULTS: A total of 46 nodules were located by MR imaging and autopsy, including 22 dysplastic nodules (DN), 9 regenerative nodules (RN), 10 early HCCs and 5 overt HCCs. Among the 22 DNs, 6 were low-grade DN (LGDN) and 16 were high-grade DN (HGDN). The average PEI of RN, DN, early and overt HCC was 205.67 ± 31.17, 161.94 ± 20.74, 226.09 ± 34.83, 491.86 ± 44.61 respectively, and their liver parenchyma nearby was 204.84 ± 70.19. Comparison of the blood perfusion index between each RN and its surrounding hepatic parenchyma showed no statistically significant difference (P = 0.06). There were significant differences in DN (P = 0.02). During the late hepatic arterial phase, the perfusion curve in DN declined. DN had an iso-signal intensity at the early hepatic arterial phase and a low signal intensity at the portal venous phase. Of the 10 early HCCs, 4 demonstrated less blood perfusion and 6 displayed minimally increased blood flow compared to the surrounding parenchyma. Five HCCs showed significantly increased blood supply compared to the surrounding parenchyma (P = 0.02).

CONCLUSION: Non-invasive MR perfusion can detect changes in blood supply of precancerous lesions.

Key words: Liver; Perfusion; Magnetic resonance imaging; Rat

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INTRODUCTION

The changes of blood flow in dysplastic and regenerative nodules in patients with hepatic cirrhosis have been evaluated in previous studies with computed tomography (CT) arterial portography (CTAP) and CT hepatic arteriography (CTHA) techniques[1-3]. It has been demonstrated that during the late stage of hepatic cirrhosis, sequential hemodynamic changes in blood supply of dysplastic nodules lead to the development of early hepatocellular carcinoma (HCC). The main portal venous blood supply of these nodules gradually changes into the hepatic arterial supply. However, invasive methods require simultaneous catheterization of hepatic artery and superior mesentery artery to inject contrast enhancement substances. Because of their invasiveness, these methods have limited clinical application. With the development of techniques, especially the reduction of scanning time, magnetic resonance imaging (MRI) following intravenous injection of extracellular contrast agents has recently been described and validated to assess perfusion parameters in liver[4-7]. With this method, the contrast agent in blood flow of arterial and portal vein can be measured non-invasively. The purpose of this study was to assess experimentally whether MR perfusion can demonstrate the hemodynamic changes of liver nodular lesions.

MATERIALS AND METHODS

Animal model

The study protocol was reviewed and approved by the Institutional Committee for Animal Care of Fudan University. Experiments were performed on 18 6-wk old male Wistar rats weighing 150 ± 10 g (Experimental Animal Center of Fudan University, Shanghai, China). All animals were housed in independent ventilating cabinets (IVC) at 18-22℃ with 55% humidity in a 12 h light/dark cycle with free access to clean diet. Lesions in rats were induced by diethylnitrosamine (DEN, 0.99 mg/mL, Sigma, USA) in order to develop hepatocellular carcinoma during carcinogenesis. The animals received DEN (10 mg/kg per day) in drinking water at 0.01% g/L from a fresh DEN solution prepared every two days for 100 d.

Magnetic resonance imaging

A 1.5 Tesla magnetic resonance (MR) system with a 40 mT/m maximum gradient capability (TwinSpeed, Excite II, double gradient field, General Electric, USA) and 3-inch surface coil was employed and two rats were scanned every week from the 12th wk to the 20th wk after they were exposed to carcinogen. The rats were deeply anesthetized with a mixture of urethane (25%) and diazepam (2 mg) before MR scanning. Plain scanning was performed first in order to show the position of nodules, including sequences of 3-planar-localization scanning, axial and coronal T2 weighted scanning (T2WI), and axial T1 weighted (T1WI) scanning (in-phase and out-phase).

MR perfusion scanning was only performed on the nodules of interest demonstrated by T2WI. Fast spoiled gradient-echo sequence (FSPGR) was selected and the time resolution was 4 sections/2 s. Major protocol parameters included TR/TE: 5.8-8 ms/4-2.7 ms; band width: 31.3-19.2 kHz; section thickness: 2 mm; intersection gap: 0.5 mm; matrix: 128 × 128; excitation time: 1; FOV: 8. The time of acquisition of perfusion-weighted images was at the 8th phase after the starting point of tail vein bolus injection of diluted gadolinium (0.1 mmol/mL, Magnevist; Schering, Guangzhou, China) (0.2 mmol/kg). The total scaning time was 2 min.

Pathological analysis

Within 24 h after MR imaging, the rats were killed and their liver was removed at autopsy. The whole liver was serially sectioned into 1 mm-thick sections corresponding to the coronal plane MR image. The sections were fixed in a 10% formalin solution for 48 h and paraffin-embedded. Standard hematoxylin-eosin staining was used to assess the nature of nodules and immunohistochemical staining was performed for reticulin protein, CD31, CD34 antibodies. Transmission electron microscopy was also performed to study the ultrastructure of hepatic cancer cells. Pathologic diagnosis was made by two specialists independently and all nodules were defined grossly and microscopically following the criteria and nomenclature of the International Working Party on the Terminology of Nodular Hepatocellular Lesions[8].

Image evaluation

Data processing was performed at a workstation with AW 4.2 software package (General Electric, USA). Signal intensity and time curves were derived from manually drawing regions of interest (ROIs) on nodules and surrounding hepatic parenchyma. Positive enhancement integral (PEI) representing the blood perfusion could be obtained by setting the start (8th) and end (12th) phases. Values were expressed as mean ± SD. Statistical analysis was performed with SPSS 11.0. Perfusion data between nodules and parenchyma nearby were compared with *t* test. $P < 0.05$ was considered statistically significant.

Table 1 Average of PEI in nodules and adjacent parenchyma

	PEI	SD	*t*	*P*
RN	205.67	31.17	5.30	0.06
DN	161.94	20.74	3.64	0.02[a]
eHCC	226.09	34.83		
HCC	491.86	44.61	3.74	0.02[a]
Parenchyma	204.84	70.19		

[a]$P < 0.05$.

RESULTS

A total of 46 nodules were located by MR imaging and autopsy, including 22 dysplastic nodules (DN), 9 regenerative nodules (RN), 10 early HCCs and 5 overt HCCs. Among the DN (2-7 mm, average 4.6 mm), 6 were low grade DN (LGDN) and 16 were high grade DN (HGDN). The average positive enhancement integral (PEI) of DN, early and overt HCCs was 205.67 ± 31.17,161.94 ± 20.74,226.09 ± 34.83, 491.86 ± 44.61 respectively, and their adjacent liver parenchyma was 204.84 ± 70.19 (Table 1).

By comparing the blood perfusion index between nodules and their surrounding hepatic parenchyma, we found no difference in RN ($t = -5.303$, $P = 0.06$) showing the same signal intensity as its adjacent parenchyma at both hepatic artery phase and portal vein phase of MR perfusion. There were significant differences in dysplastic nodules ($t = -3.63$, $P = 0.02$) which manifested decreased blood perfusion. At the later phase of hepatic artery, the perfusion curve in DN declined when compared with surrounding parenchyma. DN showed an iso-signal intensity at hepatic artery phase and a low signal intensity at portal vein phase (Figure 1).

Of the 10 early HCCs, 4 manifested low perfusion and the average PEI value was smaller than that of adjacent parenchyma, showing an iso-signal intensity at the phase of artery and a low signal intensity at portal vein phase. The PEI values were higher in 6 early HCCs than in parenchyma nearby, which had a relatively higher signal intensity at hepatic artery phase and a low signal intensity at portal vein phase (Figure 2). Five overt HCCs had a remarkably higher signal intensity at hepatic artery phase and their perfusion curves ran up when compared with the surrounding parenchyma at early artery phase. The PEI value of HCC was much higher than that of adjacent parenchyma and the difference was significant ($t = 3.74$, $P = 0.02$) (Figure 3).

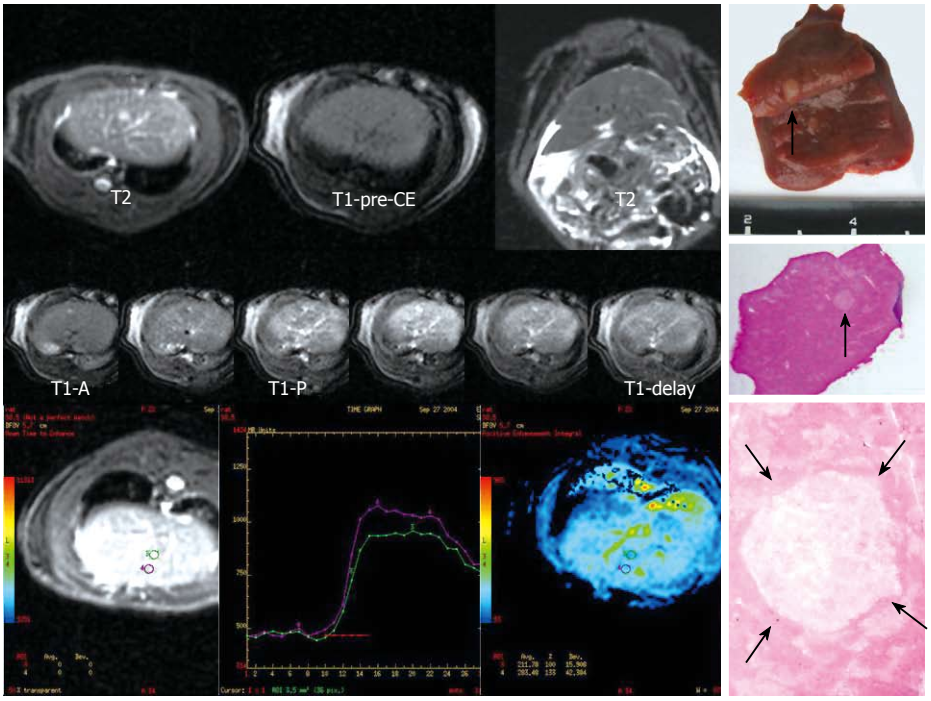

Figure 1 DN showing a high signal intensity on T2, but not on T1 and PWI.

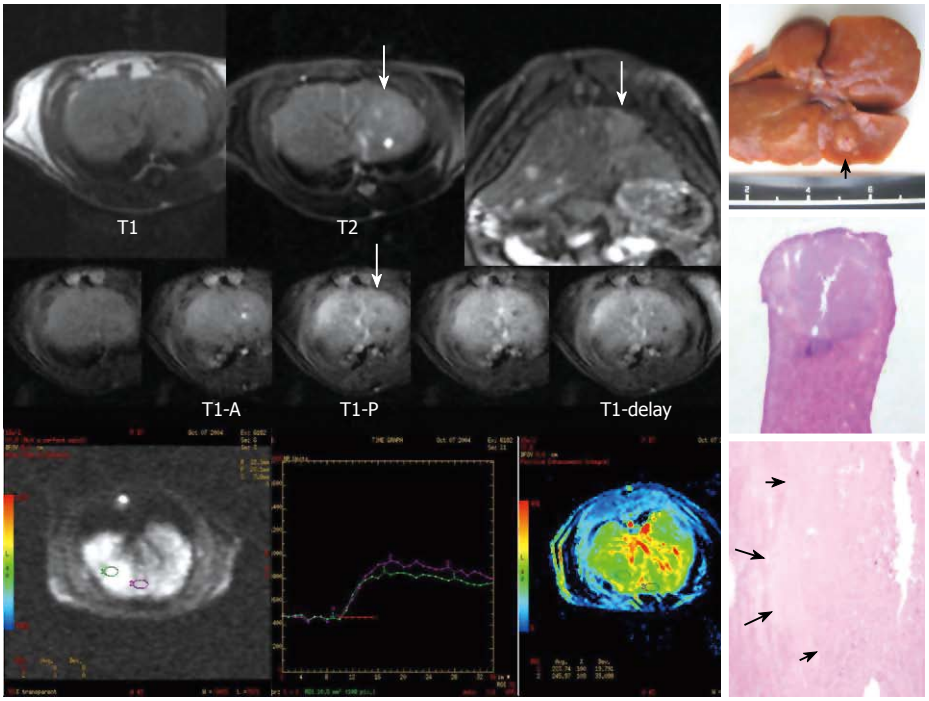

Figure 2 Early HCC showing an iso-signal intensity on T1, a high signal intensity on T2 or on T2 coronal.

DISCUSSION

There is cumulative evidence that RN at the late stage of hepatic cirrhosis can develop into HCC through an intermediate step of DN which has been considered as a precancerous lesion by the International Working Party since 1995[8,9], even though the hepatocarcinogenesis has not been clarified thoroughly. Early detection of HCC is critical to patient treatment and survival because of improved surgical techniques for resection and transplantation and new alternative therapeutic options, such as transcatheter chemoembolization or radiofrequency ablation. For surveillance of patients at risk, accurate methods capable of revealing not only HCC but also the premalignant precursor, namely DN, are required.

The imaging characteristics of DN have been previously reported[10-12]. However, the diagnosis of precancerous lesions is still difficult because of the overlapping between DN and RN, DN and early HCC on ultrasound, CT and MRI. Meanwhile, some researchers demonstrated that RN has almost the same blood flow as liver, which is mainly supplied by portal vein, and hepatic artery accounting for only a small part of total blood flow[13]. When RN progresses into DN, the blood flow in most parts of DN decreases (mainly portal vein

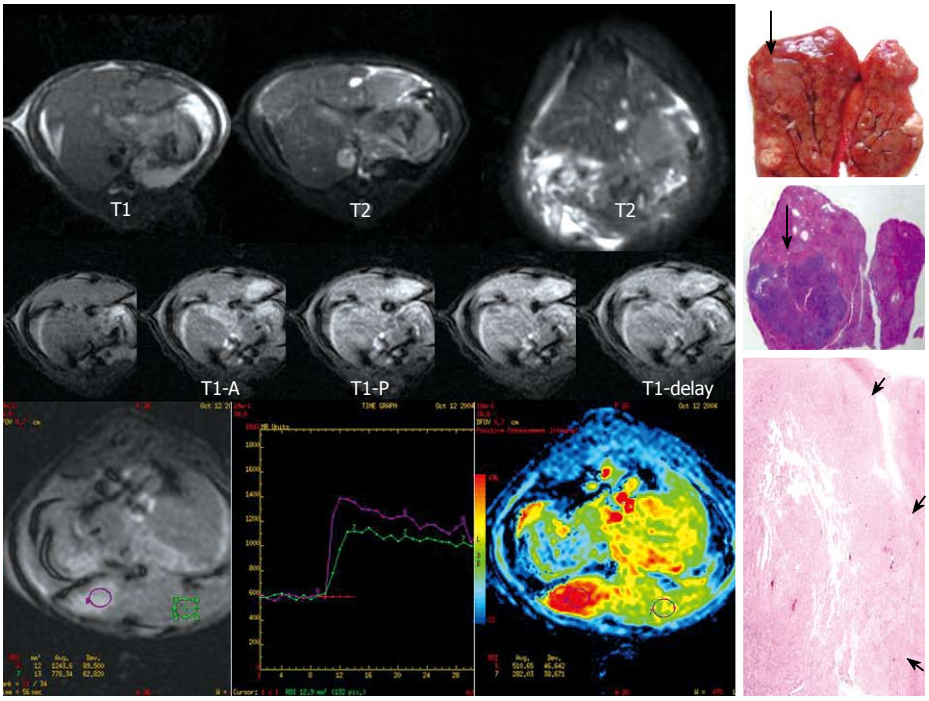

Figure 3 HCC showing an iso/low-signal intensity on T1WI, a hyper-intensity on T2.

blood supply) compared with surrounding parenchyma, and HCC always has increased blood flow (mainly hepatic artery blood supply) than its adjacent parenchyma. Until now this change has been regarded as the key-point to differentiate the nodules in liver. CTAP and CTHA are considered the best ways to make their differential diagnosis. But the application is limited in clinical practice due to their invasiveness. Non-invasive methods at present mainly focus on multi-detector spiral CT, MRI, PET and harmonic ultrasound[14-17]. To our knowledge, hemodynamic changes in hepatic nodules studied with MR perfusion have not been previously reported.

In the present study, MR perfusion demonstrated hemodynamic changes during the transition of RN to DN, early and overt HCC. The results obtained by ultra-fast MR perfusion scanning are consistent with those by invasive methods of CTAP or CTHA[18]. Moreover, hepatic artery segment of the perfusion curve in nodules declined slightly compared with surrounding parenchyma at the stage of HGDN and early HCC, suggesting that the preexisting hepatic artery is destructed in nodules. Otherwise, the positive enhancement integral representing the total blood flow would show polarization during this period. Part of them presented as increased blood flow (6/10 in early HCC; 5/16 in HGDN), but decreased blood flow was also observed at the same time in others (4/10 in early HCC; 11/16 in HGDN). The results of our pathology showed that the blood vessels in DN (including HGDN and LGDN) decreased compared with its surrounding hepatic parenchyma, and an increased number of tumor vessels were seen in HCC and even in early HCC. However, sinusoidal capillarization inside the nodules became gradually prominent during the process from RN, LGDN to HGDN and finally to HCC. This discrepancy cannot be clearly explained at present. The explanation of this phenomenon and the answer to its relationship with the alteration of nodular characteristics, and whether it corresponds with the different changes in tumor cells or in microvessles, depend on further pathologic studies.

The main advantage of MR perfusion is that this procedure provides ample information not only on the hemodynamic changes of nodular lesions in liver, but also on the parenchyma of the tumor. Therefore, this technique may be more valuable for the differential diagnosis of nodules in liver. The sequence we used in this study was fast spin gradient echo (FSPGR) which is as fast as echo-planar imaging (EPI). The ratio of time resolution was 0.5 s/section, but its signal-noise ratio (SNR) and spatial resolution were much higher than those of EPI, suggesting that this sequence is suitable for liver MR perfusion.

Nowadays, although CT perfusion (CTP) and PET are also used to study the hemodynamics, MR perfusion shows more advantages when small nodular lesions in liver and precancerous lesion of HCC are analyzed. It has been reported that MRI is more sensitive than CT and PET in revealing the small nodules in liver, especially in the hepatic parenchyma with the background of serious cirrhosis[19,20]. Moreover, MR perfusion has no side effects of radiation and has a better prospect than CT in study of hemodynamics. However, both MR imaging and CT have their technical defects at present. The scan of perfusion could only focus on ROI of one or several sections and could not cover the whole liver, while nodular lesions liver are usually multiple and located in different lobes., thus limiting its application in clinical practice. Newly developed harmonic ultrasound showing the vascular structure inside nodules more clearly, may have a broader prospect. Reports on the application of this technique in liver are few and further investigation is needed[21].

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Comethylation of *p*16 and *MGMT* genes in colorectal carcinoma: Correlation with clinicopathological features and prognostic value

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Abstract

AIM: To investigate the significance of *p*16 and O^6-methylguanine-DNA methyltransferase (*MGMT*) genes promoter hypermethylation and K-*ras* mutations on colorectal tumorigenesis and progression.

METHODS: *p*16 and *MGMT* methylation status was examined on 47 tumor samples, and K-*ras* mutational status was examined on 85 tumor samples. For methylation analysis, a methylation specific PCR (MS-PCR) method was used.

RESULTS: *p*16 and *MGMT* promoter methylation was found in 51% (24/47) and 43% (20/47) of CRCs, respectively, and the K-*ras* mutation was found in 44% (37/85) of CRCs. Comethylation of *p*16 and *MGMT* genes was significantly associated with lower aggressiveness of the disease within a two-year period of observation. Only 27% of patients with simultaneous *p*16 and *MGMT* methylation showed the detectible occurrence of metastasis and/or death, compared to 67% of patients without double methylation or with no methylation (3/11 *vs* 22/33, $P < 0.05$, χ^2-test). In addition, *p*16 and *MGMT* comethylation showed a trend toward an association with longer survival in patients with CRCs (35.5 ± 6.0 mo *vs* 23.1 ± 3.2 mo, $P = 0.072$, Log-rank test). Progression of the disease within a two-year period was observed in 66% of patients carrying the K-*ras* mutation, compared to only 19% of patients with wild type K-*ras* (29/44 *vs* 7/37, $P < 0.001$, χ^2-test). The presence of the K-*ras* mutation significantly correlated to shortened overall survival (20.0 ± 1.9 mo *vs* 37.0 ± 1.8 mo, $P < 0.001$, Log-rank test). The comethylation of *p*16 and *MGMT* genes was significantly associated with lower aggressiveness of the disease even when K-*ras* mutations were included in the analysis as an independent variable.

CONCLUSION: Our data suggest that comethylation of promoters of *p*16 and *MGMT* genes could have a prognostic value in patients with CRC. Specifically, concurrent methylation of both genes correlates with better prognosis.

Key words: Colorectal carcinoma; DNA methylation; *p*16; *MGMT*; K-*ras* mutation

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INTRODUCTION

Point mutations in the K-*ras* gene are among the most common genetic features of colorectal cancers (CRCs)[1]. In addition to mutational changes, epigenetic mechanisms also play an important role in the pathogenesis of this tumor type. The main epigenetic modification observed in the human genome is methylation of cytosine residues within CpG dinucleotides. Aberrant *de novo* methylation of CpG islands within the promoter region may lead to silencing of gene transcription through a complex process involving chromatin condensation and histone deacetylation[2,3]. Epigenetic silencing through DNA methylation can begin very early in tumor progression and may affect multiple genes involved in different cellular pathways, including cell cycle control, DNA repair and many others[4,5].

Inactivation of cell cycle regulatory genes, such as *p*16, confers a selective growth advantage to affected cells. This tumor suppressor gene encodes a cyclin-dependent kinase inhibitor, which is critical for maintaining the retinoblastoma (Rb) protein in its active,

non-phosphorylated state in the cyclin D-Rb pathway[6]. Promoter hypermethylation may lead to transcriptional inactivation of *p*16 resulting in abnormal cell cycling and uncontrolled cell growth, which are the hallmarks of cancer cells[7].

In addition, epigenetic modification can cause inactivation of DNA repair genes, such as O^6-methylguanine-DNA methyltransferase (*MGMT*)[8]. *MGMT* is a DNA repair protein that removes mutagenic and cytotoxic alkyl adducts from the O^6 position of guanine[9]. In the absence of *MGMT* activity, the adducts are not removed allowing the O^6-alkyl guanine to mispair with thymine during DNA replication, resulting in a G-to-A transition[10]. This kind of mutation may accumulate in the genes specifically associated with particular tumor types, such as K-*ras* in CRCs[9].

The literature suggests that hypermethylation of *p*16 and *MGMT* genes occurs as frequently in CRCs as previously reported for the K-*ras* gene[7,11,12]. Epigenetic changes usually begin very early in carcinogenesis, are potentially reversible, and can advance to gene alterations. For this reason, detection of aberrant methylation can be important for early diagnosis, prognosis and treatment of patients affected by this disease[5,13].

In the present study, we analyzed the methylation status of *p*16 and *MGMT* gene promoters in patients with primary CRC and compared the frequency of these changes with the occurrence of the K-*ras* gene mutations. In addition, we evaluated the correlation of these gene alterations with standard clinicopathological parameters, such as Dukes' stage, differentiation, location, histological and macroscopical type of tumor, patient gender and age. We also examined the possible correlation between these gene alterations and some immunohistochemical parameters that are known to be indicators of cell transformation, migration and metastasis of tumor cells. These parameters include altered expression of adhesive molecules E-cadherin and CD44, decrease or loss of laminin, which is a major component of epithelial basal membrane, and increased proliferate activity of the neoplastic cell. The ultimate aim of this study was to evaluate the influence of these genetic and epigenetic changes on disease progression and patient survival.

MATERIAL AND METHODS

Patients and tumor specimens

Tumor material in the form of formalin-fixed and paraffin-embedded tissue was obtained from 85 patients who underwent radical surgical resection (R0) at the Institute for Pathology and Forensic Medicine, Military Medical Academy, Belgrade. All patients gave informed consent prior to specimen collection according to institutional guidelines. None of the patients had preoperative or postoperative chemotherapy. The progression of the disease (occurrence of metastasis or/and death) was monitored during the two-year period following surgery, and survival over a five-year period was estimated. Mean follow-up was 31, 4 mo (range, 2 to 66 mo).

Tumor tissue chosen for analysis was routinely processed and microscopically examined for the regions enriched in neoplastic cells. Several 10 μm sections with neoplastic cell content greater than 85% were taken from each specimen and used for direct *in vitro* amplification of the K-*ras* gene by polymerase chain reaction (PCR). For K-*ras* mutational analysis, the sample of 47 was augmented with 38 samples from our previous study[14]. For analysis of *p*16 and *MGMT* methylation status, DNA was isolated from 47 samples.

Analysis of mutation status of the K-ras gene

Sections (10 μm thick) of the formalin-fixed and paraffin-embedded tumor specimens were de-paraffined and kept under 70% ethanol at 4℃ until PCR. *In vitro* amplification of 111-bp DNA fragments encompassing codons 12 and 13 of the K-*ras* gene was performed on tissue from sections as described by Almoguera *et al*[15]. Detection of K-*ras* mutations was performed on 85 patients with primary CRC by a highly selective oligonucleotide hybridization technique, as described earlier[14]. Two specimens were analyzed by single strand conformation polymorphism (SSCP) electrophoresis.

Analysis of methylation status of the p16 and MGMT genes

DNA methylation patterns in the promoter CpG islands of the *p*16 and *MGMT* genes were determined in 47 samples by methylation-specific PCR (MSP) following the bisulfite modification of isolated genomic DNA, as described by Herman *et al*[16], with some modifications[17]. Briefly, DNA was isolated from deparaffined tumor specimens using standard Proteinase K, phenol/chloroform/isoamyl alcohol extraction, and ethanol precipitation. Two μg of isolated DNA were denatured by NaOH (final 0.3 mmol/L) at 42℃ for 30 min and modified by sodium bisulfite (5.20-5.69 mol/L, pH 5.0, Sigma, USA) for 4 hr at 55℃. After incubation, DNA was purified using the DNA extraction KIT (MBI Fermentas, Lithuania), again treated by NaOH (final 0.3 mol/L), at 37℃ for 20 min, precipitated with ethanol/ammonium acetate and resuspended in 40 μL of 1 mmol/L Tris-HCl, pH 8.0. Aliquots of 4 μL of bisulfite modified DNA were used for MSP reactions. The PCR mixture contained 1 x PCR buffer (16 mmol/L ammonium sulfate, 67 mmol/L Tris-HCL, pH 8.8, 10 mmol/L 2-mercaptoethanol), 6.7 mmol/L $MgCl_2$, dNTP (each at 1.25 mmol/L) and primers (300 ng each per reaction) in a final volume of 50 μL. Reactions were hot-started at 95℃ for 5 min before the addition of 1.25 units of Taq polymerase (MBI Fermentas, Lithuania). Amplification was carried out in a Hybaid OmniGene temperature cycler for 35 cycles (30 s at 95℃, 30 s at the adequate annealing temperature, and 30 s at 72℃), followed by a final extension for 4 min at 72℃. Primers for a methylated and unmethylated promoter of the *p*16 gene were used from Herman *et al*[16] and for the *MGMT* gene from Esteller *et al*[8]. The annealing temperatures for unmethylated and methylated *p*16 amplification were 60℃ and 65℃, respectively, whereas the annealing temperature for both unmethylated and methylated *MGMT* amplification was 57℃. Controls without DNA were performed for each set of PCRs. Modified DNA from the human lymphoma cell line Raji (ECACC No:

85011429) served as positive control for methylated alleles, and modified DNA from normal lymphocytes as negative control for methylated alleles of *p*16 and *MGMT* genes. 10 μL of each PCR reaction were directly loaded on to nondenaturing 8% polyacrylamide gels, stained with $AgNO_3$ and visualized by Na_2CO_3, or 2% agarose gels, stained with SYBR Green I and visualized under UV illumination.

Immunohistochemistry

Immunohistochemistry was performed using the labeled streptavidin-biotin method (LSAB Kit+, Dako, Denmark) with microwave pretreatment for antigen retrieval. Endogenous peroxidase activity was blocked by incubating with 3% H_2O_2. Sections were then incubated with primary monoclonal antibodies against E-cadherin (HECD-1, Zymed Laboratories, San Francisco, USA), CD44 (Clone DF 1485, Dakocytomation, Denmark) and Laminin (4C7, Dakocytomation, Denmark) and Proliferation Cell Nuclear Antigen-PCNA (PC-10, Dakocytomation, Denmark) in optimal concentrations. Amino-ethyl carbazole was used as chromogen and finally Mayer's hematoxylin was used for counterstaining. The presence of a positive reaction in some normal tissue structures served as a positive control. Negative controls were prepared by replacing the primary antibody with Tris-buffered saline.

Immunostaining (membrane cytoplasmic immunoreactivity) for CD44 was calculated as the percentage of positive tumor cells in relation to the total number of tumor cells in representative fields. The presence of less then 10%, from 10% to 50%, and for more than 50% of positive tumor cells of CD44 were considered as low, moderate and extensive expression, respectively. Present, heterogenous and absent expression in the case of E-cadherin were distinguished. The loss of laminin immunoreactivity at the tumor-stroma borders was considered according to a three-point semiquantitive scale as follows: in more than 75%, between 25%-75% and in less than 25% of glandular structures. The PCNA index was considered to be low if less than 10% of tumor cell nuclei were positive, moderate for 10%-50%, and high for more than 50% of PCNA positive tumor cell nuclei.

Statistical analysis

Contingency tables were analyzed using Pearson's χ^2-test or Fisher's exact two-tailed test (*F*-test), when expected frequencies were lower than five. Continuous variables were compared with the use of Student's *t*-test. Overall survival distributions were estimated by the Kaplan-Meier method and differences were evaluated by the Log-rank test. The data was fitted to Cox's proportional risks regression model. In all tests, a *P* value less than 0.05 were considered as statistically significant.

RESULTS

K-ras mutations, p16 and MGMT promoter methylation in primary colorectal carcinomas

We detected K-*ras* mutations in 44% (37/85) of patients with CRC. The distribution of mutations was 57% (21/37) of G-to-T transversions, 32% (12/37) of G-to-A transitions and 11% (4/37) of G-to-C transversions. In codons 12 and 13, we detected mutations in 73% (27/37) and 27% (10/37), respectively.

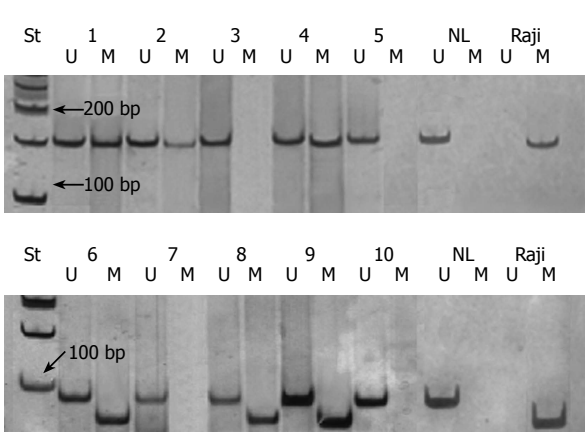

Figure 1 Analysis of *p*16 (**A**) and *MGMT* (**B**) gene methylation by MSP. The presence of a visible PCR product in lanes U indicates the presence of unmethylated genes of *p*16 (151 bp) and *MGMT* (93 bp); the presence of product in lanes M indicates the presence of methylated genes of *p*16 (150 bp) and *MGMT* (81 bp). The samples of colorectal carcinoma 1, 2 and 4 show *p*16 promoter hypermethylation, while 6, 8 and 9 show *MGMT* promoter hypermethylation. NL-normal lymphocytes as a positive control for unmethylated alleles; Raji-commercial cell line as a positive control for methylated alleles; St-molecular weight marker (50 bp).

Aberrant methylation of *p*16 and *MGMT* genes was detected in 51% (24/47) and 43% (20/47) of patients, respectively. Representative examples of the methylation analysis are shown in Figure 1A and B.

Of the total number of cases analyzed for methylation, 45% (21/47) had a mutant K-*ras* gene. Simultaneous alterations of K-*ras* and *p*16 genes were detected in 26% (12/47) of tumors. These events seemed to occur independently, as estimated by a χ^2-test (results not shown). The type of mutation at K-*ras* did not show a significant association with the *p*16 methylation by an *F*-test, although 55% (6/11) of samples with the hypermethylated *p*16 promoter carried G-to-T transversions in K-*ras*.

Simultaneous occurrence of K-*ras* mutation and *MGMT* methylation was detected in 21% (10/47) of the patients. Our results showed that *MGMT* methylation is significantly associated with a G-to-A transition in the K-*ras* gene ($P = 0.01$, *F*-test). This type of oncogene transition mutation occurs in 67% (6/9) of tumors carrying *MGMT* methylation, in contrast to only 10% (1/10) of tumors without *MGMT* methylation, but with the same type of K-*ras* mutation.

Concurrent methylation of *p*16 and *MGMT* genes was detected in 11 out of 47 (23%) cases, while simultaneous K-*ras*, *p*16 and *MGMT* alterations were present in 6 out of 47 cases (13%). Methylation of *p*16 and *MGMT* as well as K-*ras* mutation vs comethylation of *p*16 and *MGMT* genes seemed to occur independently as assessed by a χ^2-test (results not shown).

Correlation of K-ras mutations and hypermethylation of p16 and MGMT genes with clinicopathological and immunohistochemical parameters

We further examined correlation between genetic and epigenetic alterations and some clinicopathological and

Table 1 Association between *p*16 and *MGMT* methylation, K-*ras* mutations and clinicopathological features of colorectal cancer

	Methylation (%)		Mutations (%)
Variable	*p*16 (n = 46)	*MGMT* (n = 46)	K-*ras* (n = 84)
Gender			
Female	9/15 (60.0)	7/15 (46.7)	15/23 (65.2)[a]
Male	15/31 (48.4)	13/31 (41.9)	22/61 (36.1)
Dukes' stage			
A	5/9 (55.5)	5/9 (55.5)	8/21 (38.1)
B	7/10 (70.0)	4/10 (40.0)	12/24 (50.0)
C	8/15 (53.3)	8/15 (53.3)	17/39 (43.6)[4]
D	4/12 (33.3)	3/12 (25.0)	
Differentiation			
Poor	15/25 (60.0)	10/25 (40.0)	18/44 (40.9)
Moderate	8/18 (44.4)	10/18 (55.5)	16/26 (61.5)
Well	1/3 (33.3)	0/3 (0)	3/14 (21.4)
Macrosopic type[2]			
Polypoid	11/23 (47.8)	14/23 (60.7)[a]	25/48 (52.1)
Flat	13/23 (56.5)	6/23 (26.1)	12/35 (34.3)
Histological type[2]			
Mucinous	8/19 (42.1)	6/19 (31.6)	16/34 (47.1)
Tubular	16/27 (59.3)	14/27 (51.8)	21/49 (42.8)
Location[2]			
Proximal	3/3 (100)	1/3 (33.3)	5/9 (55.5)
Distal	21/43 (48.8)	19/43 (44.2)	32/74 (43.2)
Progression (2 yr)[1,3]			
Metastasis or/and death	11/25 (44.0)	9/25 (36.0)	29/44 (65.9)[b]
Without progression	12/19 (63.1)	11/19 (57.9)	7/37 (18.9)

Complete clinicopathological data were missing on one sample of colorectal cancer, and it was only included in analysis of genetic alterations; [a]$P < 0.05$; [b]$P < 0.001$ (all P values were revealed by χ^2-test); Data are missing on two[1] subjects for *p*16 and *MGMT* methylation status analysis, and on one[2] and three[3] subjects for K-*ras* mutation analysis, respectively. [4]Data for C and D Dukes' stages are considered together for K-*ras* mutation analysis.

Table 2 Association between *p*16 and *MGMT* methylation, K-*ras* mutations and immunohistochemical features of colorectal cancer

	Methylation (%)		Mutations (%)
Variable	*p*16 (n = 46)	*MGMT* (n = 46)	K-*ras* (n = 84)
E-cadherin[1,3]			
Absent	13/19 (68.4)	9/19 (47.4)	28/37 (75.7)[b]
Heterogeneous	4/14 (28.6)	7/14 (50.0)	4/23 (17.4)
Present	6/11 (54.5)	4/11 (36.4)	4/21 (19.0)
CD44 expression[1,3]			
Absent	7/11 (63.6)	6/11 (54.5)	4/22 (18.2)
Low	6/9 (66.7)	4/9 (44.4)	5/17 (29.4)
Moderate	2/7 (28.6)	4/7 (57.1)	4/10 (40.0)
Extensive	8/17 (47.0)	6/17 (35.3)	23/32 (71.9)[b]
CD44 type[2,4]			
Membranous	9/17 (52.9)	7/17 (41.2)	9/36 (25.0)
Cytoplasmic	13/26 (50.0)	12/26 (46.1)	27/44 (71.9)[b]
Laminin[1,3]			
0-25%	5/8 (62.5)	5/8 (62.5)	2/15 (13.3)
25%-75%	5/12 (41.7)	5/12 (41.7)	5/22 (22.7)
> 75%	13/24 (54.2)	10/24 (41.7)	29/44 (65.9)[b]
PCNAi[1,3]			
0	1/2 (50.0)	2/2 (100)	0/2 (0)
0-10%	5/9 (55.5)	4/9 (44.4)	3/17 (17.6)
10%-50%	6/9 (66.7)	5/9 (55.5)	3/20 (15.0)
> 50%	11/24 (45.8)	9/24 (37.5)	30/42 (71.4)[b]

Complete immunohistochemical data were missing on one sample of colorectal cancer, and it was only included in analysis of genetic alterations; [b]$P < 0.001$ (all P values were revealed by χ^2-test); Data are missing on two[1] and three[2] subjects for *p*16 and *MGMT* methylation status analysis, respectively, and on three[3] and four[4] subjects for K-*ras* mutation analysis, respectively.

immunohistochemical parameters that might be important for disease prognosis in CRC patients. The results of these analyses are summarized in Tables 1 and 2.

The presence of K-*ras* mutations was significantly more frequent in female than in male patients ($P < 0.05$, χ^2-test, Table 1). In addition, the average age of female patients carrying K-*ras* mutations was 53.3 ± 11.8 years, which was significantly lower ($P < 0.01$, t -test) than the average age for males with K-*ras* mutations (64.1 ± 8.7 years). As presented in Table 1, there was no significant association between the presence of the K-*ras* mutations and other clinicopathological features. However, the presence of K-*ras* mutations correlated significantly with the lack of E-cadherin expression, extensive expression and cytoplasmic type of CD44 antigen, decreased expression of laminin and the high PCNA index ($P < 0.001$ by χ^2-test in all cases; Table 2). These correlations were independent of the type of K-*ras* mutation, except in the case of E-cadherin, where the lack of expression of this adhesive molecule showed a significant correlation with the G-to-T transversions ($P < 0.05$, χ^2-test, results not shown).

*p*16 methylation was not associated with any of the variables considered, as presented in Table 1 and 2, though *p*16 methylation tended to be more prevalent in tumors not expressing E-cadherin ($P < 0.1$, χ^2-test). Methylation of *MGMT* gene was significantly more prevalent in polyploid than in flat colorectal carcinomas ($P < 0.05$, χ^2-test, Table 1). There was no significant association between *MGMT* methylation and any other clinicopathological features studied. In addition, comethylation of *p*16 and *MGMT* genes did not correlate with any clinicopathological or immunohistochemical parameters considered.

Survival analysis

In this study, we also monitored the progression of the disease in the two-year period following surgery. Methylation of *p*16 and *MGMT* genes, when they were considered separately, showed slight but insignificant correlation with slower progression of disease (Table 1). However, during that period, only 27% (3/11) of patients with simultaneous *p*16 and *MGMT* methylation showed the occurrence of metastasis and/or death, compared to 67% (22/33) of patients without this double or any gene methylation ($P < 0.05$, χ^2-test, Figure 2A).

This association was preserved and was even higher ($P < 0.01$, F-test, Figure 2B) when K-*ras* mutations were included as an independent variable. Namely, the progression of the disease occurred in only 2 of 6 (33%) patients with *p*16 and *MGMT* comethylation and K-*ras* mutations, while disease recurred in 13 of 14 (93%) patients with the K-*ras* mutation, but with only one methylated or both unmethylated genes. However, when both genes were unmethylated in the presence of the mutant K-*ras* gene, it was observed that all 4 patients from this group exhibited the progression of the disease within two years, compared to only 2 of 6 patients with *p*16 and *MGMT* comethylation and K-*ras* gene mutations ($P = 0.076$,

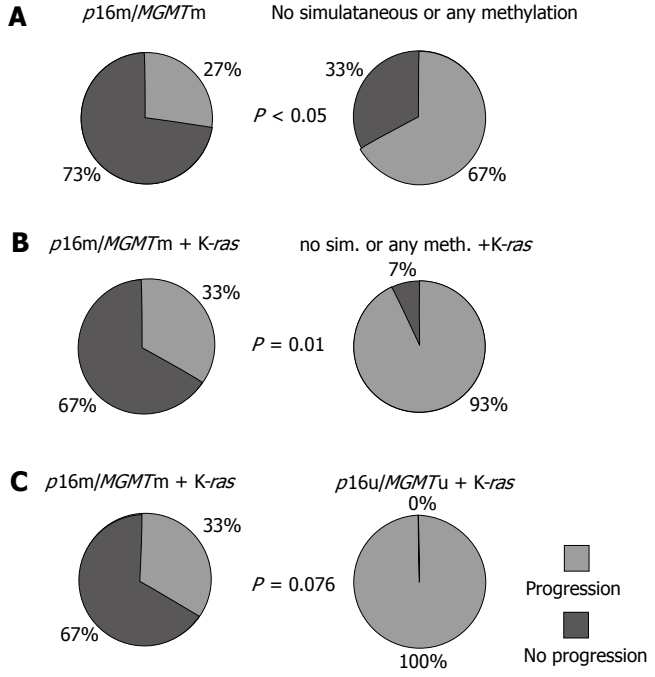

Figure 2 Graphical distribution of progression of the disease in the two-year period, as a function of simultaneous *p*16 and *MGMT* methylation status (**A**); simultaneous *p*16, *MGMT* methylation and mutated *K-ras* gene compared either to no simultaneous or any methylation of two genes (**B**) or to unmethylated both (**C**), respectively.

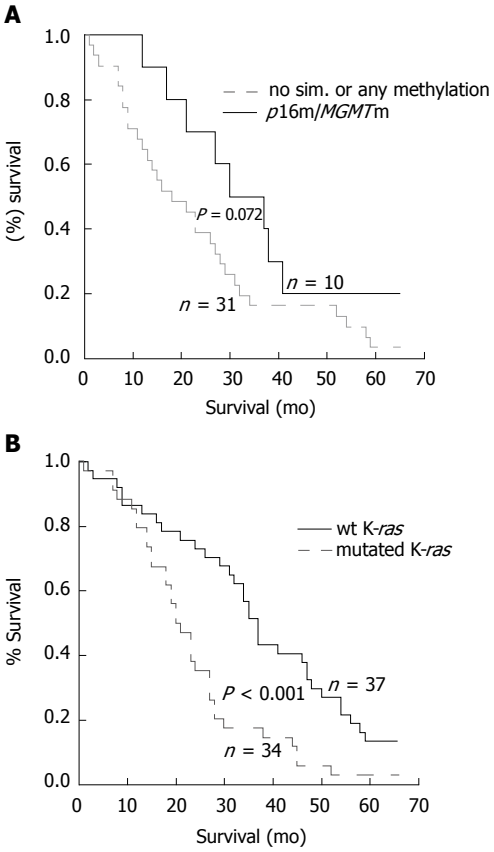

Figure 3 Overall survival among the patients with CRC according to the methylation status of *p*16 and *MGMT* genes (**A**), and mutational status of K-*ras* gene (**B**). Overall survival is longer in the group of the patients with simultaneous methylation of *p*16 and *MGMT* gene than in group with no simultaneous or any methylation ($P = 0.072$, Log-rank test). Overall survival is significantly lower in the group of patients with mutated K-*ras* than in group with unchanged, wild type K-*ras* gene ($P < 0.001$, Log-rank test). Survival curves were constructed by Kaplan-Meier method.

F-test, Figure 2C).

When the analyzed group was augmented with 38 patients from our previous study on CRC[14], we observed the progression of disease within a two-year period in 66% (29/44) of patients carrying the K-*ras* mutation, compared to only 19% (7/37) of patients with the wild type K-*ras* gene($P < 0.001$, χ^2 test, Table 1).

The Kaplan-Meier analysis of the few-year survival rate revealed a slight but insignificant influence of *p*16 and *MGMT* gene methylation on longer overall survival when the alterations of these genes were considered separately (results not shown). However, simultaneous *p*16 and *MGMT* methylation showed a trend toward association with longer survival in patients with CRCs who underwent curative surgery ($P = 0.072$, Log-rank-test, Figure 3A), as the median survival for these patients was 35.5 ± 6.0 mo in contrast to 23.1 ± 3.2 mo for patients without simultaneous or any methylation of examined genes. Multivariate analysis revealed that *p*16 and *MGMT* comethylation had no prognostic value without additional variables. By the same analysis, the presence of K-*ras* mutations significantly correlated to shortened overall survival (20.0 ± 1.9 mo *vs* 37.0 ± 1.8 mo) for 85 patients included in the analysis ($P < 0.001$, Log-rank test, Figure 3B). However, a Kaplan-Meier analysis considering simultaneous presence of K-*ras* mutations according to the methylated status of *p*16 and *MGMT* was not possible because there was a small number of samples.

DISCUSSION

The significance of *p*16 and *MGMT* methylation for tumor formation and progression, as well as correlation with the occurrence of classical genetic changes in K-*ras* genes, was the major subject of this study. While the frequency of the genetic and epigenetic alterations in these genes was similar to those previously reported[7,8,11,18,19], this is the first study to assess their cumulative and individual effects on disease progression.

While there was no clear association between methylation and standard prognostic parameters in CRC, the data obtained from survival analysis suggest that the simultaneous methylation of *p*16 and *MGMT* genes could be a better predictive factor for the course of disease. The most important finding of the current study was that comethylation of *p*16 and *MGMT* genes was significantly associated with lower aggressiveness of the disease and tended to associate with longer overall survival in patients with CRC who underwent curative surgery. More importantly, the influence of the double gene alteration was significant in spite of the simultaneous presence of K-*ras* mutations, which is another well established molecular marker of unfavorable prognosis in CRCs. Consistent with our previous findings[14] and those of the multicentric RASCAL study[20], our present results confirm that the mutated K-*ras* gene is a molecular marker for the more aggressive course of disease and was connected to reduced overall survival. In addition, we demonstrated

that K-*ras* mutation is strongly associated with parameters that are indicators of poor prognosis. These include the absence of E-cadherin expression[21], extensive expression of the cytoplasmic type of CD44 antigen[22], low expression of laminin[23] and high PCNA index[24].

The functional association between the effect of *p*16 and *MGMT* gene inactivation and K-*ras* mutation on the progression of disease is not clear. Yet, in the absence of methylation of both *p*16 and *MGMT* genes together with the mutated K-*ras*, a severe form of the disease is likely to commence. There is evidence that CRC does not evolve through a single sequence of molecular alterations, but evolves through different pathways. Each of them has distinct clinical, pathological and molecular characteristics[25]. One possible explanation for our findings could be that the colorectal tumors without comethylation of the two genes could arise through different mechanisms. These include large genetic rearrangements and chromosomal instability, with a significant degree of gene amplification and deletion[26,27] and such changes could be associated with a more aggressive course of the disease as proposed by Verma and Srivastava[13]. The other proposed pathway of colorectal carcinogenesis involves the occurrence of microsatellite instability (MSI), which is a phenotype resulting from alteration in the mismatch repair genes; e.g. *hMLH1* and *hMLH2*[28,26]. Some authors reported a relationship between MSI and CpG island methylator phenotype (CIMP+), as *hMLH1* is frequently inactivated by aberrant promoter methylation in sporadic MSI-H CRCs[29]. On the other hand, the CIMP+ phenotype is described as the occurrence of simultaneous methylation of a large number of genes, including *p*16 and *hMLH1*[29,30]. Though the biological and clinical properties of CIMP+ CRCs remain largely unknown, there is no longer doubt that those epigenetic changes mark a distinct group of tumors that have unique molecular profiles and etiology. More over, Wynter *et al*[31] demonstrated that there are at least two pathways for colorectal carcinogenesis, both implicating CIMP-high status. One group of tumors arises through *BRAF* mutations and *hMLH1* methylation, and another with K-*ras* instead of *BRAF* mutations and with *MGMT* instead of *hMLH1* promoter hypermethylation. Whitehall *et al*[32] demonstrated that methylation of *MGMT* has been linked to low levels of MSI (MSI-L) and Samowitz *et al*[33] demonstrated that the appearance of K-*ras* mutations is rare in high level MSI-H CRC. It is also known that MSI-H CRC associated with a CIMP+ phenotype is mostly located within the proximal colon[27,29]. In our study, all but three tumors have been located within the distal colon, so we can only speculate that they may not belong to the MSI-H group. We propose that in our group of analyzed patients who mostly have distal CRC, comethylation of *p*16 and *MGMT* genes together with other molecular changes suggest a pathway in signal transduction that could increase survival. Importantly this is in spite of the opposing effect of K-*ras* mutations. It would, therefore, be interesting to determine the MSI phenotype in this patient population in order to establish a possible association with observed genetic and epigenetic alteration.

Though all these questions cannot be fully resolved in the present study, our results confirm the multifactorial nature of cancer development, highlighting the molecular differences across the various classes of lesions, and exclude a single linear model of accumulating genetic alterations. In this report, we describe the association of concurrent methylation of *p*16 and *MGMT* genes with the course of disease in unselected group of CRCs. At this point, it must be noted that we did not analyze a large group of patients. However, the size of our study is still large enough to interpret the results correctly.

While some studies reported a correlation between promoter hypermethylation of the *p*16 gene and prognosis in patients with colorectal cancers[34-38], others observed no significant role of *p*16 methylation as a prognostic factor[30]. In addition, Nagasaka *et al*[39] found that methylated *MGMT* was significantly related to lower risk of recurrence in CRC. They proposed that colorectal tumors with methylated *MGMT* are less aggressive than tumors without such epigenetic change. Liang *et al*[34] proposed that geographical differences or other unknown factors supplementary to *p*16 methylation might increase tumor aggressiveness. Considering these and our previous results[14], we speculate that different geographic, environmental, and lifestyle factors could affect the genesis and clinical behavior of CRCs with different molecular profiles. Nevertheless, prospective studies that incorporate a larger number of genes and environmental factors are required to determine the relative contribution of each factor to the risk of developing CRCs with specific epigenetic and genetic profiles. We further support the notion that precise determination of molecular changes in CRCs is needed in order to overcome the risk of oversimplifying the role of a single or few genetic or epigenetic changes in tumorigenesis. Such analysis, supplemented by the conventional study of prognostic factors, introduces quality to prognostication of patients with CRC and aids the design of more efficient treatment modalities.

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COMMENTS

Background

Colorectal carcinogenesis is a multistep process in which the progressive accumulation of genetic and epigenetic changes leads to a malignant transformation of normal epithelial cells of colon and rectum. Point mutations in K-*ras* gene are among the most common genetic features of colorectal cancers. In the past decade it has been shown that methylation of the promoter region of many tumor suppressor and DNA repair genes, such as *p*16 and *MGMT* has the important role in pathogenesis of this tumor type too, but its influence on disease progression remain inconclusive.

Research frontiers

Epigenetic changes usually begin very early in carcinogenesis, they are potentially reversible, and they can advance to gene alterations. For this reason, detection of aberrant methylation can be important for early diagnosis, prognosis and treatment

of patients affected by this disease.

Innovations and breakthroughs

Comethylation of *p*16 and *MGMT* genes was significantly associated with lower aggressiveness of disease within two-year period of observation ($P < 0.05$), and showed the trend toward the association with longer survival in patients with CRCs ($P = 0.072$). On the other hand, the presence of K-*ras* mutations was associated with higher aggressiveness and shortened overall survival ($P < 0.001$). The comethylation of *p*16 and *MGMT* genes was significantly associated with lower aggressiveness of disease even when K-*ras* mutations were included in analysis as an independent variable ($P < 0.01$). This is the first attempt to assess the cumulative and individual effect of these genes on the disease progression.

Applications

The results presented in this article have demonstrated that the precise determination of molecular changes in CRCs is needed in order to overcome the risk of oversimplifying the role of single or a few genetic or epigenetic changes in tumorigenesis. In addition, this study underlines the need for considering the different geographic, environmental, and lifestyle factors that could affect the genesis and clinical behavior of CRCs with different molecular profiles. Prospective studies supplemented by the conventional study of prognostic factors, could improve the quality and accuracy of patients prognosis and aid design of the more efficient treatment modalities.

Terminology

Epigenetic changes: heritable changes in gene function that do not include the changes in DNA sequence. DNA methylation: the addition of a methyl group to a cytosine residue that lies next to guanine within CpG dinucleotides. Aberrant *de novo* methylation of CpG islands within the promoter region may lead to silencing of gene transcription through a complex process involving chromatin condensation and histone deacetylation. CpG islands: CpG rich areas located in the promoter regions of many genes. Methylation-specific PCR (MSP): amplification of modified DNA by sodium bisulfite that converts unmethylated cytosines to uracils, while methylated cytosines remains unmodified.

Peer review

The authors found some interesting findings where they showed that patients with tumors where both promoters were methylated showed less death and had longer survival rates than patients with unmethylated promoters. The data is straightforward and convincing. The authors address a clinically relevant issue on CRC prognosis. They have also demonstrated initial and unique findings examining K-*ras*, *p*16 and *MGMT* modifications and patient outcome.

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Quasispecies evolution in NS5A region of hepatitis C virus genotype 1b during interferon or combined interferon-ribavirin therapy

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Abstract

AIM: To evaluate the implication of substitutions in the hepatitis C virus (HCV) non-structural 5A (NS5A) protein in the resistance of HCV during mono-interferon (IFN) or combined IFN-ribavirin (IFN-R) therapy. Although NS5A has been reported to interact with the HCV RNA-dependent RNA polymerase, NS5B, as well as with many cellular proteins, the function of NS5A in the life cycle of HCV remains unclear.

METHODS: HCV quasispecies were studied by cloning and sequencing of sequential isolates from patients infected by HCV genotype 1b. Patients were treated by IFN-α2b for 3 mo followed by IFN-α2b alone or combined IFN-R therapy for 9 additional months. Patients were categorized intro two groups based on their response to the treatments: 7 with sustained virological response (SVR) (quasispecies = 150) and 3 non-responders (NR) to IFN-R (quasispecies = 106).

RESULTS: Prior to treatment, SVR patients displayed a lower complexity of quasispecies than NR patients. Most patients had a decrease in the complexity of quasispecies during therapy. Analysis of amino acids substitutions showed that the degree of the complexity of the interferon sensitivity-determining region (ISDR) and the V3 domain of NS5A protein was able to discriminate the two groups of patients. Moreover, SVR patients displayed more variability in the NS5A region than NR patients.

CONCLUSION: These results suggest that detailed molecular analysis of the NS5A region may be important for understanding its function in IFN response during HCV 1b infection.

Key words: Hepatitis C virus; Quasispecies; NS5A region; Interferon sensitivity-determining region; V3 domain

Veillon P, Payan C, Le Guillou-Guillemette H, Gaudy C, Lunel F. Quasispecies evolution in NS5A region of hepatitis C virus genotype 1b during interferon or combined interferon-ribavirin therapy. *World J Gastroenterol* 2007; 13(8): 1195-1203

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INTRODUCTION

In Western countries, the hepatitis C virus (HCV) is the major cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma[1]. HCV is a RNA virus belonging to the *Flaviviridae*. It has a single-stranded plus-sense genome of approximately 9.6 kb with a single open reading frame (ORF) encoding four structural (C, E1, E2 and p7) and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). Current combination therapy of pegylated interferon (IFN) alpha and ribavirin (IFN-R) is not universally effective in patients with chronic hepatitis C; patients infected with genotypes 2 or 3 show a high rate of sustained virological response (SVR) (90%) whereas the HCV genotype 1 infected patients have the lowest level of SVR (40% to 50%)[2-4]. Factors that can predict the response to antiviral therapy are not well established; the accepted predictive parameters are: age, sex, pre-treatment viral load, fibrosis stage and HCV genotype[5]. Numerous studies have been undertaken to explain the resistance of genotype 1-infected patients to IFN therapy. Causes and mechanisms are not well understood, but several viral genomic regions have been suggested to antagonize the antiviral effect of IFN alpha, such as the E2, NS3/4A and NS5A regions[6].

The non-structural 5A (NS5A) protein is the ninth

protein coded by the ORF of HCV. It has a length of 447 amino acids (aa) for genotype 1b. An interaction with the double-stranded RNA protein kinase (PKR) has been described for NS5A within the codons 2209 and 2274 ($NS5A_{2209-2274}$)[7-9]. This interaction can block the IFN signalling pathway in cultured cells, and certain authors have suggested that this mechanism may be implicated in HCV resistance to IFN therapy. In 1995, Enomoto *et al*[10] described a correlation between the number of mutations within a 40 aa sequence of the NS5A region and the response to IFN therapy in genotype 1b-infected patients. This sequence has been termed the interferon sensitivity-determining region (ISDR). These results were then confirmed by other Japanese studies[11-15], but were never in accordance with most Western European[16-20] and American studies[21-23], in which most of the ISDR sequences harbored an intermediate profile. A recent meta-analysis focusing on the number of mutations within NS5A ISDR confirmed the predictive usefulness of ISDR, but a real geographical difference between Caucasian and Asiatic patients was underlined[24].

Recently, it was suggested that NS5A protein may also inhibit the antiviral effect of IFN in a PKR-independent manner in a human hepatocytic cell line[25]. Furthermore, another domain in the NS5A region, named V3 ($NS5A_{2356-2379}$)[26], could also be linked to IFN resistance. It has been suggested that mutations in the V3 domain may correlate with IFN response[17]. However, two reports have shown that insertion and deletion around the ISDR and the V3 domain had no impact on HCV RNA replication in cell culture[27,28].

In the present study, we examined mutations in the complete NS5A region, including its ISDR, PKR binding domain (PKRbd) and V3 domain, of HCV genotype 1b from patients treated with IFN or combined IFN-R therapy. HCV quasispecies of ten patients were selected for the analysis based on their response to the treatments combined IFN-R therapy.

MATERIALS AND METHODS

Study population

We analyzed PCR products from ten HCV genotype 1b infected patients included in a therapeutic trial[29]. Seven of them had SVR - three early and four slow responders according to HCV RNA clearance during therapy at mo 3 (M3) - and three were non-responders to IFN-R (NR). Patients were all studied at baseline (D0), M3 and M6, when gene amplification was possible (positive PCR). Patients received 6 million units of IFN-α2b three times weekly. If HCV RNA was still detectable at M2, ribavirin was combined with IFN at M3 and continued up to M12. SVR was defined as undetectable HCV RNA during treatment and six months after the end of therapy (EOT). Non-SVR was defined as detectable HCV RNA during treatment and six months after the EOT.

HCV RNA quantification

Quantification of serum HCV RNA was performed using VERSANT HCV RNA 3.0 assay (Bayer Diagnostics, Emeryville, CA, USA) with a detection threshold at 615 IU/mL (2.79 log IU/mL).

Table 1 Sequences of amplification and sequencing primers

Forward primers		Positions nt
E1	5' GAGGGGGCTGTGCAGTGGATG 3'	6057-6077
I3	5' TCCGGCTCGTGGCTAAGGGA 3'	6246-6265
S2	5' GGATTTCCACTACGTGACGGG 3'	6620-6640
S4	5' GGGTCTCCCCCCTCCTTGGCC 3'	6906-6926
S31	5' GGACTACGTCCCTCCGGTGG 3'	7241-7260
Reverse primers		
AS2	5' CGTCACGTAGTGGAAATCCCC 3'	6618-6638
AS32	5' GGGACGTAGTCCGGGTCCTTC 3'	7232-7252
I4	5' GCAGCAGACGAGATCCTCAC 3'	7567-7586
E2	5' GCTGCGAGATGTTGTGGCGTA 3'	7698-7718
Cloning primers	(pMOS vector)	
SeqS1	5' GGGAAAGCTTGCATGCCTGC 3'	
SeqAS2	5' GACGTTGTAAAACGACGGCC 3'	

Nucleotide positions are numbered according to the HCV-J sequence and the NS5A region is within positions 6246-7586.

RNA extraction and amplification by RT-PCR

HCV RNA was extracted from 200 μL of serum by guanidinium thiocyanate and silica method[30]. The full-length NS5A gene was amplified by nested RT-PCR. RT was combined with the first round of PCR with outer primers E1 and E2 while second amplification was performed with inner primers I3 and I4 as previously described (Table 1)[17,31]. PCR products were then subjected to electrophoresis in a 1% agarose gel (NuSieve GTG, FMC) and visualized by ethidium bromide staining before purification. In this study, we sequenced 343 296 nts from 256 clones of the complete NS5A region. According to *Taq* error rate (0.182×10^{-4})[32], 6.247 RT-PCR-introduced errors could have been analyzed as substitutions for all clones, i.e. 0.024 RT-PCR-introduced errors per clone.

PCR products cloning

PCR fragments cut out from the agarose gel were purified using a mini-column system (Wizard PCR Preps DNA purification system, Promega) and then were ligated into 50 ng of pMOS vector (pMOS*Blue* blunt-ended cloning kit, Amersham Bioscience). Transformants were grown on LB-agar plates containing 100 μg/mL ampicillin and 15 μg/mL tetracycline. The presence of HCV insert was confirmed by plasmid amplification using SeqS1 and SeqAS2 primers (Table 1). We produced between 4 and 17 clones per patient.

DNA sequencing

Cycle sequencing was performed using the CEQ 8000 Dye Terminator Cycle Sequencing kit following the manufacturer's instructions (Beckman Coulter) before automatic electrophoresis of the sequencing products using a CEQ 8000 (Beckman Coulter). Inner PCR primers S2, S4, S31, AS2 and AS32 were used as sequencing primers (Table 1). Sequences were analyzed using the CEQ 8000 software.

Sequence analysis

Multiple nucleotide (nt) sequence alignment and ambiguity were carried out with CLUSTAL X interface[33]. The nucleotide substitution rate over sites within each set of variants was estimated by using DAMBE software. The

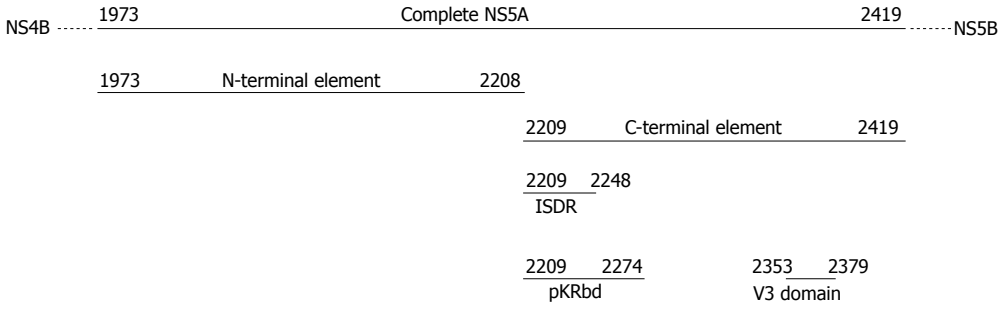

Figure 1 Schematic localisation of the six studied regions in NS5A protein. Amino acids positions are numbered according to the HCV-J polyprotein and the NS5A region is within positions 1973-2419.

Table 2 Clinical and virological characteristics of the 10 patients studied before and during treatment mean ± SD

Patient and time of treatment	Sex	Age	Viral load log IU/mL	*n*	NT substitutions/master sequence	AA substitutions/master sequence	Genetic distance
SVR1-D0	F	48	2.00	15	4.5 ± 2.3	2.5 ± 1.4	0.0065 ± 0.0008
SVR2-D0	F	53	4.55	13	5.1 ± 3.6	3.3 ± 2.1	0.0070 ± 0.0011
SVR3-D0	M	43	4.71	8	10.3 ± 3.6	4.7 ± 1.9	0.0105 ± 0.0018
SVR4-D0	M	59	6.24	16	15.4 ± 8.0	7.5 ± 4.9	0.0172 ± 0.0022
SVR4-M3	-	-	1.61	13	3.6 ± 2.1	1.8 ± 1.0	0.0051 ± 0.0008
SVR5-D0	F	65	5.44	15	10.1 ± 6.6	4.9 ± 2.0	0.0171 ± 0.0016
SVR5-M3	-	-	2.77	17	6.4 ± 2.4	3.3 ± 1.6	0.0070 ± 0.0010
SVR6-D0	M	47	7.26	17	15.9 ± 10.5	8.5 ± 5.1	0.0192 ± 0.0022
SVR6-M3	-	-	2.18	6	4.0 ± 1.7	2.4 ± 1.3	0.0062 ± 0.0012
SVR7-D0	F	51	6.61	15	29.3 ± 8.5	7.3 ± 2.7	0.0294 ± 0.0029
SVR7-M3	-	-	5.42	15	12.6 ± 4.2	4.6 ± 2.2	0.0156 ± 0.0016
NR1-D0	M	35	5.13	17	15.2 ± 4.3	3.5 ± 1.9	0.0181 ± 0.0020
NR1-M3	-	-	5.24	9	15.3 ± 4.4	4.8 ± 1.7	0.0143 ± 0.0020
NR1-M6	-	-	2.83	4	4.5 ± 3.1	2.0 ± 3.4	0.0041 ± 0.0013
NR2-D0	F	50	5.90	17	15.6 ± 6.4	5.2 ± 2.9	0.0181 ± 0.0020
NR2-M3	-	-	5.87	12	9.5 ± 3.6	3.9 ± 1.8	0.0125 ± 0.0013
NR2-M6	-	-	5.77	10	10.3 ± 3.3	4.0 ± 2.2	0.0274 ± 0.0020
NR3-D0	F	44	5.63	14	10.9 ± 3.7	4.4 ± 2.2	0.0279 ± 0.0021
NR3-M3	-	-	5.79	11	11.1 ± 2.7	4.0 ± 2.8	0.0157 ± 0.0017
NR3-M6	-	-	5.00	12	10.8 ± 3.3	4.4 ± 2.0	0.0154 ± 0.0015

F: Female; M: Male; *n*: number of PCR products analyzed; D0: beginning of the treatment; M3: 3 mo of treatment; SVR: Sustained Virological Responders; NR: Non-Responders to IFN-ribavirin therapy.

variability at each site i was measured by calculating the Shannon entropy at the site i (*Hi*) with the following formula: $Hi = -(\sum^{4}_{j=1} p_j \log_2 p_j)$, where j = 1, 2, 3, 4 corresponding to nucleotide A, C, G and T, and p_j is the proportion of nucleotide j at site i. The normalized entropy Sn was calculated as Sn = H/log N, where N is the total number of analysed sequences[34]. Types of mutational changes were also determined by means of MEGA 2.3 software. Genetic distances between pairs of sequences were performed using Kimura 2-parameters method[35]. The frequency of synonymous (dS) and non-synonymous (dN) substitutions per site were calculated with Nei-Gojobori method using the Jukes-Cantor correction to account for multiple substitution at the same site[36]. The dN/dS ratio is a measure of immunity pressure on a region. Construction of the phylogenetic tree of NS5A variants obtained from ten patients was performed using MEGA 2.3 software. The phylogenetic tree was constructed with the neighbour-joining method and bootstrap resampling (1000 replicates were used to test reliability of the tree topology)[37]. Master sequence was defined as the sequence obtained from direct sequencing of PCR products obtained before cloning.

A multiple alignment of the translated aa sequences was generated. Alignment analysis of nucleotides and proteins was performed using HCV-J (genotype 1b, D90208) as reference and HCV-H (genotype 1a, M67463) as out-group for the construction of the phylogenetic tree.

Six regions were studied: the ISDR ($NS5A_{2209-2248}$), PKR binding domain (PKRbd) ($NS5A_{2209-2274}$), V3 ($NS5A_{2353-2379}$), the N-terminal element of NS5A protein until ISDR ($NS5A_{1973-2208}$), the C-terminal element of NS5A protein from ISDR to the end ($NS5A_{2209-2419}$), and the complete sequence of NS5A ($NS5A_{1973-2419}$) (Figure 1).

Statistical analysis

Values for quantitative variables were expressed as means ± SD. Comparisons between groups were performed using the Student *T*-test or the non-parametric Mann-Whitney *U*-test or Kruskal-Wallis *K*-test for quantitative variables. The Pearson's correlation coefficient was used. A *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

Phylogenetic and heterogeneity analysis of HCV quasispecies

Characteristics of each patient are summarized in Table 2. We studied ten patients (four males and six females). Average

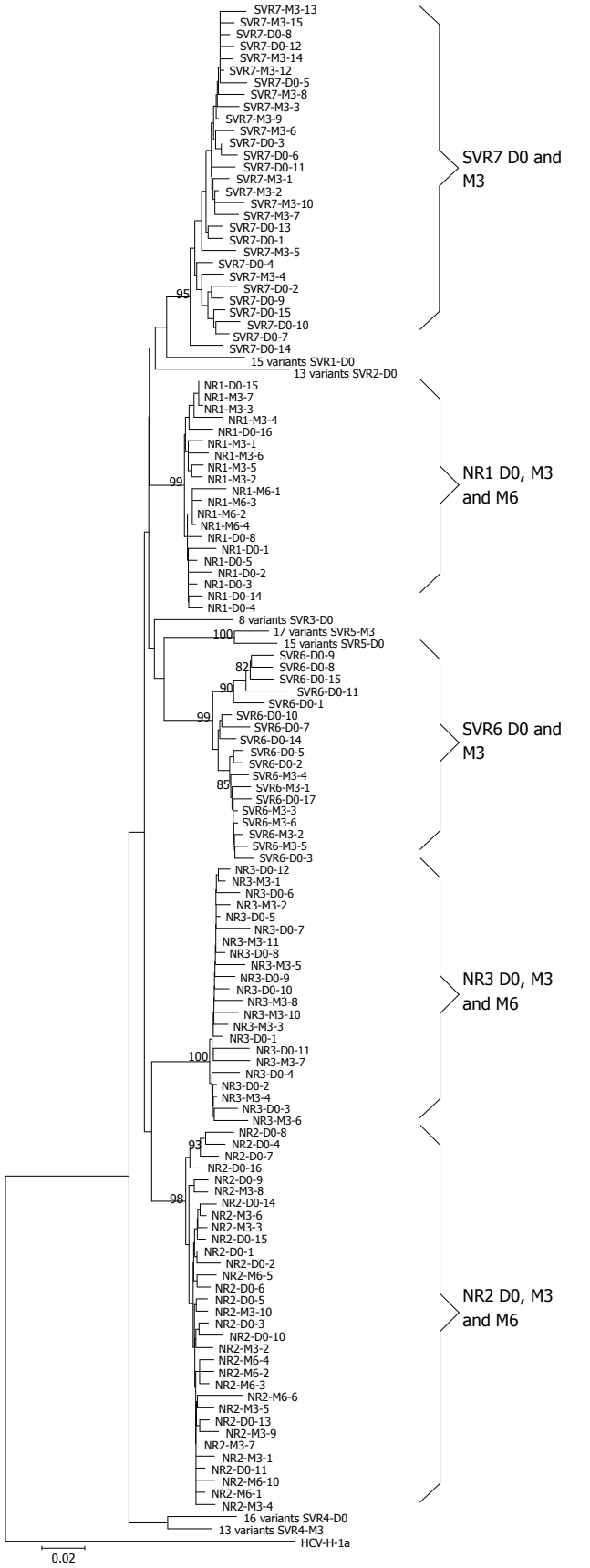

Figure 2 Phylogenetic tree of NS5A region (codon 1973-2419) of amino acids HCV variants obtained from ten patients at different time of treatment. D0: beginning of the treatment; M3: 3 mo of treatment; SVR: Sustained Virological Responders to IFN-ribavirin therapy; NR: Non-Responders to IFN-ribavirin therapy. HCV-H strain was used as outgroup. The internal node numbers represent the bootstrap values. Distance scale represents 2% of difference between sequences. When all NS5A sequences within a patient at one time point of treatment are grouped in a cluster, this cluster is presented by only one node.

Table 3 Characteristics of HCV quasispecies in pre-treatment isolates from SVR and NR patients

Region and patient type	Genetic distance	dS	dN	dN/dS	Num. amino acid mutations
NS5A					
SVR (n = 99)	0.1027	0.3239	0.039	0.120	33.9
NR (n = 48)	0.0698	0.1216	0.025	0.206	29.8
ISDR					
SVR (n = 99)	0.0834	0.3084	0.0295	0.096	1.3
NR (n = 48)	0.0765	0.2645	0.0271	0.102	1.9
PKRbd					
SVR (n = 99)	0.0934	0.2934	0.0424	0.145	5.3
NR (n = 48)	0.0815	0.2694	0.0332	0.123	5.7
V3					
SVR (n = 99)	0.1836	0.3264	0.1282	0.393	6.0
NR (n = 48)	0.1372	0.4482	0.0556	0.124	4.7

Values were calculated as described in methods; amino acids sequences were compared to HCV-J; n: number of PCR products analyzed; dS: frequency of synonymous substitutions per site; dN: frequency of non-synonymous substitutions per site; SV: Sustained Virological Responders; NR: Non-Responders to IFN-ribavirin therapy.

patient age was 49.5 years and HCV viral load was 5.35 log IU/mL (mean at baseline). We found a significant difference in viral load between the three groups of early, slow SVR and NR (P = 0.034), whereas no significant difference was found between the SVR and NR groups at baseline. The genetic variability of the NS5A region was confirmed by cloning NS5A PCR products obtained from the ten patients (7 SVR and 3 NR) into the pMOS vector. In total, PCR products from 256 clones of the NS5A region were analyzed (Table 2). No cluster in correlation with treatment response was observed in the phylogenetic tree (Figure 2). However, in two slow SVR patients (SVR4 and SVR5) two different clusters were individualized with two populations of variants (D0 and M3, respectively). In the two other slow SVR patients (SVR6 and SVR7) and in NR patients, we found mixed populations of variants at the beginning of treatment (D0) and during treatment (M3 or M6). Genetic complexity of nt and aa sequences calculated by Shannon entropy showed no significant difference between the two groups studied (Figure 3, A and B). Interestingly, when we looked at early SVR in comparison with slow SVR, we observed a significant difference between these three groups of patients, P = 0.026 and P = 0.024, respectively, for nt and aa complexity calculated by Shannon entropy. At baseline, we observed that PCR products from the SVR group displayed more frequently synonymous substitutions (dS) and non-synonymous substitutions (dN) than PCR products from the NR group along the NS5A region, ISDR and PKRbd domains, but not in the V3 domain for dS (Table 3, columns 3 and 4). dN/dS ratios were similar in the two groups in ISDR and PKRbd domains. This ratio was two times higher in SVR patients than in NR patients along the NS5A region and three times higher in the V3 domain (Table 3, column 5).

Among the aa sequences of the PKRbd and V3 domains, we observed that NR patients exhibited a higher variant complexity than SVR patients before initiation of IFN therapy in the three domains ISDR, PKRbd and V3 (Table 4). In ISDR, after three months of IFN therapy,

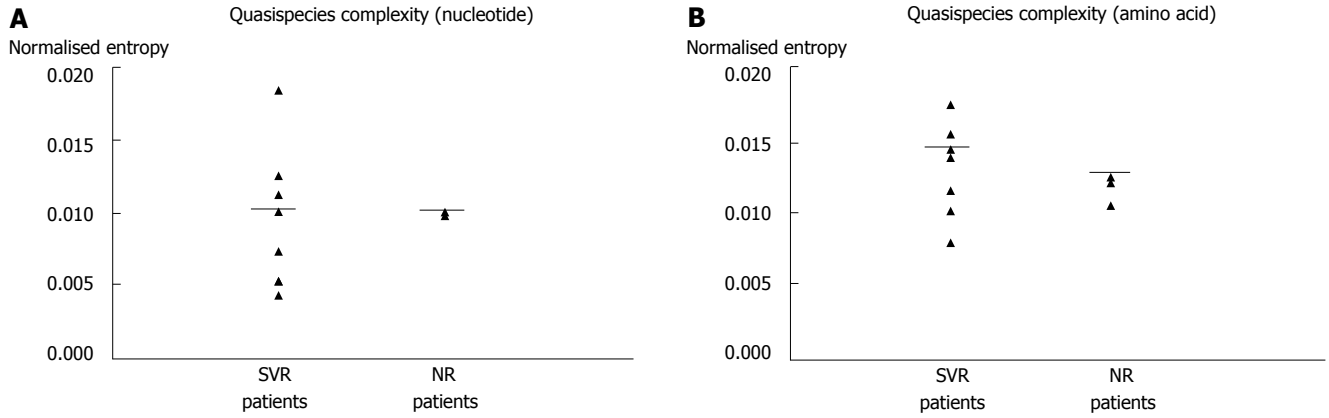

Figure 3 Genetic complexity of HCV quasispecies (**A**: nucleotide; **B**: amino acid) calculated by normalized entropy in seven sustained virological responders and three non-responders with median indicated by horizontal bar for each group. *P*-value calculated by Mann-Whitney *U*-test.

Table 4 Complexity evolution in HCV quasispecies from SVR and NR patients in ISDR, PKRbd and V3 domains

Patient	Time of treatment domains	D0 ISDR-PKRbd-V3	M3 ISDR-PKRbd-V3	M6 ISDR-PKRbd-V3
SVR1	*n* = 15	3–4–3	-	-
SVR2	*n* = 13	3–4–2	-	-
SVR3	*n* = 8	3–5–3	-	-
SVR4	*n* = 16–13	3–7–3	3–4–3	-
SVR5	*n* = 15–17	8–8–1	4–7–1	-
SVR6	*n* = 17–6	8–9–3	1–2–3	-
SVR7	*n* = 15–17	6–6–7	6–7–7	-
NR1	*n* = 17–9–4	6–8–7	3–4–4	1–3–2
NR2	*n* = 17–12–10	7–8–2	5–8–3	3–4–6
NR3	*n* = 14–11–12	6–7–4	5–7–3	4–5–2

Amino acids sequences were compared to HCV-J; *n*: number of PCR products analyzed.

two out of four slow SVR patients and the three NR patients showed a decrease in variant complexity (SVR5; SVR6; NR1; NR2 and NR10). The two other slow SVR patients displayed stability in variant complexity (SVR4 and SVR7). The three NR patients had a continuous decrease in the complexity under combined IFN-R regimen at M6 (NR1; NR2 and NR3).

Within PKRbd, after three months of IFN therapy, three out of four slow SVR patients and one out of three NR patients displayed a decrease in variant complexity (SVR4; SVR5; SVR6 and NR2) (Table 4). The two other NR patients exhibited stability in variant complexity (NR2 and NR3) and the fourth slow SVR patient showed an increase in variant complexity (SVR7). The three NR patients had a decrease in complexity under combined IFN-R therapy at M6.

After three months of IFN therapy, three out of four slow SVR patients (SVR4; SVR6 and SVR7) displayed a decrease in variant complexity in the V3 domain, as did two out of three NR patients (NR1 and NR3) (Table 4). The two other patients were found to contain an increase in variant complexity (SVR5 and NR2). Under combined IFN-R therapy, two NR patients had a low decrease in variant complexity (NR1 and NR3), whilst the third NR patient showed an increase (NR2) in variant complexity.

Variability in six different regions along the NS5A gene

Comparison to master sequence: Among the 256 PCR products studied, before and during treatment, we observed a mean of 11.7 ± 7.9 nt substitutions with a median of 10 nt substitutions.

At baseline, we observed 13.6 ± 9.1 nt substitutions (median of 12 nt substitutions) among the 147 clones analyzed, in comparison to the master sequence (Table 2, column 6). The aa variations were 4.8 ± 3.1 substitutions (with a median at 4) among all 256 clones and 5.4 ± 3.5 aa substitutions in clones analyzed before treatment, with a median of 5 aa substitutions. We also observed that all variants from SVR patients had a higher genetic diversity than all variants from NR patients in the four regions studied, including ISDR, PKRbd, V3 domain and complete NS5A (Table 3, column 2). Within patients, we observed that the 3 early SVR had a lower genetic diversity than the four slow SVR and the three NR.

We observed significantly more aa substitutions in SVR clones than in NR clones in two out of six regions (complete NS5A, $P = 0.013$; the first half of NS5A, $P = 0.032$). Interestingly, NR clones presented more aa substitutions in the V3 domain than SVR clones, but this was not statistically significant (Table 3).

During treatment, the genetic distance and numbers of substitutions (nt and aa) in SVR PCR products decreased between the baseline and M3 (Table 2, columns 6, 7 and 8). PCR products from NR patients also showed a decrease of the genetic distance, but this was lower than those from SVR patients. However, clones from NR patients had either stability or an increase in the number of aa substitutions observed under treatment. NS5A variants had a high heterogeneity without clonal selection of quasispecies and a low variation in ISDR and V3 domains during therapy.

Comparison to HCV-J sequence: Among the 256 clones analyzed, we observed a mean of 32.1 ± 5.2 aa substitutions. At baseline, a mean of 36.6 ± 5.6 aa substitutions (median at 32 aa substitutions) was observed among the 147 clones analyzed, in comparison to the

Table 5 Number of amino acids mutations observed in six different regions from NS5A protein, at baseline mean ± SD

NS5A region (aa range)	2 groups (D0) (*n* = 147)	SVR (D0) (*n* = 99)	NR (D0) (*n* = 48)	SVR (M3) (*n* = 51)	NR (M3) (*n* = 32)	NR (M6) (*n* = 26)
ISDR (2209-2248)	1.5 ± 1.6	1.3 ± 1.7	1.9 ± 1.1	0.8 ± 0.8	2.1 ± 1.0	1.8 ± 1.2
PKRbd (2209-2274)	5.4 ± 2.1	5.3 ± 2.4	5.7 ± 1.2	4.7 ± 1.6	6.1 ± 1.0	5.7 ± 1.3
V3 (2353-2379)	5.5 ± 1.4	6.0 ± 1.5	4.7 ± 0.6	6.1 ± 1.3	4.8 ± 0.7	5.1 ± 0.6
Complete NS5A (1973-2208)	32.6 ± 5.6	33.9 ± 6.0	29.8 ± 3.7	31.7 ± 5.3	30.8 ± 3.9	31.9 ± 2.9
N-terminal part of NS5A (1973-2208)	12.4 ± 3.1	12.9 ± 3.3	11.6 ± 2.4	11.7 ± 2.0	11.2 ± 2.1	12.8 ± 2.1
C-terminal part of NS5A (2209-2419)	20.1 ± 3.9	21.1 ± 4.1	18.2 ± 2.4	20.1 ± 4.2	19.6 ± 2.9	19.4 ± 3.4

n: number of PCR products analyzed; SVR: Sustained Responders; NR: Non-Responders to IFN-ribavirin therapy; D0: beginning of the treatment; M3: 3 mo of treatment; M6: 6 mo of treatment.

HCV-J sequence (Tables 2 and 5). Next, we determined for each group the level of aa substitutions in each site, with a threshold at 15 aa substitutions (this threshold was defined as half of the maximum (n = 31) of aa substitutions observed for the 256 clones; in position 2218). In ISDR, aa variations were found at positions 2218, 2224, 2232, 2234 and 2237, in the V3 domain at position 2377 and along NS5A protein at positions 2251 (PKRbd), 2280 and 2408.

At baseline, among the two group of patients (SVR and NR), the comparison of the different clone populations showed that all except one (PKRbd) of the six regions studied were able to differentiate the two groups (Table 5).

We observed more aa substitutions in SVR clones than in NR clones in four out of six regions (complete NS5A, $P < 0.001$; the first half of NS5A, $P = 0.010$; the second half of NS5A, $P < 0.001$ and V3, $P < 0.001$). Interestingly, NR clones presented more aa substitutions in ISDR than SVR clones, $P = 0.011$, and the PKRbd domain was not able to discriminate the two groups of patients. In contrast to previous findings, NR clones had more aa substitutions than SVR clones in ISDR and PKRbd regions. We did not find any correlation between HCV viral load and the number of mutations observed in ISDR and the V3 domains. A fair correlation was observed between HCV viral load and PKRbd ($r = 0.724$; $P = 0.018$). A good correlation was observed between HCV viral load and complete NS5A ($r = 0.855$; $P = 0.002$) and with the second half of NS5A ($r = 0.782$; $P = 0.008$).

During treatment, a decrease of aa substitutions occurred in the six studied regions for the two groups of patients. Only ISDR and the complete NS5A sequence showed a significant decrease in aa substitutions under treatment in comparison with populations studied at baseline ($P = 0.019$ and $P = 0.005$, respectively). In the NR clones, stability or a low decrease in aa substitutions was observed in the six regions, at baseline, M3 and M6.

Possible mutagenic effect of ribavirin during combination therapy

Among the three populations of clones from NR patients, we did not find any aa substitutions between M3 and M6 treatment points, as recently described in NS5A during ribavirin monotherapy[38]. No other specific aa substitutions were found in the NS5A protein.

DISCUSSION

Numerous viral and host factors have been described as predictors of response to therapy. HCV genotype 1, particularly genotype 1b, are most resistant to combined IFN-R therapy[5]. Among viral factors, genetic variability has been studied in many regions of the HCV genome, mainly in hypervariable region 1 (HVR1) and PKR-eIF2α phosphorylation homology domain (PePHD) of E2 region and in PKRbd (including ISDR) of NS5A[17,22,39-43]. However, few reports have studied the complete NS5A region[17,22]. Most of these studies examined patients treated by IFN monotherapy, with few studies of patients treated by combined IFN-R therapy.

A recent study examining viral sequence differences between African American and Caucasian patients treated by IFN with or without ribavirin found that the NS5A region did not cluster by race, but treatment response and IFN effectiveness did[44]. In this study, our analysis of the number of substitutions in the V3 domain showed a significant difference between the two groups of patients and a significant correlation between IFN effectiveness and a high number of mutations in the V3 domain[45].

In another study examining the second half of the NS5A region, especially ISDR, PKRbd and V3 domains, in patients treated by combined IFN-R therapy, the authors also found that the V3 domain showed more accumulation of substitutions than other domains in NS5A[45]. SVR patients had lower complexity and diversity than NR patients before treatment. During therapy, the investigators observed a decrease in complexity and diversity in SVR patients, and an increase in complexity with accumulation of non-synonymous mutations in NR patients.

In our study, we analyzed sequence variations of NS5A quasispecies in patients who received adapted therapy according to virological response during treatment. Among SVR patients, we analyzed three early SVR and four slow SVR patients compared to three NR patients. The construction of a phylogenetic tree did not allow us to distinguish the two groups of patients (SVR and NR). Before treatment, the analysis of genetic complexity and diversity did not show a significant difference between the seven SVR and the three NR patients. However, the genetic complexity and diversity were lower in early SVR than in the seven other SVR patients. The analysis of the immunity pressure in different domains of the NS5A gene did not show differences between the SVR and NR groups except for the V3 domain and the complete NS5A region where the SVR group presented a higher immunity pressure than NR patients. Generally, the analysis of mutations compared to the master sequence or HCV-J

sequences requires long fragments (a half or a complete NS5A protein) to distinguish SVR and NR. We did not observe any difference in specific aa substitutions between the two groups of patients.

During treatment, we observed a decrease of genetic complexity and diversity in slow SVR and NR patients. A very low complexity was found in the V3 domain in eight out of ten patients (6/7 of SVR and 2/3 of NR patients). In the PKRbd and V3 domains, we often observed complexity decrease or stability. Only one out of seven SVR and one out of three NR patients displayed complexity increase either under IFN therapy or under combined IFN-R therapy. Thus, we observed a different evolution of quasispecies under IFN alone or under combined therapy. This is in contrast to a recent study by Puig-Basagoti *et al*[45] where they observed a decrease in quasispecies complexity in SVR patients and a stability or increase in quasispecies complexity in NR patients. In this study, the analysis of the quasispecies was done at wk 1, 2 and 4, whereas in our study we analyzed quasispecies after a first three-month period of treatment under IFN alone and after a second three-month period under combined IFN-R therapy. We observed the same rate of appearance of aa substitutions in the ISDR domains for the two categories of patients. However, the instauration of a real modification of aa in a quasispecies was more often observed in SVR patients than in NR patients in the complete NS5A protein and in the PKRbd and V3 domains. Interestingly, in three slow SVR (SVR4-SVR5-SVR6) patients the main variant from the ISDR domain remained unchanged after three months of treatment. The same observation was made in two out of three NR patients who also exhibited complexity stability in the different NS5A regions.

Asahina *et al*[38] studied the possible mutagenic effect of ribavirin during a period of 28 days of ribavirin monotherapy before combined IFN-R therapy. During the first phase of ribavirin monotherapy, they observed an accumulation of non-synonymous substitutions located in NS5A. These substitutions were observed in ten patients and eight of them had SVR after treatment. In our study, we did not observe these substitutions in PKRbd nor in any part of NS5A. However, our treatment regimen was different and we only studied the effect of ribavirin during three months of combined therapy in NR patients. It is also possible that substitutions did arise during combined therapy, but were subsequently eliminated by the treatment.

In conclusion, our study reinforces the potential role of NS5A polymorphism, particularly in the V3 domain, in regulating HCV resistance to IFN treatment. Nevertheless, the underlying molecular mechanisms remain unclear. Mutations in the V3 domain may affect the interaction between NS5A and one or several IFN-induced antiviral effectors. Some studies found high mutation frequency in ISDR being associated with low HCV RNA titer, suggesting that NS5A may play a role in HCV replication. In our study, at baseline, we did not find any relationship between HCV viral load and the number of mutations observed in the ISDR and V3 domains. The number of mutations observed in PKRbd has shown a fair correlation with HCV viral load, but we found a better correlation when we analyzed the second half or the complete NS5A region. These results support the notion that mutations in the NS5A region may influence on HCV replication, and suggest that the entire NS5A region should be analyzed for mutations that may correlate with IFN response. Further clinical studies with new therapeutic approaches such as pegylated IFN, as well as the use of the recently developed HCV infection cell culture systems should further improve our understanding of the role of NS5A in regulation of the IFN response.

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COMMENTS

Background

In this article, we analyzed NS5A HCV quasispecies evolution during antiviral treatment. Since the NS5A protein is implicated in HCV resistance, this study should enhance the knowledge of evolution of the resistance during treatment.

Research frontiers

Many previous articles focused on a small region(s) of NS5A without studying the complete gene. We believe that an analysis of the entire NS5A region is necessary because some sections of NS5A may be important for homologous and/or heterologous RNA-protein or protein-protein interactions and thus may play a role in conferring HCV resistance to IFN therapy.

Innovations and breakthroughs

To the best of our knowledge, only two reports studied quasisipecies along the complete NS5A region and did not observe quasispecies evolution during therapy. In contrast, our results suggest that V3 domain plays a role in HCV resistance to IFN therapy.

Applications

Our study may help identify mutations within the HCV genome that can be used to predict patients' response to IFN treatment or future new antiviral modalities, as well as provide molecular hints for the mechanisms underlying HCV resistance to the therapy.

Peer review

Study is important with findings suppporting previous results as well as novel findings. The biggest problem is the very small size of the patients analyzed. The paper is well written and consise.

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VIRAL HEPATITIS

Epidemiology and transmission of hepatitis B and C viruses in Kazakhstan

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Abstract

AIM: To investigate the epidemiology of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection in the two major ethnic groups in Kazakhstan.

METHODS: A cross-sectional prospective study of HBV and HCV seroprevalence was performed among individuals born in Kazakhstan with no history of chronic hepatitis or liver disease.

RESULTS: There were 290 volunteers (140 Russians and 150 Kazakhs) aged 10 to 64 years, males accounted for 46%. Active HBV infection (HBsAg positive) was present in 3.8%, anti-HBc in 30%. The prevalence was similar in females and males (33% *vs* 25%) (P = 0.18). The prevalence of anti-HBc increased from 19% in 10-29 years old volunteers to 53% in 50-years and older volunteers. The prevalence of HBV infection was higher in married than in single adults (38% *vs* 26%, respectively) (P = 0.2) and more common in Kazakhs (35%) than in Russians (24%) (P = 0.07). HCV infection was present in 9 subjects (3.2%), 5 of them also were positive for anti-HBc in the absence of HBsAg.

CONCLUSION: The frequency of active HBV infection (3.8%) coupled with a high prevalence of HBV exposure in those > 50 years of age increases with age, which suggests that horizontal transmission likely relates to the use of contaminated needles. The low prevalence of HCV infection suggests that HBV and HCV are acquired differently in this group of subjects.

Key words: Viral hepatitis B; Viral hepatitis C; Hepatitis B virus; Transmission; Epidemiology; Sero-epidemiology; Kazakhstan

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INTRODUCTION

Kazakhstan has two major ethnic groups: Kazakhs who trace their origins back to Genghis Khan, and ethnic Russians. Each constitutes approximately 40% of the population. The different ethnic groups share similar living and socioeconomic conditions.

Kazakhstan is part of a nine-country subregion in which the mortality due to chronic liver disease and cirrhosis has been estimated to be 21.8 per 100 000 population[1]. The mortality rate of hepatocellular carcinoma is also high, with liver cancer representing approximately 4% of all malignant neoplasms[2]. Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are both common causes of hepatocellular carcinoma. We conducted a cross-sectional study in Kazakhstan to examine the epidemiology and risk factors associated with HBV and HCV infections. We gave particular emphasis on possible relationships between chronic hepatitis B or C virus infections and a history of intravenous injections, blood transfusions, vaccinations, needle sticks, surgeries, having tattoos, and shared use of tooth brushes, body brushes and towels.

MATERIALS AND METHODS

We performed a cross-sectional seroepidemiologic study in Almaty, Kazakhstan. Both Russians and Kazakhs were entered if they were unrelated and born in Kazakhstan with no prior history of clinical hepatitis or chronic liver disease. An attempt was made to obtain an equal number

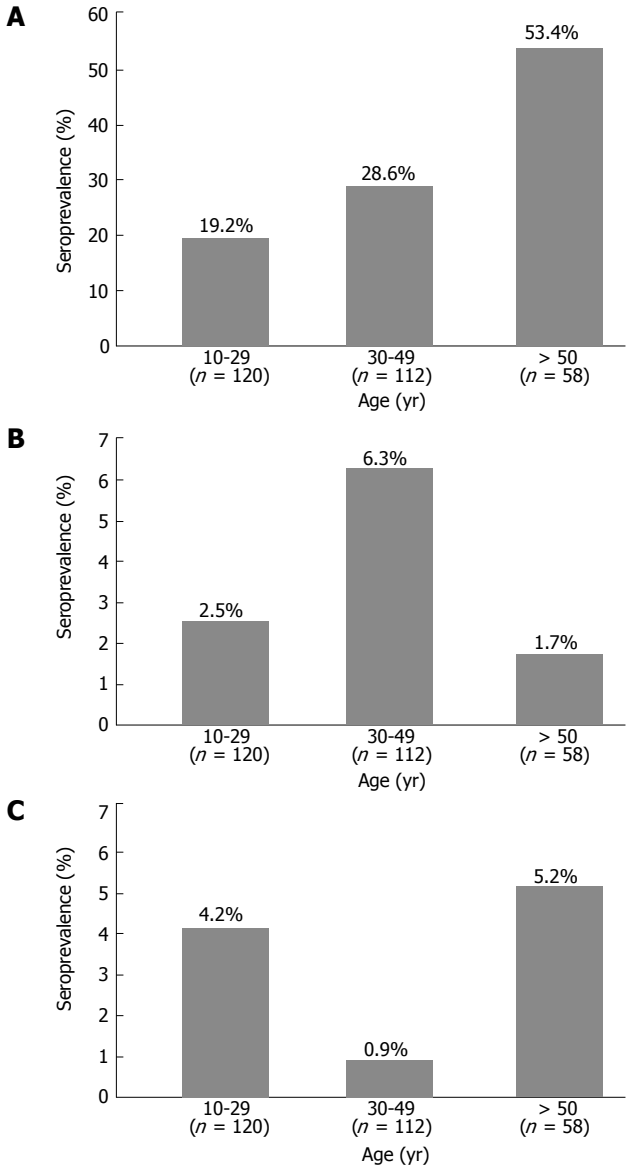

Figure 1 Prevalence of anti-HBc antibodies (**A**), HBsAg (**B**), and anti-HCV antibodies (**C**) in relation to age in Kazakhstan.

of volunteers per decade from each of these two major ethnic groups.

A trained physician interviewed each volunteer and completed a detailed questionnaire. Demographic data focusing on the environment during the subject's childhood included social and economic data (parent's education, occupation and family income) as well as histories of blood transfusions, intravenous injections, vaccinations, needle sticks, surgeries, having tattoos, and the shared use of tooth brushes, body brushes or towels. In women, we obtained data about miscarriages and abortions.

Serologic methods

Each volunteer provided a blood sample. Sera were stored at -20℃ until analyzed. Serum samples were tested for hepatitis A and B serology using kits (Abbott Diagnostic Laboratories, Abbott Park, IL), including enzyme immunoassays (EIA) for the qualitative detection of total antibody to hepatitis A virus (anti-HAV; HAVAB EIA), hepatitis B surface antigen (HBsAg; AUSZYME Monoclonal), and total antibody to hepatitis B core antigen (anti-HBc; Corzyme). Antibody to hepatitis B surface antigen (anti-HBs; AUSAB, human subtypes ad and ay) was evaluated by solid phase radioimmunoassay. Antibody to hepatitis C (anti-HCV; HCV Version 3.0, Ortho-Clinical Diagnostics, Inc, Raritan, NJ) was detected by EIA. The interpretation of the results was done according to the manufacturer's instructions.

Table 1 Concentration of anti-HBs in four HBsAg and anti-HBc positive subjects

I.D.	mIU/mL
11	16.1
180	3.4
249	4.0
143	5.7

RESULTS

Two hundred and ninety volunteers were enrolled in the study including 150 Kazakhs and 140 ethnic Russians ranging in age from 10 to 64 years. One hundred and thirty-four (46%) of the subjects were men.

Risk factors and prevalence of HBV

Total anti-HBc was found in 30% of the population, being similar in women (33.3%) and men (25.4%) ($P = 0.18$). There was an age-specific significant increase in the prevalence of anti-HBc antibody: from 19.2% in 10-29 years old individuals to 53.4% in those who were 50- years old and older (Figure 1A) ($P < 0.01$). The prevalence of anti-HBc was more common in Kazakhs (34.7%) than in Russians (24.3%) ($P = 0.07$). Among those who were 21-years old and older, the prevalence of hepatitis B infection was similar in married and single adults (38% *vs* 26%, $P = 0.2$) (data not shown).

The overall seroprevalence rate of active HBV infection (HBsAg positive) was 3.8% (11 of 290 subjects), none of these individuals tested was positive for anti-HCV. There was no significant difference in the prevalence of detectable HBsAg in men and women (3.7% of men *vs* 3.8% of women) ($P = 0.7$) nor between ethnic groups (4.3% of Russians *vs* 3.3% of Kazakhs) ($P = 0.9$). The prevalence of HBsAg peaked in those whose age was 30-49 years (6.3%) and was lower in those whose age was 10-29 years (2.5%) ($P = 0.3$) and lowest in those whose age was 50 years and more (1.7%) ($P = 0.4$, Figure 1B). Four of the 11 HBsAg positive individuals (36%) were anti-HBs positive. However, the concentrations of antibodies were low in this group of individuals (Table 1). Three of four individuals with concurrent HBsAg and anti-HBs positivity were Kazakhs, but gender distribution was equal.

Table 2 shows unadjusted and adjusted (for socio-economic status, gender, and ethnicity) incidence rates and risk factors possibly associated with acquisition of hepatitis B. The highest risks associated with hepatitis B infection were having been a blood donor or having a history of dental surgery. The risk factors for positive anti-HBc and

Table 2 Effect of selected risk factors on the incidence rate of anti-HBc in a study population from Kazakhstan (n = 86)

Risk factor	Unadjusted OR	P	95% CI	Adjusted[1] OR	P	95% CI
Blood transfusion	2.11	0.19	0.67-6.48	1.26	0.72	0.36-4.40
Blood donor	1.77	0.03	1.05-2.98	1.38	0.48	0.56-3.42
Tattoo	1.67	0.19	0.77-3.65	1.26	0.62	0.51-3.12
Ear piercing	1.43	0.16	0.87-2.39	1.20	0.57	0.64-2.24
Dental surgery	1.93	0.02	1.10-3.31	1.29	0.29	0.80-2.08

[1]Adjusted OR for socioeconomic status, ethnicity and gender.

Table 3 Effect of selected risk factors on the incidence rate of anti-HCV in a study population from Kazakhstan (n = 9)

Risk factor	Unadjusted OR	P	95% CI	Adjusted[1] OR	P	95% CI
Blood transfusion	2.48	0.41	0.29-21.20	1.42	0.76	0.15-13.38
Tattoo	4.02	0.05	0.90-16.44	14.40	0.01	1.76-118.32
Ear piercing	2.58	0.18	0.65-10.18	3.05	0.29	0.38-24.4
Dental surgery	1.65	0.48	0.42-6.52	1.46	0.64	0.29-7.26
Towel sharing	3.25	0.09	0.82-12.83	3.87	0.07	0.91-16.4

[1]Adjusted OR for socioeconomic status, ethnicity and gender.

HBsAg included dental surgery (73%), frequent injections (64%), and ear piercing (54%).

Risk factors and prevalence of viral hepatitis C

Anti-HCV was detected in only 9 subjects (3.2%) including 3 Kazakh women and 6 Russians (three women and three men). Five of the 9 subjects also had detectable levels of anti-HBc, but were negative for anti-HBs. Figure 1C and Table 3 display age-specific prevalence rates of anti-HCV in this study population and selected risk factors associated with anti-HCV seropositivity. A history of tattoos was a common risk factor for hepatitis C with a trend in risk toward towel sharing.

DISCUSSION

Almost 30% of the study population examined had evidence of current or past HBV infection which is similar to the results of a prior study in northwestern Kazakhstan, showing that 22% (n = 579) of the healthy population had detectable anti-HBc antibodies[3]. The prevalence of anti-HBc seropositivity in Kazakhstan is substantially lower than that reported in 110 Bukharian Jews who immigrated from Uzbekistan and Tajikistan (former Soviet Union) to Israel, 66% of them had evidence of exposure to HBV[4]. However, the prevalence of past or current hepatitis B infection is much higher in Central Asia than in Pakistan, Ethiopia, Sweden, England and Wales[5-8].

The primary mode of transmission of HBV in Kazakhstan is unsettled. The prevalence of infection increasing with age could result from either birth cohort effects and continuing gradual horizontal acquisition, or both. Demographic factors associated with risk of acquisition (i.e. history of blood donation and dental surgery) found in a recent study in Pakistan are blood transfusions and use of contaminated syringes, and contaminated dental and surgery equipment are the main risk factors for acquiring HBV infections[9]. However, in our study, adjustments for socioeconomic status, ethnicity and gender eliminated these significantly associated risk factors (Table 2).

In countries of European Region C, including Kazakhstan, the annual number of injections was reported to be 11.3 per person in 2000 and this estimate is the highest among countries recognized as having a global burden of disease. The same is true for the Russian Federation where the annual number of injections per person is also 11.3 and different from neighboring countries such as Kyrgyzstan and Uzbekistan where this estimate is 5.2 or China where the annual number of injections per person is only 2.4[10]. Several studies conducted in Russia showed that 85%-99% of injections are unnecessary with a ratio of 20:1 for injections being given with therapeutic intent compared to those for disease prevention[11,12]. In addition, the proportion of equipments being reused was reported to be 11%. Of interest in this study, the common factor for having both anti-HBc and HBsAg was a history of frequent injections.

In the current study, 4 of the 11 HBsAg positive subjects also had anti-HBs antibodies (36%). It was reported that the rate of concurrent HBsAg and anti-HBs positivity is between 21% and 32%[13-16] and is associated with evidence of viral replication and features of active inflammation[13] or with progressive liver disease[14]. Most probably, these discordant serologic results reflect a low concentration of non-complexed anti-HBs that fails to recognize insertions or deletions that occur in the pre-S/S region of the HBV genome[16,17]. Heterotypic antibody is often observed in these situations[13,15].

The prevalence of anti-HCV in this study population was 3.2% which is somewhat higher than the prevalence of anti-HCV antibodies observed in northwestern Kazakhstan (1.7%; P = 0.3)[3], or in Siberian natives from the Kamchatka Peninsula of Russia (1.4%, n = 348)[18]. In contrast, in the Republic of Azerbaijan[19], 8.7% of screened serum samples are anti-HCV reactive[20]. We feel certain that virtually all of our reactive samples represented a past or present infection with HCV based on strong EIA ratios of 3.0 or greater. However, we did not do HCV RNA testing to confirm its active infection. It also is possible that we underestimated the prevalence of hepatitis C in that a proportion of the population may have resolved their infection and subsequently lost their anti-HCV[21].

The risk factors related to HCV infection (Table 3) have very wide confidence intervals indicative of the small sample size which precludes making firm conclusions regarding transmission. However, the low prevalence of hepatitis C infection compared with the relatively high prevalence of HBV infection suggests that different modes of transmission are active in this population due to the sexual or vertical transmission of HBV and the

parenteral transmission of HCV. Overall, the use of unsafe (contaminated) needles remains of great concern in this population as a vehicle for transmission of these viruses.

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BASIC RESEARCH

Antisense oligonucleotide targeting midkine suppresses *in vivo* angiogenesis

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Abstract

AIM: To evaluate the effect of antisense oligonucleotide targeting midkine (MK-AS) on angiogenesis in chick chorioallantoic membrane (CAM) and *in situ* human hepatocellular carcinoma (HCC).

METHODS: An *in situ* human hepatocellular carcinoma (HCC) model and CAM assay were used in this experiment. The effect of MK-AS on angiogenesis was evaluated by cell proliferation assay and hematoxylin-eosin (HE) staining.

RESULTS: MK-AS significantly inhibited human umbilical vein endothelial cells (HUVEC) and *in situ* human HCC growth. At the same time, MK-AS suppressed the angiogenesis both in human hepatocellular carcinoma cell line (HEPG2)-induced CAM and *in situ* human HCC tissues.

CONCLUSION: MK-AS is an effective antiangiogenesis agent *in vivo*.

Key words: Midkine; Angiogenesis; Antisense oligonucleotide; Tumor; Chick chorioallantoic membrane assay; Hepatocellular carcinoma

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INTRODUCTION

Angiogenesis is a process leading to formation of new blood vessels, which plays a central role in survival of cancer cells, growth of local tumors, and development of distant metastasis[1]. It is believed that tumor cells require the ability to stimulate angiogenesis, and angiogenesis induced by tumor cells, leads to hypervascularization of tumor tissue[2]. More recently, it has been found that tumor cells also produce angiogenesis inhibitors[3]. The angiogenic phenotype of a solid tumor is accepted as a result of the net balance between the activities of angiogenesis promoters and inhibitors[4]. Preclinical testing of these antiangiogenic factors in animal models suggested that they can effectively suppress the growth and/or metastasis of experimental tumors[5-7]. Inevitably, identification of angiogenic inhibitors and demonstration of their angiostatic functions would provide rational foundations for the development of tumor-specific antiangiogenic therapy.

Up to now, different growth factors, such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), transforming growth factor-alpha (TGF-α) and midkine (MK) have been identified as positive regulators of angiogenesis and are secreted by cancer cells to stimulate normal endothelial cell growth through paracrine mechanisms[8-11]. MK was first identified in early stage embryonal carcinoma cells during retinoic acid-induced differentiation[12]. MK and pleiotrophin comprise a family of heparin-binding growth/differentiation factors, which are different from other heparin-binding growth factors such as fibroblast growth factor and hepatocyte growth factor[13-15]. MK is over-expressed in various malignant tumors[16-24], and low or undetectable in normal adult tissues[13,24]. It is accepted that MK promotes the survival[25,26], growth[27,28] and migration[29-31] of many cells, involved in activating mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases 1 and 2 or protein kinase B (PKB/AKT) pathways[25,32]. Recently, it was reported that midkine binds to anaplastic lymphoma kinase (ALK) and acts as a growth factor for different cell types[33].

There is evidence that MK plays an important role in angiogenesis, which is an important event in tumor development and progression. It was reported that enhanced tumor growth in MCF-7 breast carcinoma cells due to high MK expression is correlated with increased vascular density and endothelial proliferation, implicating an angiogenic role of MK in tumor growth[11]. Additionally, MK, an angiogenic factor, is expressed in bladder cancer,

and its over-expression correlates with a poor outcome in patients with invasive cancers[20]. In fact, we have confirmed that MK-AS transfer can significantly inhibit the growth of hepatocellular carcinoma cells, which is associated with increased Caspase-3 activity[34]. The present study was to determine whether MK-AS can suppress angiogenesis, which is an important mechanism underlying inhibition of tumor cell proliferation.

MATERIALS AND METHODS

Antisense oligodeoxynucleotides

MK-AS (5'-CCCCGGGCCGCCCTTCTTCA-3') and MK-SEN phosphorothioate oligonucleotide (5'-TGAAGAAG GGCGGCCCGGGG-3') were synthesized with an applied biosystem model 391 DNA synthesizer using Oligo Pilot Ⅱ DNA (Amersham-Pharmacia, Piscataway, NJ, USA) and purified by high-performance liquid chromatography (HPLC) (Waters Delta Prep 4000, Milfordd, MA, USA) with SOURCE 15Q (Amersham Pharmacia, Piscataway, NJ, USA) as previously described[34].

Cultured cell lines

Human umbilical vein endothelial cells (HUVECs), a gift of the Chinese Academy of Medical Sciences, Beijing, China, were pooled after collagenase type Ⅰ treatment and seeded on cell culture plates. The cells were grown in RPMI 1640 medium (Invitrogen Corporation, CA, USA) supplemented with 20% fetal calf serum (FBS; GIBCO BRL, Grand Island, NY, USA), 100 U/mL penicillin and 100 μg/mL streptomycin at 37℃ in an atmosphere containing 5% CO_2.

Cell proliferation assay

A total of 3×10^3 cells were seeded in each well of a 96-well microtiter plate and allowed them to attach overnight. Oligonucleotides at the concentrations of 0.2, 0.4, 0.8 μmol/L were transfected into the cells with Lipofectin (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Transfection medium was replaced by normal culture medium after 6 h. The effects of antisense oligodeoxynucleotide (ASODN) on cellular viability were measured by 3-[4, 5-dimethythiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt (MTS) assay. After 48 h of incubation following transfection, 20 μL MTS (Sigma, St Louis, MO, USA) was added to each well and incubated at 37℃ for 2 h. The absorbance value was determined at 490 nm by a MR600 microplate reader (Wallac 1420 Multilable counter, Wallac, Turku, Finland).

In vivo tumor studies

In situ HCC models were established as previously described[35]. Two days after *in situ* HCC models were established, mice were injected intravenously with saline (vehicle control) and MK-AS (25, 50 and 100 mg/kg per day) for 20 d. Body weight and general physical status of the animals were recorded daily. At the endpoint of the study, mice were killed by cervical dislocation and tumors were removed and weighed. Tumor sizes were monitored with calipers, the tumor volume (V, mm^3) was calculated as $(L \times W^2)/2$, where L = length (mm) and W = width (mm). The percentage of tumor growth inhibition was calculated as: inhibitory rate (%) = $(W_{control}-W_{treat})/W_{control} \times 100$.

Immunohistochemistry analysis for microvessel formation

Tumor specimens were fixed and frozen in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC). Five-μm thick cryosections were cut and stained with HE for histopathological analysis. To analyze the microvessel formation in tumors, sections were stained with anti-CD34 monoclonal antibody (DAKO Corp., Carpinteria, CA) and subsequently with the avidin-biotin-peroxidase (ABC) method. Positively stained vascular endothelial cells (brown) were visualized and imaged using a digital camera attached to an Olympus microscope. Microvessel density was determined as previously described[36]. Briefly, regions of the highest vessel density ("hot spot" regions) were scanned at low magnification (× 40-100) and counted at higher magnification (× 200). Three such "hotspot" fields were counted in each tumor section, and the mean microvessel density value was recorded. Any endothelial cell or endothelial cell cluster that was clearly separated from adjacent microvessels was considered a single, countable microvessel. Positively stained vascular endothelial cells were visualized and imaged using a Magnifire camera (Olympus, Melville, NY) attached to an Olympus Provis microscope.

Western blot analysis

After transfection with MK-AS for 24 h, cells in the microplates were collected into 1.5 mL Eppendorf tubes on ice and centrifuged at 2000 r/min for 5 min at 4℃. Pellets were then lysed with lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 0.5 mmol/L EDTA, 0.5% NP40, and 150 mmol/L NaCl] in the presence of protease inhibitors. Lysates were centrifuged at 12000 × *g* for 15 min to remove debris. Proteins (30 μg) were separated from samples by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto hybond-polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). Midkine protein was identified using the primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The reactive band was visualized with an ECL-plus detection kit (Amersham Biosciences, Piscataway, NJ) and scanned by Gel Doc 1000 (Bio-Rad CA, USA). β-actin was used as a control.

Chick chorioallantoic membrane assay

Angiogenesis assay in chick chorioallantoic membranes (CAM) was performed as previously described[37]. Fertilized eggs were incubated at 38℃. On d 7, a window was opened on the eggshell to expose the CAM, and the window was covered with a tape for further incubation. Filter paper discs (0.5 cm in diameter) containing HepG2 cells (18000/disc) or PBS was placed on the surface of each CAM on d 8. After 24 h, 100 μL of 0.4 μmol/L MK-AS was given using a 30-gauge needle. Two days later, the CAM were fixed in 3.7% formaldehyde. Pictures were taken with a stereoscope, and the number of vessel

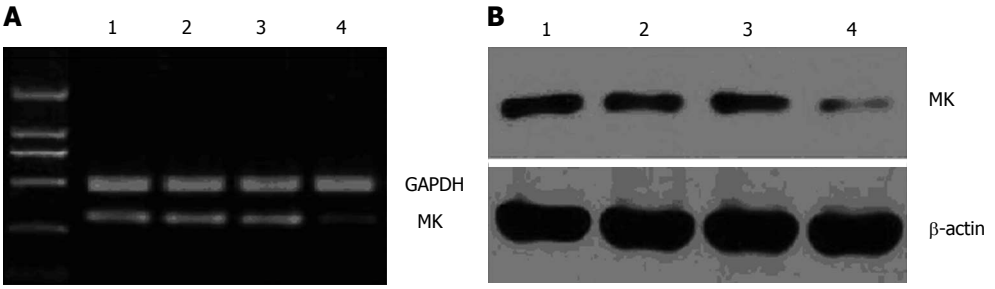

Figure 1 RT-PCR (**A**) and Western-blotting analysis (**B**) of MK expression in human umbilical vein endothelial cells (HUVEC) after transfection with 0.4 μmol/L MK-AS (lane 4), MK-SEN (lane 3), Lipofectin alone (lane 2) for 24 h. Lane 1 represents cells without transfection. GAPDH and β-actin were used as control respectively.

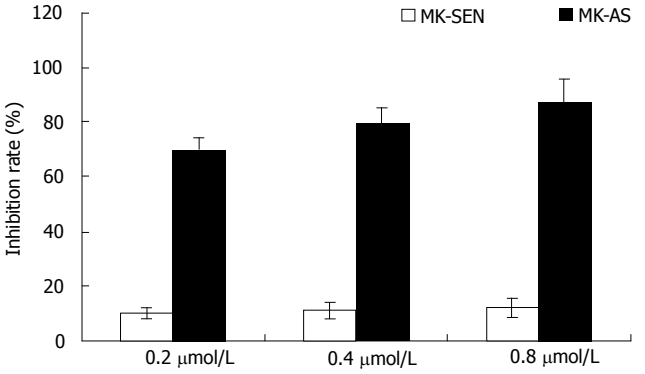

Figure 2 Effect of MK on growth of HUVEC. Cells after transfection with 0.4 μmol/L MK-AS or MK-SEN for 24 h were analyzed by MTS assay. Data were expressed as mean ± SD from four independent experiments.

branches was counted. Ten eggs were used for each experimental condition.

Statistical analysis

Data were expressed as mean ± SD, statistical analysis was carried out using Student's *t*-test (two tailed), $P < 0.05$ was considered statistically significant.

RESULTS

Effect of MK-AS on MK expression in HUVEC

Our previous studies showed that antisense oligonucleotide targeting MK down regulates MK expression in hepatocellular carcinoma cells[34]. In this study, we measured the effect of MK-AS on MK expression in HUVEC. After MK compounds were transfected for 24 h, total RNA and protein of HUVEC were extracted for analysis of the effect of MK-AS transfer on MK expression. Our results indicated that MK-AS could efficiently decrease the mRNA and protein content of MK in HUVEC (Figure 1A and B).

Effects of MK-AS treatment on HUVEC growth

To investigate the effect of MK-AS on endothelial cell proliferation, we analyzed the growth of HUVEC transfected with MK-SEN or MK-AS. MK-AS transfer significantly inhibited HUVEC proliferation (Figure 2), suggesting that MK signaling might directly contribute to endothelial cell growth. In contrast, no significant inhibition of HUVEC transfected with MK-SEN was observed.

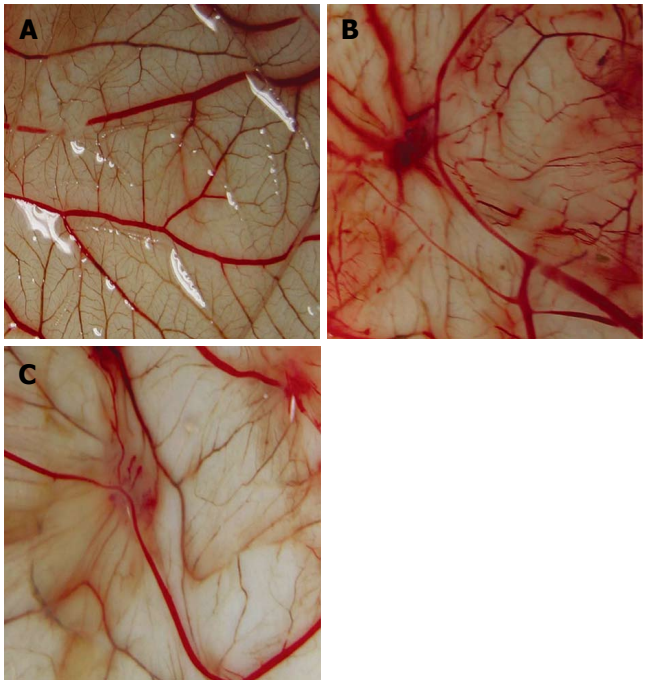

Figure 3 Angiogenesis in normal chick CAM (**A**), HepG2-induced CAM (**B**), and HepG2-induced CAM treated with 0.4 μmol/L MK-AS (**C**). Chick CAM assays were used to assess the impact of MK-AS on angiogenesis *in vivo*.

MK-AS inhibited angiogenesis in chick CAM

It is widely accepted that tumors can stimulate angiogenesis by releasing angiogenesis stimulators such as bFGF and VEGF[8,9]. HepG2 induced noticeable angiogenesis (Figure 3B). When paper discs absorbing PBS alone were placed onto the CAM, a few vessels occupied the CAM area covered by the paper discs (Figure 3A). Interestingly, when CAM implanted with HepG2 were treated with MK-AS (0.4 μmol/L), the number of vessels under the paper discs was significantly reduced (Figure 3C). Similar results were obtained when the mean vessel length was measured (data not shown). Chick embryos treated with MK-AS were alive and showed the same extent of motility as embryos from PBS-treated eggs at the end of the experiment.

Effects of MK-AS treatment on in situ HCC xenograft growth

We have reported that MK-AS inhibits tumor proliferation[38]. In the present study, an *in situ* HCC model of mice was used to evaluate the effect of MK-AS on *in vivo* tumor proliferation. MK-AS at 25 mg/kg, 50 mg/kg, 100

Table 1 Effect of MK-AS on weight and inhibition of *in situ* HCC

Treatment group	Tumor weight (g)	Inhibition rate (%)
Saline	1.29 ± 0.13	-
MK-AS 100 mg/kg per day	0.44 ± 0.18[b]	65.89
MK-AS 50 mg/kg per day	0.64 ± 0.18[b]	50.39
MK-AS 25 mg/kg per day	0.70 ± 0.14[b]	45.74
MK-Sen 50 mg/kg per day	1.04 ± 0.14[a]	19.38

[a]$P < 0.05$, [b]$P < 0.01$ *vs* control group.

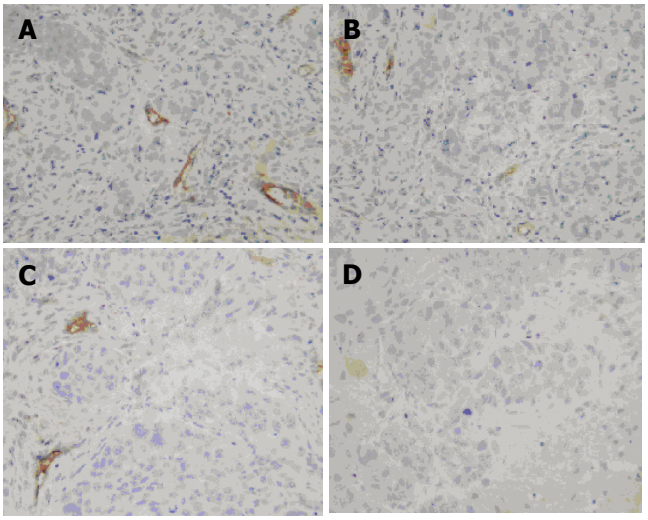

Figure 4 Immunohistochemical analysis of HCC xenograft angiogenesis after treatment with MK-SEN (**A**), 25 mg/kg of MK-AS per day (**B**), 50 mg/kg of MK-AS per day (**C**), and 100 mg/kg of MK-AS per day (**D**). Representative views of anti-CD34 antibody-stained vascular endothelium (brown) (x 200).

mg/kg per day and saline were intravenously administered for 20 d. Tumors were removed, measured and weighed. MK-AS treatment significantly inhibited tumor growth compared to saline treatment (Table 1). The highest inhibitory efficacy was 65.89% in MK-AS treatment group compared to saline treatment group. The effects of MK-AS on tumor growth are shown in Table 1. The final tumor volumes after 20-d treatment are shown in Table 2 ($P < 0.01$). No difference was found in the mean body weight of mice between the groups during the study (data not shown).

Effects of MK-AS treatment on in situ HCC xenograft angiogenesis

To determine whether the antitumor effect of MK-AS therapy is associated with angiogenesis suppression, the status of vessel formation in *in situ* HCC xenograft was assessed by immunohistochemistry with endothelial cell-specific CD34 staining. A representative result of immunostaining is shown in Figure 4. High density of microvessels was present in untreated tumor samples, whereas MK-AS treatment significantly reduced vascularization (Figures 4 and 5).

Table 2 Effect of MK-AS on growth of *in situ* human HCC

Treatment group	Tumor volume (mm^3)
Saline	807.75 ± 195.19
MK-AS 100 mg/kg per day	294.50 ± 70.66[b]
MK-AS 50 mg/kg per day	532.00 ± 121.57[b]
MK-AS 25 mg/kg per day	552.75 ± 84.38[b]
MK-Sen 50 mg/kg per day	630.88 ± 188.76[a]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* control group.

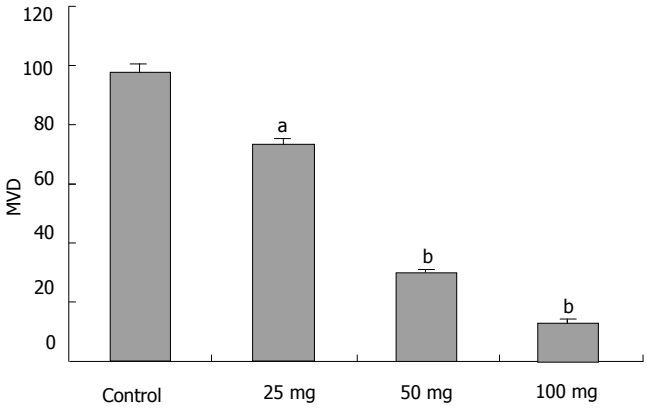

Figure 5 Tumors resected from mice at the experimental end point. Resected tumors were sectioned and stained as described in the text. Microvessel densities (MVD) were determined by counting CD34-positive endothelial cells in the sections and presented as mean ± SE positive cells/field from the three "hot-spot" fields. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group.

DISCUSSION

Tumor-induced neovascularization plays a key role in the development, progression and metastasis of neoplasm[39]. Angiogenesis is regulated by several endogenous stimulators and inhibitors of endothelial cell migration, proliferation and tube formation[4]. Tumor growth progression is correlated to the serum concentration of angiogenic mediators[40]. Furthermore, vascular density of tumor tissues is correlated to the clinical course of tumors[2]. MK is heparin binding to cytokines involved in neurogenesis, cell migration and mesoderm-epithelial interactions[41]. Choudhuri *et al*[11] reported that in vitro tumor cell growth is not affected by these growth factors, while *in vivo* tumor cell growth is correlated to increased vascular density of tumors. Since MK plays an important role in angiogenesis, it may be a possible therapy target to suppress angiogenesis of tumor proliferation.

Under physiological conditions the vasculature is quiescent in normal adults. Only 0.01% of endothelial cells in normal adult vessels are in the cell division cycle at any given time[42]. However, in response to an appropriate angiogenic stimulus, endothelial cells can be activated to grow new vessels. In the present study, down regulation of MK expression could restrain HUVEC proliferation, suggesting that endothelial cells can also inhibit angiogenesis *in vivo*. Chick CAM assay showed that MK-AS could inhibit local CAM vasculogenesis.

Furthermore, we developed an *in situ* HCC model of mice to determine the effect of MK-AS on tumor proliferation and angiogenesis *in vivo*. Our results indicate that MK-AS administration significantly inhibited tumor growth in the *in situ* HCC model of mice (Tables 1 and 2). Furthermore, Mk-AS administration also suppressed tumor angiogenesis *in vivo* (Figures 4 and 5). It should be noted that the tumor cell line originated from human HCC maintained the complete characteristics of human HCC tissue, such as AFP secretion, drug sensitivity. In addition, the pathological evidence also suggests that this model exhibits various features seen in clinical HCC patients, indicating that the information provided by this model can reflect the true clinic results of patients. In fact, angiogenesis suppressed by transfection with SiRNA targeting MK in human prostate cancer cell line (PC-3) has also been reported[43].

It has been well established that tumor growth and metastasis depend on the induction of new blood supply[44,45]. Angiogenic activity as determined by MVD, has been shown to correlate with worse prognosis in a number of solid tumors[46-48]. Therefore, antiangiogenic agents targeting either these factors or vascular endothelial cells can be used as promising therapeutic modalities in treatment of tumors[49]. In the present study, significant inhibition of angiogenesis was achieved using MK-AS, indicating that MK-AS is an effective antiangiogenesis agent.

COMMENTS

Background

Midkine (MK) is a 13kDa protein, a heparin-binding growth factor. Subsequent studies suggest that MK plays an important role in carcinogenesis.

Research frontiers

MK plays an important role in angiogenesis, which is an important event in tumor development and progression. Antisense oligonucleotide targeting MK is employed in cancer therapy as it can inhibit hypervascularization of tumors and is of great clinical interest.

Innovations and breakthroughs

In this study, MK inhibited angiogenesis in chicken chorioallantoic membrane and human hepatocellular carcinoma (HCC) xenograft. Meanwhile MK inhibited proliferation of human umbilical vein endothelial cells (HUVEC) and growth of HCC xenograft.

Applications

In this study, we addressed the potential therapeutic effect of MK on angiogenesis. Significant inhibition of angiogenesis was achieved using MK, indicating that MK is an effective anti-angiogenesis agent.

Peer review

The manuscript by Li-Cheng Dai *et al* describes the successful use of midkine to inhibit angiogenesis in chicken chorioallantoic membrane and human HCC xenograft. The results were found to be important for HCC therapy. The findings are of clinical interest.

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BASIC RESEARCH

Tetrandrine stimulates the apoptosis of hepatic stellate cells and ameliorates development of fibrosis in a thioacetamide rat model

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Abstract

AIM: To investigate the therapeutic effect of tetrandrine on liver fibrosis induced by thioacetamide in rats *in vivo* and *in vitro*.

METHODS: *In vitro* study: we investigated the effect of tetrandrine on the apoptosis of rat hepatic stellate cells transformed by simian virus 40 (T-HSC/Cl-6), which retains the features of activated cells. *In vivo* study: hepatic fibrosis was induced in rats by thioacetamide. Tetrandrine was given orally to rats at doses of 5, 10 or 20 mg/kg for 4 wk compared with intraperitoneal injection of interferon-r.

RESULTS: *In vitro* study: 5, 10 or 25 μg/mL of tetrandrine-induced activation of caspase-3 in t-HSC/Cl-6 cells occurred dose-dependently. *In vivo* study: tetrandrine treatment as well as interferon-r significantly ameliorated the development of fibrosis as determined by lowered serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (T-Bil) and the levels of liver hydroxyproline (Hyp), hyaluronic acid (HA), laminin (LN) and also improved histological findings. The effects of tetrandrine at the concentration of 20 mg/kg were better than the other concentration groups.

CONCLUSION: Tetrandrine promotes the apoptosis of activated HSCs *in vitro*. Tetrandrine administration can prevent liver fibrosis and liver damage induced by thioacetamide in rats *in vivo*, indicating that it might exert a direct effect on rat HSCs.

Key words: Tetrandrine; Apoptosis; Caspase-3; Liver fibrosis; Thioacetamide

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INTRODUCTION

Fibrosis represents the response of the liver to diverse chronic insults such as chronic viral infection, alcohol, immunological attack, hereditary metal overload, parasitic diseases and toxic damage. Because of the worldwide prevalence of these insults, liver fibrosis is common and ultimately results in cirrhosis that is associated with significant morbidity and mortality. Hepatic fibrosis, regardless of the causes, is characterized by an increase in extracellular matrix constituents, leading to complications of portal hypertension, esophageal varices and hepatic failure. The excessive accumulation of extracellular matrix is characteristic of hepatic fibrosis[1-3]. It is accepted that hepatic stellate cells (HSCs) play a central role in the development and resolution of liver fibrosis. HSCs are localized within the space of Disse and function to store retinoids in normal liver. In response to liver damage, HSCs are "activated" to a myofibroblast-like phenotype. It has recently been shown that recovery from established experimental fibrosis can occur through apoptosis of HSCs and is associated with reductions in liver collagen and expression of the tissue inhibitor of metalloproteinases. During chronic liver diseases, HSCs are activated into proliferative, fibrogenic, and smooth muscle α-actin (α-SMA) positive myofibroblasts, resulting in liver fibrosis[4,5]. Recently it was reported that during recovery from acute human and experimental liver injury, a number of activated HSCs undergo apoptosis, and there is a significant decrease in the extent of fibrosis within the same livers in association with this HSC apoptosis[6].

Therefore, induction of HSC apoptosis might be an important therapeutic strategy for hepatic fibrosis, while inhibition of HSC activation might have a role in the prevention of hepatic fibrosis. The development of approaches to prevent fibrotic changes in the liver or reverse the fibrosis is important[7]. However, therapeutic antifibrotic drugs are still at an experimental stage[8,9]. The major problems of antifibrotics are toxicity owing to the chronic administration and the lowered therapeutic effects of the agents used clinically as compared with *in vitro* studies. Therefore, developing antifibrotics from the natural products used in traditional medicine with little acute toxicity, may improve therapies[10].

Tetrandrine is a bis-benzyl isoquiniline alkaloid from the Chinese herb radix *Stephania tetrandra* S Moore. This compound has been characterized pharmacologically to exhibit hypotensive, anti-inflammatory, and immunosuppressive properties, to inhibit lipid peroxidation, and to have an antifibrogenic activity against pulmonary fibroblasts and an inhibitory effect on type I and Ⅲ collagen gene mRNA levels in the lung tissue of rats[11-13]. The dried root of *S. tetrandra* is one of the traditional Chinese medicines that have long been used to treat human liver fibrosis and cirrhosis[14]. Tetrandrine also shows a blocking action of calcium channels, which are known to play an important role in the regulation of hepatic stellate cell contractility, a marked phenotype of activated HSCs[15,16]. For many years, our laboratory has been screening candidate antifibrotic agents from natural products that have been used in traditional medicine to treat liver disease[10,17]. It was previously reported that tetrandrine has an antifibrotic function on liver fibrosis in rats induced by bile duct ligation and scission and tetrandrine exerts a direct inhibitory effect on rat HSCs[18]. We were intrigued to know if tetrandrine could improve the liver injury induced by thioacetamide (TAA). In a preliminary assay, we found that tetrandrine did induce apoptosis in HSCs. The aim of the present study was to explore the sequential pattern of apoptosis and the antifibrotic effect of tetrandrine on hepatic fibrosis induced by TAA in rats. Our results suggest that tetrandrine ameliorates development of fibrosis in a TAA rat model, accompanied by activation of caspase-3 and reduced number of activated HSCs.

MATERIALS AND METHODS

Reagents

Tetrandrine was purchased from Sigma-Aldrich (St Louis, MO, USA) and was dissolved in dimethyl sulphoxide (DMSO). The concentration of tetrandrine used for *in vitro* experiment was prepared by dilution with Williams' medium E (WME; Sigma-Aldrich). DMSO in cells was maintained at 0.5%. Thioacetamide (TAA) was also from Sigma-Aldrich. Hyaluronic acid RIA (HA) kit and laminin RIA (LN) kit were purchased from Shanghai HaiYan Medical Biotechnology Center. Interferon-r was from Livzon Biotechnology Co., Zhuhai, China.

Isolation of hepatic stellate cells and establishment of t-HSC/Cl-6 cell line

The transformed rat hepatic stellate cell line was generated as described by Kim *et al*[19] with some modifications. t-HSC/Cl-6 cells were cultured in WME medium supplemented with 10% fetal bovine serum (FBS; US Biotechnologies Inc., Parkerford, PA, USA) and 100 units/mL penicillin G and 100 μg/mL streptomycin (Gibco-Invitrogen Corp., Grand Island, NY, USA) and maintained at 37℃ with 5% CO_2/95% O_2. Cells were routinely passaged before reaching confluence.

Assay of caspase activity

After different treatments, cells were collected and washed with ice-cold phosphate-buffered saline (PBS). Cell lysates were prepared with caspase colorimetric assay kits (R&D Systems Inc.), according to the manufacturer's instructions. Protein concentrations in t-HSC/Cl-6 cell lysates were determined with a Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). All of the samples were assayed in three independent experiments.

Tetrandrine treatment for TAA induced liver injury

Male Wistar rats (initial body weight 200-220 g) were purchased from the Animal Center of Changchun Agriculture University. All rats were housed and treated in accordance with the recommendations of the American Physiological Society (Council of Europe, 1982). On receipt, they received normal chow and water ad libitum and were maintained at 20℃-25℃, relative humidity of 50%-60% with a 12 h light/dark cycle throughout the experimentation. The rats were divided into six groups of 10 rats each. The hepatic fibrosis was established with TAA, which was diluted and administered to the rats by intraperitoneal injection (200 mg/kg mixed with water) twice weekly. Rats were given orally by gavage at a daily dose of 5, 10 or 20 mg/kg of tetrandrine group. Interferon-r group was given intraperitoneal injection of 5×10^4 U per rat every day. The normal and control groups received equal amounts of saline for 28 d. Three days after the last dose of TAA, the rats were anesthetized and sacrificed. Then blood was obtained by cardiac puncture for serum biochemical testing. Blood samples were kept at room temperature for 1 h and centrifuged at 3000 r/min for 30 min to obtain sera. The livers were immediately removed and a slice of the right lobe was fixed in formaldehyde solution. The rest were divided into equal parts, frozen in liquid nitrogen and stored at -70℃.

Determination of serum and liver biochemical indices

The level of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (T-bil) were measured on an Autodry chemistry analyzer (Spotchem SP4430, Arkray, Kyoto, Japan). While serum hyaluronic acid (HA) and laminin (LN) content in the liver were determined by radioimmunoassay. All procedures were in accordance to the instructions of the manual.

Determination of hydroxyproline contents in liver

The hydroxyproline content in the liver was determined by the method described by Jamall *et al*[20]. Briefly, specimens of the liver were weighed and completely hydrolyzed in 6 mol/L HCl. A fraction of the sample was derivatized using chloramine T solution and Erhlich's reagent, and optical

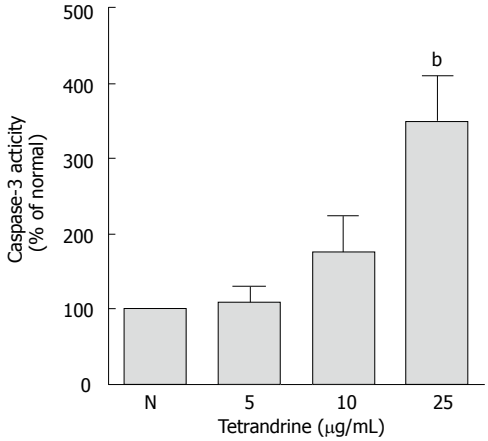

Figure 1 Caspase activity analysis. The caspase activity of normal cells was set at 100% and relative changes in activity are shown in association with drug doses. Values represent the results of three separate experiments. $^{b}P < 0.001$, *vs* normal control values.

density was measured at 558 nm. A standard calibration curve was prepared using trans-4-hydroxy-L-proline. The level of hydroxyproline reflects collagen in the liver.

Histological and immunohistochemical examinations

Liver specimens were preserved in 4% buffered paraformaldehyde and dehydrated in a graded alcohol series. Following xyline treatment, the specimens were embedded in paraffin blocks and cut into 5-μm thick sections, which were placed on plain glass microscopic slides. The sections were then stained with haematoxylin and eosin (HE) or Sirius-red staining, and observed under a light microscope.

Alpha-smooth muscle actin (α-SMA) for detection of activated HSCs was assessed immunohistochemically by the Polymer Detection System using an immunohistological staining kit and anti-α-SMA monoclonal antibodies.

Statistical analysis

All values are expressed as means ± SD. The results were evaluated by one-way ANOVA and Tukey's multiple comparison tests. Statistically significant differences between groups were defined as *P* values less than 0.05. Calculations were performed with the GraphPad Prism program (Graphpad Software, Inc, San Diego, USA).

RESULTS

Caspase-3 activation in tetrandrine -induced apoptosis

To assess the involvement of caspases in t-HSC/Cl-6 cell apoptosis, we detected the enzymatic activity of caspases-3, a downstream caspase. Activation of caspase-3 occurred dose dependently induced by the 5, 10 or 25 μg/mL of tetrandrine (Figure 1).

Liver/weight index

The conditions of rats did not change in control group, but the activity level of the rats was reduced. The general states of rats in other groups were much better than those in control group. Liver/weight index in model group was slightly higher than that in other groups, with no statistical significant difference (data not shown).

Table 1 Serum biochemical values in fibrotic rats induced by TAA treated with tetrandrine for 4 wk

Group	AST (IU/L)	ALT (IU/L)	T-Bil (μmol/L)
Normal	102 ± 24	34 ± 11	11.10 ± 1.29
TAA (200 mg/kg)	356 ± 70	198 ± 32	18.63 ± 2.50
TAA + tetrandrine (5 mg/kg)	266 ± 38[a]	142 ± 29[b]	15.70 ± 1.42[a]
TAA + tetrandrine (10 mg/kg)	259 ± 45[a]	139 ± 29[b]	15.11 ± 2.26[b]
TAA + tetrandrine (20 mg/kg)	227 ± 49[d]	115 ± 30[d]	14.89 ± 0.89[b]
TAA + interferon-r (5×10^4 U)	265 ± 38[a]	137 ± 25[b]	15.43 ± 1.36[a]

$^{a}P < 0.05$; $^{b}P < 0.01$; $^{d}P < 0.001$, *vs* control group.

Serum biochemical parameters

In rats treated with thioacetamide (TAA; 200 mg/kg, ip), serum levels of AST, ALT and T-Bil were increased to 349%, 618% and 168%, respectively, compared with that of normal rats (Table 1). Tetrandrine (5, 10 or 20 mg/kg per day, po, for 4 wk) significantly lowered serum AST, ALT and T-Bil levels in rats intoxicated by TAA. In 5 mg/kg tetrandrine-treated rats, serum AST, ALT and T-Bil levels were lowered to 74.7% ($P < 0.05$), 71.7% ($P < 0.01$) and 84.0% ($P < 0.05$) compared with that of control fibrotic rats, respectively. In 10 mg/kg tetrandrine treated rats, AST, ALT and T-Bil levels were lowered to 72.7% ($P < 0.05$), 70.2% ($P < 0.01$) and 81.1% ($P < 0.01$) compared with that of control fibrotic rats, respectively. In 20 mg/kg tetrandrine treated rats, AST, ALT and T-Bil levels were lowered to 63.7% ($P < 0.001$), 58.1% ($P < 0.001$) and 79.9% ($P < 0.01$) compared with that of control fibrotic rats, respectively. In interferon-r treated fibrotic rats, AST, ALT and T-Bil levels were lowered to 74.4% ($P < 0.05$), 69.1% ($P < 0.01$) and 82.8% ($P < 0.05$) compared with that of control fibrotic rats, respectively.

Hydroxyproline matrix contents in the liver

As shown in Figure 2A, liver contents of hydroxyproline increased to 229%, 4 wk after TAA-treatment ($P < 0.001$). Compared with the control fibrotic rats, in fibrotic rats treated with 5, 10 or 20 mg/kg tetrandrine, the hydroxyproline contents in the liver reduced to 73.3% ($P < 0.05$), 67.8% ($P < 0.01$) or 61.5% ($P < 0.001$), respectively. In interferon-r treated fibrotic rats, the hydroxyproline contents in the liver reduced to 64.6% ($P < 0.001$).

Extracellular matrix content in serum

As shown in Figures 2B and 2C, serum contents of HA and LN increased to 251% and 233%, 4 wk after TAA treatment ($P < 0.001$). In the fibrotic rats treated with 5, 10, 20 mg/kg tetrandrine or interferon-r, HA content in the serum reduced to 68.2% ($P < 0.05$), 62.32% ($P < 0.01$), 50.73% or 47.09% ($P < 0.001$), respectively, compared with the control fibrotic rats. Compared with the control fibrotic rats, in fibrotic rats treated with 5, 10, 20 mg/kg tetrandrine or interferon-r, LN content in the serum reduced to 53.59% ($P < 0.05$), 55.09% ($P < 0.01$), 65.09% ($P < 0.01$) or 68.30% ($P < 0.05$), respectively.

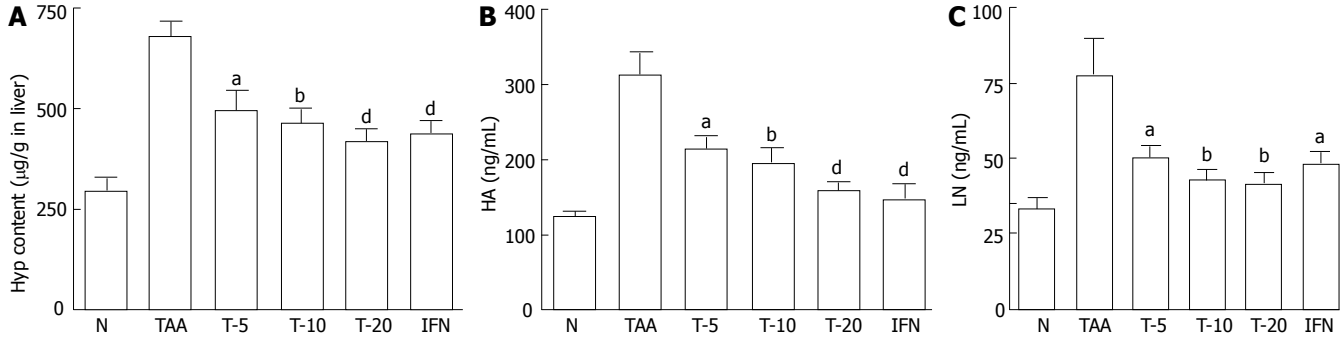

Figure 2 Liver hydroxyproline (**A**); HA (**B**) and LN (**C**) content of the TAA rats treated with tetrandrine. T-5: treated with 5 mg/kg tetrandrine; T-10: treated with 10 mg/kg tetrandrine; T-20: treated with 20 mg/kg tetrandrin; IFN: treated with interferon-r 5 × 10^4 U. Results represent the mean ± SD. $^aP < 0.05$; $^bP < 0.01$; $^dP < 0.001$, *vs* control group.

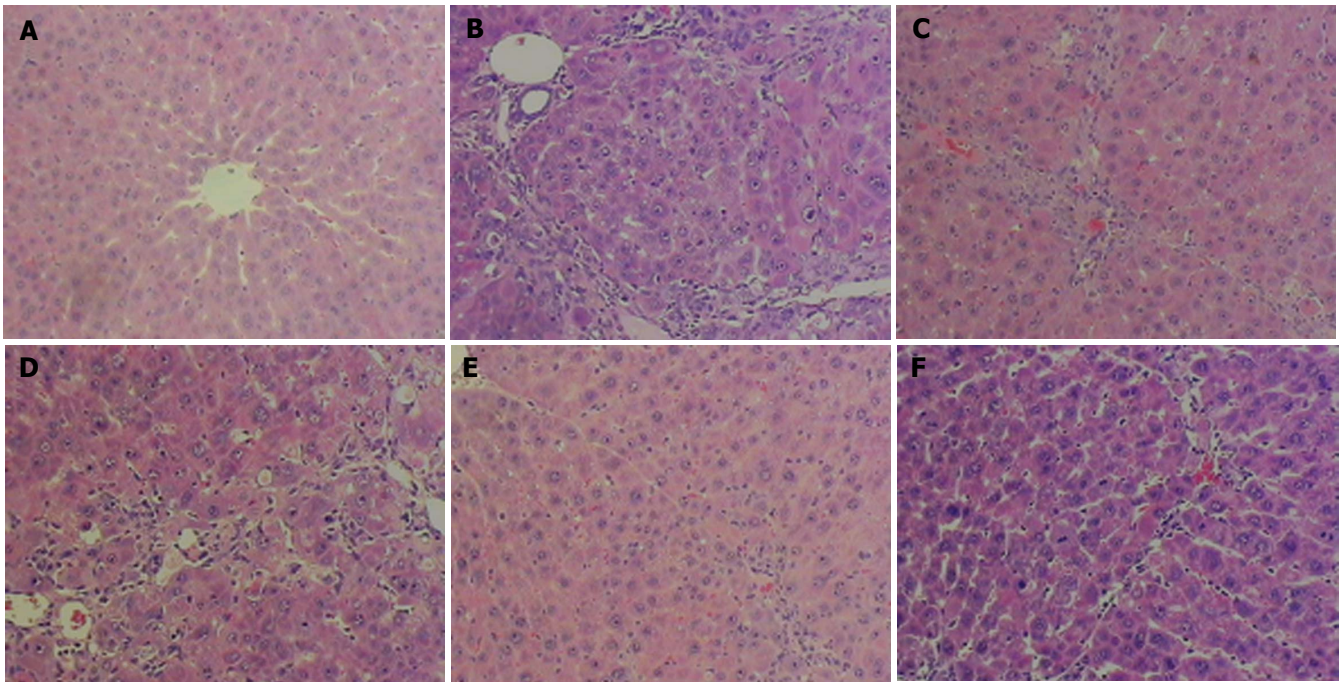

Figure 3 Light microscopic appearance (HE × 100) of fibrotic rat liver induced by TAA treated with tetrandrine for 4 wk. **A**: normal group; **B**: control group; **C**: TAA group with tetrandrine (5 mg/kg); **D**: TAA group with tetrandrine (10 mg/kg); **E**: TAA group with tetrandrine (20 mg/kg); **F**: TAA group with interferon-r (5 × 10^4 U).

Morphological changes in the liver

In control fibrotic group, the liver stained with HE and Sirius-red showed inflammation and an extensive accumulation of collagens (Figures 3B and 4B), fibrotic septa were increased and seen between areas of portal to portal and portal to central vein in some parts of liver lobules, and in some serious units pseudolobules presented. In tetrandrine and interferon-r treated fibrotic rats, there was a tendency towards less pronounced destruction of the liver architecture, compared with control fibrotic rat liver (Figure 3C-F, and Figure 4C-F).

As shown in Figures 3 and 4, TAA treatment for 4 wk resulted in liver injury, with loss of normal lobular architecture and a marked increase of collagen deposition throughout the liver. However, tetrandrine treatment resulted in reduced collagen deposition in the liver of TAA-treated rats.

HSC activation was examined immunohistochemically by determining α-SMA-positive areas in injured rat livers treated with TAA. Considerable expression was detected in areas of centrilobular and periportal fibrotic bands in TAA treated rats (Figure 5B). In normal livers, collagen was observed only around the central and portal veins (Figure 5A). Activated HSCs were characterized by expression of α-SMA. In tetrandrine-treated or interferon-r groups, the expression of α-SMA was much lower than that in control fibrotic group and the distribution of α-SMA positive cells was similar to that of collagen in the liver (Figure 5C-F). In contrast, 20 mg/kg tetrandrine-treated rats notably reduced the positive areas of α-SMA in the livers of rats treated with TAA.

DISCUSSION

Thioacetamide (TAA) is a thiono-sulfur containing compound, undergoing an extensive metabolism to acetamide and TAA-S-oxide. Whereas acetamide is devoid of liver-necrotizing properties, TAA-S-oxide

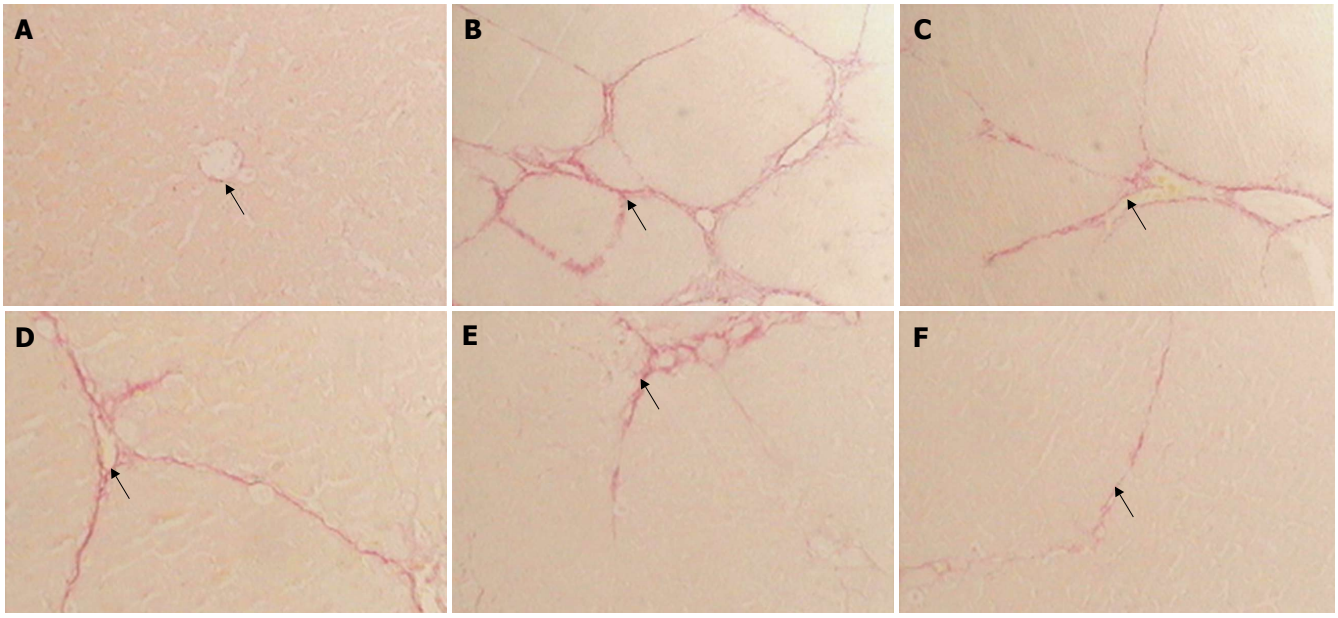

Figure 4 Light microscopical appearance (sirius red staining) of fibrotic rat liver induced by TAA treated with tetrandrine for 4 wk. Sirius red stains most hepatic collagens. **A**: normal group; **B**: control group; **C**: TAA group with tetrandrine (5 mg/kg); **D**: TAA group with tetrandrine (10 mg/kg); **E**: TAA group with tetrandrine (20 mg/kg); **F**: TAA group with interferon-r (5×10^4 U).

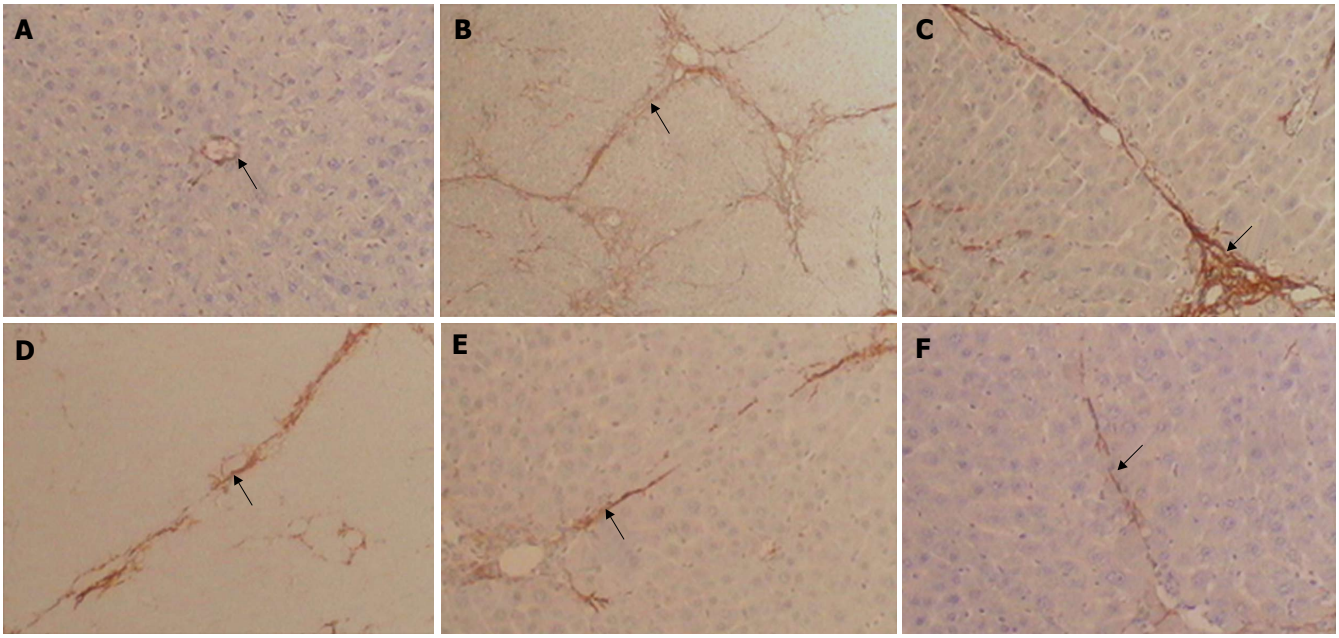

Figure 5 Representative liver section showing reaction of alpha-smooth muscle cell like actin (α-SMA) with mouse anti-α-SMA monoclonal antibody. α-SMA immunoreactivity (arrows) was seen in stellate cells in the fibrotic area. **A**: normal group; **B**: control group; **C**: TAA group with tetrandrine (5 mg/kg); **D**: TAA group with tetrandrine (10 mg/kg); **E**: TAA group with tetrandrine (20 mg/kg); **F**: TAA group with interferon-r (5×10^4 U).

is further metabolized, at least in part, by cytochrome P-450 monooxygenases to other products, including a polar product that is thought to be the sulfene, TAA-S-dioxide, a highly reactive compound[21]. Therefore, TAA is a typical hepatotoxin and causes centrilobular necrosis by generation of ROS. Although TAA is known mainly to cause necrosis in the liver, it has been reported recently that TAA induced apoptosis in rat liver within a few hours after its administration[22]. The present study showed that a low dose of TAA induced apoptosis in the pericentral areas, whereas a high dose of TAA induced necrosis. Excessive ROS causes necrosis.

The mechanism by which tetrandrine inhibits rat hepatic stellate cell activation is not clear. Tetrandrine administration has been shown to block the calcium channels in various cell types[23]. Moreover, activated hepatic stellate cells have receptors for a number of vasoconstrictor substances such as endotheline, angiotensin Ⅱ, vasopressin, thrombin and thromboxan A2[24-26]. Therefore, it is likely that the inhibitory effect of tetrandrine on hepatic stellate

cell activation is, at least in part, through the blocking of calcium channels. However, further study is needed to understand the precise mechanism of tetrandrine on activated hepatic stellate cells.

Here we showed that tetrandrine is toxic against t-HSC/Cl-6 cells and antifibrotic. Previous studies demonstrated that tetrandrine induced apoptosis in t-HSC/Cl-6 cells, and activated rat hepatic stellate cells transformed by SV40. The morphologies of tetrandrine-treated cells revealed the typical features of apoptosis and these apoptotic phenomena were further confirmed by DNA laddering and annexin v-propidium iodide double staining[27]. Previous reports have demonstrated that the caspase family proteases play essential roles in the process of apoptosis[28]. Caspase-3, which has a short prodomain and is localized near nuclei, cleaves substrates at the downstream end of the cascade[29]. This study demonstrated that the activation of caspase-3 is involved in tetrandrine-induced cell death including apoptosis. Moreover, we have shown that tetrandrine is also effective in mediating HSC apoptosis *in vivo* after the development of fibrosis. It suggests that apoptosis of activated HSCs plays a key role in resolution of hepatic fibrosis.

Our data provide evidence that tetrandrine will effectively induce HSC apoptosis. In addition, we have demonstrated evidence that induction of apoptosis in HSCs enhances the resolution of experimental fibrosis. Taken together, these observations suggest that a strategy based on inducing HSC apoptosis may be an effective antifibrotic approach. The effects of tetrandrine at 20 mg/kg dose are better than the other groups. In conclusion, our results demonstrate that tetrandrine administration could prevent liver fibrosis and liver damage induced by TAA in rats. In this study, tetrandrine reduced the degree of hepatocellular injury as determined by lower serum levels of AST, ALT, T-bil and liver hydroxyproline, serum HA and LN, and also improved morphological structure of the fibrotic liver. Our study shows a direct inhibitory effect on rat HSC activation and an antifibrogenic potential of tetrandrine in fibrotic rats induced by TAA. Tetrandrine may have therapeutic effects in human liver fibrosis.

COMMENTS

Background

Fibrosis represents the response of the liver to diverse chronic insults such as chronic viral infection, alcohol, immunological attack, hereditary metal overload, parasitic diseases and toxic damage. It is accepted that HSCs play a central role in the development and resolution of liver fibrosis. Recently it was reported that during recovery from acute human and experimental liver injury, a number of activated HSCs undergo apoptosis, and there is a significant decrease in the extent of fibrosis within the same livers in association with this HSCs apoptosis.

Research frontiers

Tetrandrine promotes HSC apoptosis in hepatic fibrosis. The study of its mechanism might lead to a new approach for fibrosis therapy. Further studies of the roles and regulation of HSC apoptosis and its possible mechanism are important for understanding the mechanism of resolution of liver fibrosis.

Innovations and breakthroughs

In this research, we investigated the effect of tetrandrine on the apoptotic death of rat HSCs *in vitro* and whether tetrandrine can prevent thioacetamide-induced fibrosis *in vivo*. The results showed that tetrandrine promotes the apoptosis of activated HSCs *in vitro* and prevent liver fibrosis and liver damage induced by thioacetamide in rats *in vivo*.

Applications

The results provide significant evidence illustrating the key feature of recovery from liver fibrosis is HSC apoptosis.

Terminology

HSCs: hepatic stellate cells; Caspase: Caspases, closely associated with apoptosis, are aspartate-specific cysteine proteases and members of the interleukin-1b-converting enzyme family. The activation and function of caspases, involved in the delicate caspase-cascade system, are regulated by various kinds of molecules, such as the inhibitor of apoptosis protein, Bcl-2 family proteins, calpain, and Ca^{2+}.

Peer review

In this manuscript by Yin *et al*, the authors report effect of tetrandrine on the treatment of liver fibrosis-induced by thioacetamide in rats *in vivo* and *in vitro*. They employed assay of caspase activity to observe the enzymatic activity of caspases-3, a downstream caspase, occurred dose-dependently and investigated tetrandrine treatment as well as interferon-r significantly ameliorates the development of fibrosis as determined by lowered serum levels of AST, ALT, T-Bil and the levels of liver hydroxyproline, HA, LN and also improved histological findings. Their data may be critical for the study of the mechanism of resolution of rat liver fibrosis and shed new light on the liver fibrosis therapy.

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CLINICAL RESEARCH

Role of CARD15, DLG5 and OCTN genes polymorphisms in children with inflammatory bowel diseases

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Abstract

AIM: To investigate the contribution of variants of CARD15, OCTN1/2 and DLG5 genes in disease predisposition and phenotypes in a large Italian cohort of pediatric patients with inflammatory bowel diseases (IBD).

METHODS: Two hundred patients with Crohn's disease (CD), 186 ulcerative colitis (UC) patients, 434 parents (217 trios), and 347 healthy controls (HC) were studied. Polymorphisms of the three major variants of CARD15, 1672C/T and -207G/C SNPs for OCTN genes, IGR2096a_1 and IGR2198a_1 SNPs for the IBD5 locus, and 113G/A variant of the DLG5 gene were evaluated. Potential correlations with clinical sub-phenotypes were investigated.

RESULTS: Polymorphisms of CARD15 were significantly associated with CD, and at least one variant was found in 38% of patients (15% in HC, OR = 2.7, $P < 0.001$). Homozygosis for both OCTN1/2 variants was more common in CD patients (1672TT 24%, -207CC 29%) than in HC (16% and 21%, respectively; $P = 0.03$), with an increased frequency of the TC haplotype (44.8% *vs* 38.3% in HC, $P = 0.04$). No association with the DLG5 variant was found. CD carriers of OCTN1/2 and DLG5 variants more frequently had penetrating disease ($P = 0.04$ and $P = 0.01$), while carriers of CARD15 more frequently had ileal localization ($P = 0.03$). No gene-gene interaction was found. In UC patients, the TC haplotype was more frequent (45.4%, $P = 0.03$), but no genotype/phenotype correlation was observed.

CONCLUSION: Polymorphisms of CARD15 and OCTN genes, but not DLG5 are associated with pediatric onset of CD. Polymorphisms of CARD15, OCTN, and DLG5 genes exert a weak influence on CD phenotype.

Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; CARD15; DLG5; Carnitine/organic cation transporter gene

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INTRODUCTION

The inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC) have become increasingly common causes of morbidity in children[1,2]. Their prevalence has been on rise with children and adolescent currently accounting for approximately 30% of IBD patients[3,4]. Although the precise etiology of IBD remains elusive, both animal models and human studies point towards a strong genetic susceptibility.

The characterization of the NOD2/CARD15 gene at the IBD1 locus (16q12) in 2001 as the first gene conferring susceptibility to CD represented a milestone observation[5-7]. Although the role of NOD2 protein in CD remains under evaluation[8], it is surely involved in the innate immune response to bacterial pathogens[9,10]. CARD15 major variants are mostly associated with ileal disease and stricturing behaviour[7]. In pediatric series, a correlation with ileal localization[11-14], stricturing behaviour[12,13,15] early surgery[12,13] growth delay[13,14], and higher disease activity[13,16] has been reported, although with conflicting findings[17-19].

Functional polymorphisms in the carnitine organic cation transporter cluster (OCTN1/2)[20] on chromosome 5q31 and mutations in disc large gene 5 (DLG5)[21] on

the long arm of chromosome 10 (10q23) have also been reported to be associated with CD. Linkage on the 5q31 genomic area, the so-called IBD5 risk haplotype, was first reported by Rioux *et al*[22]. This 250-kilobase region contains 5 genes and multiple genetic variants with strong linkage disequilibrium[22]. A number of studies have confirmed the association of the IBD5 risk haplotype with CD[23-28] and UC[28,29]. Peltekova *et al*[20] found two novel single nucleotide polymorphisms (SNPs) with functional mutations, generating a 2-alleles risk haplotype (TC) predisposing to CD. Replication studies have found inconsistent genotype/phenotype correlation[30-34] with some concordance for presence of perianal fistulae[31,33], which is similar to that observed for the IBD5 risk haplotype[25,28]. More importantly, although Peltekova *et al*[20] in preliminary functional studies demonstrated that the OCTN1/2 variants resulted in impaired transport function of various organic cations and carnitine, but the precise link with IBD pathogenesis remains unexplained. All subsequent replication studies were unable to confirm the association of TC haplotype in the absence of the IBD5 risk haplotype[30-33], making the assumption of OCTN variants as causal genes still disputable[35].

Concerning the DLG5 gene, Hampe *et al*[36] initially described a susceptibility locus on chromosome 10 in a genome linkage study. Stoll *et al*[21] further narrowed down this risk region and identified two distinct haplotypes associated with IBD and CD. More specifically, the "risk haplotype D" was defined by a single non-synonymous SNP 113G→A; this nucleotide change resulting in the amino acid substitution R30Q, probably impedes scaffolding of the protein evaluated in silico analysis. A collaborative study[37], investigating two independent case-control cohorts and one family-based collection confirmed the association in 2 of the 3 cohorts, although with a modest effect on the relative risk to IBD (OR = 1.25). Other groups, however, reported negative findings[30,31,38]. More recently, a more thorough evaluation of the previously reported cohorts with other large control populations, has demonstrated that the DLG5 gene is a risk factor for CD only for men (OR = 2.49, CI 1.5-4), but not for women (OR = 1.01)[39]. Intriguingly, this difference is driven by a gender-dependent transmission ratio distortion among healthy controls (frequency of A allele: men 5.2%, women 11.3%).

In young patients, IBD might offer the opportunity to understand the pathopysiology of the diseases in a form that is closer to their underlying cause and mechanisms than in adult, because of a lower influence of environmental risk factor (i.e. smoking). Paradoxically, in contrast to the data in adults, there are studies mainly looking at CARD15 polymorphisms[11-19,40], while being scarce and conflicting on OCTN1/2 and DLG5 genes[41-44]. Moreover, many pediatric series are flawed by a small sample size[11,12,15,16,18,19,43,44], little information on UC patients[13,15,41], and lack of control populations[11,12,16,17,19].

In this study we have investigated the contribution of variants of CARD15, OCTN1/2 and DLG5 genes in IBD predisposition in a large Italian pediatric cohort. We also examined the genotype/phenotype relationships and gene-gene interactions.

MATERIALS AND METHODS

Study population

Patients under age of 18 at diagnosis were recruited into this study from 16 tertiary pediatric and gastroenterologic centres in Italy, in cooperation with a multicenter study endorsed by the SIGENP (Italian Society for Pediatric Gastroenterology, Hepatology and Nutrition). Ethical approval was obtained at all participating centres. All study participants were Caucasian and their parents gave a written informed consent.

The diagnosis of CD or UC was established by conventional clinical, radiological, endoscopic and histological criteria[45]. Indeterminate colitis was excluded from the study.

The recruited population was comprised of 200 patients with CD and 186 patients with UC. Blood samples were also taken from 434 parents, making 217 complete family trios (108 CD and 109 UC, respectively) available for analysis.

A total of 347 adult healthy unrelated blood donors (184 male, mean age 32 years, range 20-45) who served as controls were randomly recruited from their sites to minimize the potential geographic differences among San Giovanni Rotondo (Southern Italy, $n = 147$), Rome (Centre, $n = 100$) and Padova (Northern Italy, $n = 100$).

Data collection

Retrospective data were collected on patients using a standardized questionnaire obtaining information on patient and parental smoking details (at least one cigarette/d), ethnicity, and IBD family history. Additional clinical data were collected on patient demographics, age at IBD diagnosis, medications, extra-intestinal manifestations and need for resective surgery. Standard investigations employed in these patients were upper GI endoscopy, ileo-colonoscopy, and barium examination. CD disease location and behavior were categorized according to the Vienna classification[46]. Presence of perianal fistulae was analysed separately according to the Montreal's proposal[47].

In all patients, weight and height percentiles were calculated at diagnosis. Presence of growth retardation was defined as a reduction below the 5th percentile for weight, height or both.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes according to standard protocols[48], and genotyped in the laboratory of San Giovanni Rotondo Hospital.

Genotyping for Arg702Trp and Leu1007fsinsC common CARD15 variants was performed by DHPLC (denaturing high performance liquid chromatography, Wave System, Transgenomic Ltd, UK) and restriction fragment length polymorphisms (RFLP) assay was used for Gly908Arg (G/C). The 380 base pairs PCR product was digested with Hha I (New England Biolabs, Ipswich, MA), yielding 2 fragments of 138 and 242 base pairs in the presence of C allele and visualized on 2% (w/v) agarose gel. One hundred random samples were also confirmed by sequencing on ABI 310 DNA sequencer (Applied

Table 1 Primers sequences, methodology, and restriction enzymes used for genotyping

Gene locus	SNPs		Sequence	Methods/enzyme
CARD15	R702W	For	5′ACCTTCAGATCACAGCAGCC3′	DHPLC
		Rev	5′GCTCCCCCATACCTGAAC3′	
	G908R	For	5′AAGTCTGTAATGTAAAGCCAC3′	RFLP/Hha Ⅰ
		Rev	5′CCCAGCTCCTCCCTCTTC3′	
	L1007fsinsC	For	5′CTCACCATTGTATCTTCTTTTC3′	DHPLC
		Rev	5′GAATGTCAGAATCAGAAGGG3′	
IBD5	IGR2198a_1	For	5′GGGGCAATTCTATGAGGACA3′	RFLP/Nla Ⅲ
		Rev	5′CCAGAGACACTGGGACATCA3′	
	IGR2096a_1	For	5′GTAGCGAGAGGCTCCACAGT3′	RFLP/Dra Ⅰ
		Rev	5′TCCTCCATGCTACTGCTCTG3′	
OCTN1	C1672T	For	5′GGGTAGTCTGACTGTCCTGATTG3′	TaqMan
SLC22A4		Rev	5′TCTGGAAGAGTCATTCCCAAACTTTC3′	
		VIC	5′AAGGGTGAGGATTC3′	
		FAM	5′AAGGGTGAAGATTC3′	
OCTN2	G207C	For	5′CCGCTCTGCCTGCCA3′	TaqMan
SLC22A5		Rev	5′GCGGCTGGCCTTACATAGG3′	
		VIC	5′CAGGCCCGGAACC3′	
		FAM	5′CAGGCCCGCAACC3′	
DLG5	R30Q		C_7432738_10	TaqMan

DHPLC: Denaturing high performance liquid chromatography; RFLP: Restriction fragment length polymorphisms; TaqMan: ABI Prism 7700 sequencer detector.

Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations.

For OCTN1/2 genotyping, the SLC22A4 1672C/T and SLC22A 5-207G/C primers were designed using Primer Express V 1.5 (Applied Biosystems). SNPs were genotyped using the Taqman system ABI PRISM 7700 (Applied Biosystems, Foster City, CA, USA) as previously described[33].

For the IBD5 locus, the IGR2096a_1 and IGR2198a_1 SPNs were selected from the haplotype originally described by J. Rioux[22] (http://www.genome.wi.mit.edu/IBD5). Genotyping was performed by restriction fragment analysis as previously described[28]. Results were confirmed by direct sequencing of representative samples for each genotype, using ABI cycle sequencing kit V 1.1 and the ABI 310 genetic analyzer.

For the DLG5 gene, we genotyped the 113 G→A variant (rs1248696) tagging the haplotype D, the over-transmitted haplotype in the study by Stoll *et al*[21], with 7700 TaqMan bi-allelic discrimination system. PCR reactions (15 mL) were performed in 1 × TaqMan Universal PCR Master Mix, 1 × Genotyping Assay Mix, and 50 ng of genomic DNA. After 2 min at 50℃, and 10 min at 94℃ initial denaturation, reaction was amplified for 40 cycles: 15 s at 94℃, and 60 s at 60℃.

A summary of primer sequences and methods is depicted in Table 1.

Detection of antibodies

Whenever serum samples were available, anti-nuclear cytoplasmatic antibodies (ANCA) were tested by a standard immuno-fluorescence technique[49]. In addition, presence of anti-saccharomyces cerevisiae antibodies (ASCA) was investigated by means of a commercial ELISA assay (Quanta Lite™ ASCA, Inova Diagnostics Inc, San Diego, USA)[50].

Data analysis

Comparison of allele and genotype frequencies was performed by Chi-square and Fisher exact tests when appropriate. Student's *t* test was used to compare means of continuous variables with the SPSS software ver. 11.5. Tests for Hardy-Weinberg equilibrium, linkage disequilibrium, haplotype frequency analysis and transmission disequilibrium were performed by the Haploview Software ver. 3.2 (http://www.broad.mit.edu/personal/jvbarret/haploview).

Power calculation was performed using the PS software (http://biostat.mc. vanderbilt. edu/twiki/bin/view/Main/PowerSampleSize).

Genotype-phenotype associations were analysed by means of univariate and multivariate stepwise logistic regression with the SPSS software. This approach allowed to take into account a dose-response effect (heterozygote or homozygote), the possible interactions between genes, and the effect of potential confounding variables (duration of follow-up, disease localization, etc.). The obtained *P* values were corrected (exact *P* value) for multiple comparisons. A possible interaction between CARD15, OCTN1/2, and DLG5 genes was also investigated.

RESULTS

Clinical findings

Main clinical characteristics of patients included in the study are depicted in Table 2.

The 200 CD patients had a mean age of 21 ± 8 years (range 1-18) with a slight male predominance (male/female = 115/85). The 186 UC patients had a mean age of 19 ± 9 years (range 1-18) with a female predominance (73/113). The mean age at diagnosis and the mean duration of follow-up (8-9 years) were similar between the two groups of patients. Eighteen percent

Table 2 Clinical and demographic characteristics of patients with CD and UC as evaluated in last follow-up (mean ± SD)

	Crohn's disease (n = 200)		Ulcerative colitis (n = 186)
Age (yr)	21 ± 8		19 ± 9
Age at diagnosis (yr)	12 ± 4		11 ± 5
Duration of follow-up (yr)	9 ± 7		8 ± 8
Sex (male/female)	115/85		73/113
Localization CD: n (%)			
- Ileum	38 (19)		
- Ileo-colon	116 (58)		
- Colon	45 (23)		
- Upper G-I tract	35 (18)[1]		
Localization UC: n (%)			
- Rectum-sigmoid			44 (24)
- Left colon			54 (29)
- Pancolitis			88 (47)
Disease type CD: n (%)	(Vienna)	(Montreal)	
- Inflammatory	104 (52)	135 (67)	
- Stricturing	40 (20)	45 (23)	
- Penetrating	56 (28)	20 (10)	
Growth delay: y/n (%)	60/84 (42)		21/126 (14)
Surgery: y/n (%)	51/149 (26)		13/137 (7)
EIM: y/n (%)	79/121 (40)		46/140 (25)
Perianal fistulae: y/n (%)	36/164 (18)		1/185 (0.5)
Family history: y/n (%)	18/182 (9)		22/164 (12)
ASCA: y/n (%)	77/32 (71)		18/57 (24)
ANCA: y/n (%)	18/86 (17)		65/50 (57)
Smoking y/n (%)	44/156 (22)		40/146 (21)

EIM: Extra-intestinal manifestations. [1]One patient had upper G-I involvement only.

of CD had an upper G-I involvement. The incidence of resective surgery (26% *vs* 7%, $P < 0.01$), extra-intestinal manifestations (40% *vs* 25%, $P = 0.002$), growth delay (42% *vs* 14%, $P < 0.001$) and perianal fistulae (18% *vs* 0.5%, $P < 0.001$) was significantly higher in CD than in UC patients. In the latter group, almost half of the patients had a pancolitis (47%). The incidence of ASCA and ANCA was 71% and 17% in CD patients, and 24% and 57% in UC patients, respectively, with a significant difference ($P < 0.0001$ and $P < 0.0001$, respectively).

Genotyping findings

The error rate of genotyping (defined as the percentages of disagreement in genotypes among 200 random samples tested in duplicate) was < 1% for all SNPs.

All genotypes in patients and healthy controls were in Hardy-Weinberg equilibrium. A strong linkage disequilibrium (D' > 91) was identified between the two OCTN1/2 variants, and between each OCTN1/2 variant and the IBD5 risk haplotype markers as well ($r^2 > 0.62$, $P < 0.001$) (Figure 1).

Table 3 summarizes the distribution of genotypes for the CARD15 variants (Arg702Trp, Gly908Arg and Leu1007fsinsC), OCTN1/2 variants (SLC22A4 and SLC22A5), the IBD5 risk haplotype SNPs (IGR2096a_1 and IGR2198a_1), and DLG5 gene polymorphism (113G→A).

Regarding the CARD15 gene, all three variants were frequently seen in CD patients as compared to controls ($P < 0.001$). By combining the three variants,

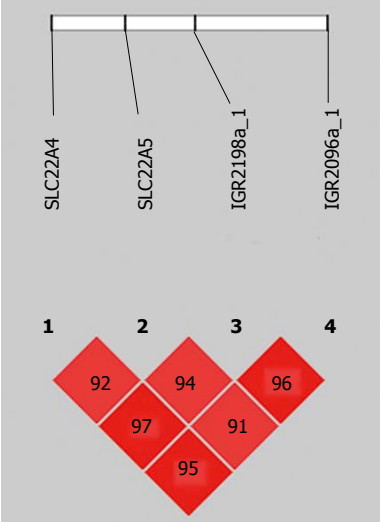

Figure 1 Evaluation of linkage disequilibrium in control subjects between IBD5 risk haplotype SNPs and OCTN1/2 variants ($r^2 > 0.62$; $P < 0.001$).

38% of CD patients had at least one mutation, and 15% had two mutations (homozygosis or compound heterozygosis). Respective figures in healthy controls were 15% and 1%. In carriers of one mutation, the odd risk was 2.7 (CI = 1.7-4.2; $P < 0.001$) while that for two mutations was 17 (CI = 5-58; $P < 0.001$).

As for the OCTN1 and OCTN2 variants, they were both significantly increased in CD patients for the homozygous genotype (1672TT: 24% *vs* 16% in controls, $P = 0.03$) (-207CC: 29% *vs* 21% in controls, $P = 0.03$). Moreover, the two-point haplotype, consisting of the 1672T and -207C alleles (OCTN-TC), was associated with CD (44.8% *vs* 38.3% in controls, $P = 0.043$). The 1672TT (22% *vs* 16% in controls) and -207CC (29% *vs* 21% in controls) genotypes were also increased in UC patients, the difference being statistically significant ($P = 0.10$ and $P = 0.05$, respectively). Accordingly, the frequency of TC haplotype was significantly increased also in UC patients (45.4% *vs* 38.3 in controls, $P = 0.03$).

The markers of IBD5 (IGR2096a_1 and IGR2198a_1) risk haplotype were both increased for the homozygous genotype in CD patients compared to controls, although with this sample size, only the AA genotype of the SNP IGR2096a_1 was significantly increased (23% *vs* 14% of controls, $P = 0.01$). Among individuals lacking the IBD5 risk haplotype (homozygous with respect to the non-risk associated allele of IGR2096a_1), only 7% of patients with CD and 5% of controls carried the TC haplotype, while in presence of the IBD5 risk haplotype, the TC haplotype was found in 61.9% of CD patients and 58.7% of controls. Similar data were obtained in UC patients (data not shown, available on request).

The distribution of 113G→A polymorphism of DLG5 was similar in patients and controls. No significant differences in allele or genotypes frequencies were noted. We also attempted to analyse the data based on gender of cases and controls based on the recent findings of male predominance[39], but results did not differ.

TDT analysis

TDT analysis of UC trios did not show significant transmission distortion of any genetic markers. As expected, a significant over-transmission towards affected

Table 3 Genotype distributions of CARD15, OCTN1-2, DLG5 genes, and IBD5 locus polymorphisms

SNPs	Genotype	Crohn's disease	*P* value OR 95% IC	Controls	*P* value OR 95% IC	Ulcerative colitis
CARD15			TT and CT *vs* CC			
R702W	TT	5 (3)	$P < 0.001$	1 (0)	NS	0 (0)
	CT	38 (19)	3.47 (2.06-5.86)	25 (7)		17 (9)
	CC	153 (78)		321 (93)		167 (91)
G908R	CC	2 (1)	CC and CG *vs* GG	0 (0)	NS	1 (1)
	CG	25 (13)	$P < 0.001$	18 (5)		12 (7)
	GG	170 (86)	2.90 (1.55-5.42)	329 (95)		171 (93)
1007fsInsC	CC	1 (1)	CC and CG *vs* GG	0 (0)	NS	0 (0)
	CG	25 (13)	$P = 0.0005$	11 (3)		10 (5)
	GG	172 (87)	4.62 (2.23-9.57)	336 (97)		176 (95)
OCTN1/2						
C1672T	TT	42 (24)	TT *vs* CT and CC	57 (16)	$P = 0.10$	36 (22)
	CT	79 (46)	$P = 0.03$	178 (51)		76 (47)
	CC	52 (30)	1.63 (1.04-2.56)	112 (32)		49 (30)
-G207C	CC	50 (29)	CC *vs* CG and GG	72 (21)	$P = 0.05$	46 (29)
	CG	84 (49)	$P = 0.035$	183 (53)		77 (48)
	GG	38 (22)	1.57 (1.03-2.38)	92 (27)		38 (24)
IBD5						
IGR2198a_1	GG	40 (21)	GG *vs* CG and CC	55 (16)	$P = 0.09$	39 (22)
	CG	93 (49)	$P = 0.13$	175 (50)		86 (48)
	CC	58 (30)		117 (34)		54 (30)
IGR2096a_1	AA	44 (23)	AA *vs* AC and CC	50 (14)	NS	32 (18)
	AC	89 (47)	$P = 0.01$	176 (51)		91 (51)
	CC	58 (30)	1.78 (1.13-2.79)	121 (35)		56 (31)
DLG5						
113G/A	AA	1 (1)	NS	2 (1)	NS	1 (1)
	AG	16 (10)		57 (16)		26 (15)
	GG	148 (90)		288 (83)		144 (84)

Table 4 Analysis of possible interaction between OCTN TC-haplotype and DLG5 variant with presence (or absence) of at least one CARD15 variant

		CD		UC		Controls	
CARD15		DLG5	OCTN TC haplotype (%)	DLG5	OCTN TC haplotype (%)	DLG5	OCTN TC haplotype (%)
CARD15+	DLG5+	5 (3)	42.6	7 (4)	35.2	11 (3)	44.8
	DLG5-	56 (34)		27 (16)		38 (12)	
CARD15-	DLG5+	12 (7)	46.2	20 (12)	48.7	44 (13)	39.1
	DLG5-	92 (56)		116 (68)		241 (72)	

DLG5-positive was defined as the AA or AG genotypes. Data expressed as absolute values or percentages (between brackets). All comparison had a *P* value > 0.05.

offspring was observed for CARD15 variants in CD trios: the Arg702Trp (transmitted/untrasmitted [T/U], 24/8, $P = 0.004$) and Leu1007fsinsC variants (T/U, 14/2, $P = 0.027$) were significantly over-transmitted. We did not observe distortion of transmission towards affected offspring for the Gly908Arg CARD15 variant, OCTN1/2 variants, and for the two IBD5 risk-haplotype-tagging SNPs IGR2198a_1 and IRG2096a_1. Regarding the DLG5 variant, an over-transmission of the wild-type allele of the 113G→A SNP towards affected offspring was found (T/U, 21/9, $P = 0.02$).

Gene-gene interaction

For the evaluation of gene-gene interactions, all subjects were stratified according to their CARD15 genotype. Genotypes and allele frequencies for the investigated variants of OCTN1/2 and TC haplotype, and for G113A variant of DLG5 were not different between CARD15-positive (carrying at least 1 CARD15 variant) and CARD15-negative subjects (Table 4). Similarly, no interactions between OCTN1/2 and DLG5 variants were found (data not shown, available on request).

Genotye-phenotype analysis

For the analysis of possible correlation between genotype and phenotype, patients were classified on the basis of presence or absence of at least one CARD15 variant, TC haplotype, and DLG5 risk genotype (carriers of the A allele). Data are shown in Table 5 and Table 6. CD patients carrying the CARD15 variants had more frequently ileal localization and less frequently a colonic localization (OR = 2.8, $P = 0.029$). Carriers of TC haplotype of OCTN1/2 variants had more occurrence of a penetrating disease (48.7% *vs* 43.5%) as compared with inflammatory/stricturing behaviour; this difference was statistically significant when perianal fistulae were not taken into account (62% *vs* 43%; $P = 0.038$) according to Montreal's classification. Similarly, a more frequent penetrating disease

Table 5 Genotype-phenotype correlation in CD patients evaluated by comparisons of carriers and non-carriers of at least one CARD15 variant (CARD15+), AA/AG genotypes of DLG5 variant (DLG5+) and presence of TC haplotype of OCTN1/2 variants (TC+)

CD *n* = 200	CARD15+ (*n* = 75)	CARD15- (*n* = 121)	DLG5+ (*n* = 17)	DLG5- (*n* = 148)	OCTN TC haplotype (%) +	-
Localization CD, *n* (%)						
Ileum	17 (23)	21 (17)	1 (6)	33 (22)	46	54
Ileo-colon	48 (64)	64 (53)	13 (76)	80 (54)	47	53
Colon	10 (13)	35 (29)	3 (18)	34 (23)	39	61
Upper G-I tract	16 (21)	18 (15)	3 (18)	29 (20)	53	47
Ileo *vs* colon	*P* = 0.029 OR 2.83 (1.10-7.33)					
Disease type CD, *n* (%)	Vienna-Montreal	Vienna-Montreal	Vienna-Montreal	Vienna-Montreal	V-M	V-M
- Inflammatory	37 (50) 48 (64)	66 (54) 86 (71)	7 41) 9 (52)	87 (59) 109 (74)	45/44	55/56
- Stenosing	19 (25) 21 (28)	19 (16) 22 (18)	4 (24) 4 (24)	26 (17) 30 (20)	40/41	60/59
- Penetrating	19 (25) 6 (8)	36 (30) 13 (11)	6 (35) 4 (24)	35 (24) 9 (6)	49/62	51/38
F *vs* S+ I (according to Montreal)			*P* = 0.011 OR 4.75 (1.29-17.57)		*P* = 0.038 OR 2.13 (1.03-4.4)	
Growth delay y/n, (%)	24/35 (41)	36/47 (43)	4/5 (44)	54/62 (47)	49/44	51/56
Resective surgery y/n, (%)	23/52 (31)	27/94 (22)	5/12 (29)	26/122 (18)	42/46	58/54
EIM y/n, (%)	27/48 (36)	49/72 (41)	9/8 (53)	58/90 (39)	40/48	60/52
Perianal fistulae y/n, (%)	13/62 (17)	23/98 (19)	2/15 (12)	26/122 (18)	45/45	55/55
ASCA (pos/neg), (%)	31/8 (79)	46/24 (66)	8/3 (73)	63/29 (68)	46/58	54/42
ANCA (pos/neg), (%)	5/26 (16)	13/59 (18)	1/11 (8)	15/66 (19)	44/49	56/51
Steroids need, y/n (%)	48/27 (64)	86/35 (71)	12/5(71)	96/52 (65)	43/48	57/52

P values are corrected for multiple comparisons.

Table 6 Genotype-phenotype correlation in UC patients evaluated by comparison of carriers vs non-carriers of at least one CARD15 variant (CARD15+), the AA/AG genotypes of DLG5 variant (DLG5+), and presence of TC haplotype of OCTN1/2 variants (TC+)

UC *n* = 186	CARD15+ (*n* = 36)	CARD15- (*n* = 148)	DLG5+ (*n* = 27)	DLG5- (*n* = 144)	OCTN TC haplotype (%) +	-
Localization UC, *n* (%)						
Rectum-sigmoid	7 (19)	36 (24)	9 (33)	28 (19)	45	55
Left colon	11 (31)	43 (29)	6 (22)	44 (31)	44	56
Pancolitis	18 (50)	69 (47)	12 (45)	72 (50)	47	53
Growth delay y/n, (%)	3/26 (10)	18/98 (16)	5/14 (26)	16/101 (14)	43/46	57/54
Resective surgery y/n, (%)	4/32 (11)	9/139 (6)	1/26 (4)	9/135 (6)	31/46	69/54
EIM y/n, (%)	9/27 (25)	37/111 (25)	4/23 (15)	40/104 (28)	51/44	49/56
ASCA (pos/neg), (%)	4/10 (29)	14/46 (23)	5/10 (33)	13/46 (22)	42/46	58/54
ANCA (pos/neg), (%)	13/6 (68)	52/43 (55)	11/8 (58)	52/40 (57)	43/40	57/60
Steroids need, y/n, (%)	26/10 (72)	109/39 (74)	21/6 (78)	105/39 (73)	46/46	54/54

(not including perianal fistulae) was found in CD patients with DLG5 risk variant (24% *vs* 6%; $P = 0.011$). No other significant differences were found between CD and UC patients (Table 6). No further correlation at this sample size could be observed when considering patients with two CARD15 variants, homozygous for TC haplotype or DLG5 variant. Similarly, no correlation could be found when evaluating subjects with all three concomitant gene variants (at least one CARD15 variant, OCTN TC-haplotype and DLG5 113G/A).

The correlations between at least one CARD15 variant with ileal localization (OR = 2.8, CI = 1.1-7.5; $P = 0.031$), OCTN TC-haplotype and DLG5 113G/A variant (OR = 4.05, CI = 1.05-15.5; $P = 0.041$) with penetrating disease were also confirmed at the stepwise logistic regression, after correction for potential confounders (duration of follow-up).

DISCUSSION

This study reports in the largest available cohort of pediatric IBD patients the contribution of variants of CARD15, OCTN1/2 and DLG5 genes on disease predisposition and correlation with phenotype. Reports of pediatric series to date available have been limited to mainly CARD15 variants[11-19], small number of subjects[11,12,15,16,18,19], and scarce information on UC patients[13,15,41].

The carriage rate of CARD15 variants in our pediatric population was 38% for CD patients and 20% for UC patients, not significantly different from figures in adult Italian population (36% for CD and 15% for UC, respectively)[51]. This finding is in agreement with previously reported frequencies in CD pediatric series ranging form 8.6% up to 60%, with lowest incidence in Swedish and Scottish population, similarly to the rates reported in corresponding adult population. Accordingly, this figure does not support the hypothesis of a stronger effect of CARD15 variants in early onset of CD. The relative contribution of each of the three common variants in our data is consistent with data from adult populations of IBD patients: 1007finsC confers the greatest risk (OR = 4.6, CI = 2.2-9.5) and G908R the least

(OR = 2.9, CI = 1.5-5.4). More specifically, the 1007finsC and R702W variants were associated with susceptibility to CD both in case control and family-based analysis, while the G908R variant was associated only in case control studies.

Homozygous carriers of OCTN1/2 variants were significantly more frequent in CD patients as compared to controls (OR = 1.6, $P = 0.03$). The TC haplotype was found in 44.8% of CD patients and 38.3% of controls ($P = 0.04$). No evidence of interaction with CARD15 gene was found since the frequency of TC haplotype was similar in CARD15-positive and CARD15-negative patients. Furthermore, a trend towards an increase of the homozygous carriers of OCTN1/2 variants was noticed in UC patients ($P = 0.05$), with a significant increase of TC haplotype (45.4%; $P = 0.03$), with similar finding in Italian adult population[33] and two other pediatric cohorts[41,42]. In contrast, no correlation of OCTN1/2 variants has been found in other pediatric series[43,44], perhaps for the smaller sample size of patients and controls. Our study confirmed the strong linkage disequilibrium between OCTN1/2 variants and SNPs tagging the IBD5 risk haplotype. Contrary to the Peltekova's study[20], the large majority of homozygous carriers of OCTN1/2 variants and TC haplotype were found in the background of IBD5-positive risk allele[35]. Although the possible causal role of the OCTN1/2 variants could not be excluded, further functional studies are needed to confirm and explain the role of these genes in IBD.

The DLG5 variant (G113A) was not correlated with pediatric IBD, even after stratifying patients and controls for gender in both the association study and family-based analysis. In addition, the A (risk) allele was significantly under-transmitted ($P = 0.02$), similarly to another adult series[31]. This might reflect a reduced effect of this variant in pediatric onset of IBD (5% *vs* > 10% in corresponding adult population)[39] and/or an insufficient power of the study sample. Based on the observed frequency of this variant in the control population (9%), our study sample could detect a ± 4.5% difference with a power of 90% and significance of 0.01.

No interaction between DLG5 variant and CARD15 or OCTN1/2 variants was found.

When the genotype-phenotype correlation was explored, there was only a weak correlation with ileal disease for CARD15 variants ($P < 0.03$) and penetrating disease for OCTN1/2 and DLG5 ($P < 0.04$, and $P < 0.02$, respectively). All these correlations were confirmed in the stepwise logistic regression analysis.

The correlation of CARD15 variants with ileal disease has been widely reported in most studies of adult[7] and child[11-14,41,44] Caucasian populations. Although some explanations for this consistent association have been put forward (e.g. presence of NOD2 in Paneth cells in terminal ileum, impaired regulation of Paneth-cell mediated antimicrobial response, perhaps through impaired production of α-defensin)[52], the clinical implications are unclear yet. Less than 50% of CD patients with ileal localization in this and other studies carry one CARD15 variant; moreover, carriers of this variant do not have a disease course and response to therapy, being different from non-carriers[53].

The association of OCTN1/2 and DLG5 variants with penetrating disease is intriguing since both genes are involved in maintaining mucosal integrity and permeability. A correlation between OCTN1/2 variants and penetrating disease has been reported in adult population[31-33], but not in the only two available studies in children with CD, in which no correlation[44] or only a correlation with growth delay was found[41]. Obviously, difference in selection of patients and duration of follow-up may explain this discrepancy. In our series, patients with penetrating disease were more than double than in the above-mentioned studies[41,44]. Moreover, our definition of growth delay (below 5th percentile) was more stringent than that of the Scottish study[41] (below 9th percentile). Nevertheless, when considering patients with both height and weight delay below the 5th percentile, a trend towards a correlation with TC-haplotype was found in our study ($P = 0.07$).

Regarding the correlation of DLG5 variant with penetrating disease, no reports are available so far in children with IBD. Moreover, data in adult population are very conflicting with no clear genotype-phenotype correlation demonstrated.

Finally, no correlation with age at diagnosis, family history, active and passive smoking, previous appendectomy, presence of ANCA and ASCA, growth delay, use of steroids, or need for surgery could be demonstrated in our series. Moreover, despite a significant increase of TC-haplotype of OCTN1/2 variants in UC patients, no correlation with any clinical phenotype was found. A potential limitation of this study lies in the large number of centres involved and the possible lack of reproducibility of clinical classification (disease behaviour). This choice allowed, however, to accomplish the study in the largest population of pediatric IBD patients so far for genetic purpose. Moreover, all patients were enrolled at academic referral centres and sorting of clinical features was obtained through a previously validated standardized questionnaire.

In conclusion, this study has demonstrated that the three common CARD15 variants and the recently described OCTN1/2 variants, but not the 113G/A DLG5 variant, are associated with susceptibility to CD in children. Furthermore, all three investigated genes exert a weak influence on clinical expression of CD; CARD15 variants (Arg702Trp, Gly908Arg and Leu1007fsinsC) are slightly more frequent in subjects with ileal localization, while OCTN (1672C/T and -207G/C) and DLG5 (113G/A) polymorphisms correlate with presence of penetrating behaviour.

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CLINICAL RESEARCH

Molecularly defined adult-type hypolactasia among working age people with reference to milk consumption and gastrointestinal symptoms

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Abstract

AIM: To study milk consumption and subjective milk-related symptoms in adults genotyped for adult-type hypolactasia.

METHODS: A total of 1900 Finnish adults were genotyped for the C/T$_{-13910}$ variant of adult-type hypolactasia and filled in a structured questionnaire concerning milk consumption and gastrointestinal problems.

RESULTS: The C/C$_{-13910}$ genotype of adult-type hypolactasia was present in 18% of the study population. The prevalence of the C/C$_{-13910}$ genotype was higher among subjects who were undergoing investigations because of abdominal symptoms (24%, $P < 0.05$). Those with the C/C$_{-13910}$ genotype drank less milk than subjects with either the C/T$_{-13910}$ or the T/T$_{-13910}$ genotype of lactase persistence (18% *vs* 38%; 18% *vs* 36%, $P < 0.01$). Subjects with the C/C$_{-13910}$ genotype had experienced more gastrointestinal symptoms (84%) during the preceding three-month period than those with the C/T$_{-13910}$ (79%, $P < 0.05$) or the T/T$_{-13910}$ genotype (78 %, $P < 0.05$). Only 9% (29/338) of the subjects with the C/C$_{-13910}$ genotype consumed milk and reported no symptoms from it.

CONCLUSION: Gastrointestinal symptoms are more common among adults with the C/C$_{-13910}$ genotype of adult-type hypolactasia than in those with genotypes of lactase persistence.

Key words: Lactase persistence; Lactose malabsorption; C/T$_{-13910}$ genotype; Abdominal symptoms; Milk consumption

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INTRODUCTION

Gastrointestinal symptoms are a frequent cause for seeking medical advice in adult working age population[1]. Adult-type hypolactasia, characterised by the down regulation of lactase enzyme activity in the intestine during development is the most common enzyme deficiency in humans[2]. The symptoms caused by undigested lactose, bloating, diarrhoea and bowel gas are unspecific making the assessment of the diagnosis of adult-type hypolactasia a challenge to clinical practice. Symptoms vary greatly in severity and depend on the amount of lactose ingested and on individual sensitivity and may overlap with those of other gastrointestinal diseases such as irritable bowel syndrome (IBS) or diseases presenting with secondary lactose malabsorption, i.e. celiac disease[3]. Cases of individuals suspecting they have lactose intolerance are more common than the true prevalence of adult-type hypolactasia[4-9]. It has been shown that lactose-restricted diets improve symptoms markedly for example in IBS patients with lactose malabsorption and reduce the number of visits to the outpatient clinics[10]. Accurate diagnosis of lactose malabsorption, which is easily treatable by diet modification, would therefore be cost effective and time saving.

Diagnosis of adult-type hypolactasia has been based

on indirect methods, the lactose tolerance test (LTT) or breath hydrogen test (BHT). Specificity of the LTT ranges from 77% to 96%, and that of BHT from 89% to 100%. Sensitivities vary from 76% to 94% for LTT and from 69% to 100% for BHT[11]. These methods are time consuming for the patient and need substantial assistance by medical personnel. The determination of disaccharidase activities and lactase/sucrase ratios from the intestinal biopsy specimen would be the most accurate diagnostic method[12] but this invasive method is not suitable for everyday clinical practice.

It has been demonstrated that lactase non-persistence is inherited as an autosomal recessive trait. A proportion of the human population can digest lactose (lactase persistence) due to a mutation retaining lactase activity[13]. A single nucleotide polymorphism C to T located 13910 base pairs upstream the lactase gene is associated with the persistence/non-persistence trait in the Finnish families[14]. Analyses of several hundreds of intestinal biopsies have demonstrated that the C/C-13910 genotype is associated with low lactase activity (< 10 U/g/protein) and the C/T-13910 and T/T-13910 genotypes with high activity[14-16]. The prevalence of the C/T-13910 variant is compatible with the previously published figures for adult-type hypolactasia in European, Asian, African-American and Northern African populations[14-19]. It has been suggested, however, that in some African tribes the frequency of T-13910 allele does not parallel with the prevalence of lactase persistence[18]. Lactase mRNA transcribed from the C-13910 allele declines in the intestinal mucosa of children around five years of age[20], paralleling with the age of developmental down regulation of lactase enzyme activity[16]. *In vitro* -studies of the C/T-13910 variant have demonstrated greater increase in lactase promoter activity by the T-13910 variant[21,22]. This could possibly be explained by the recent finding of the T-13910 allele binding more strongly the transcription factor Oct-1 compared to the C-13910 allele[23]. Thus, the obtained functional data has given evidence for the use of the C/T-13910 variant as a robust marker for adult-type hypolactasia.

Here we have genotyped 1900 working-age people attending primary health care for the C/T-13910 variant associating with adult-type hypolactasia, and addressed the question about the prevalence and frequency of gastrointestinal symptoms as well as consumption of milk products.

MATERIALS AND METHODS

Participants

1902 adults from the capital area of Finland attending laboratory investigations in primary health care were asked to give a blood sample for genotyping of the C/T-13910 variant of adult-type hypolactasia and to fill in a questionnaire concerning the daily consumption of dairy products (normal and delactated milk, sour milk, yoghurt or ice cream, cheese) and possible milk related symptoms. The questions asked concerned whether or not a specific dairy product is included in the diet. Each participant was asked whether he/she had experienced any gastrointestinal complaints during the preceding three-month period. Frequency of gastrointestinal symptoms was also evaluated on daily and weekly basis. The questions on gastrointestinal symptoms covered: (1) the frequency of gastrointestinal symptoms such as flatulence, bloating, diarrhoea, heartburn, and constipation (2) the relation of their symptoms to meals, (3) correlation of the symptoms to different types of milk products or other foods, (4) previous diagnosis of lactose intolerance or a GI-disease such as colon cancer, celiac disease, or Helicobacter pylori infection. The age of the participants was ticked in questionnaire in three age groups (18-35 years, 36-51 years, and 52-64 years) due to the extensive number of study population. The formulated questionnaire was pre-tested on a small group of healthy adults. The collection of the questionnaires and blood samples occurred during a three-month period from February to May 2004. The study was approved by the Ethical Committee of the Helsinki University Central Hospital. All the subjects signed their written informed consent.

Genotyping

DNA of the study subjects (n = 1900) was isolated from peripheral blood samples by submerging a blank strip (Merck, Darmstadt, Germany) into EDTA blood. Strips were dried and heated in 1 × PCR reaction buffer (Dynazyme; Finnzymes, Espoo, Finland) containing 10 mmol/L Tris HCl (pH 8.8 at 25℃), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, and 0.1% Triton X-100 to degrade proteins. After centrifuging 10 μL of the supernatant containing the DNA, the sample was amplified in a total volume of 50 μL, containing primers (one biotin-labeled 5'-(Biotin) CCTCGTTAATACCCACTGACCTA-3') (5 μmol/L) and one unmodified 5'-GTCACTTTGATATG ATGAGAGCA-3' (50 μmol/L), dNTPs (1000 μmol/L), 0.5 U of Taq polymerase (Dynazyme, Finnzymes) in a standard buffer. The PCR cycle conditions used were as following: an initial round of denaturation at 94℃ for 4 min, then 35 cycles at 94℃ for 30 s, 53℃ for 30 s, 72℃ for 1.15 min, and a final extension of 72℃ for 10 min. The resulting PCR products were analyzed by 1.5% agarose gel electrophoresis to verify the amplification and the size of the PCR product.

The C/T-13910 single nucleotide polymorphism was analysed using the solid-phase mini-sequencing method[24] that is based on the detection of tritium-labeled T-13910 and C-13910 alleles in the PCR reaction and measurement of their ratio using scintillation counter that directly reflects the ratio between the two sequences in the original sample. Briefly, in the mini-sequencing of the C/T-13910 single nucleotide polymorphism, two 10-μL aliquots of the biotin-labeled PCR product were captured to streptavidin coated microtitre wells (Thermo Electron, Helsinki, Finland). The reaction mixture contained 10 pmol of the detection primer (5'-GGCAATACAGATAAGATAATGT AG-3'), 0.1 μL of either ^{3}H-dCTP or ^{3}H-dTTP (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and 0.05 U of DNA polymerase (Dynazyme Ⅱ, Finnzymes, Espoo, Finland). The reactions were allowed to occur for 15 min at 56℃ before washing off the unattached

label. Finally, the attached detection primer was eluted by NaOH treatment and the radioactivity measured in a liquid scintillation counter (Rackbeta 1209; Wallac, Turku, Finland).

Two samples of the 1902 were disqualified after being found infected with hepatitis B.

Statistical analyses

Statistical analyses were conducted using Tixel (version 8.1), which is a VBA-program for Excel. Descriptive analyses were conducted with simple logistic regression. Proportions were compared by using Chi-squared tests with continuity correction or Fisher's exact test when appropriate. Two-sided significance tests were used throughout.

RESULTS

The frequency of the C/C-13910 genotype associated with lactase non-persistence among the participants was 18% (Table 1). This figure corresponds to the earlier published prevalence of adult-type hypolactasia in the Finnish population[14-16], implying that the study population is representative for the general population. There was no difference in genotype distribution among male and female subjects, and no difference in separate age groups (data not shown).

The response rate for the questionnaire was high; a total of 99% (n = 1885) of the participants returned the questionnaire. A total of 42% of the patients reported a new, not gastrointestinal related disease as the reason for their laboratory visit. Earlier diagnosed diseases (25%) were the second most common cause for the visit, followed by gastrointestinal symptoms that were reported by 19% (348/1787) of the participants. Of these 348 subjects, the adult-type hypolactasia genotype C/C-13910 was observed in 24% (84/348), which was significantly more than in the study population ($P < 0.05$, Table 1).

Subjects with the C/C-13910 genotype had experienced more gastrointestinal symptoms (84%; 280/330) during the preceding three-month period than those with the C/T-13910 (79%; 698/885; $P < 0.05$) or the T/T-13910 genotype (78%; 501/641; $P < 0.05$, Table 2). Those with the C/C-13910 genotype reported more frequently gastrointestinal symptoms compared to C/T-13910 or T/T-13910 genotypes, but the difference was not significant (Table 2).

Gastrointestinal symptoms associated with lactose intolerance, namely flatulence, bloating and diarrhoea, were frequent among all genotype groups (Table 3). Flatulence was the only symptom significantly more frequent among the subjects with lactase non-persistent genotype compared to those with genotypes of lactase persistent ($P < 0.05$). Those with the C/C-13910 genotype experienced more bloating (61%) than those with the T/T-13910 genotype (55%) and C/T-13910 genotype (58%) but the difference did not reach statistical significance (Table 3).

As many as 45% of the participants informed having experienced gastrointestinal symptoms after drinking milk (Figure 1) and 25% of the participants reported symptoms from food containing milk. It is notable, that only 18% (60/338; $P < 0.01$)) of the subjects with the C/C-13910 genotype of adult-type hypolactasia informed drinking milk with meals, which is significantly less ($P < 0.01$) than those with the lactase persistent genotypes C/T-13910 (38%; 333/894) and T/T-13910 (36%; 236/653; Figure 1). The fat content of the milk was not questioned, but in Finland the vast majority of people use low-fat or fat-free milk as a drink. The number of people using low lactose containing milk as a drink was marginal according the questionnaire.

Table 1 Frequency of the C/T-13910 genotypes

Genotype	% (n)	GI-complaints % (n)	P
C/C-13910	18 (341)	24 (84)	< 0.05
C/T-13910	47 (901)	43 (148)	NS
T/T-13910	35 (658)	33 (116)	NS
Total	100 (1900)	100 (348)	

Only 9% (29/338) with the C/C-13910 genotype of adult-type hypolactasia consumed milk daily and reported no symptoms from milk. However, one third (18/60) of the milk drinkers with the C/C-13910 genotype did not answer the question about milk related symptoms. Most people with C/C-13910 genotype reporting GI-problems from milk (69%; 190/274) did not drink milk. The respective number of people with lactase persistence genotypes who reported milk-related problems and did not drink milk was 54% (299/554) for the C/T-13910 genotype and 50% (207/411) for the T/T-13910 genotype.

Cheese caused gastrointestinal symptoms for 11% of the participants according to their own judgment: for 17% of those with the C/C-13910 genotype, 10% of those with the C/T-13910 genotype and 9% of the ones with the T/T-13910 genotype ($P < 0.05$, Figure 2). Among all participants 14% experienced symptoms from cereal or bread, and 37% from ingested fat (Figure 1) and these were not related to the genotype of adult-type hypolactasia.

Among the study population, 15% (245/1649) reported having had a pathological LTT earlier: 36% (109/299) of those with the C/C-13910 genotype, 11% (82/777) with the C/T-13910 and 9% (54/573) with the T/T-13910 genotype.

A previous, pathological LTT was reported by 19% (64/341) of participants with the C/C-13910 genotype, by 10% (89/901) with the C/T-13910, and by 14% (91/658) with the T/T-13910 genotype. Five out of these 180 subjects with the C/T-13910 or T/T-13910 genotype with a pathological LTT reported a previously diagnosed possible secondary cause for hypolactasia i.e. celiac disease. An undiagnosed celiac disease in two other participants of these 180 subjects was suggested due to elevated level of transglutaminase antibodies in their sera[25].

DISCUSSION

The inability to absorb lactose is frequently suspected to underlie gastrointestinal symptoms in populations with high prevalence of adult-type hypolactasia and frequent dairy consumption. In this study we show that subjects with the C/C-13910 genotype, having low lactase activity in the intestinal wall[14-17,20] indeed do seek medical advice for

Table 2 The frequency of gastrointestinal symptoms according to the three genotype groups of lactase persistence/nonpersistence during previous three months

Abdominal complaints	C/C % (*n*)	C/T % (*n*)	T/T % (*n*)	OR C/C *vs* C/T (95% CI)	*P*	OR C/C *vs* T/T (95% CI)	*P*
During previous three months	84 (280/332)	79 (698/885)	78 (501/641)	1.44 (1.03-2.02)	< 0.05	1.50 (1.06-2.14)	< 0.05
Daily	23 (78/338)	22 (199/891)	19 (127/652)	1.04 (0.77-1.41)	NS	1.24 (0.90-1.71)	NS
Every other day	21 (72/338)	16 (145/891)	20 (129/652)	1.39 (1.02-1.91)	< 0.05	1.10 (0.79-1.52)	NS
Once a week	22 (74/338)	20 (176/891)	18 (115/652)	1.14 (0.84-1.55)	NS	1.31 (0.94-1.82)	NS
More seldom than once a week	17 (58/338)	20 (177/891)	21 (138/652)	0.84 (0.60-1.16)	NS	0.77 (0.55-1.08)	NS
No complaints	16 (52/332)	21 (187/885)	22 (140/641)	1.44 (1.03-2.02)	< 0.05	1.50 (1.06-2.13)	< 0.05

Table 3 The type of gastrointestinal symptoms among three genotype groups of lactase persistence/nonpersistence

Gastrointestinal symptoms	C/C % (*n* = 294)	C/T % (*n* = 725)	T/T % (*n* = 524)	OR C/C *vs* C/T (95% CI)	*P*	OR C/C *vs* T/T (95% CI)	*P*
Flatulence	79 (232)	73 (531)	73 (380)	1.37 (0.99-1.89)	< 0.05	1.42 (1.01-1.99)	< 0.05
Diarrhoea	40 (119)	38 (275)	40 (207)	1.11 (0.84-1.47)	NS	1.04 (0.78-1.39)	NS
Constipation	24 (72)	21 (152)	20 (107)	1.22 (0.89-1.68)	NS	1.26 (0.90-1.78)	NS
Bloating	61 (178)	58 (423)	55 (287)	1.10 (0.83-1.44)	NS	1.27 (0.94-1.69)	NS
Heartburn	32 (94)	37 (265)	38 (198)	0.82 (0.61-1.09)	NS	0.77 (0.57-1.04)	NS

Figure 1 Frequency of gastrointestinal symptoms experienced by various foods in the study population.

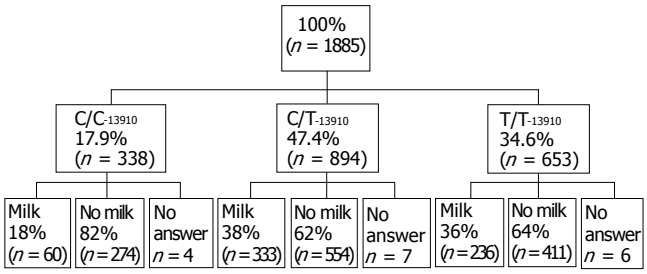

Figure 2 Flow-chart of the use of cow's milk as a drink in different genotype groups.

abdominal symptoms more often than subjects with high lactase activity. This occurred although they already had self restricted their milk consumption.

The response rate in this study was extremely high, 99%, thus the data about GI-symptoms was comprehensive. This exceptionally high response rate was achieved by the sound motivation of both the study subjects and the laboratory personnel. As expected, gastrointestinal symptoms were very common (80%) during the previous three months comparable to the earlier findings[1]. Flatulence was the only symptom that in this study was significantly more common among subjects with low lactase (C/C-13910 genotype). Diarrhoea and bloating, which according to earlier published studies are more common among lactose malabsorbers[4] were not more frequent among those with adult-type hypolactasia. These symptoms, however, were very common in the study population (40%-60%) pointing out the high prevalence of functional GI-symptoms[26].

The great majority of the study subjects did not drink milk at all. Milk consumption was rarest in the group of lactase non-persistent subjects: only 18% reported drinking milk daily, which might indicate that a natural aversion of milk has been developed by the subjects with low lactase activity. This is in agreement with our findings in a paediatric population in which children with the C/C-13910 genotype consumed less milk than children with a non-C/C-13910 genotype[16,27]. The majority of the children with the lactase non-persistent genotype C/C-13910 reported that they never drank milk[16]. At the age of 8-9 years, more than 40% of the subjects with the C/C-13910 genotype reported drinking less than one dl of milk daily, compared to 20% of the subjects with the C/T-13910 and T/T-13910 genotype, respectively[26].

Only 9% of those with the C/C-13910 genotype used daily milk and did not experience symptoms from ingested milk. This is in line with other studies[6,8,9] showing that not all individuals with lactose malabsorption report symptoms from food containing lactose. The colonic micro biota is variable[28] and the individual sensitivity to feel distension of the colon and to sense discomfort varies[29]. Accordingly, the recognition of the possible link between milk consumption and abdominal symptoms is not always easy[30]. The meal content has an effect on emptying of the stomach and thereby affects the lactose load in the

intestine[31,32]. Most lactose malabsorbers seem to tolerate small amounts of milk especially during meals[5,33]. Further studies are warranted to evaluate the amount of lactose tolerated by different genotype groups.

In this study population, 10% of those with the genotypes associated with lactase persistence (the C/T-13910 genotype or T/T-13910 genotype) reported that they had been earlier diagnosed lactose intolerant based on results of LTT. A possible cause for secondary hypolactasia, i.e. celiac disease, was found in 3% of these individuals. These findings imply that LTT, a commonly used method for diagnosis of lactose intolerance, produces high numbers of false positive test results as has been earlier observed[11]. One third of those with the C/C-13910 genotype associated with low lactase reported that they had previously had a positive LTT. Due to the way our question on earlier diagnosis of lactose intolerance was formulated, we do not know the extent of subjects with the C/C-13910 genotype who might have had a negative result for their LTT.

In conclusion, gastrointestinal symptoms are more common among adults with the C/C-13910 genotype of adult-type hypolactasia than in those with genotypes of lactase persistence. This was seen although individuals with the C/C-13910 genotype had restricted their milk consumption. Genotyping of the C/T-13910 polymorphism is a practical means for defining adult-type hypolactasia.

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RAPID COMMUNICATION

Clinical investigation of 41 patients with ischemic colitis accompanied by ulcer

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Abstract

AIM: To investigate the relationship among the presence of ulcer lesions, underlying disease, and clinical course in patients with ischemic colitis.

METHODS: The subjects were 41 patients (10 male and 31 female; mean age 70 years) with ischemic colitis who were admitted to and received treatment in our hospital from 2000 to 2006. We compared their characteristics and analyzed the mean lengths of admission and fasting for 9 patients with ulcer lesions (ulcer group) and 32 without (non-ulcer group).

RESULTS: The groups with presence and absence of ulcer differed significantly only in white blood cell (WBC) count. Lengths of fasting and admission were 7.9 d and 17.9 d for the ulcer group and 4.4 d and 10.7 d for the non-ulcer group, respectively, and significantly longer in the ulcer group (P = 0.0057 and 0.0001). There was no correlation between presence of ulcer and presence of underlying diseases.

CONCLUSION: Lengths of fasting and admission were significantly longer in patients with ischemic colitis with ulcer than for those without ulcer.

Key words: Ischemic colitis; Ulcer; Fasting; Admission; White blood cell

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INTRODUCTION

Ischemic colitis was first reported as a result of reversible vascular occlusion of the colon in 1963 by Boley *et al*[1], and in 1966 Marston *et al*[2] suggested the name "ischemic colitis" for it and clarified its clinical and histopathological features.

Marston *et al*[2] classified this disease into 3 forms, transient, strictures, and gangrene. It is defined as a "reversible condition caused by obstruction of blood flow in intestinal tract mucosa without apparent blockage of the main artery". In general, it is common in the elderly[3], and its prevalence increases with age, although it is seen in younger patients as well[4-6]. Its causes include vascular factors such as ischemia and embolus[7-9], intestinal factors such as constipation[5,10], irritable bowel syndrome[11,12] and history of intestinal surgery[13,14] as well as administration of drugs such as alosetron[11], cocaine[15], non-steroidal anti-inflammatory drugs[16], oral contraceptives[17] and oral laxatives[18].

While surgery is indicated for the gangrenous form of this disease, transient and stricturing forms are often ameliorated by bowel rest, fasting, and parenteral fluid administration alone. The lengths of fasting and admission vary among individuals. Significant prolongation of healing is sometimes observed, especially in patients with ulcer. We therefore examined the associations among etiology, clinical course, and presence of ulcer lesions in patients with transient and stricturing forms of ischemic colitis.

MATERIALS AND METHODS

The subjects were 41 patients (10 male and 31 female; mean age 70 years) with ischemic colitis who were admitted to and received treatment in our hospital from 2000 to 2006. Patients who developed ischemic colitis during admission for other diseases were excluded. Ischemic colitis was diagnosed comprehensively based on the essential criteria including lack of antibiotic administration, negative bacterial culture of feces or biopsy, lack of history of inflammatory bowel disease (IBD), as well as endoscopic and histopathological findings on biopsy. Age and mode of disease onset, symptoms and affected sites were included as complementary parameters.

Patients were divided into 2 groups by the presence of ulcer lesions and compared for age, gender, affected sites, hematological findings, performance status (0-4, 0: asymptomatic; 1: symptomatic but completely ambulant; 2: symptomatic, < 50% in bed during day time; 3:

Table 1 Clinical features of ischemic colitis patients: Comparison between ulcer and non-ulcer groups (mean ± SD)

Clinical features	Ulcer group (n = 9)	Non-ulcer group (n = 32)	Statistical significance
Age (mean, range, yr)	68 ± 19 (35-87)	71 ± 11 (42-92)	NS
Percentage of females (%)	77.8	75.0	NS
Lesion location: left colon (%)	88.9	100	NS
Performance status 0-1 (%)	77.8	87.5	NS
WBC ($\times 10^9$/L)	12.27 ± 4.67	8.44 ± 3.17	P = 0.0065
CRP (mg/dL)	2.6 ± 4.1	1.5 ± 2.3	NS
K (mEq/L)	3.8 ± 0.4	4.0 ± 0.5	NS
Total cholesterol (mg/dL)	179.3 ± 30.6	191.4 ± 35.6	NS
TG (mg/dL)	93.3 ± 41.0	73.2 ± 23.4	NS
LDH (IU/L)	190.0 ± 42.8	203.8 ± 37.8	NS
CPK (IU/L)	80.7 ± 38.9	82.7 ± 39.5	NS

Percentage of females and lesion location were evaluated with an χ-square test; Other data were evaluated with Student's t test after skewing with log transformation. CRP: C-reactive protein; K: potassium; TG: triglyceride; LDH: lactate dehydrogenase; CPK: creatinine phosphokinase.

Table 2 Underlying diseases: Comparison between ulcer and non-ulcer groups n (%)

	Ulcer group (n = 9)	Non-ulcer group (n = 32)	Statistical significance
Hypertension	6 (66.7)	19 (59.4)	NS
Hyperlipemia	0	6 (18.8)	NS
Diabetes mellitus	1 (11.1)	3 (9.4)	NS
Atrial fibrillation	1 (11.1)	1 (3.1)	NS
Cerebral infarction	1 (11.1)	2 (6.3)	NS
Chronic constipation	5 (55.6)	19 (59.4)	NS
Medications			
NSAIDS	0	1 (3.1)	NS
Antihypertensive agents	5 (55.6)	18 (56.3)	NS
Others	2 (22.2)	5 (15.6)	NS
History of abdominal surgery	4 (44.4)	14 (43.8)	NS

Percentages were evaluated with an χ-square test. NSAIDS: non-steroidal anti-inflammatory agents.

symptomatic, > 50% of time in bed, but not bed bound; 4: bed bound, using the WHO score criteria), underlying disease (hypertension, hyperlipemia, diabetes, chronic atrial fibrillation, cerebral infarction, constipation, and history of abdominal surgery), oral medications, and mean lengths of fasting and admission.

All patients underwent endoscopy of the lower gastrointestinal tract within 3 d after admission and 5 d after disease onset. As treatment, fasting and bowel rest with fluid replacement were used in all patients. Twenty-two (53.7%) patients received oral antiflatulents and 13 (31.7%) patients a combination with antibiotics. Total parenteral nutrition *via* a central venous line was performed in none of the patients. After disappearance of abdominal pain, patients were allowed to begin oral ingestion. Patients were discharged if they exhibited no relapse of symptoms after restarting oral ingestion.

Statistical analysis

Data are expressed as mean ± SD or percentage. Percentage of females, lesion location, performance status and underlying systemic diseases were evaluated with an X-square test. Other data were evaluated with Student's t test after skewing variable with log transformation. All data analysis was performed with the StatView 5.0. Statistical significance was set at $P < 0.05$.

RESULTS

Forty-one patients were analyzed (10 male and 31 female; mean age 70 years). Affected sites included the sigmoid colon (70.7%), descending colon (51.2%), transverse colon (12.2%), rectum (4.9%), and ascending colon (2.4%), with some overlap. The disease developed in 92.7% of the patients for the first time, and the remaining 7.3% for the second time. The disease was accompanied by ulcer in 9 (22.0%) patients.

Nine patients had ulcer (2 male and 7 female; mean age 66, range 35-87 years), the affected site was the left hemicolon in 8 patients. On the other hand, 32 patients had no ulcer (8 male and 24 female; mean age 71, range 42-92 years), the affected site was in the left hemicolon in all of them. In both groups, underlying diseases including hypertension, hyperlipemia, diabetes, atrial fibrillation, cerebral infarction, chronic constipation, and history of abdominal surgery, as well as oral medications, performance status, and hematological findings were examined as parameters. The significant difference was found between groups only in white blood cells (WBC), which was higher in the ulcer group (12.27×10^9/L) than in the non-ulcer group (8.44×10^9/L, P = 0.0065; Table 1). There were no statistically significant differences between groups in other parameters (Table 1 and Table 2).

Lengths of fasting and admission were 7.9 d and 17.9 d for the ulcer group and 4.4 and 10.7 d for the non-ulcer

Table 3 Durations of fasting and hospitalization: Comparison between ulcer and non-ulcer groups (mean ± SD)

	Ulcer group (n = 9)	Non-ulcer group (n = 32)	Statistical significance
Fasting (d)	7.9 ± 4.1	4.4 ± 2.9	P = 0.0057
Hospitalization (d)	17.9 ± 6.8	10.7 ± 3.6	P = 0.0001

group, respectively, being significantly longer in the ulcer group (P = 0.0057 and 0.0001; Table 3).

DISCUSSION

The etiology of ischemic colitis remains unclear. However, it may involve combination of vascular factors such as hypertension or hyperlipemia and intestinal factors such as chronic constipation and history of abdominal surgery. These may cause reduction of blood flow in the mucosa or wall of the intestinal tract, leading to ischemia. In the present study, patients with hypertension (61%), chronic constipation (58.5%), and history of abdominal surgery (43.9%) exhibited higher prevalence of ischemic colitis, which are consistent with previous reports[19,20].

Tohda *et al*[7] detected the correlation between arteriosclerosis and ischemic colitis using pulse wave velocity (PWV) as an indicator related to vascular factors, and reported higher PWV levels for the elderly. Similarly, we compared the contributions of vascular and intestinal factors to the occurrence of ischemic colitis in age, and observed that 2 (25%) patients developed the disease because of vascular factors and 7 (87.5%) patients because of intestinal factors among the eight younger patients aged less than 60 years. This indicates that vascular factors contribute predominantly to development of ischemic colitis.

In the present study, only WBC at admission was correlated with presence of ulcer. Notably, no difference in number of WBC between *H pylori*-infected patients with gastritis and those with ulcer has been noted[21]. Additionally, little difference has been reported in number of WBC between those with active disease and those with inactive disease in patients with inflammatory bowel disease[22]. Bjornestad *et al*[23] performed three routine laboratory tests (white blood cell count, hemoglobin concentration and erythrocyte volume fraction) in patients with four acute abdominal diseases (acute mesenteric ischemia: AMI, perforation of colon, perforation of peptic ulcer and intestinal obstruction). The WBC in patients with AMI was significantly higher than the normal range. The three variables were higher in AMI patients than in the other patients ($P < 0.001$). The discriminant analysis of the variables classified 80% of the patients correctly into AMI and non-AMI groups. This difference may reflect the inflammatory response in the acute stage and biological reactions associated with continued chronic inflammation.

This is the first report on lengths of fasting and admission in patients with ischemic colitis. In the present study, these lengths were 7.9 d and 17.9 d for the ulcer group and 4.4 d and 10.7 d for the non-ulcer group, respectively, being significantly longer in the ulcer group. Although we did not evaluate patient quality of life (QOL), stress on patients due to fasting and prolonged admission may cause a decrease in QOL. In addition, the costs associated with prolonged admission also causes a problem.

Strategies for the treatment of ischemic colitis are determined based on the severity of ischemia. Surgery is indicated for the gangrenous form of the disease. On the other hand, for transient and stricturing forms of the disease, younger patients may obtain prompt relief solely with follow-up, while older patients or those who have severe abdominal pain will require hospitalization, fasting, and management using parenteral fluid administration.

Lengths of fasting and admission were significantly longer in patients with ischemic colitis with ulcer than in those without ulcer. Ulcer associated with ischemic colitis is often localized in the left hemicolon. Noninvasive and local ulcer treatment such as use of enema preparations in addition to conventional treatment centering on bowel rest should be considered for ischemic enteritis accompanied by ulcer.

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RAPID COMMUNICATION

Effect of preoperative biliary drainage on outcome of classical pancreaticoduodenectomy

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Abstract

AIM: To investigate the role of preoperative biliary drainage (PBD) in the outcome of classical pancreaticodu odenectomy.

METHODS: A 10-year retrospective data analysis was performed on patients (n = 48) undergoing pancreaticoduodenectomy from March 1994 to March 2004 in department of surgery at SMS medical college, Jaipur, India. Demographic variables, details of preoperative stenting, operative procedure and post operative complications were noted.

RESULTS: Preoperative biliary drainage was performed in 21 patients (43.5%). The incidence of septic complications was significantly higher in patients with biliary stent placement ($P < 0.05$, 0 *vs* 4). This group of patients also had a significantly higher minor biliary leak rate. Mortality and hospital stay in each group was comparable.

CONCLUSION: Within this study population the use of PBD by endoscopic stenting was associated with a high incidence of infective complications. These findings do not support the routine use of biliary stenting in patients prior to pancreatico-duodenectomy.

Key words: Whipple's operation; Preoperative stenting; Sepsis; Preoperative biliary drainage

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INTRODUCTION

Pancreaticoduodenectomy is a challenging operation and is associated with high morbidity[1]. In the last two decades peri-operative mortality has decreased to less than 5% in published series from specialized centers[2,3]; although the morbidity remains high (25%-50%). The impact of jaundice on post-operative morbidity and mortality is well known. However the routine use of pre-operative biliary drainage (PBD) remains controversial[4]. The potential advantages of preoperative stenting include improved nutritional, metabolic and immune function, and the possibility of reduced postoperative morbidity and mortality rates[5-7]. Opponents of PBD argue that it increases infective complications and morbidity[8,9]. There are, however, certain clinical situations such as acute suppurative cholangitis and severe malnutrition where urgent biliary drainage is indicated and can be life-saving[10]. It is not clear whether the procedure itself or its complications influence the morbidity after pancreaticoduodenectomy. The optimal duration of preoperative drainage also remains unknown. Experimental studies have indicated that a period of 6 wk is necessary to obtain clinical benefit with PBD[11]. On the other hand, a clinical study has shown a higher incidence of morbidity and mortality when surgery was carried out within 6 wk of stent placement[12]. Although several reports have been published, still there are no clear guidelines regarding use of PBD in these patients. The aim of this study was to evaluate the effect of endoscopic PBD on morbidity and mortality after pancreatico-duodenectomy in a low volume center.

MATERIALS AND METHODS

Details of 48 patients who underwent Kosch-Whipple's pancreaticoduodenectomy for periampullary tumors (excluding tumor of the pancreatic head) from March 1994 to March 2004 were entered into a database that included patient characteristics, details of biliary stenting and stent-related infective complications, surgery, morbidity and mortality. Pancreaticoduodenectomy was performed in the Department of Surgery, S.M.S. Hospital, Jaipur by a single surgical team using a standard Kosch-Whipple's

Table 1 Patient variables in the stented and non stented groups

	PBD	No PBD	Significance
Patients (*n*)	21	27	0.453
Median age (yr)	50 (25-68)	48 (30-72)	0.446
Sex ratio (M:F)	10:11	15:12	0.588
Mean serum bilirubin (mg/dL)	7.85 (SD ± 5.59)	11.83 (SD ± 9.05)	0.561
Albumin (mg/dL)	3.34 (SD ± 0.7)	3.17 (SD ± 0.82)	0.872
Location of tumor			
Ampulla	18	25	0.439
Bile duct	0	1	0.373
Duodenum	3	1	0.188

Table 2 Morbidity and mortality in the two groups

	PBD	No PBD	Significance
Sepsis	4	0	0.018[a]
Wound infection	5	1	0.037[a]
Minor bile leak	3	0	0.043[a]
Pancreatic leak	6	2	0.106
Bleeding	1	0	0.252
Lymphorrhea	2	4	0.580
Median hospital stay (d)	13 (1-147)	12 (9-32)	0.943
Mortality	3	5	0.690

[a]$P < 0.05$, PBD *vs* No PBD.

technique. Before year 2000 most reconstructions involved a pancreatojejunostomy ($n = 17$), but after year 2000 pancreatogastrostomy ($n = 31$) was performed according to the surgeon's preference. Patients with chronic and benign diseases were excluded from this study. The data were collected retrospectively as well as prospectively and were reviewed.

Pancreatic leak was defined according to the criteria described by Yeo *et al*[9]: pancreatic fistula was diagnosed when more than 50 mL of drainage fluid with an amylase concentration of more than 3 fold the normal upper limit in serum was obtained on or after postoperative d 5, or when pancreatic anastomotic disruption was shown radiologically. Biliary leak was defined as more than 50 mL bile per day in the drain for at least three consecutive days after postoperative d 7. Biliary leak less than 50 mL per day in drain after postoperative d 3 was considered minor bile leak. Infectious morbidity was defined as any complication with evidence of associated localized or systemic infection indicated by fever, leucocytosis and positive culture.

Statistical analysis

Results are expressed as medians and ranges or as numbers and percentages of patients. Two tailed *t* test, chi-square test and binary logistic regression analysis were used for data analysis. SPSS statistical package was used (SPSS v13.0, Chicago, USA) for analysis. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Forty eight patients had Whipple's operation for periampullary tumors. Twenty five patients (52.1%) were male and 23 (47.9%) were female. Median duration between onsets of symptoms to presentation was 3 mo (range 1 mo to 17 mo). The median age at presentation was 49 years (range 25 to 72). At admission 44 patients (91.7%) had jaundice [mean preoperative total bilirubin level was 10.09 mg/dL (SD ± 7.91)], 33 (68.8%) had abdominal pain, and 6 (12.5%) had a history of vomiting. Twenty-five (52.1%) patients noticed weight loss, and 27 (56.3%) had anemia. Preoperative liver function tests and other investigations are outlined in Table 1.

Forty three (89.6%) patients underwent side-viewing endoscopy for diagnosis. Preoperative histological diagnosis was obtained in 29 (60.4%) patients. Twenty one (43.8%) underwent preoperative biliary stenting at the time of preoperative instrumentation with drainage established successfully in all stented patients. All patients in stented group (21) underwent stenting by an endoscopical approach and plastic stents were used for drainage. There were no statistically significant differences in the demographics and pre-operative variables between patient groups with or without PBD (Table 2). Mean pre-operative serum bilirubin in the PBD group was 7.85 (SD ± 9.05) and 11.83 (SD ± 5.59) in non PBD group ($P \geqslant 0.05$).

Thirty one (64.6%) patients had pancreatico-gastrostomy and 17 (35.4%) patients had pancreatico-jejunostomy. The majority of the patients (43; 89.6%) had ampullary (ampula of Vater) carcinoma, whilst 4 (10.4%) patients had duodenal carcinoma and one patient had cholangiocarcinoma. Histopathological examinations of specimens revealed well differentiated tumors in 43 patients (89.6%); whilst other 5 were poorly differentiated. Only 8.3% of patients had lymph node involvement identified in surgical specimens and only 3% of the patients had positive resection margins.

All postoperative complications and subpopulation analyses between PBD and non-PBD groups are shown in Table 2. Postoperative sepsis occurred in 8.3% (4/48) of patients. Median hospital stay was 13 d (1-147 d) and overall mortality was 16.67%. One patient had postoperative bleeding that required re-exploration. There were 6 (12.5%) patients with wound infection and lymphorrhoea each and one patient developed delayed gastric emptying. Subpopulation analysis demonstrated a higher occurrence of infection and wound infection in PBD group ($P \geqslant 0.05$).

DISCUSSION

Pancreatico-duodenectomy is recognized as an acceptable surgical option in patients with pancreatic malignancies although the routine use of PBD remains controversial. In general, our unit's protocol is against PBD but within this series almost half of the patients (21/48) were already stented as they were referred from other centers. This may be reflected by the late presentation of patients in this series (the duration of symptoms varied from 1 mo to over one year).

There were some changes in the surgical technique in our study over the study period. During the initial years, classical pancreaticoduodenectomy was done with

pancreatico-jejunal (Dunking) anastomosis and 17 patients had pancreaticojejunal anastomosis (35.4%). Later on pancreatico-gastric direct duct to mucosa anastomosis was favoured in 31 patients. The morbidity and mortality rates were not significantly affected by the technique of pancreatic anastomosis to the gastrointestinal tract.

Half of the patients had bilirubin levels more than 10 mg% and only 4 cases (8%) presented without jaundice at the time of admission to our surgical unit. An average bilirubin level of 10 mg% at presentation has been reported in other studies. Earlier studies reported that there is an increased morbidity and mortality associated with severe jaundice (> 10 mg%)[13,14]. On the contrary, some recent studies have reported that the severity of jaundice has no influence on postoperative morbidity and mortality[15]. Povoski *et al*[8] concluded in their study that PBD, but not preoperative biliary instrumentation alone, was associated with increased morbidity and mortality rates in patients undergoing pancreatico-duodenectomy. In a prospective review, Sohn *et al*[9] reported the incidence of wound infection and pancreatic fistula to be significantly higher in stented patients.

The overall morbidity in our study of 48 cases was 35.41% (17 cases) and mortality was 16% (8 cases). In the first 5 years of our experience with pancreatico-duodenectomy, the operative mortality rate was 17.6%, which reduced to about 9% in last five years. Two patients died because of non surgical complications; one from dengue fever and one of myocardial infarction at the time of discharge. In our study, PBD was associated with increased morbidity following pancreatico-duodenectomy while mortality rates were unchanged. Although recent reports have shown that there is no change in infectious complication rates, notably wound infection, after PBD[16,17], our experience was different. The stented group had significantly higher morbidity (41%) in comparison to the non-stented group (30.7%). Wound infection, sepsis and minor bile leak were found to be main complications in the stented group. The wound infection rate was 27.2%, and fever 18.1%, both significantly higher in the stented group ($P \geqslant 0.05$). Minor biliary leak was significantly higher in the stented group ($n = 3$) ($P \geqslant 0.05$) (Table 2). Further regression analysis showed that PBD was associated with infective morbidity arising from stent placement. Larger prospective studies are needed to resolve the issue of duration of PBD. Other complications such as pancreatic leak, hemorrhage and lymphorrhoea were not influenced by stenting. The mean hospital stay in the two groups was comparable in spite of septic complications.

Some published studies suggest that stenting may have an effect on bile duct bacterial colonization and cause more infective complications[8,12]. Supporting the data above, our study confirms that PBD may have a negative impact on postoperative outcome after pancreatico-duodenectomy. However, judicious use may reduce the incidence of septic complications in selective patients. Randomized controlled trials in patient groups such as those with severe obstructive jaundice are required to address these controversies and improve outcomes and avoid unnecessary morbidity and mortality associated with PBD.

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RAPID COMMUNICATION

Effects of the myeloperoxidase 463 gene polymorphisms on development of atrophy in *H pylori* infected or noninfected gastroduodenal disease

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Abstract

AIM: To investigate the relationship between myeloperoxidase polymorphisms as a host-related factor and atrophy caused by *H pylori*.

METHODS: Our study enrolled 77 patients. Biopsy materials obtained during gastrointestinal endoscopies were evaluated for the presence of *H pylori*. Polymerase chain reaction-restriction fragment length polymorphism assay was used to characterize myeloperoxidase genotypes.

RESULTS: Forty four patients (57.1%) were *Hp* (+) and 33 (42.9%) were *Hp* (-). Sixty six (85.7%) had GG genotype, 10 (12.9%) had GA genotype and 1 (1.29%) had AA genotype. The change in atrophy in relation to neutrophil infiltration was significant in *Hp* (+) patients (P = 0.0001). The change in atrophy in relation to neutrophil infiltration in patients with GG genotype was significant (P = 0.002). However, the change in atrophy in relation to neutrophil infiltration was not significiant in patients with *Hp* (+) GG genotype (r = 0.066, P = 0.63).

CONCLUSION: Myeloperoxidase genotype is critical for development of atrophy in relation to the severity of inflammation. However, it is interesting to note that, *H pylori* does not show any additive effect on development of atrophy.

Key words: Gastritis; Gastroduodenal ulcer; Gastric cancer; Myeloperoxidase; *H pylori*

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INTRODUCTION

H pylori is an agent that produces chronic infection in more than half of the world population[1]. The most important characteristic of *H pylori* infection is that it causes chronic active inflammation in the gastric mucosa, which involves neutrophils and monocytes[2]. While 100% of the persons carrying this microbe develops gastritis, the lifetime risk of peptic ulcer is 15%-20% and that of gastric cancer is 1%-3%[1].

The role of host-related factors in the pathogenesis of diseases caused by *H pylori* has been greatly ignored up to date[3,4]. It was demonstrated that the ability of the host to regulate the production of cytokines is influenced by the presence of polymorphisms in the promoter region of the relevant genes[5]. The polymorphisms in the genes affecting the production of cytokines might be one of the factors that lead to the interpersonal differences in the severity of gastric inflammation[3,5].

Myeloperoxidase (MPO) is a lysosomal enzyme found in the azurophilic granules of polymorphonuclear leukocytes (PNL)[6-9]. There are two promoter regions affecting MPO; -463G/A and -129G/A[10]. Allele A of this polymorphism reduces mRNA expression and thus, tissue damage in local inflammation is decreased[8,9,11]. Allele G has 25 times more transcriptional efficacies compared to allele A. The G/G genotype confers higher risk for persistent *H pylori* infection[12,13].

H pylori activates the oxidative metabolism in neutrophils[14]. Reactive oxygen products such as free oxygen radicals released from neutrophils, (O^{2-}), H_2O_2 and hydroxyl ion (OH) reacts with MPO and hypochloric acid (HOCI) is formed. Monochloroamines (NH_2CI) are formed when HOCI reacts with the ammonium produced by the urease enzyme of *H pylori*. Monochloroamines are oxidizing agents that are able to induce DNA fragmentation[4,8,14-16]. Thus, it has been advocated that

myeloperoxidase is involved in gastric damage induced by *H pylori*[4,11,17]. We assessed the association between neutrophil infiltration and atrophy caused by *H pylori* and MPO gene polymorphisms.

MATERIALS AND METHODS

We enrolled 77 patients (46 males, 31 females) who had undergone endoscopic examinations due to epigastric pain, dyspepsia, nausea and vomiting or weight loss. Patients who received antibiotic therapy, proton-pump inhibitors or non-steroid anti-inflammatory drugs within 3 mo prior to endoscopies were excluded.

Based on the endoscopic findings, 24 patients had gastritis, 26 had ulcers (duodenal or gastric ulcers) and 27 had gastric cancers. Diagnosis of patients who showed malignant findings in their endoscopies was confirmed by pathologic examination. In the light of endoscopic findings, two biopsies were obtained each from the antrum, angulus and corpus mucosa of the tissue adjacent to ulcer region and regions distant to malignant lesions. Biopsy materials were transferred into 3 separate small bottles containing formaldehyde and sent to the laboratory for evaluation of *H pylori* and pathologic examinations. Also, peripheral blood samples (3 mL) were collected from enrolled patients simultaneously and transferred into EDTA hemogram tubes. The tubes were transferred to the genetic laboratory under appropriate conditions avoiding coagulation.

Histopathologic examinations

The materials brought to the pathology laboratory were embedded in paraffin after follow-up procedures. Subsequently, cross-sections of 3-4 microns thick were obtained from the paraffin blocks. These cross-sections were stained by hematoxylin-eosin, Giemsa and Warthin-Starey stains, respectively. Preparations were divided into two groups as negative or positive based on the presence of *H Pylori* during direct visualization under a light microscope. Biopsy materials were staged according to neutrophilic activity, chronic inflammatory cell infiltration, glandular atrophy, intestinal metaplasia and *H pylori* density using criteria from the modified Sydney classification system. Each feature was evaluated as none (0), mild (1), moderate (2) or apparent (3)[18]. After all patients were scored according to 4 parameters of Sydney classification, sum of the parameters was calculated for three biopsy regions.

Myeloperoxidase genotyping

Patient blood samples transferred into EDTA tubes were used for DNA isolation. DNA isolation was performed using the column method (Gentra DNA isolation kit). Myeloperoxidase polymorphism analysis was performed by PCR and restriction fragment length polymorphism (RFLP) methods. Primers to be used were designed to detect Codone 463 of the myeloperoxidase gene.

Preparation of DNAs for PCR

Twenty microlitres DNA from each patient was transferred into 0.2 mL Eppendorf tubes. After addition of two different primers (forward pimer 5'-CCGTATAGGCAGA GAATGGTGAG-3' and reverse primer 5'-GCAATGGT TCAAGCGATTCTTC-3'), 1.5 μL; dNTP mix, 1 μL; Taq DNA polymerase, 1 μL; PCR buffer, 10 μL and distilled water, 15 μL, these tubes were placed into a PCR machine. PCR conditions were primer annealing at 56℃ for 1 min, polymerization at 72℃ for 1 min, and denaturation at 94℃ for 1 min. Thirty cycles were carried out[19]. The PCR product (amplicon) was incubated with *Acl*-I enzyme at 37℃ for 1.5 h and separated on a 2% agarose gel. DNA fragments on the gel were visualized after staining with 0.5 μg/mL ethidium bromide (EtBr).

Table 1 Distribution of myeloperoxidase genotypes in *Hp* (+) and *Hp* (-) patients according to endoscopic findings

	Hp (+)	*Hp* (-)	Total
n (%)	44 (57.1)	33 (42.9)	77 (100.0)
Gastritis	13	11	24
Ulcer (gastric or duodenum)	18	8	26
Gastric cancer	13	14	27
Myeloperoxidase GG	37	29	66
Myeloperoxidase GA	7	3	10
Myeloperoxidase AA	-	1	1
Neutrophil infiltration	2.80 ± 1.94[1]	0.88 ± 1.24	1.97 ± 1.91

Hp (+): *H pylori* positive group; *Hp* (-): *H pylori* negative group; $P < 0.0001$, *vs* the *Hp* (-) group. [1]Mean ± SD of a score of 0-3 according to the updated Sydney system.

Statistical analysis

Statistical analyses were performed using SPSS 11.0 for Windows statistical software, Kendall's Tau test and Linear regression analysis. P values < 0.05 were considered significant.

RESULTS

The mean age of the patients was 54.9 ± 14.1 years (19-82). Pathologic examination revealed that 44 of them were *Hp* (+) and 33 were *Hp* (-). Based on MPO gene polymorphism, 66 patients (85.7%) had GG genotype, 10 (12.9%) had GA and 1 (1.29%) had AA genotype. The patient with genotype A was an *Hp* (-) ulcer patient (Table 1).

There was a difference between *H pylori* (+) and *H pylori* (-) patients with respect to PNL infiltration (P = 0.0001). The change in atrophy in relation to neutrophil infiltration was significant in *Hp* (+) patients (y = 0.700x + 0.051) (P = 0.0001) (Figure 1). No such correlation was found for *Hp* (-) patients (y = 0.1214x + 0.50) (y: atrophy, x: neutrophil infiltration) (Figure 2) .

We found a significant correlation between atrophy and neutrophil infiltration in patients with GG genotype (n = 66) (y = 0.252x + 0.239) (P = 0.002). There was an insignificant correlation between atrophy and neutrophil infiltration in patients with GA genotype (n = 10) (y = 0.15x + 1.387) (P = 0.56).

Among the *Hp* (+) patients, the correlation between atrophy and neutrophil infiltration was insignificant for patients with both GG (n = 37) and GA genotypes (n = 7) (r = 0.066, P = 0.63; r = -0.474, P = 0.18, respectively).

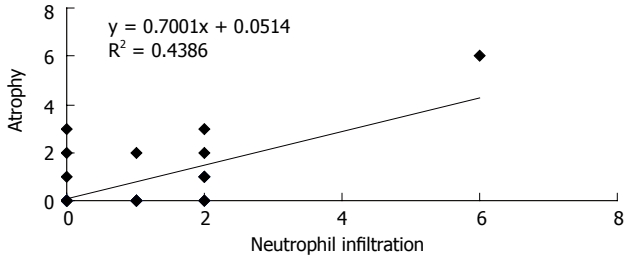

Figure 1 Changes in the atrophy in relation to neutrophil infiltration in *Hp* (+) patients.

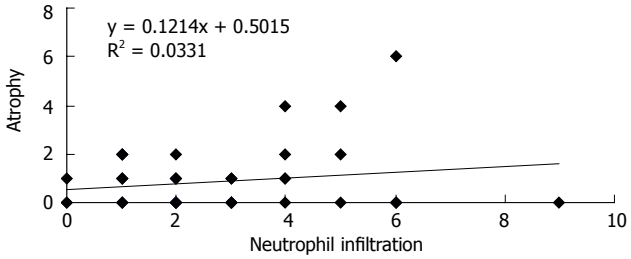

Figure 2 Changes in the atrophy in relation to neutrophil infiltration in *Hp* (-) patients.

Among the patients with *Hp* (-), we found an insignificant correlation between atrophy and neutrophil infiltration in patients with both GG (n = 29) and GA genotypes (n = 3) (r = 0.316, P = 0.06; r = 0.816, P = 0.22, respectively). Since our sample size was small, a statistical analysis for MPO AA (n = 1) genotype could not be performed.

DISCUSSION

About 60% of the world population is infected with *H pylori*[1]. Hyperproliferation induced by *H pylori* gastritis has been assumed as a starting point for events that trigger gastric cancer. Moreover, this hyperproliferation has been suggested to initiate changes in DNA[4,20,21]. The World Health Organization (WHO) has declared *H pylori* as the leading carcinogenic factor. In a cohort study conducted by WHO in 3 different regions, anti-*Hp* antibody levels were measured in blood samples obtained within the last 24 years from patients diagnosed with gastric cancer. It was stated that *H pylori* seropositivity increased the relative risk for developing gastric cancer[1]. Yamagato *et al*[22] reported that 3% of 1721 patients with *H pylori* developed gastric cancer during 9 years of follow up. Also, Uemura *et al*[23] reported that 2.9% of 1246 *Hp* (+) patients developed gastric cancer during 7.6 years of follow up. While there was a significant difference in *Hp* (+) patients with respect to atrophy and neutrophil infiltration, no such correlation was found for *Hp* (-) patients. Thus, inflammation induced by *H pylori* could be one of the predisposing factors in carcinogenesis.

Recently studies have been published on cytokine production in several conditions caused by *H pylori* infection as a host response and genetic polymorphisms[4]. Zambon *et al*[3] found that bacterial and virulence factors were associated with mucosal inflammation and severity of the illness. They found that *H pylori* virulence genes and a host genotype of IL-1 RN were directly correlated with peptic ulcer and intestinal metaplasia and they suggested that the interaction between cytokine genotypes and bacterial virulence factors are fundamental to development of *H pylori*-related lesions. Nardone *et al*[24] reported that, first atrophy localized in the antrum regresses or disappears subsequent to *H pylori* eradication. The changes seen in these lesions as a result of eradication show that, neutrophil infiltration plays a major role in these events above all.

Polymorphisms, defined as known changes in the genomic sequence, occur frequently throughout the human genome. It is known that in some cases, they modify the expression or function of gene products. Any polymorphism could affect susceptibilities to and outcome of an illness through the interaction of environment and genetics[25].

The question of how the variations in the genes associated with inflammation affect the inflammatory response induced by *H pylori* and accompanying gastric pathologies is an interesting one[26]. It has been stated that MPO itself might modulate susceptibilities to clinical outcomes of several illnesses where neutrophils are involved[10]. In a number of studies, the association between MPO polymorphisms and several conditions including chronic granulomatous illness, Alzheimer' s disease, malignancies such as the lung or pharyngeal cancer, some types of leukemia, atherosclerosis, periodontal diseases, multiple sclerosis and cystic fibrosis were reported[4,8,11-13]. Hamajima *et al*[12] investigated a myeloperoxidase genotype with low expression, which was negatively correlated with *H pylori* infection in 241 patients having complaints of dyspepsia, without a history of cancer. They found 79.7% of GG genotype, 19.5% of GA genotype and 0.8% of AA genotype. In another study assessing the association between MPO polymorphisms, atrophy and neutrophil infiltration, Roe *et al*[4] found 81.9% of GG genotype, 18.1% of GA genotype and 0.0% of AA genotype. It was indicated that while there was a positive correlation between the degree of atrophy and neutrophil infiltration in individuals with GG polymorphism, no such association was found in individuals with GA polymorphism. Also, in our study we found that while the correlation between atrophy and neutrophil infiltration was significant in patients with GG genotype, it was insignificant in patients with GA genotype.

Our results show that generally myeloperoxidase GG genotype is a critical host-related factor for development of atrophy that occurs in the setting of inflammation, and is considered as a cancer precursor. However, it is interesting to note that on the contrary to what is expected, GG genotype and *H pylori* positivity does not potentiate each other for development of atrophy. More studies are needed to elucidate this point in larger patient groups.

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RAPID COMMUNICATION

Prevalence of gastric varices and results of sclerotherapy with N-butyl 2 cyanoacrylate for controlling acute gastric variceal bleeding

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Abstract

AIM: To study the prevalence, predictors and control of bleeding following N-butyl 2 cyanoacrylate (NBC) sclerotherapy of gastric varix (GV).

METHODS: We analyzed case records of 1436 patients with portal hypertension, who underwent endoscopy during the past five years for variceal screening or upper gastrointestinal (GI) bleeding. Fifty patients with bleeding GV underwent sclerotherapy with a mean of 2 mL NBC for control of bleeding. Outcome parameters were primary hemostasis (bleeding control within the first 48 h), recurrent bleeding (after 48 h of esophago-gastro-duodenoscopy) and in-hospital mortality were analyzed.

RESULTS: The prevalence of GV in patients with portal hypertension was 15% (220/1436) and the incidence of bleeding was 22.7% (50/220). Out of the 50 bleeding GV patients, isolated gastric varices (IGV-I) were seen in 22 (44%), gastro-oesophageal varices (GOV) on lesser curvature (GOV-Ⅰ) in 16 (32%), and GOV on greater curvature (GOV-Ⅱ) in 15 (30%). IGV-Ⅰ was seen in 44% (22/50) patients who had bleeding as compared to 23% (39/170) who did not have bleeding (P < 0.003). Primary hemostasis was achieved with NBC in all patients. Re-bleeding occurred in 7 (14%) patients after 48 h of initial sclerotherapy. Secondary hemostasis was achieved with repeat NBC sclerotherapy in 4/7 (57%). Three patients died after repeat sclerotherapy, one during transjugular intrahepatic portosystemic stem shunt (TIPSS), one during surgery and one due to uncontrolled bleeding. Treatment failure-related mortality rate was 6% (3/50).

CONCLUSION: GV can be seen in 15% of patients with portal hypertension and the incidence of bleeding is 22.7%. NBC is highly effective in controlling GV bleeding. In hospital mortality of patients with bleeding GV is 6%.

Key words: Gastric varices; Portal hypertension; N-butyl cyanoacrylate; Bleeding; Sclerotherapy

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INTRODUCTION

Gastric varix (GV) and its association with portal hypertension were first described in 1913[1]. Since then, there have been reports on different aspects of gastric varices including prevalence, bleed tendency and treatment options. The prevalence of GV in patients with portal hypertension varies from 18% to 70%[2,3], although the incidence of bleeding from gastric varices is relatively low ranging from 10% to 36%[3].

Management of GV presents is a challenging problem, because (1) since there is no consensus regarding the optimum treatment of GV, treatment tends to be empiric; (2) GV is not a homogeneous entity, and accurate classification defines its natural history and dictates its management; (3) although GV bleeding occurs less frequently than esophageal varix (EV) bleeding[4,5], whenever bleeding occurs it tends to be more severe and requires more red blood cell transfusions and has a higher mortality than EV bleeding[5]; (4) after control of acute bleeding, GV has a high rebleeding rate of 34% to 89%[6]; and (5) there is no consensus regarding subsequent sclerotherapy for prophylaxis against recurrent bleeding in GV patients. Optimal management of GV requires a multidisciplinary approach and close cooperation between gastroenterologists, interventional radiologists and the surgical team.

Sohendra *et al*[7] first reported in 1986 that bleeding from gastric varices could be controlled by sclerotherapy

using the tissue adhesive agent butyl cyanoacrylate. Since then several authors have used different sclerosing agents to achieve hemostasis in bleeding gastric varices, including N-butyl-2 cyanoacrylate (histoacryl)[4,8], 2-octyl cyanoacrylate[9], ethanolamine oleate injection[10], gastric variceal banding[11], thrombin[12], sodium tetradecyl sulfate[13]. However, N-butyl 2 cyanoacrylate (NBC) is the only promising agent.

Most reports on endoscopic treatment of bleeding gastric varices are small series, case reports, or retrospective reviews[14,15]. Not more than 1000 patients with bleeding GV have been treated with different sclerosing and coagulating agents. Cyanoacrylate injection can achieve primary hemostasis in 70% to 95% of patients with acute GV bleeding, with an early rebleeding rate ranging from 0% to 28% within 48 h[5,7,16]. Different doses of cyanoacrylate are used by different gastroenterologis ts[16,17]. Moreover, dilution ratio of NBC to lipoidal is different[18,19]. However, there is no consensus regarding effective dose and dilution of sclerosing agents.

This study was to analyze patients with GV in order to establish predictors of bleeding GV, and the efficacy and safety of NBC in treatment of bleeding GV.

Table 1 Clinical characteristics of patients with gastric varices

Characteristics	*n*	(%)
Number of patients	220	
Male:Female	141:79	(65:35)
Mean age (yr)	50 ± 11	
Etiology of GV		
HCV cirrhosis	155	(70)
HBV cirrhosis	38	(17.5)
NBNC	27	(12.5)
NCPHTN	9	
Alcoholism	5	
Budd-Chiari syndrome	1	
Wilson's disease	1	
Child-Pugh classification (A/B/C)	39/109/72	(18/49/33)
Types of gastric varices		
GOV-1	78	(35)
GOV-2	56	(25)
IGV-1	59	(27)
IGV-2	6	(03)
GOV-1 + GOV-2	14	(06)
GOV-2 + IGV-2	3	(1.5)
GOV 1+IGV 2	2	(01)
GOV 1 + IGV 1	2	(01)

MATERIALS AND METHODS

From March 2000 to March 2005, 1036 patients with portal hypertension underwent endoscopy in our hospital. Out of 220 GV patients with or without esophageal varices, 50 had active bleeding gastric varices which were treated with N-Butyl cyanoacrylate injection.

The cause of portal hypertension was defined on the basis of ultrasound and/or computed tomographic scan of abdomen along with Doppler ultrasound to study the patency and spleno-portal axis in all patients with bleeding GV.

The location of gastric varices was determined according to the classification described by Sarin and Kumar[3] and divided into gastroesophageal varices type 1 (GOV-1) GV continuing as an extension of esophageal varices on the lesser curve of the stomach), gastro-esophageal varices type 2 (GOV-2) on the greater curvature or fundal varices communicating with esophageal varices. Isolated gastric varices type 1 (IGV-1) and fundal varices within a few centimeters of the gastric cardia, or isolated gastric varices type 2 (IGV-2) and isolated ectopic gastric varices. Active bleeding was defined as bleeding or oozing of blood from a gastric varix, a clot or blackish ulcer or rent over a gastric varix, or the presence of distinct large gastric varices and absence of esophageal varices or other cause of gastrointestinal (GI) bleeding.

The clinical characteristics of patients including age, gender, Child-Pugh classification, type of varix, etiology and complications in patient with gastric varices were recorded and compared between groups with or without bleeding (Table 1).

Endoscopic sclerotherapy with n-butyl cyanoacrylate and lipoidal injection was performed by experienced endoscopists using a GIFQ160 gastroscope (Olympus, Tokyo, Japan). The sclerosant was injected into bleeding gastric varices using the 23 G disposable needle injector (Wilson Cook Medical Inc., Winston-Salem, NC).

The injection needle was primed with lipoidal (approximately 0.5 mL) to fill the dead space within the injection catheter. After the gastric varix lumen was punctured with the needle, 2 mL of NBC (histoacryl 100%) in aliquots of 1 mL, diluted in 0.5 mL lipoidal, was injected over 45-90 s, thus a dilution of 2:1 was achieved in all cases (Figure 1 and Figure 2). Distilled water was used to flush the injection needle before and after sclerotherapy. Additional NBC was not injected if bleeding was immediately controlled in patients. All patients received octreotide infusion (50 mg/per hour) at admission and continued for 3 d. All patients were given a proton pump inhibitor, initially intravenously for 48 h and then orally for 4-6 wk as per our endoscopy unit protocol.

Outcome measures

Primary hemostasis was defined as stability of vital clinical signs, no drop in hemoglobin concentration and no recurrent bleeding within the first 48 h after the initial sclerotherapy[16]. Re-bleeding was defined as hematemesis and/or melena, hypotension (a drop in systolic blood pressure of > 20 mmHg from baseline), fall in 2 gm/dL of hemoglobin and/or transfusion requirement of $\geqslant$ 2 units of packed red cells within that time.

Data analysis

Statistical interpretation of data was performed using Statistical Program for Social Sciences (SPSS) version 13. Results were expressed as mean ± SD for all continuous variables (e.g., age, gender, hospital stay, units of packed cells *etc.*) and numbers (percentage) for categorical data (e.g., gender, Child's class, *etc.*). Analysis was performed by using the independent *t*-test, chi-square test and Fisher's exact test wherever appropriate. $P < 0.05$ was considered statistically significant.

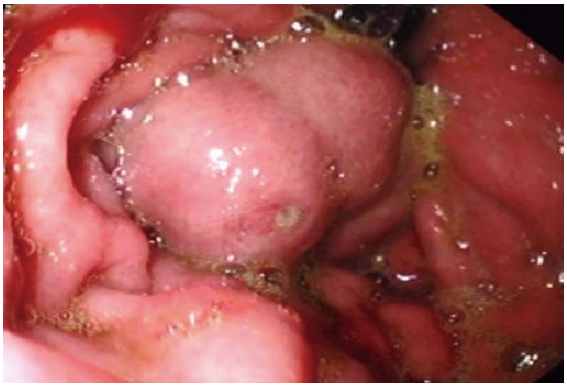

Figure 1 Isolated gastric varices with a nipple suggesting recent bleeding.

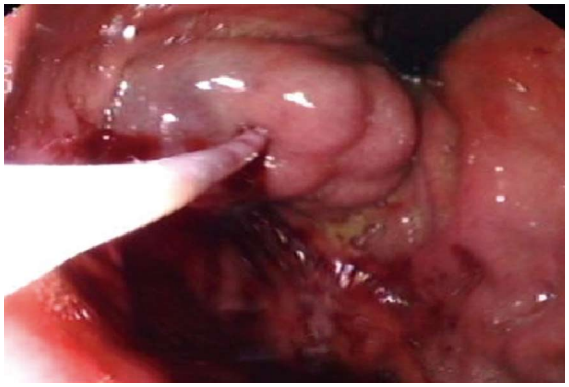

Figure 2 Injector needle with N- butyl cyanoacrylate in a large gastric fundal varix.

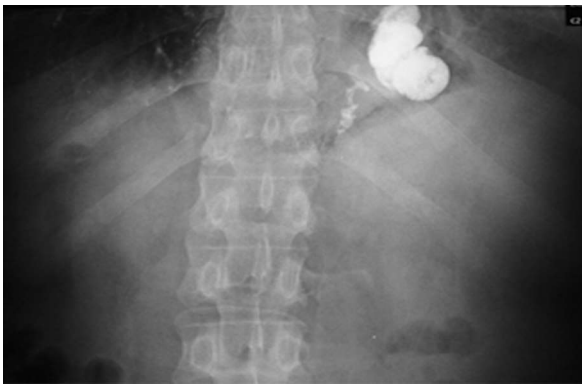

Figure 3 X-ray showing N-butylcyanoacrylate deposition in a gastric varix after sclerotherapy.

Table 2 Comparison between bleeding and non bleeding varices

Characteristics	Bleeding GV *n* (%)	Non bleeding GV *n* (%)	*P*
Age	50.48 ± 11.2	47.52 ± 12.7	0.14
Gender			
Male	30 (60)	111 (65.3)	
Female	20 (40)	59 (34.7)	0.49
Child Pugh class.			
A	12 (24)	27 (15.9)	
B	26 (52)	83 (48.8)	0.22
C	12 (24)	60 (35.3)	
Type of GV			
GOV- I	16 (32)	77 (45.3)	0.09
GOV-Ⅱ	15 (30)	58 (34.1)	0.58
IGV- I	22 (44)	39 (22.9)	< 0.01
IGV-II	0 (0)	11 (6.5)	0.06
Etiology of PHTN			
HBV	6 (12)	29 (17.1)	0.39
HCV	34 (68)	121 (71.2)	0.76
NBNC	6 (12)	21 (12.4)	0.94
Alcoholic	02 (2)	03 (1.8)	0.48
Non cirrhotic PHTN	01 (2)	08 (4.7)	0.59
Wilson's disease	01 (2)	00 (0)	0.06

RESULTS

The prevalence of bleeding gastric varices in patients with portal hypertension was 15% (220/1436) and the incidence of bleeding was 22.7% (50/220). The mean age of our patients with gastric varices was 50 ± 11 years, male patients accounting for 65% (141). The main etiology of bleeding gastric varices was hepatitis C-related cirrhosis in 34 (68%), followed by hepatitis B virus in 6 and non B non C cirrhosis in 6 (12%) patients, respectively. Alcoholic liver disease was found in 2 patients (4%), non cirrhotic portal hypertension in 1 patient and Wilson's disease in 1 patient, respectively. Twelve patients (24%) belonged to Child-Pugh A, 26 (52%) to Child-Pugh B, and 12 (24%) to Child-Pugh C.

Out of the 50 bleeding GV patients, IGV- I was seen in 22 patients (44%), GOV- I in 16 patients (32%), and GOV-Ⅱ in 15 patients (30%), concomitant GOV- I with GOV-Ⅱ in 3 patients. N-butyl cyanoacrylate (2 mL diluted in 1 cc lipoidal) was injected intravariceally in all patients with bleeding GV. The characteristics of bleeding and non bleeding gastric varices are shown in Table 2.

Among these 50 patients, 43 (86%) had active bleeding and 7 (14%) showed evidence of recent bleeding. Most varices (n = 48) were large (F3) according to Hashizume classification[20]. We compared bleeding and non bleeding GV and found that IGV- I was seen in 22/50 (44%) patients who had bleeding and in 39/170 patients (23%) patients who did not have bleeding (P < 0.003). Similarly, IGV-Ⅱ was observed in 0/50 (0%) and 11/170 (6.4%), GOV- I in 16/50 (32%) and 77/170 patients (45.2%) who did not have bleeding, GOV-Ⅱ in 15/50 (30%) and 50/170 patients (29.4%) who did not have bleeding from gastric varices, respectively.

The overall success rate for achieving primary hemostasis with NBC was 100% without recurrent bleeding within 48 h. A mean of 2.0 ± 0.5 cc NBC was injected in each patient.

Re-bleeding occurred in 7 (14%) patients after 48 h. Secondary hemostasis with repeat NBC sclerotherapy was achieved in 4 (57%) patients. Three patients died after repeat sclerotherapy: one during transjugular intrahepatic portosystemic stem shunt (TIPSS), one during surgery and one due to uncontrollable torrential bleeding. Treatment failure-related mortality rate was 6 % (3/50).

No major side effects occurred in most patients during and after NBC sclerotherapy. Three patients reported mild retrosternal chest pain which subsided within the next 24 h (Figure 3).

DISCUSSION

The overall incidence of gastric varices in patients with portal hypertension is 2%-70%[3]. Furthermore, we found that the incidence of bleeding from gastric varices in our patients is 22.7%, which is also similar to other reports[5].

In this study, the rate for primary hemostasis with NBC injection is consistent with the reported rate of 90% to 97% in other studies[8,14,16,21-23]. In our study, sclerotherapy with 2 mL NBC diluted in 1 mL lipoidal was effective in control of bleeding in all our patients during the first 48 h.

We used a higher dose of NBC with 2:1 dilution. Our technique avoids dilution of NBC, thus decreasing chances of migration and embolization as instantaneous polymerization is delayed.

It was reported that a hemostasis rate of 95% can be achieved without dilution of NBC, but serious side effects such as embolization may occur[24].

The risk of embolization increases with over dilution of cyanoacrylate which can slow down the process of polymerization. We avoided this problem by diluting 2 mL of NBC in 1 mL of lipoidal which was injected slowly over 45-90 s. Although the volume of injection was large, the side effects of a large volume were avoided and good hemostais was achieved by keeping a balanced dilution.

We also studied the predictors of the first gastric variceal bleeding, showing that the increased risk of bleeding is associated with larger varices including predominant IGV- I and GOV- I [5]. Kim *et al*[23] found that advanced Child-Pugh class and varix > 5 mm in size are associated with an increased risk of bleeding. Unlike esophageal varices, a high porto-systemic pressure > 12 mmHg does not cause GV bleeding, which is probably related to the high frequency of spontaneous gastro-renal shunts[17].

Cyanoacrylate therapy can improve and control re-bleeding. The high mortality rate is primarily a reflection of underlying advanced liver disease unaffected by the injection of cyanoacrylate[25,26]. This is further explained by a study of Kim *et al*[23] in which they prospectively followed a cohort of patients with gastric varices who did not undergo endoscopic therapy and found that the mortality rate is 35% over a median of 15 mo. Therefore, the role and efficacy of sclerotherapy for prophylaxis against recurrent bleeding are questionable.

In the present study, re-bleeding occurred after 48 h in 7 (14%) patients and was controllable in 4 (57%) with repeat injection of NBC. It was reported that the incidence of re-bleeding ranges from 23% to 35%[8,10,16]. However, Kind *et al*[14] reported that the incidence of re-bleeding is 15.5%.

Treatment failure-related mortality in our study was 6%, which is consistent with the reported mortality[16]. Another study showed that the mortality is 12.5% due to re-bleeding immediately after NBC sclerotherapy[8]. Kind *et al*[14] reported that the hospital mortality rate is 19.5% in patients with bleeding gastric varices.

In conclusion, endoscopic injection of NBC at a dilution of 2:1 appears to be effective and safe for the control of hemostasis in patients with bleeding gastric varices. If NBC is injected slowly, a large amount of NBC in proper dilution can be used without serious side effects.

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RAPID COMMUNICATION

Detection of small hepatocellular carcinoma: Comparison of dynamic enhancement magnetic resonance imaging and multiphase multirow-detector helical CT scanning

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Abstract

AIM: To compare the gadolinium-enhanced multiphase dynamic magnetic resonance imaging (MRI) and multiphase multirow-detector helical CT (MDCT) scanning for detection of small hepatocellular carcinoma (HCC).

METHODS: MDCT scanning and baseline MRI with SE T1-WI and T2-WI sequence combined with FMPSPGR sequence were performed in 37 patients with 43 small HCCs. Receiver operating characteristic (ROC) curves were plotted to analyze the results for modality.

RESULTS: The areas below ROC curve (Az) were calculated. There was no statistical difference in dynamic enhancement MDCT and MRI. The detection rate of small HCC was 97.5%-97.6% on multiphase MDCT scanning and 90.7%-94.7% on MRI, respectively. The sensitivity of detection for small HCC on MDCT scanning was higher than that on dynamic enhancement MRI. The sensitivity of detection for minute HCC (tumor diameter $\leqslant$ 1 cm) was 90.0%-95.0% on MDCT scanning and 70.0%-85.0% on MRI, respectively.

CONCLUSION: MDCT scanning should be performed for early detection and effective treatment of small HCC in patients with chronic hepatitis and cirrhosis during follow-up.

Key words: Hepatocellular carcinoma; X-ray computed tomography; Magnetic resonance imaging

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignancy tumor of the liver. Dual-phase CT scanning is a sensitive method for the detection of HCC. The developments in rapid magnetic resonance imaging (MRI) in combination with gadolinium-enhanced multiphase multirow-detector helical CT (MDCT) scanning can obviously improve the detection of small HCC. However, MRI and MDCT have a lower sensitivity for detecting small HCC, especially minute HCC. MDCT scanning has a higher sensitivity for detection of small HCC[1-5]. The purpose of this study was to compare the dynamic enhancement MRI and MDCT for the detection of small HCC in patients with chronic liver disease and cirrhosis and to value the clinical role of new imaging technology in detection of small HCC.

MATERIALS AND METHODS

Patient Sample

Between October 2002 and December 2004, 37 patients (29 men, 8 women, mean age: 56 years, rang: 29-70 years) with chronic hepatic disease and cirrhosis who were suspected of having HCC during postoperative follow-up were included in this study. All the patients underwent multiphase contrast enhanced dynamic MDCT and gadolinium-enhanced dynamic MRI at 7 d intervals.

Lesion confirmation

Small HCC was confirmed in 24 of 37 patients. Fifteen of the 24 patients with 43 small HCCs underwent surgery and pathologic examination. Of the 15 patients undergoing surgery, small HCC was found in 2 by needle biopsy, in

Table 1 Area under the ROC curve and *P* value for MRI and MDCT in detecting HCC

	Observer 1		Observer 2	
Imaging techniques	A_z value	95% CI	A_z value	95% CI
MDCT	0.983	0.950-1.013	0.99	0.968-1.012
MRI	0.951	0.901-1.001	0.94	0.882-0.999
t value	-0.425		-0.956	
P value	0.672		0.348	

5 by digital subtraction angiography (DSA), and in 2 by iodized oil CT and elevated serum α-fetoprotein level (> 400 ng/mL) and ultrasound examination. Two out of 37 patients undergoing multiphase dynamic contrast enhanced MDCT and dynamic gadolinium enhancement MRI respectively, were suspected of having small HCC. Their serum α-fetoprotein level was decreased. Ultrasound examination one month after follow-up and multiphase dynamic contrast enhanced MDCT examination three months after follow-up showed that the lesions remained unchanged. Five out of 37 patients with liver cirrhosis undergoing gadolinium enhancement dynamic MRI scanning and multiphase dynamic contrast enhanced MDCT imaging were suspected of having small HCC. However, small HCC could not be found. Small HCC was not found in 3 of 5 patients who were followed up for 2-3 mo by contrast-enhanced MDCT, and in 2 of 5 patients who were followed up for 3 mouths by enhancement dynamic MRI. Multiphase dynamic contrast-enhanced MDCT and MRI examination displayed no new liver lesions in another 6 patients who were not diagnosed having small HCC during the tree-month follow-up period. The size of small HCC was ⩽ 1 cm in 20, and > 1 cm or ⩽ 3 cm in 23 of 43 small HCCs, respectively.

MDCT

All CT examinations were performed using a commercially available multidetector CT scanner (Marconi Mx 8000) with 0.5-0.75 s gantry rotation speed, 23.3 mm/s table speed, 5.0 mm-thick section, reconstruction interval 2.5 mm, 120 Kv and 200-250 mA.

Entire pre-contrast hepatic scanning was followed by a nonionic contrast enhancement (Omnipaque 300 mg I/mL) with 1.5 mL/kg and injection rate of 3 mL/s *via* an antecubital vein. Multiphase acquisitions were performed. The scanning delay set for early arterial phase (EAP), late arterial phase (LAP) and portal venous phase (PVP) was 21 s, 34 s and 85 s, respectively. Each of the entire liver scanning in cephalad-caudal orientation was completed in 4-8 s with patient's breath held.

MRI

MRI was performed with a GE Signa 1.5 T MR imaging system. A standard whole-body coil was used as the receiver coil for examinations. All the 37 patients underwent baseline MR imaging, including breath-hold spin-echo T1- weighted imaging(TR 500-700 ms, TE 14-20 ms), fat-suppressed fast spin-echo T2-weighted imaging (TR 2000-4000 ms, TE 80-120 ms) and gadolinium-enhanced triphasic dynamic gradient-recalled echo imaging with a fast multiplanar spoiled-gradient-recalled echo breath-hold imaging (FMPSPGR TR/TE/Flip Angle = 100-150 ms/4.6 ms/60-90°), matrix (256 × 128), 7 mm-thick section, and 1 acquisition. MR imaging was performed before and after gadolinium-enhanced dynamic gradient-recalled echo imaging (Gd-DTPA, Magnevist, Germany, NJ). Dynamic imaging was performed with a fast multiplanar spoiled-gradient-recalled echo breath-hold imaging. The contrast material was 0.2 mL/kg of body weight administered as a rapid IV bolus. After un-enhanced imaging, arterial phase image was obtained during 20-25 s. The second and third sets of images were obtained after approximately 60-90 s and 3 min, respectively.

Imaging analysis

All MRI and MDCT images were reviewed by two experienced radiologists, who knew that patients with liver cirrhosis were at risk of developing HCC, but were unaware of the presence and location of liver lesions and the result of other imaging examinations. The size and number of lesions were analyzed for the various multiphase dynamic contrast-enhanced MR sequences and MDCT imagines (early arterial, later arterial, portal phases) of the 37 patients.

The readers scored each imagine for the presence or absence of focal hepatic lesions, and assigned confidence levels to their observation: 1 = definite presence, 2 = probable presence, 3 = equivocal, 4 = probable absence, 5 = definite absence[6].

Statistical analysis

For each imaging method, a binomial receivor operationg characteristic (ROC) curve was fitted to each radiologist' s confidence rating using maximum likelihood estimation. The diagnostic accuracy of each imaging set for each observer and the composite data were calculated by measuring the area under the alternative free response ROC curve. The differences between imaging sets in terms of the mean Az value, were statistically analyzed using the two-tail Student's t test for paired data. The sensitivity and positive predictive values for each image set were then calculated. The sensitivity of each observer was determined by detecting the number of lesions assigned a confidence level of 1 or 2 from 42 HCCs. The degree of inter-observer agreement was calculated with chance-corrected kappa statistics. In general, a kappa statistic value greater than 0.75 is considered excellent agreement, 0.4-0.75 good agreement, and less than 0.4 poor agreement[6-8]. Statistical analyses were performed using the SPSS Statistical Programs, version 10.0. $P < 0.05$ was considered statistically different (Figure 1).

RESULTS

The kappa values were excellent between observers 1 and 2 for multiphase dynamic contrast-enhanced MDCT (κ value = 0.883) and MRI (κ value = 0.812). The A_z values calculated by each observer with multiphase dynamic contrast-enhanced MDCT and MRI for 42 lesions are shown in Table 1. For detection of lesions, two observers achieved a slightly higher diagnostic performance with

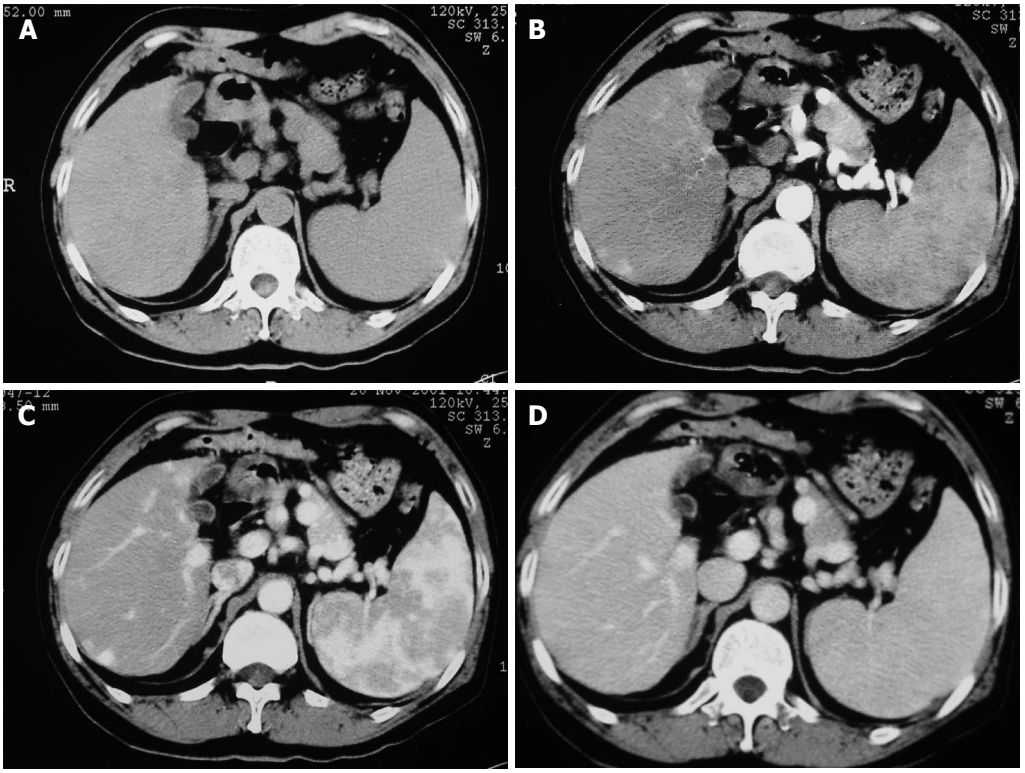

Figure 1 A 42-yr old man with pathologically proven hepatocellular carcinoma. **A**: Plan scanning; **B**: early arterial phase MDCT image showing slightly enhanced nodule in right lobe; **C**: later arterial phase MDCT image showing obvious enhanced lesion; **D**: on the portal venous phase MDCT image showing slight low attenuation.

Table 2 Sensitivity and positive predictive value for MRI and MDCT in detecting small HCC *n* (%)

Imaging Technique		Sensitivity (%)			Positive predictive value (%)
		≤ 1 cm SHCC *n* = 20	> 1 cm ≤ 3 cm SHCC *n* = 23	Total SHCC *n* = 43	*n* = 43
Observer 1	MDCT	19 (95.0)	22 (95.7)	41 (95.31)	97.6
	MRI	17 (85.0)	22 (95.72)	39 (90.7)	90.7
Observer 2	MDCT	18 (90.0)	21 (91.31)	39 (90.79)	97.5
	MRI	14 (70.0)	22 (95.7)	36 (83.7)	94.7

MDCT than with MRI, but the difference in the mean Az values of both imagine sets was not significant. The chance-corrected kappa values indicated the confidence levels for the imagine interpretation of the ROC analysis between the two observers.

The sensitivity and positive predictive values obtained with dynamic contrast-enhanced MDCT and MRI are shown in Table 2. For less than or equal to 1 cm minute HCC, the sensitivity of MRI was 85% and 70% , respectively for the two observers, but the sensitivity of MDCT for the two observers was higher than that of MRI (95% and 90%, respectively). The positive predictive value for MDCT was 97.5%-97.6% for MDCT and 90.7%-94.7% for MRI, respectively. There were less false-positive findings on MDCT.

DISCUSSION

Most HCCs occur in cirrhotic liver. Oka *et al*[9] in a 6-year follow-up study of 140 patients with cirrhosis, Oka *et al*[9] found that the cumulative incidence of HCC is 39% and its per year occurrence rate is 5.3%-8.8%. Small HCC results from cirrhosis. Early detection and diagnosis of small HCC are important in its effective treatment. Lim *et al*[10] have analyzed the sensitivity and specificity of double phase SCT in detecting small HCC. It was reported that imaging technology should be used in detecting small HCC[9,11].

Multiphase dynamic CT using a helical scanner has become the standard technology for detection and diagnosis of small HCC. The detection of hepatic nodular lesions by CT depends on the attenuation difference in the normal parenchyma and enhancement nodule lesions. Multiphase dynamic CT scanning can show the change between the tumor and its surrounding parenchyma. This may in part be a result of the difference in uptake and secretion of contrast material by hepatocytes of the normal liver parenchyma and HCC cells. Because the arterial phase is shorter than the portal venous phase, the scanning time of hepatic lesions is not the optimal time of tumors. Therefore, the dual-phase scanning is difficult to detect small HCC[7,9,11,12].

MRI is a less invasive and more feasible technique. The spin-echo sequence and gadolinium-enhanced triphasic dynamic breath-hold imaging are more effective. The advantage of contrast enhancement MRI is to deliver a small contrast material volume over a shorter period of time, which results in a tighter bolus. MRI has several advantages over dual-phase SCT in detection and characterization of small HCC, including greater soft-tissue contrast and sensitivity to intravascular contrast agent, and more types of sequences[6,14,16].

Tang *et al*[17] reported that 94% of small HCC can be found in cirrhotic liver by dynamic gadolinum-enhanced MRI. Yan *et al*[18] reported that 94.12% of small HCC (diameter < 1 cm) can be detected by MRI. Kim *et al*[19]

reported that 67% of small HCC (diameter < 1 cm) can be displayed by MRI.

The optimal imaging technology can detect most of small HCC. MDCT scanner recently has been introduced into clinical CT practice, and allows faster Z-axis coverage speed and hepatic imaging with thin image thickness in a very short time. MDCT can scan the entire liver during the double arterial phase. In addition, it improves the sensitivity of depicting small HCC and increases conspicuity for hypervascular lesions, and sensitivity of detecting small HCC[4,5,24].

The ROC curve analysis revealed that there was a higher validity for two imaging methods. Two observers achieved slightly higher diagnostic efficiency with multiphase contrast-enhanced MDCT than with dynamic enhanced MRI, but the difference in the areas below the ROC cure was not statistically significant (observer 1, $P = 0.672$; observer 2, $P = 0.348$). Multiphase contrast-enhanced MDCT had slightly higher predictive values than dynamic contrast enhanced MRI. The positive predictive value for MDCT and MRI by observer 1 was 97.6% and 90.7%, and 97.5% and 94.7% by observer 2, respectively. There were less false-positive findings on MDCT, but whether the diagnostic efficiency of multiphase contrast-enhanced MDCT is superior to dynamic contrast enhancement MRI needs further study with a large sample.

It was reported that the sensitivity of MRI in detection mall HCC is lower[20-22]. However, several factors can affect the visualization of primary focal hepatic lesions during contrast-enhanced or un-enhanced MRI, including the functionality of normal hepatic parenchyma, as well as the dimension, composition, and degree of visibility of the lesions, and the residual hepatic functionality of neoplastic cells themselves[23,24]. Since these factors tend to vary from patient to patient, it is often difficult to predict the behavior of a given lesion. There is a very good correlation between the blood supply and the degree of pathologic characteristics of hepatic lesions.

In our study, the sensitivity of MRI in detecting small HCC ($\leqslant$ 1 cm) was 70% and 85%, respectively. Seventy percent of 20 small HCCs showed isointensity, 4 demonstrated the signal change from isointensity to hyperintensity during arterial phase, portal venous phase, and delay phase. The other lesions displayed the signal change from hyperintensity to isointensity. These different findings may be due to the following reasons. First, cirrhotic liver has homogeneous or nonhomogeneous density, the signal intensity of cirrhotic liver parenchyma is higher than normal liver parenchyma, and the high patching signal intensity on T1-weigted imagines is due to fatty deposition or hepatitis. Second, since the degree of the liver parenchyma is lower because of hemosiderin deposition and large fibrous tissue in the liver parenchyma, the impaired hepatic cells could not absorb contrast material. Third, the portal hypertension splits liver blood flow into collateral vessels, thus reducing the contrast material in the liver parenchyma. Forth, since the time window of arterial phase is narrower and the imaging time of MRI is fixed at the arterial phase 20-25 s, portal venous phase 60-90 s, and delayed phase 120 s, it is difficult to show a real arterial phase[4].

In conclusion, small HCC often manifests as relatively small lesions in cirrhotic livers. There is an obviously enhanced small nodule in hepatic arterial phase whether it has homogeneous or nonhomogeneous density. The signal intensity and contrast enhancement patterns cannot be used in the final diagnosis of small HCC. It is important for patients with cirrhosis to undergo follow-up imaging more frequently. Nodule growth is highly predictive of small HCC[21,26]. Further study is needed since our study has some limitations.

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RAPID COMMUNICATION

Aberrant expression of ether à go-go potassium channel in colorectal cancer patients and cell lines

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Abstract

AIM: To study the expression of ether à go-go (Eag1) potassium channel in colorectal cancer and the relationship between their expression and clinico-pathological features.

METHODS: The expression levels of Eag1 protein were determined in 76 cancer tissues with paired non-cancerous matched tissues as well as 9 colorectal adenoma tissues by immunohistochemistry. Eag1 mRNA expression was detected in 13 colorectal cancer tissues with paired non-cancerous matched tissues and 4 colorectal adenoma tissues as well as two colorectal cancer cell lines (LoVo and HT-29) by reverse transcription PCR.

RESULTS: The frequency of positive expression of Eag1 protein was 76.3% (58/76) and Eag1 mRNA was 76.9% (10/13) in colorectal cancer tissue. Expression level of Eag1 protein was dependent on the tumor size, lymphatic node metastasis, other organ metastases and Dukes' stage ($P < 0.05$), while not dependent on age, sex, site and degree of differentiation. Eag1 protein and mRNA were negative in normal colorectal tissue, and absolutely negative in colorectal adenomas except that one case was positively stained for Eag1 protein.

CONCLUSION: Eag1 protein and mRNA are aberrantly expressed in colorectal cancer and occasionally expressed in colorectal adenoma. The high frequency of expression of Eag1 in tumors and the restriction of normal expression to the brain suggest the potential of this protein for diagnostic, prognostic and therapeutic purposes.

Key words: Colorectal cancer; Adenoma; Ether à go-go gene; Potassium channels

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INTRODUCTION

Ion channels play a vital role in the function of diverse cell types. In recent years, the concept of ion channels as therapeutic targets and diagnostic and prognostic biomarkers has attracted increasing interest, and trigged a wave of experimental results, identifying individual ion channels relevant with specific cancer types.

In 1969, several mutants from the fruit fly Drosophila melanogaster were produced following the treatment of adult males with ethyl methane sulfonate[1]. The mutants presented shaking of the legs under ether anesthesia and independent gene loci were found to be involved. One of these mutants exhibited a slow and rhythmic leg-shaking behavior; the locus involved was then named ether à go-go. Homology screening helped to identify other two sequences closely related to eag: the eag-related gene (erg) and the eag-like gene (elk); thus the EAG channels family comprises three subfamilies: EAG, ERG and ELK[2]. Two members for the eag subfamily, three for erg and two for elk are differentially expressed in different species including rat, bovine and humans. Two members of the eag subfamily are Eag1 (KCNH1, Kv10.1) and Eag2 (KCNH5, Kv10.2)[3].

Eag1 is detected only in the brain and placenta in the process of myoblast fusion, indicating that the channel is not expressed in differentiated peripheral tissues[4]. On the other hand, eag1 is expressed in several cell lines derived from human malignant tumors, such as neuroblastoma[5,6], melanoma[7], breast[5], and cervical carcinoma[5]. In these cell lines, Eag1 enhances the proliferation of the cells, and is required for the maintenance of growth. One of the most intriguing aspects of human eag1 channels is its relationship to cellular transformation. Cells transfected with Eag1 are able to grow in the absence of serum, lose contact inhibition, and induce aggressive tumors when implanted into immune-depressed mice[5]. Recently, functional expression of Eag1 has been described in clinical samples of cervical carcinoma[8]. Similarly, aberrant

expression of the channel has also been detected in sarcomas[9] and other tumors from diverse origins[12], while the surrounding tissues are devoid of Eag1 expression. Moreover, specific inhibition of Eag1 expression by antisense technique[5], siRNA[9,10] or by non-specific blockers[7,11] leads to a reduction in tumor cell proliferation *in vitro*.

The membrane protein Eag1 is accessible from the extracellular side and is predominantly present in tumor cells, represents a potential tumor marker and an interesting therapeutic target for cancer. For any potential clinical application it is an essential prerequisite that samples from human tumors overexpress the target Eag1. Colorectal cancer is a malignancy with a worldwide distribution, and the actual frequency of expression of Eag1 in colorectal tissue remained unknown. In this study, we explored the expression of Eag1 potassium channel in colorectal cancer and the relationship between their expression and clinicopathological features.

MATERIALS AND METHODS

Patients

Samples were obtained from Renmin Hospital of Wuhan University, according to a protocol approved by the Committee for the Conduct of Human Research. These patients had surgery between 2004 and 2005. Resected tissues were formalin-fixed and paraffin-embedded in the Department of Pathology. For immunohistochemical analysis, colorectal cancer tissue with paired non-cancerous matched tissue (NCMT) (n = 76) as well as colorectal adenoma tissue (from endoscopic biopsy) (n = 9) were obtained. For reverse transcription polymerase chain reaction, 13 colorectal cancer tissues with NCMT as well as 4 colorectal adenoma tissues (obtained from endoscopic biopsy) were examined during March to June 2006. These fresh specimens were kept in liquid nitrogen immediately after excision until use. Two pathologists screened histological sections and selected areas of the representative tumor cells. Tumor stage was classified according to Dukes' criteria.

Immunohistochemistry

For immunohistochemical analysis, 5 μm sections were sliced and mounted on poly-L-lysine-coated slides the day before use. Immunohistochemistry was conducted according to instructions of HistostainTM-Plus kits (Beijing Zhongshan Golden Bridge Biotechnology Co., LTD). The primary antibody Eag1 (Sigma-Aldrich, USA) was diluted 1:200 with 0.1% bovine serum albumin. As negative controls, the slides were treated by replacement of primary antibody with non-immune serum.

TTo achieve a semi-quantitative estimation of Eag1 expression levels, we used an immunohistochemical score method: Scores were 0, less than 10% of the tumor cells stained; 1+, faint staining in more than 10% of the cells; 2+, moderate staining in more than 10% of tumor cells; and 3+, strong staining in more than 10% of the cells. The immunohistochemical score was evaluated as negative (0), weakly positive (1+), and strongly positive (2+, 3+). Each stained slide was scored by two independent observers. There were no major disagreements regarding scoring and the average scoring was reported.

Cell culture

HT29 and LoVo cells (obtained from Cell Bank, Chinese Academy of Sciences) were maintained in T75 flasks in a humidified atmosphere at 37℃ with 50 ml/L carbon dioxide and passaged every 4-5 d. The HT-29 line was isolated from primary tumor, and LoVo line was isolated from metastatic tumor nodules in the left supraclavicular region. HT29 cells were cultured in McCoy's 5a medium (modified) with 1.5 mmol/L L-glutamine adjusted to contain 2.2 g/L 90% sodium bicarbonate, 10% fetal bovine serum. LoVo cells were grown in Ham's F12K medium with 2 mmol/L L-glutamine adjusted to contain 1.5 g/L 90% sodium bicarbonate, 10% fetal bovine serum. All media were also supplemented with 100 units/mL penicillin plus 100 μg/mL streptomycin.

RNA preparation and reverse transcription PCR

Total RNA was isolated from colorectal tissue and HT29 and LoVo cells using TRIZOL® reagent (Invitrogen Corporation, USA) following instructions of the TRIZOL kit.

We designed specific primers for Eag1 (Genbank accession: AF078741) and β-actin. The primers were as follows: For Eag1 (bp966-bp1441, 475 bp), sense primer 5' -GCTTTTGAGAACGTGGATGAG-3'; antisense primer 5'-CGAAGATGGTGGCATAGAGAA-3'. For β-actin (479 bp): sense primer 5'-TGACGGGGTCACCCACACT GTGCC-3'; antisense primer: 5'-CTGCAFCCTGTCGG CAATGCCAG-3' (479 bp). The primers were synthesized by Shanghai Sangon (China).

One step reverse transcription PCR (RT-PCR) was performed using One Step mRNA Selective PCR Kit 1.1 (TaKaRa Dalian, China) according to the manufacturer's specifications. The RT-PCR reaction mixture contained 25 μL of 2 × mRNA selective PCR buffer reaction buffer I, 10 μL of $MgCl_2$, 5 μL of dNTP/analog mixture, 1 μL of RNase Inhibitor, 1 μL of AMV Rtase XL, 1 μL of AMV-Optimized taq, 1 μL sense primer (20 μmol/L), 1 μL of antisense primer (20 μmol/L), 1 μL of total RNA, 4 μL of RNase free dH_2O to a final volume of 50 μL. Reactions without template RNA were used as a negative control. The RT-PCR for β-actin was used to check the quality of the RNA extraction and RT-PCR. The following RT-PCR conditions were used for Eag1: 1 cycle of 45℃ for 25 min; 30 cycles of 88℃ for 30 s, 50℃ for 45 s, and 72℃ for 1 min; and a final cycle of 72℃ for 7 min. The conditions for β-actin: 1 cycle of 50℃ for 15 min; 30 cycles of 85℃ for 30 s, 45℃ for 45 s, and 72℃ for 1 min; and a final cycle of 72℃ for 7 min.

Fifteen microliters of PCR products were analyzed by electrophoresis on a 1.5% agarose gel. DNA fragments were visualized and photographed under UV light after ethidium bromide staining. The expected band for each marker was identified by comigration of a DNA marker ladder electrophoresed in an adjacent lane.

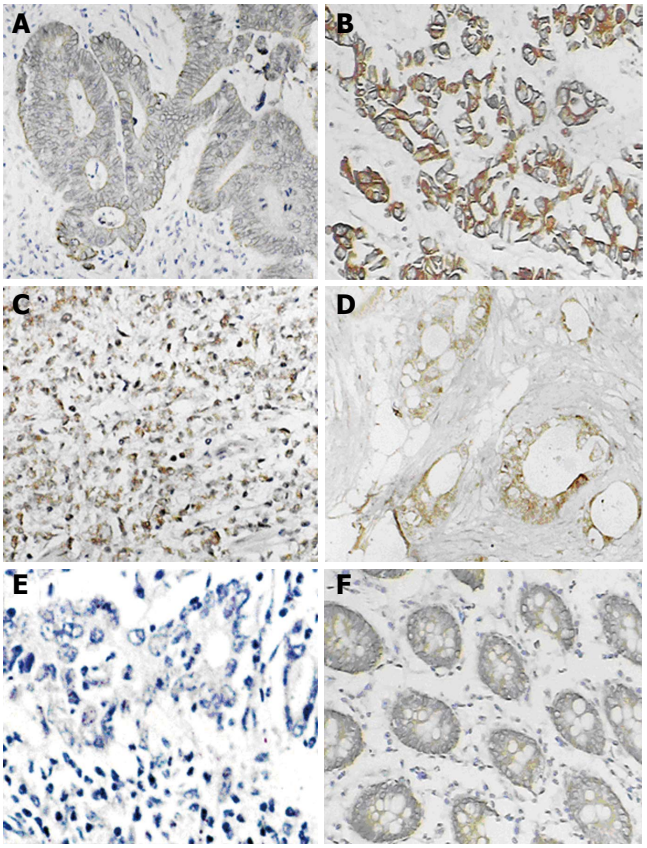

Figure 1 Typical immunohistochemical staining for Eag1 in colorectal cancer and adenoma. **A**, **B**, **C**: Positive staining of colorectal cancers; **D**: Positive staining of metastasis tissue from greater omentum; **E**: Negative control of colorectal cancer; **F**: Positive staining from one case of colonic adenoma.

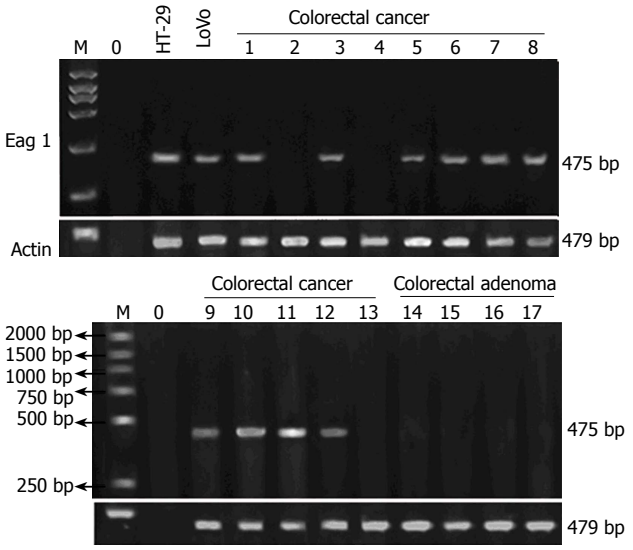

Figure 2 Eag1 mRNA expression in colorectal cancer and adenoma. Eag1 mRNA positively expressed in LoVo and HT29 cell lines and in 10/13 of colorectal cancer tissues, but negatively expressed in NCMT(not shown) and colorectal adenoma. M, molecular weight marker; 0, water.

Table 1 Eag1 immunoreactivity in the colorectal cancers

Stage	Eag1 expression[1]			Positive cases
	Negative	Weak	Strong	*n* (%)
A	3	2	2	4/7 (57.1)
B	13	14	12	26/39 (66.7)
C	2	5	9	14/16 (87.5)
D	0	5	9	14/14 (100)
Total	18	26	32	58/76 (76.3)

[1]Intensity of expression was interpreted as negative, weakly positive, and strongly positive, $P < 0.05$.

Statistical analysis

The χ^2-test was used for analysis. Differences between the values were considered significant when $P < 0.05$.

RESULTS

Eag1 channel is aberrantly expressed in colorectal cancer

Immunopositivity for Eag1 protein was clearly marked on the cytoplasm and/or membrane of cells. Using immunohistochemistry, aberrant expression of Eag1 was detected in tissue of colorectal cancer and negative expression was detected in NCMT. The frequency of expression of Eag1 in colorectal cancer averaged 76.3% (58/76) of the cases studied. Figure 1 shows representative positive expression of neoplastic tissue of colorectal cancer and metastatic cancer. Again, using reverse transcription PCR, positive expression of Eag1 was found in 10/13 (76.9%) of colorectal cancer samples and negative expression was found in 13 cases of NCMT (Figure 2, negative expression of NCMT was not shown here). Positive expression of Eag1 was also detected in HT29 and LoVo colorectal cancer cell lines (Figure 2).

Eag1 channel is occasionally expressed in colorectal adenoma

Nine cases of colorectal adenoma were also studied by immunohistochemistry, which were negative for Eag1 protein in most of the cases except that in 1 case weak positive expression was detected (Figure 1). In addition, Eag1 mRNA expression was not detected in 4 samples of colorectal adenomas by RT- PCR (Figure 2).

Relationship of Eag1 expression and clinic-pathological features

The immunohistochemistry results showed that no association was detected between Eag1 expression and sex, age, site, differentiation, while expression levels of Eag1 protein were found to be dependent on the tumor size, lymphatic node metastasis, other organ metastases and Dukes' stage ($P < 0.05$). Especially, all the tissues from metastatic cancer of other organ or Dukes' D stage cancer showed an intensively positive immunostaining (Figure 1 and Table 2). Table 1 summarizes the relationship of Eag1 protein expression with clinico-pathological features.

DISCUSSION

Because of its restricted distribution in normal tissue and its more ubiquitous distribution in cancer cells and its oncogenic properties, Eag1 channels have gained interest

Table 2 Clinico-pathological features and Eag1 protein expression

Clinico-pathological features	Number of tumor *n* (%)	Positive Eag1 expression *n* (%)	χ^2 value
Sex			0.70
Male	44 (57.9)	32(72.2)	
Female	32 (42.1)	26(81.3)	
Age (yr)			0.002
< 60	51 (67.1)	39 (76.5)	
⩾ 60	25 (32.9)	19 (76.0)	
Location			4.52
Recta	28 (36.8)	22 (78.6)	
Left colon	21 (27.6)	16 (76.2)	
Transverse colon	9 (11.8)	6 (66.7)	
Right colon	18 (23.7)	14 (77.8)	
Differentiation			2.09
High	18 (23.7)	12 (66.7)	
Moderate	22 (28.9)	16 (72.7)	
Low	29 (38.2)	24 (82.8)	
Undifferentiated	7 (9.2)	6 (85.7)	
Size			6.41[a]
< 5 cm	48 (63.2)	32 (66.7)	
⩾ 5 cm	28 (36.8)	26 (92.9)	
Lymphatic node metastasis			5.14[a]
N0	46 (60.5)	31 (67.4)	
N1	30 (39.5)	27 (90.0)	
Other organs metastasis			4.59[a]
M0	62 (81.6)	44 (71.0)	
M1	14 (18.4)	14 (100)	
Dukes' stage			8.89[a]
A	7 (9.2)	4 (57.1)	
B	39 (51.3)	26 (66.7)	
C	16 (21.1)	14 (87.5)	
D	14 (18.4)	14 (100)	

[a]$P < 0.05$.

as promising tools for the development of novel diagnostic and therapeutic methods of cancers.

Recently, a few published papers have documented the contribution of potassium channels in the pathological status of cells other than neural or lymphoid origin. A paper of Pardo's group represents a milestone in the link between Eag1 and cancer[5]. Furthermore, they found that Eag1 expression was limited outside the CNS, but was frequently expressed in clinic tumors from diverse origins, including colon carcinoma[12]. They reported that 6/8 of colon carcinomas were positively expressed. However, the actual frequency of expression of hEAG1 in colorectal cancer remained unknown. Here, we have provided that EAG1 protein and Eag1 mRNA were negatively expressed in normal colorectal tissue, but aberrantly expressed in a large fraction of colorectal cancer tissue and colorectal cell lines. These results strongly suggest the possibility of the use of Eag1 as a tool for the differential diagnosis of malignant transformation together with morphological criteria.

We also found that EAG1 protein was occasionally expressed in colorectal adenoma. Similar, Farias's group found one of negative pap smears of control cervical samples that presented human papilloma virus infection (the most important etiological factor for cervical cancer) was positive for Eag1 expression[8]. A plausible scenario is that the increase in Eag1 expression in precancer pathological changes could be an early sign of tumor development, namely, Eag1 may be involved in the early development of cancer.

We analyzed relationship of Eag1 expression detected by immunohistochemistry with clinico-pathological features, and found that there was no association between Eag1 expression and sex, age, site, differentiation, while expression of Eag1 was dependent on the tumor size, lymphatic node metastasis, metastases to other organs and Dukes' stage. Especially, all the tissues from metastatic cancers of other organ showed intensively positive immunostaining. Such a correlation would obviously be of great interest, since the channel could thus serve as a prognostic marker. These conclusions are somewhat different from opinions held by the Pardo's group. They found there were no correlations between Eag1 expression and demographic factors (sex, age, grade and site of tumor) of soft tissue sarcoma[9]. However, they also found that the tumors from patients who died over the two-year follow up period were much more likely to express significant levels of Eag1. To address this question precisely, quantitative studies of Eag1 expression and prospective studies with larger samples and longer time period will be needed.

Studies of Eag1 in cancer treatment have also provided some promising conclusions. Recently, the Pardo's group reported siRNA sequences acting specifically on Eag1, reproducibly induced a significant decrease in the proliferation of tumor cell lines while did not trigger any observable non-specific responses[10]. So, siRNA would serve as tools in the future to facilitate the elucidation of both the physiological and pathophysiological functions of this intriguing protein. We are also investigating the roles of Eag1-siRNAs served as tools in colorectal cancer therapy, and trying to clarify the effect of silencing the channel activity on tumor cells.

In conclusion, Eag1 potassium channels are aberrantly expressed in colorectal cancer patients and cell lines. These findings strongly suggest that Eag1 might be used as a potential diagnostic and prognostic marker as well as a potential membrane therapeutic target for colorectal cancer.

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RAPID COMMUNICATION

Preventive effect of gelatinizedly-modified chitosan film on peritoneal adhesion of different types

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Abstract

AIM: To comparatively study the preventive effect of gelatinizedly-modified chitosan film on peritoneal adhesions induced by four different factors in rats.

METHODS: Chitosan was chemically modified by gelatinization, and made into films of 60 μm in thickness, and sterilized. Two hundred Sprague-Dawley rats were randomly divided into five groups, Sham-operation group (group A), wound-induced adhesion group (group B), purified talc-induced adhesion group (group C), vascular ligation-induced adhesion group (group D), and infection-induced adhesion group (group E), respectively. In each group, the rats were treated with different adhesion-inducing methods at the cecum of vermiform processes and then were divided into control and experimental subgroups. Serous membrane surface of vermiform processes were covered with the films in the experimental subgroups, and no films were used in the control subgroups. After 2 and 4 wk of treatments, the abdominal cavities were reopened and the adhesive severity was graded blindly according to Bhatia's method. The cecum of vermiform processes were resected for hydroxyproline (OHP) measurement and pathological examination.

RESULTS: Adhesion severity and OHP level: After 2 and 4 wk of the treatments, in the experimental subgroups, the adhesions were significantly lighter and the OHP levels were significantly lower than those of the control subgroups in group B (2 wk: 0.199 ± 0.026 *vs* 0.285 ± 0.041 μg/mg pr, $P < 0.001$; 4 wk: 0.183 ± 0.034 *vs* 0.276 ± 0.03 μg/mg pr, $P < 0.001$), D (2 wk: 0.216 ± 0.036 *vs* 0.274 ± 0.040 μg/mg pr, $P = 0.004$; 4 wk: 0.211 ± 0.044 *vs* 0.281 ± 0.047 μg/mg pr, $P = 0.003$) and E (2 wk: 0.259 ± 0.039 *vs* 0.371 ± 0.040 μg/mg pr, $P < 0.001$; 4 wk: 0.242 ± 0.045 *vs* 0.355 ± 0.029 μg/mg pr, $P < 0.001$), but there were no significant differences in groups A (2wk: 0.141 ± 0.028 *vs* 0.137 ± 0.026 μg/mg pr, $P = 0.737$; 4 wk: 0.132 ± 0.031 *vs* 0.150 ± 0.035 μg/mg pr, $P = 0.225$) and C (2 wk: 0.395 ± 0.044 *vs* 0.378 ± 0.043 μg/mg pr, $P = 0.387$; 4 wk: 0.370 ± 0.032 *vs* 0.367 ± 0.041 μg/mg pr, $P = 0.853$); Pathological changes: In group B, the main pathological changes were fibroplasias in the treated serous membrane surface and in group D, the fibroplasia was shown in the whole layer of the vermiform processes. In group E, the main pathological changes were acute and chronic suppurative inflammatory reactions. These changes were lighter in the experimental subgroups than those in the control subgroups in the three groups. In group C, the main changes were foreign body giant cell and granuloma reactions and fibroplasias in different degrees, with no apparent differences between the experimental and control subgroups.

CONCLUSION: The gelatinizedly-modified chitosan film is effective on preventing peritoneal adhesions induced by wound, ischemia and infection, but the effect is not apparent in foreign body-induced adhesion.

Key words: Chitosan; Gelatinization; Chemical modification; Peritoneum; Adhesion

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INTRODUCTION

Peritoneal adhesion is a kind of defensive reaction to

peritoneal injury. However, it can also result in intestinal obstruction and cause severe clinical disorders. Therefore, it is important to prevent peritoneal adhesion in abdominal surgical operations. Unfortunately up to now, there have been no ideal methods to prevent peritoneal adhesion in clinical practice. Chitosan is a deacetylated derivate from chitin. Many previous studies showed that chitosan has effects of haemostasis, and sterilization, facilitates epithelial reparation and inhibits fibroblastic growth[1-3]. Chitosan has been used to prevent tissue adhesions, such as peritoneal adhesion, tendon adhesion and synarthrophysis[4-6]. In clinical application, it was found that the gel flows easily and is difficult to stay in the target regions for sufficient time, and the gel also degrades so fast that it could only maintain effectiveness for a short duration. In order to delay the degradation and decrease the fluidity of the gel, in our previous study, we processed the chitosan to films and transplanted it into the abdominal cavity of rats. But the film degraded too slowly and 8 wk after the transplantation, most of the films resided in the cavity. The residual film was encapsulated by the surrounding tissue and the peritoneal adhesion was worsened. In order to overcome these disadvantages, we mixed gelatin to chitosan and produced blending films. The blending film degraded much faster than the previous pure chitosan film, but it also produced foreign body reactions and formed severe foreign body granuloma around the blending film[7].

To solve the above problems and develop a useful chitosan film to prevent the peritoneal adhesion, in the present study we chemically modified the chitosan by gelatinization to develop a new sort of chitosan film, and comparatively studied the preventive effect of the film on peritoneal adhesions induced by wound, infection, ischemia and foreign body in rats.

MATERIALS AND METHODS

Animals and grouping

Two hundred Sprague-Dawley rats, one half for each gender, weighing 200-250 g, were provided by the Experimental Animal Center of Zhejiang Province. They were randomly divided into five groups: sham-operation group (group A), wound-induced adhesion group (group B), purified talc-induced adhesion group (group C), vascular ligation-induced adhesion group (group D), and infection-induced adhesion group (group E). Each of the above groups was subdivided into two subgroups, experimental and control subgroups (20 rats for each subgroup and one half for each gender). All the rats were fed under the same conditions: at 24-26℃ of environmental temperature, about 40% humidity, with an alternate 12 h light/dark cycle, and free access to food and water.

Preparation of chitosan film

The chitosan (from Yuhuan Aoxing Chitin Ltd., Zhejiang Province, China) was dissolved, purified, gelatinizedly modified, filtered and made into films. The films were dried and dissected to patches of 10 cm × 10 cm in size and 60 μm in thickness, and the film patches were sterilized by radiation of ^{60}Co for later use.

Surgical operation

Under general anaesthesia with intraperitoneal injection of 3% amobarbital (60-90 mg/kg), the rats were immobilized in dorsal position, routinely degermed, abdominally incised through a median incision of 2-3 cm long, and their vermiform processes were searched and pulled out of the incisions, then the terminal vermiform processes within a length of 3 cm were treated as follows: In group A, the vermiform processes were exposed to air for 5 min; in group B, the anterior surface of serous membrane was scraped slightly with surgical blade till obvious congestion and small bleeding drops appeared; in group C, 10 mg of talc powders were evenly smeared over the anterior surface of serous membrane; in group D, the vermiform artery stem was ligated with 0# surgical thread at a point of 3 cm from the dead end in the following way: loosely knotting the first loop, thrilling a thread with equivalent diameter to the vermiform artery stem through the first loop, tightening the first loop, pulling out the thrilled thread, and knotting and tightening the second loop of the ligation knot. The ligation resulted in a stricture of vermiform artery, which induced ischemia of the distal vermiform tissue from the ligation point. In group E, the dead end of the vermiform process was poked out with a hole using a 16# needle, a drop of intestinal content was extruded out and evenly smeared over the anterior surface of serous membrane, and then the remaining content in the vermiform process was pushed to the cecum. After the above treatments, for the experimental subgroups, the treated serous membranes were covered with the prepared chitosan films, and the vermiform processes were put back into the abdominal cavity, which were then closed. For the control subgroups, all the treatments were the same as those of the experimental subgroups except that the chitosan film was not placed. The duration from opening to closing the abdominal cavity was 5 min, so that the duration of exposure of intestines to air was the same for each rat.

At 2 and 4 wk after the surgery, 10 rats (5 female and 5 male) in each subgroup were randomly selected respectively and their abdominal cavities were reopened under anaesthesia, and the grades of peritoneal adhesion were evaluated, which existed between the treated vermiform processes and intestines, mesenteries and abdominal walls. After that, the vermiform processes with adhesions were resected and washed with normal saline, and then were divided into two segments for each resected process. The proximal segments were fixed with formalin and histopathologically examined, and the distal segments were stored at -80℃ for measurement of hydroxyproline (OHP).

Grading standard for peritoneal adhesion

According to Bhatia's[8] grading method of 5 levels and considering the characteristics of peritoneal adhesion in rats, we formulated the following grading standard:

Table 1 Comparison of peritoneal adhesive grades between experimental and control subgroups within each group

Group	Control group (n = 10)					Experimental group (n = 10)					U	P
	0	I	II	III	IV	0	I	II	III	IV		
Group A: sham-operation												
2 wk	9	1	0	0	0	10	0	0	0	0	45.000	0.317
4 wk	10	0	0	0	0	9	1	0	0	0	45.000	0.317
Group B: wound-induced adhesion												
2 wk	0	5	4	1	0	6	3	1	0	0	14.500	0.005
4 wk	0	5	5	0	0	7	3	0	0	0	7.500	0.001
Group C: purified talc-induced adhesion												
2 wk	0	0	1	3	6	0	0	1	2	7	45.500	0.687
4 wk	0	0	1	4	5	0	0	0	4	6	43.000	0.547
Group D: vascular ligation-induced adhesion												
2 wk	0	4	4	2	0	3	6	1	0	0	18.000	0.009
4 wk	1	4	4	1	0	5	4	1	0	0	21.500	0.023
Group E: infection-induced adhesion												
2 wk	0	0	1	4	5	0	4	5	1	0	5.500	0.001
4 wk	0	0	1	5	4	0	6	4	0	0	2.000	< 0.001

Grade 0: no adhesions; Grade I: the ratio of adhesive area/total treated area in the vermiform processes is < 50%, and the adhesion is easily to be dissected bluntly; Grade II: the ratio is ≥ 50%, and the adhesion is easily to be dissected bluntly; Grade III: area of the adhesion is out of consideration. Although blunt dissection for the adhesion can be carried out, it is difficult and the intestinal wall will be impaired after the blunt dissection; Grade IV: area of the adhesion is out of consideration. The adhesion is fast and cannot be bluntly dissected. Each rat was graded by three referees blindly and the average grade of the three was accepted as the adhesive grade of the rat.

Determination of total protein and OHP

The levels of total protein and OHP in the adhesive tissue were determined using the corresponding kits supplied by Nanjing Jiancheng Bioengineering Institute, China. The determining processes completely followed the instructions of the kits. Contents of OHP in the adhesive tissue were calculated as micrograms of OHP in each milligram of total protein (μg/mg pr).

Statistical analysis

All the data were processed with SPSS10.0. Mann-Whitney U test of non-parametric statistics for independent samples was used to analyze differences in the peritoneal adhesive grades and t-test was used to analyze differences in OHP levels between the experimental and control subgroups within each group.

RESULTS

Gross findings

Abdominal incisions healed in first grade in all rats of all groups. There was no obvious postoperative abdominal infection in groups A, B, C and D at 2 and 4 wk after the surgical operations. There was no residual chitosan film in the abdominal cavities of rats in all experimental subgroups.

Comparison of peritoneal adhesion grade

As it shows in Table 1, within group A (sham-operation group) and group C (talc-induced adhesion group), there was no significant difference in peritoneal adhesion between the experimental and control subgroups both at 2 and 4 wk ($P > 0.05$). Within group B (wound-induced adhesion group), group D (vascular ligation-induced adhesion group) and group E (infection-induced adhesion group), the peritoneal adhesion grades of experimental subgroups were significantly lower than those of corresponding control subgroups ($P < 0.05$) both at 2 and 4 wk after the surgical treatments. It indicates that the gelatinizedly modified chitosan film has remarkable effect on preventing peritoneal adhesions induced by wound, ischemia and infection, but no obvious effect on adhesion induced by talc powders. From the results in Table 1, we also concluded that in group E, the mean decreased adhesion grades were 1.7 and 1.4 from experimental to control group at 2 and 4 wk respectively. While in group B, the mean decreased grades were 1.1 and 1.2, and in group D, the mean decreased grades were 1.0 and 0.9. It suggests that the modified chitosan film is more effective on preventing infection-induced peritoneal adhesion than on wound and ischemia induced adhesion.

Comparison of OHP levels in adhesive tissue

As it shows in Table 2, in groups A and C, there were no significant differences in OHP levels between the experimental and control subgroups both at 2 and 4 wk ($P > 0.05$). In groups B, D and E, the OHP levels of experimental subgroups were significantly lower than those of corresponding control subgroups ($P < 0.05$) both at 2 and 4 wk after the surgical treatments. The changes in OHP levels were concordant with the changes in the adhesive grades, and it was confirmed to have a peritoneal adhesion-preventive effect when the gelatinizedly modified chitosan film was applied to regions with wound, ischemia and infection in abdominal surgical operations.

Comparison of pathological changes

In group A, there were no obvious pathological changes in vermiform processes of all rats. In groups B and D, there were obvious fibroplasias and sporadic infiltration of lymphocytes in serous membrane (group B) and the whole

Table 2 Comparison of OHP levels between experimental and control subgroups within each group (μg/mg pr)

Group	Control group (n = 10)	Experimental group (n = 10)	t	P
Group A: sham-operation				
2 wk	0.137 ± 0.026	0.141 ± 0.028	0.331	0.737
4 wk	0.150 ± 0.035	0.132 ± 0.031	1.217	0.225
Group B: wound-induced adhesion				
2 wk	0.285 ± 0.041	0.199 ± 0.026	5.602	< 0.001
4 wk	0.276 ± 0.038	0.183 ± 0.034	5.768	< 0.001
Group C: purified talc-induced adhesion				
2 wk	0.378 ± 0.043	0.395 ± 0.044	0.874	0.387
4 wk	0.367 ± 0.041	0.370 ± 0.032	0.182	0.853
Group D: vascular ligation-induced adhesion				
2 wk	0.274 ± 0.040	0.216 ± 0.036	3.408	0.004
4 wk	0.281 ± 0.047	0.211 ± 0.044	3.438	0.003
Group E: infection-induced adhesion				
2 wk	0.371 ± 0.040	0.259 ± 0.039	6.34	< 0.001
4 wk	0.355 ± 0.029	0.242 ± 0.045	6.675	< 0.001

layer (group D) of the adhesive vermiform processes at 2 wk, and the main pathological change was fibroplasia at 4 wk after the surgical treatments. The above pathological changes were milder in experimental subgroups than those in control subgroups except for changes in group D and at 4 wk. In group C at 2 wk, there occurred obvious foreign-body giant cell reactions, granuloma, fibroplasias and sporadic infiltration of lymphocytes at the treated serous membranes of adhesive vermiform processes, and the granuloma and fibroplasias became severer at 4 wk. There were no significant differences in the above pathological changes between the experimental and control subgroups. In group E, the main pathological change in the treated region was acute suppurative inflammation at 2 wk, and chronic inflammatory reaction characterized with granulation and fibroplasias at 4 wk after the surgical treatments. The above inflammatory reactions were milder in experimental subgroup than those in control subgroup both at 2 and 4 wk.

DISCUSSION

Chitosan is chemically termed β-(1, 4)-2-amino-2-deoxy-D-dextran, and its main component is glucosamine. Glucosamine is also a sort of internal substance in human bodies, therefore, chitosan is biocompatible. If the chitosan is introduced into animal or human bodies, it will be degraded into small molecules of oligosaccharides and absorbed by the bodies without causing any acute or chronic toxicity. Chitosan is a derivant of deacetylized chitin, and the chitin is the major component of outer shells of invertebrates. Because of these characteristics of chitosan, it is widely and deeply researched in areas of pharmaceutical preparations and medical polymer synthesis[9].

Peritoneal adhesion occurs in more than three fourths of patients following laparotomy. The outcomes of adhesion are unpredictable and diverse, causing a significant health care burden. Intestinal obstruction, infertility, problems at reoperative surgery and cumulative costs of care over many years are the key concerns[10]. The peritoneal adhesion develops only several hours after the abdominal surgical operations. At first, the serous fluid exudes from the injured sides of intestinal wall, and then fibrinogen in the serous fluid transforms to fibrin and coagulates, thereby membranous peritoneal adhesion in the injured intestinal wall is formed. After that, the fibrinolytic system is activated and the fibrin is lyzed, thereby the membranous peritoneal adhesion is gradually eliminated. If the fibrin cannot be totally lyzed, the left fibrin will be organized and develop fibrinous adhesion, which usually forms at 2 wk after the surgical operation[11]. Based on the mechanisms through which the chitosan prevents peritoneal adhesion: inhibiting growth of fibroblasts, facilitating reparation of the epithelium, and disinfection, it is concluded that the chitosan can only prevent against pre-fibrinous adhesion. Once the fibrinous adhesion is formed, chitosan is useless. Therefore, when we use chitosan film to prevent peritoneal adhesion, the optimal duration for the film to stay in the abdominal cavity is within 2 wk. Firstly, the film can exert an effect of mechanical isolation in a solid state before it is completely degraded; On the other hand, when the film is degraded, the released chitosan monomers can also take anti-adhesive effect. In the present study, we gelatinizedly modified the chitosan to develop a new sort of chitosan film, which can be slowly dissolved in water. The experiments demonstrated that, within 2 wk after the film was transplanted into the abdominal cavity of the rats, it was completely degraded and absorbed without any foreign-body granuloma reaction. This suggests that the gelatinizedly modified chitosan film has the potential to be biomaterial for adhesion-prevention.

There are many factors that can induce peritoneal adhesions, of which the main factors are wound, ischemia, infection and foreign bodies. In most cases of clinical peritoneal adhesion, the adhesions are caused by combined factors, among which one or several factors play major roles[11,12]. The present study utilized rat models to investigate effects of the gelatinizedly modified chitosan film on peritoneal adhesions induced by four different factors. The results demonstrated that, at 2 and 4 wk after the surgical operations, the chitosan films significantly reduced the adhesion grades in groups of wound, ischemia

and infection-induced adhesions. This suggests that the chitosan films have obvious preventing effects on wound, ischemia and infection-induced peritoneal adhesions. The results also demonstrated that the films are more effective on infection-induced peritoneal adhesion. The mechanism may be as follows: The chitosan film prevents infection-induced peritoneal adhesion not only through promoting the epithelium recovery and inhibiting the growth of fibrous tissue, but also through its anti-infection effect by inhibiting hyperplasia of granulation and fibrous tissue. Through the double pathways the chitosan may inhibit the adhesion more strongly. It also seemed that healing of the abdominal incisions is not obviously affected by the chitosan transplantation. With respect to effects of the chitosan film on intra-abdominal anastomotic stoma healing, it needs to be clarified further.

Within the talc powder-induced adhesion group, there were no significant differences in adhesive grades between the experimental and control subgroups both at 2 and 4 wk. This showed that the gelatinizedly modified chitosan film has no obvious preventive effect on foreign body-induced peritoneal adhesion. The reason is as follows: For the talc powder-induced adhesion, the main pathological changes are foreign-body granuloma complicated with a large quantity of fibroplasias, and these changes will maintain as long as the foreign bodies exist. However, the chitosan film degraded in a relative fast rate in the abdominal cavity and there was no obvious residual film at 2 wk after the transplantation. Therefore, the fast degraded film cannot exert a strong effect on a chronic and persistent foreign-body granuloma reaction.

There is a high content of OHP in collagen protein, a very low content in elastin protein, and none in other sorts of proteins. Ozogul *et al*[13] reported that there existed positive correlation between adhesive grades and OHP levels in the adhesive tissue, and concluded that OHP is a significant index to measure the adhesive degree of tissue, which is more sensitive and objective than the index of gross adhesive grade. In the present study, the OHP changing tendency within each group and differences in OHP levels between subgroups were concordant to those of adhesive grades. This further confirms the preventive effect of gelatinizedly modified chitosan film on wound, ischemia and infection-induced peritoneal adhesions.

COMMENTS

Background

Peritoneal adhesion can cause intestinal obstruction and other severe clinical disorders, so it is very important to prevent peritoneal adhesion in abdominal surgical operations. But up to now, there are still no ideal methods to prevent peritoneal adhesion in clinical practices. Chitosan is a deacetylated derivate from chitin. Many studies reported that chitosan was applied to prevent tissue adhesions, such as peritoneal adhesion, tendon adhesion and synarthrophysis, but the effect was not satisfactory.

Research frontiers

Chitosan is a sort of natural biological material and it has been processed into many forms for medical use. In the area of prevention of peritoneal adhesion with chitosan, the research hotspot is how to modify the chitosan by chemical and physical methods to improve its effectiveness on preventing the adhesion, and simultaneously reduce its adverse reactions.

Innovations and breakthroughs

In the previous application of chitosan gels to the prevention of peritoneal adhesion, it was found that the gel was much fluid and was difficult to stay in the target places for sufficient time, and moreover, the gel degraded so fast that it could only maintain the effectiveness for a short duration. In order to delay the degradation and decrease the fluidity of the gel, we processed pure chitosan into films, but the film degraded too slowly and the residual film was encapsulated by surrounding tissue and the peritoneal adhesion was worsened. In order to overcome these disadvantages, we mixed chitosan with gelatin and produced blending films. The blending film degraded much faster than the previous pure chitosan film, but it also created foreign body reaction and formed severe foreign body granuloma around the blending film. In the present study we chemically modified the chitosan in gelatinization to develop a new sort of chitosan film, and showed that the film is remarkably effective on preventing peritoneal adhesions induced by wound, ischemia and infection except the foreign body-induced adhesion.

Applications

The study results suggest that the gelatinizedly-chitosan film is a potential therapeutic material that could be used in preventing peritoneal adhesions induced by wound, ischemia and infection.

Terminology

Peritoneal adhesion: Peritoneal adhesion is a sort of defensive reaction to the peritoneal injury mainly including wound, infection, ischemia and foreign body, but it can also develop intestinal obstruction and cause severe clinical disorders; chitosan: Chitosan is a deacetylated derivate from chitin, and chitin is the second most abundant natural biopolymer derived from exoskeletons of crustaceans and also from cell walls of fungi and insects.

Peer review

This is a good descriptive study in which authors analyze the preventive effect of gelatinizedly-modified chitosan on peritoneal adhesions induced by different factors in rats. The results are interesting and suggest that gelatinizedly-chitosan is a potential therapeutic substance that could be used in preventing peritoneal adhesions induced by wound, ischemia and infection.

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CASE REPORT

Endoscopic resection of an ampullary carcinoid presenting with upper gastrointestinal bleeding: A case report and review of the literature

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Abstract

Ampullary carcinoid is a rare tumor that can present with gastrointestinal bleeding, obstructive jaundice or pancreatitis. Some of these tumors are associated with Von Recklinghausen disease. The usual surgical options are a biliary-enteric anastomosis, Whipple procedure or rarely a local resection. The mean survival does not appear to be much different after a pancreaticoduodenectomy versus local surgical excision. We report a very rare case of a non-metastatic ampullary carcinoid causing upper gastrointestinal bleeding, which was managed by endoscopic ampullectomy.

Key words: Carcinoid; Ampulla of Vater; Ampullary tumor; Ampullectomy; Gastrointestinal bleeding

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INTRODUCTION

Despite being rare, carcinoids are the most common neuroendocrine tumors of the gastrointestinal (GI) tract[1,2]. Carcinoid of the ampulla is an extremely rare tumor (0.05%) for which the correct pre-operative diagnosis is very low (14%) and very rarely they present with an ulceration of the overlying duodenal mucosa[2]. Less than 3% of ampullary carcinoids patients experience carcinoid syndrome[3]. These tumors generally have an indolent course, but more aggressive behavior in the form of distant metastases has been reported. We report a case of an ampullary carcinoid that manifested as an upper gastrointestinal bleeding and was managed with endoscopic snare resection of the ampulla of Vater.

CASE REPORT

A 71-year old man underwent upper endoscopy for the work up of a recent history of epigastric pain, melena and a 4 g decrease in hemoglobin. The esophagogastroduodenoscopy (EGD) was unremarkable except for a prominent ampullary area. A sideviewing scope showed an approximately 1.5 cm mass with an overlying superficial ulcer (Figure 1A). Biopsy specimen revealed a carcinoid tumor involving the muscularis propria (Figure 2A and B). The tumor lacked mitotic activity and psammoma bodies (sometimes associated with duodenal somatostatinomas). The tumor was positive for chromogranin, synaptophysin and somatostatin, and negative for gastrin, insulin, glucagon and human pancreatic polypeptide. The patient denied flushing, diarrhea, weight loss or wheezing. His liver enzymes were normal. A CT and an octreotide scan were unremarkable. EUS revealed no regional lymph nodes and tumor free pancreatic and common bile ducts. A serum serotonin level was normal at 80 ng/mL (22-180 ng/mL) and urinary 5-HIAA was 6.1 mg/24 h (normal up to 6 mg/24 h).

The patient declined aggressive surgical options but agreed to undergo an endoscopic ampullectomy with its modest risk of complications. After submucosal saline injection in the ampullary area, a snare assisted ampullectomy was performed with blended current (cut to coagulation ratio 5:1) at a setting of 150:30 watts using an electrosurgical unit (ERBE ICC-200, Tubingen, Germany). The tumor was removed piecemeal. A 5 Fr, 3 cm pancreatic stent was placed to prevent pancreatitis. The procedure was uneventful except for mild oozing which promptly stopped with local infiltration of 4 cc of 1/10000 epinephrine. The patient remained stable except for an asymptomatic transient elevation of transaminases and was discharged the next evening. The pancreatic stent was removed two weeks later and there was no residual tumor seen at this examination (Figure 1B). He did not have any further episodes of GI bleeding in the last three years. His hemoglobin and liver related tests were normal. Subsequent follow-up endoscopies using a side-viewing scope showed no residual tumor and biopsies (using jumbo

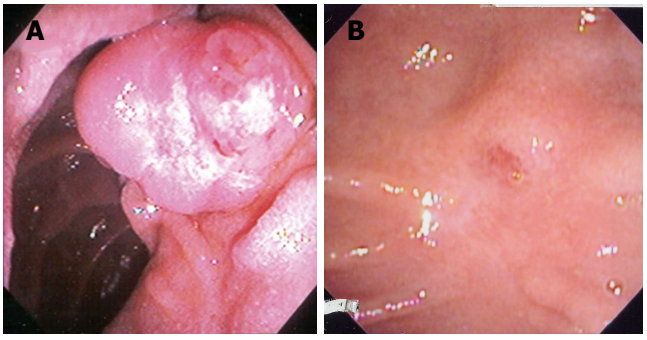

Figure 1 Endoscopic view showing an ampullary tumor (**A**) and the same area weeks after endoscopic resection of the tumor (**B**).

forceps) of the area remained negative for tumor cells. A follow-up EUS and two CT scans were also unremarkable.

DISCUSSION

Neuroendocrine neoplasms only rarely occur at the ampulla of Vater, and consist mostly of carcinoids and occasionally of poorly differentiated endocrine carcinomas (small cell carcinomas)[4]. Up to 26% of patients may have associated Von Recklinghausen disease[5]. There is a higher incidence (46%) of finding metastatic disease at diagnosis in carcinoids of the ampullary area. Jaundice (53%), pain (24%), pancreatitis (6%) and weight loss (3.6%) are common presenting symptoms[6] but upper GI bleeding from ulceration is rare[7]. Carcinoids have an intact overlying mucosa which may explain a high rate of false negative biopsies[2,6]. Examination using a side-viewing scope and obtaining deep excavating biopsies is essential towards establishing the diagnosis. The possibility of the bile duct involvement should be ascertained with ERCP and/or EUS. In detecting smaller tumors and for local angio-invasion, EUS could be extremely sensitive[8]. CT scan and MRI have a low sensitivity for the primary lesion[8]. Somatostatin receptor scan is 86% sensitive for duodenal carcinoids of $\geqslant$ 1 cm, in detecting both primary and metastatic disease[9]. A capsule endoscopy should be considered to rule out synchronous small bowel lesions.

Re-classified by WHO, carcinoids are now called neuroendocrine tumors. Ampullary carcinoids range from highly differentiated (probably benign) to well differentiated endocrine carcinomas and poorly differentiated carcinomas. The overall survival appears to be excellent at 90% for well differentiated tumors[2], but poor when these are not so well differentiated[10].

Choosing the best treatment option for ampullary carcinoids could be challenging. Although tumor size is not a good prognostic predictor[2,10], most experts still recommend either a Whipple or a pylorus preserving pancreatico-duodenectomy (PPPD) for tumors over 2 cm in size[11]. Metastatic disease has been found in 48% and 40% of the patients with tumor sizes of more than 2 cm and less than 2 cm, respectively[12]. Thus, some experts recommend a Whipple pancreatico-duodenectomy for all size tumors. On the other hand long-term survival has been achieved with local resection only[2,13]. In a study of

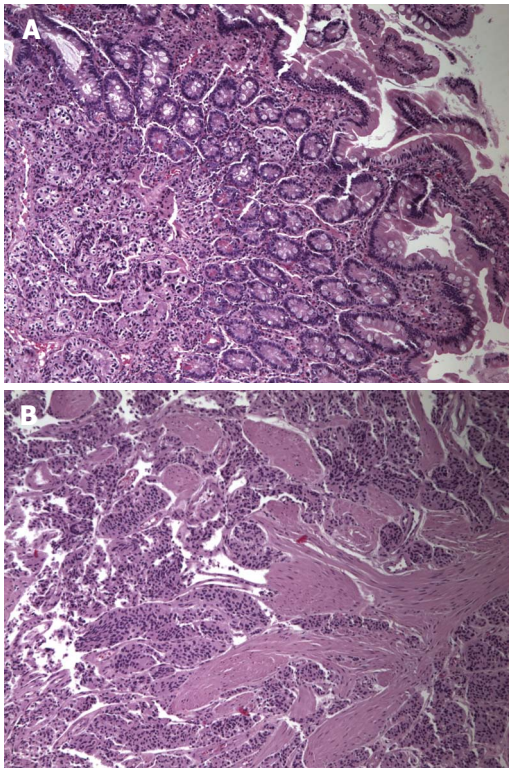

Figure 2 Histological examination of the resected specimen revealing carcinoid in the mucosa and submucosa, composed of discrete solid nests of round tumor cells with central nuclei and occasionally with gland-like lumina (**A**) and solid nests of tumor cells involving the musclaris propria (**B**) (HE, x 100).

90 patients with ampullary carcinoids, 52 had a PPPD (majority > 2 cm), while 22 underwent a local resection (majority < 2 cm). Post-operative mortality was 3/52 in the PPPD group compared to 0/22 in the group with local resection[14]. Therefore, less radical approaches should be considered in highly differentiated, slow growing tumors. In relatively high surgical risk patients with a small, non-metastatic tumor, local excision seems to be a reasonable operative choice, whereas the least invasive approach should be reserved for those who are not surgical candidates. An endoscopic ampullectomy which seems to be an effective treatment in the management of ampullary adenomas, could also be a viable option in selected patients with tumors *in situ* (Tis)[15] and carcinoids of the ampulla of Vater, without vascular invasion.

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CASE REPORT

Multistep hepatocarcinogenesis from a dysplastic nodule to well-differentiated hepatocellular carcinoma in a patient with alcohol-related liver cirrhosis

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Abstract

We describe a rare case of the transformation of a dysplastic nodule into well-differentiated hepatocellular carcinoma (HCC) in a 56-year-old man with alcoholrelated liver cirrhosis. Ultrasound (US) disclosed a 10 mm hypoechoic nodule and contrast enhanced US revealed a hypovascular nodule, both in segment seven. US-guided biopsy revealed a high-grade dysplastic nodule characterized by enhanced cellularity with a high N/C ratio, increased cytoplasmic eosinophilia, and slight cell atypia. One year later, the US pattern of the nodule changed from hypoechoic to hyperechoic without any change in size or hypovascularity. US-guided biopsy revealed well-differentiated HCC of the same features as shown in the first biopsy, but with additional pseudoglandular formation and moderate cell atypia. Moreover, immunohistochemical staining of cyclase-associated protein 2, a new molecular marker of well-differentiated HCC, turned positive. This is the first case of multistep hepatocarcinogenesis from a dysplastic nodule to well-differentiated HCC within one year in alcohol-related liver cirrhosis.

Key words: Multistep hepatocarcinogenesis; Dysplastic nodule; Well-differentiated hepatocellular carcinoma; Alcohol-related liver cirrhosis; Cyclase-associated protein 2

Kim SR, Ikawa H, Ando K, Mita K, Fuki S, Sakamoto M, Kanbara Y, Matsuoka T, Kudo M, Hayashi Y. Multistep hepatocarcinogenesis from dysplastic nodule to well-differentiated hepatocellular carcinoma in a patient with alcohol-related liver cirrhosis. *World J Gastroenterol* 2007; 13(8): 1271-1274

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INTRODUCTION

In Western countries and in Japan, hepatocellular carcinoma (HCC) usually develops in close association with pre-existing liver cirrhosis (LC). The cirrhotic liver is therefore believed to have a precancerous lesion recognizable as a small nodule, referred to as the dysplastic nodule[1] or adenomatous hyperplasia[2]. The mean size of most dysplastic nodules is 7-8 mm, seldom larger than 20 mm in diameter. Among LC cases, the dysplastic nodule is usually hepatitis C virus (HCV)-related, rarely hepatitis B virus (HBV)-related, and has not been reported as alcohol-related. HCC usually develops in chronic liver diseases such as HCV and HBV. In Japan, HCC occurs in 81% of HCV and 14% of HBV carriers[3], but seldom in alcohol-related LC, whereas in the United States HCC occurs in 30% of alcohol-related LC[4]. Herein we describe a rare case of multistep hepatocarcinogenesis from a dysplastic nodule to well-differentiated HCC in a patient with alcohol-related LC.

CASE REPORT

A 56-year-old man was admitted to Kobe Asahi Hospital in April 2005 for further examination of a 10 mm hypoechoic nodule in the liver. In April 2001, histologic analysis of tissue obtained at biopsy because of his abnormal liver function had revealed alcohol-related LC (Figure 1); no nodule was detected. His alcohol consumption over 35 years was 120 mL/d. A physical examination on admission in April 2005 showed no remarkable abnormalities. There was no evidence of

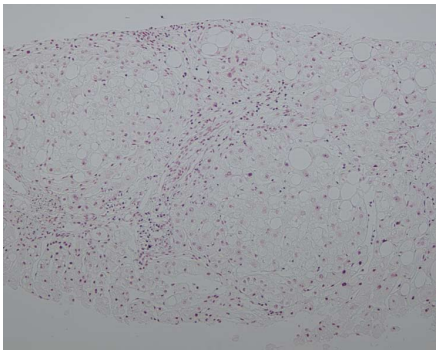

Figure 1 Histological features of liver biopsy (April 2001), alcohol-related liver cirrhosis, micronodular liver cirrhosis with fatty change (HE × 100).

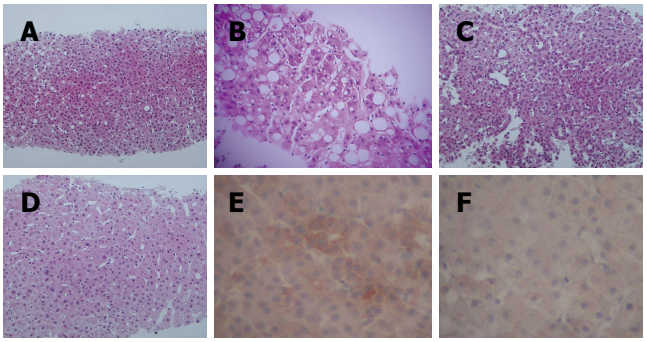

Figure 3 Histological features of US-guided liver biopsy of the liver. **A**: High grade dysplastic nodule (April 2005): increased cellularity with a high N/C ratio, increased cytoplasmic eosinophilia, and slight atypia (HE × 200); **B**: Non nodular lesion (HE × 200) (April 2005), well-differentiated HCC (May 2006): increased cellularity with a high N/C ratio, increased cytoplasmic eosinophilia, moderate atypia, and pseudoglandular formation (arrow) (HE × 200); **C**: Non-nodular lesion (May 2006); **D**: Immunohistochemical finding (May 2006) (HE × 200), CAP2 positive HCC cells are observed (more than 30%); **E**: Immunohistochemical finding (May 2006) (HE × 200): Liver cirrhosis is negative for CAP2; CAP2 positive HCC cells are observed (more than 30%); **F**: Immunohistochemical finding (May 2006) (HE × 200): Liver cirrhosis is negative for CAP2.

lymph adenopathy or splenomegaly. The serum was negative for HCV antibody (anti-HCV), hepatitis B surface antigen (HBsAg), and hepatitis B core antibody (anti-HBc). Laboratory data on admission in April 2005 disclosed the following abnormal values: platelets 7.1 × 10^4/μL (normal 13.1-36.2), aspartate aminotransferase 88 IU/L (8-38), γ-glutamyl transpeptidase (γ-GTP) 677 IU/L (16-84), total bilirubin 1.4 mg/dL (0.2-1.0), and indocyanine green (ICG) 15 min retention rate 35% (0%-10%). Alpha-fetoprotein (AFP), lens culinaris agglutinin A-reactive fraction of alpha fetoprotein (AFP L3) and protein-induced vitamin K absence (PIVKA Ⅱ) were within normal ranges. Immunological examinations revealed the following: IgG 1452 mg/dL (870-1700), IgM 235 mg/dL (33-190), IgA 207 mg/dL (110-410), B cell 8% (14%-13%), T cell 83% (66%-89%), CD4/CD8 ratio 2.08 (0.40-2.30), and NK cell activity 50% (18%-40%). Ultrasound (US) disclosed a 10 mm hypoechoic nodule in segment seven (S7) (Figure 2A). Plain computed tomography (CT) did not show any nodule, and contrast enhanced CT and magnetic resonance imaging (MRI) did not reveal any enhanced nodule. MRI revealed an isointensive nodule at both T1- and T2-weighted sequences. CT hepatic arteriography (CTA) did not show any enhanced nodule, and CT during arterial portography

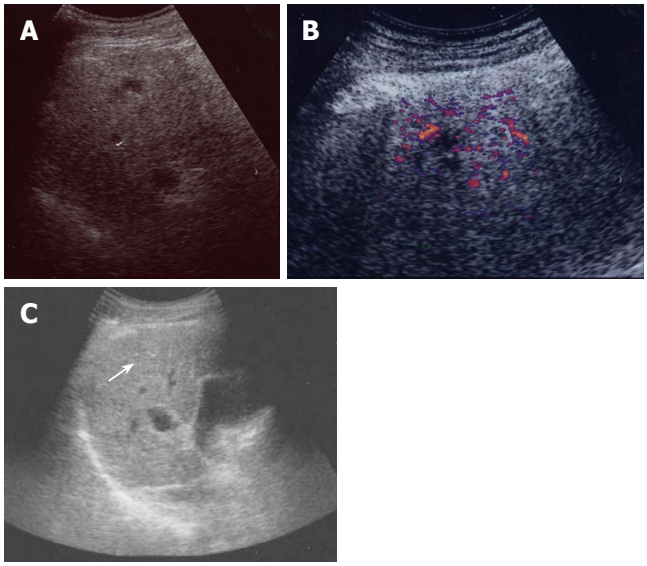

Figure 2 **A**: US imaging (April 2005). A 10 mm hypoechoic nodule in S7; **B**: contrast enhanced US (April 2005). Hypovascularity in the early phase; **C**: US imaging (May 2006). A 10 mm hyperechoic nodule in S7.

(CTAP) did not show any perfusion defect. Contrast enhanced US revealed a hypovascular nodule (Figure 2B). A US-guided biopsy revealed a high grade dysplastic nodule characterized by increased cellularity with a high N/C ratio, increased cytoplasmic eosinophilia, and slight cell atypia (Figure 3A and B). Immunohistochemical staining of both heat shock protein (HSP) 70 and cyclase-associated protein (CAP) 2 was negative.

In May 2006, the US pattern of the nodule changed from hypoechoic to hyperechoic (Figure 2C), although the size of the nodule did not change. Constant enhanced US revealed a hypovascular nodule, and AFP, AFP L3, and PIVKA Ⅱ were within normal ranges. The findings of imaging studies including contrast enhanced CT and MRI were all the same as in April 2005. A US-guided biopsy revealed well differentiated HCC of the same features as those in April 2005 with additional pseudoglandular formation and moderate cell atypia (Figure 3C and D). Immunohistochemical staining of CAP2 was over 30% positive (Figure 3E and F), although HSP70 staining was negative.

DISCUSSION

According to the classification by the International Working Party of the World Congress of Gastroenterology, hepatic nodules in patients with chronic liver diseases are subdivided into regenerative nodules (mono acinus and multi acinus), low-grade dysplastic nodules, high-grade dysplastic nodules, well-differentiated HCC, moderately-differentiated HCC, and poorly-differentiated HCC, in an ascending order of histologic grades, representing a sequence of multistep hepatocarcinogenesis[1].

It is often difficult-even for the hepatopathologist - to differentiate among regenerative nodules, precancerous lesions, and early HCC, especially from the examination of biopsy specimens. Therefore, the uncovering of an

objective molecular marker that would help to standardize histological diagnosis of early HCC and to lead to appropriate treatment is eagerly anticipated. Also, the molecular mechanisms of hepatocarcinogenesis are far from clear. A molecular understanding of multistep hepatocarcinogenesis is an important step toward the identification of additional biomarkers and new therapeutic targets of a greater specificity for HCC management. Diagnosis is based on histologic analysis of liver samples for identifying cytoarchitechtural features (cell atypia, increased cellularity, increased cytoplasmic eosinophilia, fatty change, pseudoglandular formation, trabecular thickness, etc) and on information from immunocytochemical staining, such as that with HSP70[5], and CAP2[6].

Chuma *et al*[5] have found several up-regulated genes involved in HCC progression by comparing expression profiles among early and progressed components of seven nodule-in-nodule-type HCCs (1 HBV-positive case, 5 HCV-positive cases and 1 negative for both B and C) and their corresponding non-cancerous liver tissues, with the use of an oligonucleotide array: HSP70 (a molecular marker of early HCC) and CAP2, with up-regulated expression in a stepwise manner in multistage hepatocarcinogenesis[6].

Immunohistochemically, HSP70 is significantly overexpressed in early HCC compared with its expression in dysplastic nodules, reaching 80% in most cases of well-differentiated HCC[5].

All cases of dysplastic nodules were negative or focally positive (about 5%-10% of the lesions) for CAP2; in contrast, most cases of HCC (27 of 29 cases) were positive for CAP2, to some extent. Of the lesions, 70%-100% were positive in the progressed components, and the positivity of well-differentiated HCC ranged from 10% to 100%[6].

In our case, although CAP2 staining of the first biopsy (April 2005) was negative, that of the second (May 2006) was positive over 30%. Histopathologically, the distinct difference between the two biopsies was the pseudoglandular formation and the moderate cell atypia of the second biopsy that are specific to well-differentiated HCC.

Consequently, we made the definite diagnosis of a high-grade dysplastic nodule after the first biopsy and a well-differentiated HCC after the second biopsy.

In our case, immunohistochemical staining of HSP70 was negative in the second biopsy, whereas that of CAP2 was positive. One explanation for this discrepancy is the difference in the positivity of immunohistochemical staining of well-differentiated HCC between HSP70 (72%)[5] and CAP2 (93%)[6].

It is well accepted that HCC can in a multistep manner develop from a dysplastic nodule[7,8] into HCC. Among LC cases, the dysplastic nodule is usually HCV-related, rarely HBV-related, and has not been reported as alcohol-related. Similarly, multistep hepatocarcinogenesis from a dysplastic nodule to well-differentiated HCC is usually HCV-related, rarely HBV-related, and has not been reported as alcohol-related[4].

In April 2001, no nodule was detected; in April 2005, a 10 mm hypoechoic nodule with the background of alcohol-related LC appeared in S7. Imaging studies by contrast enhanced US, CT, MRI, CTA, and CTAP revealed a hypovascular nodule compatible with a dysplastic nodule or well-differentiated HCC. The histopathological findings revealed a high-grade dysplastic nodule. In May 2006, the US pattern of the nodule changed from hypoechoic to hyperechoic without any change in size, and the imaging findings other than US were all the same as those in April 2005. Tumor markers such as AFP, AFP L3 and PIVKA Ⅱ were within normal ranges. The histopathological findings of the hyperechoic nodule revealed well-differentiated HCC. Alcohol abuse in some cases induces hyperplastic nodules that are usually associated with hypervascularity[9-11] and that are often misdiagnosed as HCC from imaging studies, as are dysplastic nodules from histopathological findings. Hyperplastic nodules do not, however, transform to HCC and often disappear subsequent to discontinued alcohol intake. The dysplastic nodule in our case is, in this respect, different from the hyperplastic nodule associated with hypervascularity. The clinical course of the patient showed multistep hepatocarcinogenesis from a dysplastic nodule to well-differentiated HCC within one year with the background of alcohol-related LC. The mechanism by which alcohol causes HCC is not known, but has been hypothesized to include oxidative stress, changes in retinoic acid metabolism and DNA methylation, decreased immune surveillance and genetic susceptibility[4]. The former three factors are common in patients with alcohol abuse. The latter two factors are specific to patients with individual differences of alcohol intake. In this case immunological examinations including immunoglobulin, the percentage of B cells and T cells, CD4/CD8 ratio, and NK cell activity were within almost normal limits. Therefore, one explanation for the transformation from a dysplastic nodule to an HCC may be genetic susceptibility that was not examined at the time.

To the best of our knowledge, this is the first case of multistep hepatocarcinogenesis from a dysplastic nodule to a well-differentiated HCC in alcohol-related LC.

Further study is needed to clarify the objective criteria for the definite diagnosis of dysplastic nodules and well-differentiated HCC, and the precise mechanism of hepatocarcinogenesis in alcohol-related LC.

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CASE REPORT

Common bile duct schwannoma: A case report and review of literature

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Abstract

Schwannoma is a myelin sheath tumor complicated with neurofibroma, neurofibromatosis and neurogenic sarcoma. Peripheral nerve sheath tumors represent 2%-6% of gastrointestinal tract stromal tumors (GIST), but there are deficient data about location of neurogenic tumors in the biliary system and only nine cases of schwannoma of the extrahepatic biliary tract have been reported. These tumors are clinically non-specific. They are usually symptomatic by compressing the close or adjacent structures when being retroperitoneal, and their preoperative diagnosis is extremely difficult. This paper reviews the literature data and describes a case of schwannoma of the common bile duct associated with cholestasis in a healthy young woman, diagnosed and treated in our department. This case is of interest on account of the complexity of its diagnosis and the atypical macroscopic growth pattern of the tumor.

Key words: Schwannoma; Neurinoma; Common bile duct; Biliary tract

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INTRODUCTION

Myelin sheath tumors comprise schwannoma, neurofibroma, neurofibromatosis and neurogenic sarcoma. More than 90% are benign[1] and present in young to middle-aged subjects (twice in women than in men). They are usually asymptomatic and hence discovered incidentally[2]. Schwannoma is more common than neurofibroma. Its origin is often single. The 10% with a multiple origin[3] are classified as neurofibromatosis II, a disorder of autosomal dominant inheritance[4]. When located in gastrointestinal tract, schwannoma, leiomyoma, leiomyosarcoma and leiomyoblastoma constitute the gastrointestinal stromal tumors (GIST)[5]. Peripheral nerve sheath tumors account for 2%-6% of the GIST, but there are few reports of neurogenic tumors in the biliary system. The retroperitoneum is rarely involved[6]. Schwannomas arise only occasionally in the extrahepatic ducts and provoke symptoms by compressing adjacent structures[7]. Their symptoms were so varied to make their preoperative diagnosis very difficult. A cholestatic syndrome in a healthy woman is described in this report.

CASE REPORT

The patient was a 41-year-old woman who presented with ingravescent pruritus lasting one month, a 4 kg weight loss and scleral subicterus. Her clinical and family history were devoid of significance. There was no history of drug intake. The physical examination evidenced a normal spleen and the absence of lymphadenopathies in the explorable sites. There was evidence of diffuse scratching lesions on the lower limbs and subicteric sclerae.

Laboratory results: RBC 4.49 × 10^6/L (4.2-5.4), WBC 4.76 × 10^3/L (4-10), hemoglobin 14.4 g/dL (12-16), ESR 21 mm/s, PCR 0.76 mg/L (< 3), LDH 412 U/L (313-618), total proteins 8.7 g/dL (6.3-8.2), total bilirubin 2.1 mg/dL (0.2-1.3), conjugated bilirubin 1.4 mg/dL (0-0.4), bile acids 6.8 μmol/L (0-6), AST 90 U/L (< 40), ALT 161 U/L (9-56), GGT 290 U/L (12-58), ALPh 192 U/L (38-126), amylase 60 U/L (30-110), antimitocondrial antibody negative, CEA 1.3 ng/mL (< 5), CA 19-9 48.5 U/mL (< 37), CA-125 39.6 U/mL (< 35).

A CT scan (Figure 1A) revealed a lesion between the head of the pancreas and the gall bladder infundibulum near to the middle segment of the common bile duct. The mass was heterogeneous with a diameter of 3 cm. The upstream bile ducts were slightly dilated. The gall bladder was overdistended and contained a 2 cm stone in the fundus. Cholangio-MRI with the secretin test (Figure 1B-D) dislcosed a hypointensive signal in the weighted

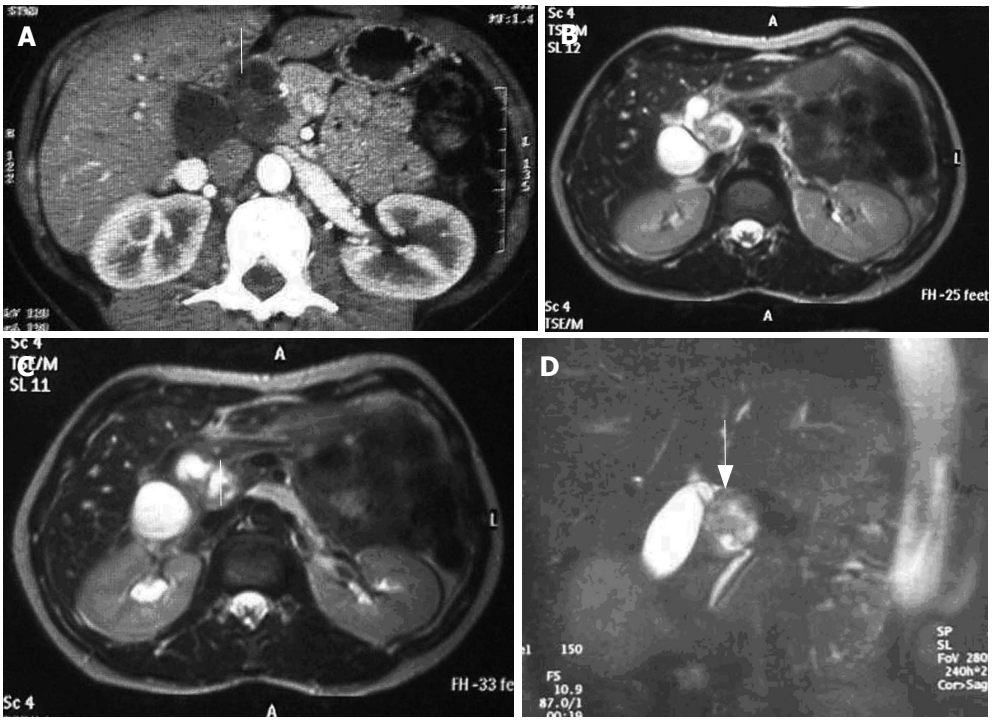

Figure 1 **A**: CT scan showing a very heterogeneous formation with a liquid content in close contiguity to the CBD (white arrow); **B**, **C**, **D**: Cholangio-MRI reveals a solid formation with a hypointensive signal in the weighted T1 sequences at the pancreatic isthmus (white arrows) with dilation of the upstream bile ducts.

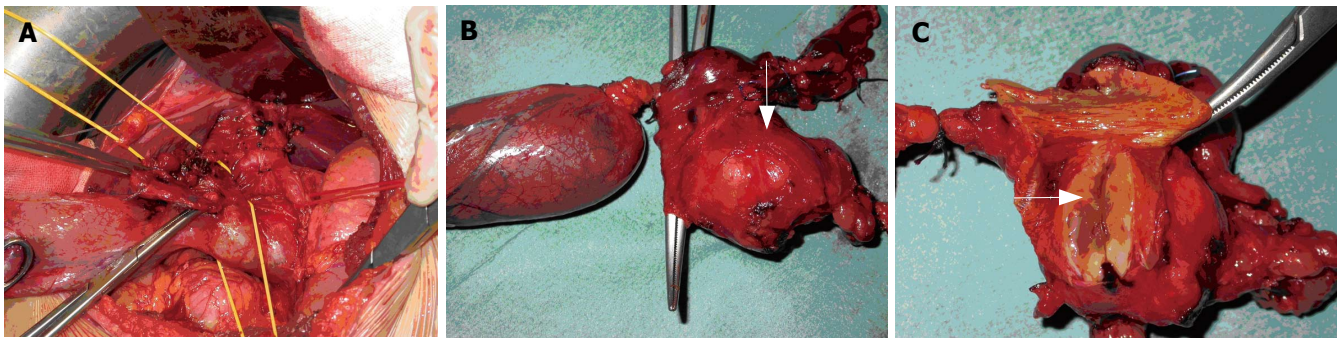

Figure 2 Intraoperative view of involvement of the intermediate tract of the common bile duct between its confluence with the cystic duct (**A**) and the prepancreatic tract (**B**) and (**C**) Gross inspection showing that the lesion (arrows) is solid and situated intramurally and below the mucosa in close contiguity with the CBD.

T1 sequences. The mass was assigned to the pancreatic isthmus. Its size and lack of uniformity were confirmed and a neoplastic origin was suspected. A good response to secretin showed that the function of the pancreas was unimpaired. Biliopancreatic echoendoscopy ruled out involvement of the pancreas, confirmed distention of the gall bladder and disclosed a solid, non-vascularised formation with many anaechogenic areas. Its contiguity with the portal axis rendered percutaneous biopsy inadvisable and exploratory laparotomy was performed. The formation involved the intermediate tract of the common bile duct between its confluence with the cystic duct and the prepancreatic tract (Figure 2A). The middle segment of the common bile duct was resected, together with the gall bladder (overdistended, but uninflamed) and the lymph nodes. A bilio-digestive Roux-en-Y anastomosis was performed on the loop. Gross inspection showed an intramural lesion under the mucosa with a diameter of 4 cm (Figure 2B and C). Histological examination revealed the proliferation of interwoven bundles of fused cells, occasionally separated by slacker, oedematous areas with a pseudocystic appearance (Figure 3A). There were also a few cells with an hyperchromic, atypical and bizarre nucleus (Figure 3B), ectatic, congested and partially thrombosed vessels (Figure 3C), and signs of perivascular hyalinisation (Figure 3D).

Immunohistochemical investigation showed that the tumor cells were vimentin and protein S-100 positive, and alpha-actin, desmin, CD34, cytokeratin pan (clone ARE1/E3) and CD117 negative. The postoperative course was uneventful. The cholestatic and hepatic cytolytic indices normalised after two months, with definite disappearance of pruritus, which continue to be normal after one year .

DISCUSSION

Schwannoma of the bile ducts is particularly rare, and it usually arises in the head, neck, spinal cord and the extremities[8], but rarely in retroperitoneum (6% of primary retroperitoneal tumors)[9]. It is usually located in the para-vertebral regions or in the pre-sacral pelvic zone[2,10]. The bladder and abdominal wall are occasionally involved.

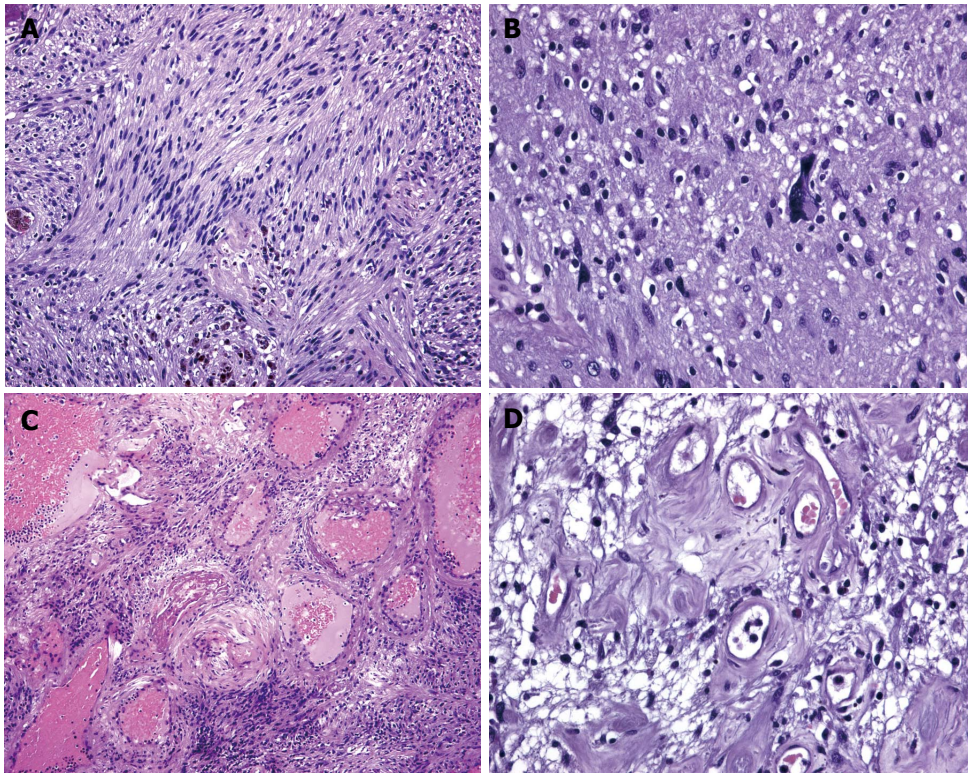

Figure 3 **A:** Histological examination showing that inter-woven bundles of fused cells separated by slacker, oedematous areas with a pseudocystic appearance (Antoni A schwannoma growth pattern); **B**: Histological examination showing a few cells with hyperchromic, atypical and bizarre nuclei.Histological examination showing a few cells with hyperchromic, atypical and bizarre nuclei; **C**: Histological examination showing hyalinisation and ectatic and partially thrombosed vessels; **D**: Histological examination showing perivascular hyalinisation.

Liver, rectal colon and esophageal involvement has been described[11-14], whereas only nine cases of schwannoma of the extrahepatic biliary tract and one case of benign schwannoma in the hepatoduodenal ligament have been reported since the 1970s[7,15-23]. Determination of tumor location must be done with great care when mass exists between the liver and the retroperitoneum, especially when the mass is hypovascular. Pre-surgical diagnosis of the tumor is very difficult because early clinical detection is limited until it gives a palpable mass or compresses the surrounding organs[6].

Diagnosis is usually entrusted to CT and MRI. The gastrointestinal schwannomas appeared as a round or oval homogeneously attenuating, well-defined mass with frequent signs of degeneration, such as cysts and calcifications on CT[24]. Recently it was reported that CTs do not reveal tumor capsule, cystic change, necrosis or calcification in any of the observed schwannomas[25]. Weighted T1 images disclose masses with low-medium signal intensity, whereas this is high in the weighted T2 images owing to alternation of the Antoni A and B areas and secondary degeneration[26].

In the present case, CT illustrated a hypodense and very heterogeneous formation initially referable to duodenal diverticulum. The MRI images were marked by slight weighted T2 hyperintensity due to the partially fluid content. A good response to secretin showed that the function of the pancreas was unimpaired.

We did not employ PET with fluorodeoxyglucose (FDG) uptake since it has been widely used to quantify the metabolism of malignant bone and soft-tissue malignant tumors, whereas little is known about FDG uptake in benign lesions. FDG PET is of limited value for the preoperative differentiation of schwannoma versus sarcoma[27]. Thus, a high FDG uptake is rather common in schwannoma[28].

The macroscopic growth pattern of the tumor mass was atypical. The two schwannoma growth patterns are called Antoni A and Antoni B. Elongated cells form an irregular, but compact palisade and the tissue arrangement is loose and there are cystic spaces between the cells[29]. In some cases, insufficient vascularisation of the mass may lead to degeneration in the form of cysts, calcifications, haemorrhages and hyalinisation[30]. These degenerative tumors are called "ancient schwannomas". Morphologic and immunophenotypic features of the lesion pointed to a so-called "ancient schwannoma", with reactive lymph nodes as described in the literature[31]. Digestive tract schwannomas are different from their soft tissue counterpart; they are not encapsulated and have an intramural growth pattern. Moreover, they are different both histologically and immunohistochimically from peripheral schwannomas. Gastrointestinal schwannomas, as in our case, are usually negative for CD34, CD117 and muscle cell markers, whereas they are strongly positive for vimentin and S100 protein. This typical combination differentiates schwannomas of the digestive tract from GIST. This differentiation is of practical importance. Gastrointestinal schwannomas are benign and associated with a good prognosis since post-surgical recurrences are unusual[32], even when treated only with enucleation. Gastrointestinal stromal tumors, on the other hand, present a negative course in more than 50% of the cases[33,34].

The distinctive feature of this case is the complexity of its diagnosis. Conventional radiography was unable to provide a precise picture of the nature and location of the mass. A diagnosis deduced from the instrumental finding often awaits intraoperative and definitive histological

confirmation[6]. Despite its complications, resection remains the treatment of choice. In the present case, only explorative laparotomy was able to demonstrate the seat and the nature of the lesion.

Lastly, in our case the postoperative course was uneventful and the cholestatic symptoms regressed two months after the operation.

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CASE REPORT

Foregut duplication cysts of the stomach with respiratory epithelium

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Abstract

Gastrointestinal duplication is a congenital rare disease entity. Gastric duplication cysts seem to appear even more rarely. Herein, two duplications cysts of the stomach in a 46 year-old female patient are presented. Abdominal computed tomography demonstrated a cystic lesion attached to the posterior aspect of the gastric fundus, while upper gastrointestinal endoscopy was negative. An exploratory laparotomy revealed a non-communicating cyst and a smaller similar cyst embedded in the gastrosplenic ligament. Excision of both cysts along with the spleen was performed and pathology reported two smooth muscle coated cysts with a pseudostratified ciliated epithelial lining (respiratory type).

Key words: Gastric; Cyst; Foregut; Duplication; Respiratory

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INTRODUCTION

Gastrointestinal duplications are rare and even more exceptional are those occurring in the stomach. As a general definition, a gastrointestinal duplication is a spherical hollow structure with a smooth muscle coat, lined by a mucous membrane and attached to any part of the gastrointestinal (GI) tract from the base of the tongue to the anus[1]. These malformations are believed to be congenital and are formed before differentiation of the lining epithelium and, therefore, are named for the organs with which they are associated[2]. Foregut duplications may or may not communicate with the GI tract and usually are diagnosed in a younger age; in adults non-specific symptoms delay diagnosis, which is established during surgical exploration[3]. Herein, two gastric duplication cysts with respiratory epithelium are reported.

CASE REPORT

A 46-year-old female patient was admitted to our Surgical Department from another medical center, where the patient was evaluated for an episode of loss of consciousness. Past medical history, present status and physical examination were non disease-specific. Diagnostic workup included an abdominal computed tomography (CT) which demonstrated a cystic lesion measuring 6 cm × 6 cm × 7 cm attached to the posterior wall of the gastric fundus, was well circumscribed and had no contrast enhancement (Figure 1). Interestingly CT coronary sections revealed a pulmonary sequestration in the basal segment of the left lower lobe (Figure 2). Moreover, upper GI endoscopy showed a bulging deformation of the gastric fundus by an extrinsic mass, without any mucosal abnormalities. An exploratory laparotomy was performed and revealed two cystic lesions; the first was attached to the fundus of the stomach and the second was embedded within the gastrosplenic ligament, close to the spleen. Therefore, intact excision of both cysts and splenectomy were carried out. Postoperative course was uneventful and the patient has been asymptomatic since then.

Pathologic examination of the surgical specimens reported a large cyst, measuring 8 cm × 5.5 cm in diameter and 0.3 cm thick and a smaller cyst (removed from the gastrosplenic ligament), measuring 3 cm in diameter and 0.2 cm thick. Both consisted of a smooth muscular wall, were lined by a pseudostratified, ciliated and columnar (respiratory type) epithelium and contained a clear, gelatinous fluid. Sub-epithelium seromucous glands were microscopically identified (Figure 3), a histologic appearance reminiscent of bronchial wall; however no cartilage was present.

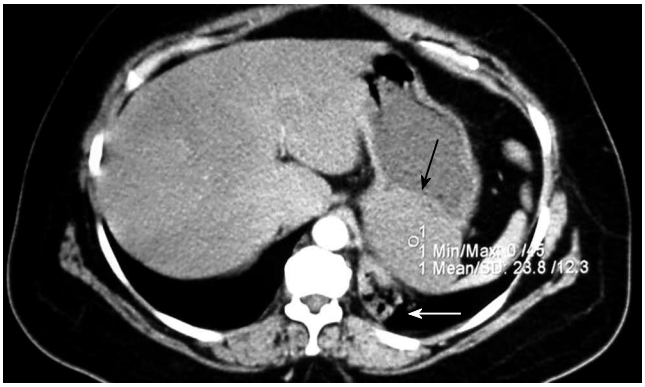

Figure 1 Abdominal CT scan demonstrating a cystic lesion attached to the posterior gastric wall (black arrow) and a pulmonary sequestration in the left pulmonary base (white arrow).

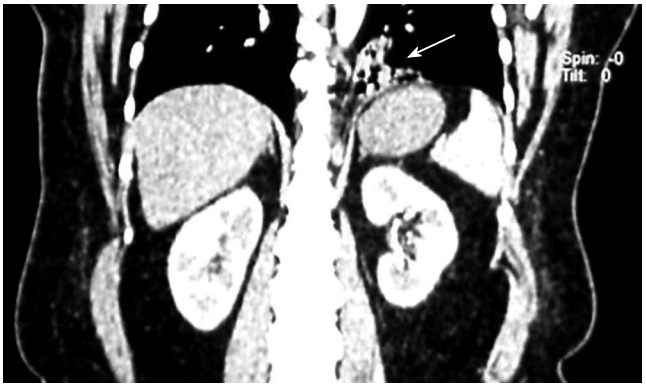

Figure 2 Coronary CT plate showing the pulmonary sequestration of the basal segment of the left lower lobe (arrow).

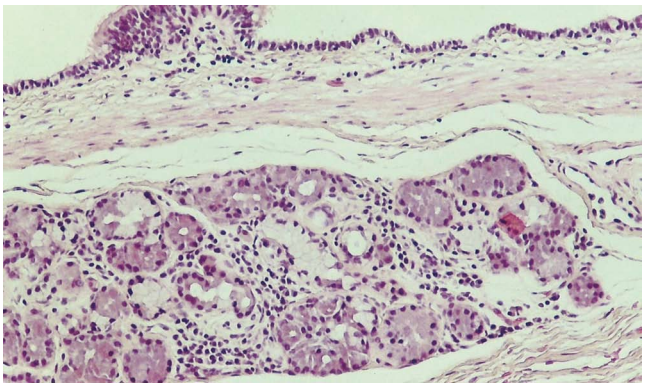

Figure 3 Histological section of the cyst wall lined by a respiratory-type epithelium and containing seromucinous glands (HE, x 100).

DISCUSSION

Gastric duplications account for between 3% and 20% of gastrointestinal duplications[2,4,5] and occur twice as frequent in females as in males. Controversy exists over the embryological origin of these anomalies. Duplications of the stomach are usually single, less than 12 cm in diameter and located on the greater curvature or on the posterior or anterior gastric wall[2]. Wieczorek *et al*[6] reported that gastric duplications can be tubular or cystic; the cystic type does not communicate with the gastric lumen (about 80% of gastric duplication cysts[7]). The first case report of a gastric duplication was published in 1911 by Wendel[8] and approximately 150 cases have been reported since then[2].

Due to their position and mass effect, gastric duplications cysts are usually diagnosed in a younger age. However, in adults diagnosis may be difficult. A wide range of symptoms and signs have been reported and vary from asymptomatic to non disease-specific presentations, e.g. vague abdominal complaints, nausea, vomiting, epigastric fullness, weight loss, anemia, dysphagia, dyspepsia, etc. Vomiting usually occurs due to partial or complete gastric outlet obstruction, while even more emergent cases have been reported such as pancreatitis[9], hemoptysis[10] and gastrointestinal hemorrhage[11]. In our case, the female patient presented with loss of consciousness due to dehydration occurring as a result of excessive vomiting and concomitant anemia. Associated pathologic conditions including pulmonary sequestration[12], multicystic kidney[13] and neoplasias[14] have been reported. The presence of respiratory type mucosa and seromucinous glands along with the presence of pulmonary sequestration in the lower lobe of the ipsilateral lung in our case supports the theory of embryologic origin from supernumerary foregut buds.

Foregut duplications cysts of the stomach are usually incidentally diagnosed. Although upper gastrointestinal series demonstrate external pressure on the stomach, preoperative workup mainly includes abdominal ultrasonography and computed tomography scans, as well as endoscopic ultrasonography and magnetic resonance imaging[15,16].

Due to malignant transformation and the report of gastric cancer arising in duplications of the stomach[17], surgical excision is considered to be the best treatment. Complete resection of the cyst is the ideal technique achieved with both open and laparoscopic[18] approaches. Unsuccessful approaches including percutaneous or endoscopic aspiration of cystic fluid have been reported[19], but are associated with complications, such as fistula formation and hemorrhage. When the recommended complete excision is not possible, converting both stomachs into one using a stapling device[20] or performing a segmental gastrectomy[21] are the possible alternatives.

In conclusion, foregut duplication cysts of the stomach are rare entities diagnosed incidentally and usually intraoperatively in adults and should be treated surgically by complete resection.

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CASE REPORT

Melena: A rare complication of duodenal metastases from primary carcinoma of the lung

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Abstract

Small bowel metastases from primary carcinoma of the lung are very uncommon and occur usually in patients with terminal stage disease. These metastases are usually asymptomatic, but may present as perforation, obstruction, malabsorption, or hemorrhage. Hemorrhage as a first presentation of small bowel metastases is extremely rare and is related to very poor patient survival. We describe a case of a 61- year old patient with primary adenocarcinoma of the lung, presenting with melena as the first manifestation of small bowel metastasis. Both primary tumor and metastatic lesions were diagnosed almost simultaneously. Upper gastrointestinal endoscopy performed with a colonoscope revealed active bleeding from a metastatic tumor involving the duodenum and the proximal jejunum. Histological examination and immunohistochemical staining of the biopsy specimen strongly supported the diagnosis of metastatic lung adenocarcinoma, suggesting that small bowel metastases from primary carcinoma of the lung occur usually in patients with terminal disease and rarely produce symptoms. Gastrointestinal bleeding from metastatic small intestinal lesions should be included in the differential diagnosis of gastrointestinal blood loss in a patient with a known bronchogenic tumor.

Key words: Melena; Duodenal metastases; Lung cancer

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INTRODUCTION

Metastases affecting the small bowel and originating from carcinoma of the lung are rare, but recent reports suggest that they may be more frequent than previously thought as they rarely produce symptoms[1-3].

The majority of patients with metastases of the small bowel reported in the literature, present with bowel perforation[2-4]. Overt gastrointestinal hemorrhage has been described in a few cases as a prelude to bowel perforation, whilst it has been described only rarely as the main presentation[5-7].

We present a case of a 61-year old patient with upper gastrointestinal bleeding due to small bowel metastases from a primary adenocarcinoma of the lung with secondary deposits in the abdominal lymph nodes. Metastatic involvement of the duodenum presenting with melena has been described rarely in the literature.

CASE REPORT

A 61-year old man was admitted to our hospital with a two-day history of melena. He reported a four-month history of weakness, anorexia, 5 kg loss of weight, cough, dyspnea on exertion, and blood-stained sputum, and a two-month history of fatigue and altered bowel habits. He was a cigarette smoker with chronic obstructive airway disease and arterial hypertension.

On examination he was pale, with normal blood pressure and pulse rate. He had wheezy breathing and decreased breath sounds in the upper right lung region. Melena was proved by digital examination.

Hematocrit was 35% and hemoglobin 9.5 g/dL. Chest x-ray demonstrated a density in the upper region of the right lower lobe. Endoscopy of the upper gastrointestinal tract up to the second part of the duodenum revealed erosive antral gastritis, but no active bleeding site. CT scan of the thorax disclosed an irregularly-shaped nodular mass measuring 4 cm in diameter, in the upper region of the right lower lobe of the lung. Bronchial biopsy revealed the presence of blood clots and desquamated bronchial epithelial cells, without signs of malignancy. The results of cytological examination of bronchial excretions, however, proved positive for non-small cell carcinoma of the lung. Staging of the disease followed by CT scan of the upper and lower abdomen and the retroperitoneal space showed multiple, enlarged bilateral para-aortic, and mesenteric

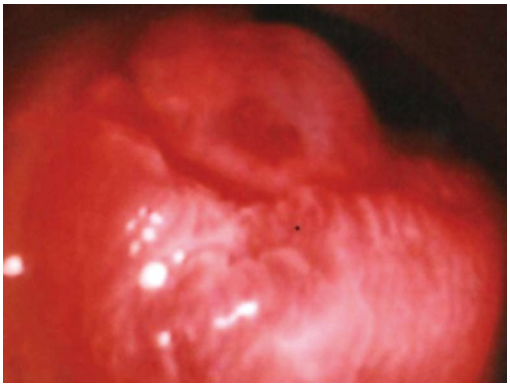

Figure 1 Endoscopic appearance of a large hemorrhagic polypoid mass at the proximal jejunum, representing metastatic lung carcinoma.

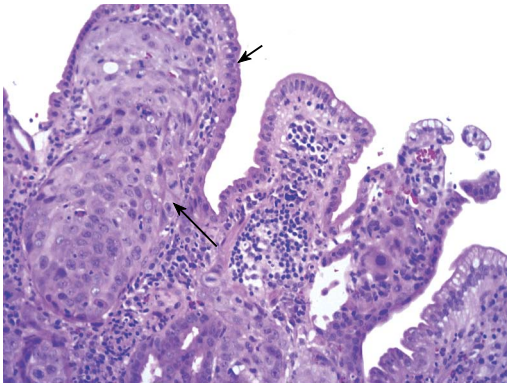

Figure 2 Gatherings of poorly-differentiated neoplastic cells invading the mucosal bed, and extending into the intestinal villi (long arrow), with sparing of the superficial epithelium (short arrow).

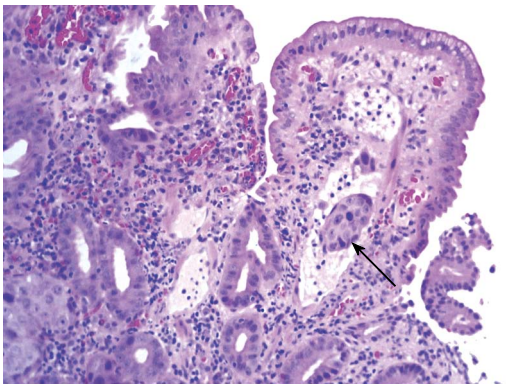

Figure 3 Embolus of neoplastic cells in lymph vessels of the intestinal villi.

lymph nodes.

The patient was started on combined chemotherapy with cisplatin and hydrochloric gemsitabine. His bowel motions became normal by the third day of hospitalization and he was subsequently discharged, denying further investigation.

During admission for the second round of chemotherapy and one month after diagnosis, the patient complained of diffuse abdominal pain and tenderness. Shortly afterwards this was accompanied with hemodynamic instability and tachypnea. Melena was proved by digital examination. Hematocrit fell from 34% to 26%, hemoglobin from 9 g/dL to 6 g/dL, and serum urea/creatinine ratio was greater than 40 (urea being 60mg/dL and creatinine 1.3 mg/dL). Plain abdominal X-ray showed no abnormalities and the patient underwent endoscopy of the upper and lower gastrointestinal tract using a Pentax© 160 cm colonoscope for both examinations. In the 4th part of the duodenum three irregular, nodular protrusions of the mucosa measuring 0.5-1.5 cm in diameter were observed. They were friable and slightly hemorrhagic. At the proximal jejunum there was a larger hemorrhagic polypoid mass about 2.5 cm in diameter (Figure 1). Biopsies of these lesions revealed gatherings of poorly-differentiated neoplastic cells invading the mucosal bed and extending into the intestinal villi with sparing of the superficial epithelium (Figure 2). Infiltration of the lymph vessels was also noted (Figure 3). These findings were consistent with metastatic disease. Immunohistochemical staining for keratins 8, 18, 19 and thyroid transcription factor 1 (TTF 1) as well as histochemical staining for periodic acid schiff (PAS) strongly supported the diagnosis of poorly-differentiated metastatic adenocarcinoma of the lung. Radiological examination of the small bowel was not completed due to patient discomfort. The patient was transfused with 3 units of packed erythrocytes and 3 units of fresh frozen plasma.

Only four cycles of chemotherapy were carried out due to deterioration of the patient. In addition, he received 4000 U of erythropoietin per week. Three months after his last episode of melena the patient continued to have occult upper gastrointestinal bleeding and was admitted on several occasions for blood transfusion. The patient died seven months after the diagnosis of end stage cancer of the lung.

DISCUSSION

Small bowel metastases usually originate from primary carcinomas of the gastrointestinal or genital organs, and more specifically from the large bowel, uterus, cervix, ovaries, and testes. Thoracic malignancies metastasize less frequently to the small bowel. Apart from carcinoma of the lung, these also include malignant melanoma, carcinoma of the breast, carcinoma of the salivary glands, carcinoma of the esophagus, and rhabdomyosarcoma of the lung[1,8,9].

Our patient had histologically-proven small intestinal lesions originating from primary lung adenocarcinoma. A review of the literature revealed few studies of patients with lung cancer and metastases to the small bowel. An extensive eleven-year study of patients with primary lung cancer revealed that at autopsy, 46 out of 431 patients (10.6%) had secondary deposits in the small bowel[1]. These patients had an average of 4.8 metastatic sites. A more recent study of patients with non-small cell carcinoma of the lung revealed small bowel metastases in 4.6% of cases[9]. It should be emphasized that all these cases had adenocarcinomas, with a disease stage greater than IIIA, as well as a minimum of two other metastatic sites before the development of bowel metastases. Another

large study involved 1399 patients with lung cancer who underwent surgical resection of the primary tumor[2]. This study revealed a much smaller percentage (0.5%) of symptomatic patients having small bowel metastases. In direct contrast to the previous study, which concludes that adenocarcinoma is the cell type resulting more frequently in small bowel metastasis, this study concludes that squamous cell carcinoma is the more frequent cause, accounting for 61% of cases.

Patients with primary lung cancer with metastases to the small bowel are usually asymptomatic[10,11]. Less frequently these metastases can cause symptoms which vary according to the way the metastatic tumor invades the bowel wall. Rapid tumor growth leads to symptoms of obstruction, although it seems that perforation occurs more commonly probably due to central tumor necrosis. Most cases of primary lung cancer and small bowel metastasis present with small bowel perforation[3,4,10]. According to a recent study, 98 cases of perforated lung cancer metastasis to the small intestine have been described in the literature since 1960[3]. When ulceration of the mucosa occurs the metastatic tumor may bleed, whilst large extension of tumor may lead to symptoms of malabsorption[1].

Hemorrhage from a metastatic tumor in the small bowel is uncommon, explaining the absence of large studies on this issue. A small number of isolated cases, however, have been described. They describe patients over the age of 55 years, with primary lung carcinoma of large or small cell types, who have already developed distant metastases, and these patients usually present with acute hemorrhage from ulceration of the metastatic tumor, and less frequently with iron deficiency anemia, or with non-specific abdominal symptoms before the onset of melena[12-15]. In one case report, the patient presented with iron-deficiency anemia and melena, and the diagnosis of primary lung cancer was made after surgical resection of the intestinal metastasis, as no tumor evidence was found on chest x-ray[2]. The presence of small bowel metastases in this patient was confirmed by CT scanning, as upper and lower gastrointestinal endoscopies were negative.

A very small number of cases of upper gastrointestinal bleeding due to duodenal metastases from lung cancer have been reported[5-7,12]. Small intestinal metastases have been reported to occur more frequently in the jejunum and terminal ileum than the duodenum[2,3,16]. As in our case, metastatic lesions of the small bowel from lung carcinoma are usually multiple[1,11].

Our patient is one of the very few cases described where melena as the first manifestation of small bowel metastasis, has arisen from the duodenum and jejunum[5-7,12,16]. Also it is interesting to note that the time interval was short between diagnosis of cancer and metastasis to the small bowel, as the patient presented with melena before the establishment of the diagnosis of lung carcinoma and was still in a good general state of health. The diagnosis of metastatic involvement of the small bowel was suspected, but not confirmed at the beginning. This emphasizes the importance of considering the presence of small bowel metastases in a patient with lung cancer displaying symptoms of hemodynamic instability, melena, or abdominal symptoms such as dyspepsia, distention and pain, even if the time elapsed from the time of diagnosis of cancer is short. It should be noted that occult gastrointestinal bleeding must be suspected if laboratory investigations reveal iron deficiency anemia, or a fall in hematocrit or hemoglobin, even in an asymptomatic patient.

Gastrointestinal hemorrhage can usually be managed conservatively with intravenous fluids and red blood cell transfusion until the patient is hemodynamically stable and hemorrhage ceases. However, cases requiring surgical resection of small bowel in order to control massive hemorrhage have also been reported[12,16]. Only a few patients have survived more than 9 mo after surgical resection of intestinal metastases, with the exception of one patient who survived 22 mo after peritonitis [2]. Perioperative mortality has decreased considerably, and the question of whether surgery should be considered as palliative therapy not only for symptomatic patients remains to be solved. After hemorrhage from small bowel metastases, patient survival varies between several weeks to several months[6,13]. It seems that hemorrhage originating from small bowel metastases, is related to a very poor patient survival. The patient we presented, survived for a considerably long time (7 mo after his first episode of bleeding). During this period of time he continued to have microscopic bleeding requiring occasional blood transfusion. Active gastrointestinal bleeding from small bowel metastases has never been described as the cause of death in patients suffering from carcinoma of the lung.

In conclusion, small bowel metastases from primary carcinoma of the lung occur usually in patients with terminal disease and rarely produce symptoms. Hemorrhage as a first presentation of small bowel metastases is extremely rare, especially when these are located in the duodenum, with a poor prognosis. Gastrointestinal bleeding from metastatic small intestinal lesions should be included in the differential diagnosis of gastrointestinal blood loss in a patient with a known bronchogenic tumor.

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CASE REPORT

Liver toxicity of rosuvastatin therapy

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Abstract

We report here a case of clinically significant liver toxicity after a brief course of rosuvastatin, which is the first statin approved by the regulatory authorities since the withdrawal of cerivastatin. Whether rosuvastatin has a greater potential compared with other statins to damage the liver is unclear and the involved mechanisms are also unknown. However, rosuvastatin is taken up by hepatocytes more selectively and more efficiently than other statins, and this may reasonably represent an important variable to explain the hepatotoxic potential of rosuvastatin. Our report supports the view that a clinically significant risk of liver toxicity should be considered even when rosuvastatin is given at the range of doses used in common clinical practice.

Key words: Rosuvastatin; Liver; Toxicity; Hepatitis; Statins

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INTRODUCTION

Liver toxicity is a well recognized adverse effect of treatment with statins[1]. However pre-marketing studies have suggested that rosuvastatin may have a lesser potential to cause liver toxicity as compared with other statins[2]. We report here a case of clinically significant liver toxicity after a brief course of rosuvastatin.

CASE REPORT

A 64-year-old man presented with a seven-days history of malaise, anorexia, upper abdominal discomfort, and jaundice. Four months earlier the patient had an acute myocardial infarction that was treated with angioplasty and stenting of a culprit lesion in the right coronary artery; liver function tests were normal and he was discharged on clopidogrel, aspirin, metoprolol, ramipril, and atorvastatin (40 mg daily). One week later, rosuvastatin (10 mg daily) was prescribed instead of atorvastatin as the patient reported an itching skin rash that developed soon after he took the second tablet of atorvastatin; at that time serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were 55 U/L and 45 U/L (normal range 10-36 U/L for both), respectively, with bilirubin, γ-glutamyltransferase (γ-GT), and alkaline phosphatase (AP) within the normal limits.

At the present admission, he was fully alert and oriented, apyrexial, with mild cutaneous and scleral jaundice, and no flapping tremor or stigmata of chronic liver disease; the remaining physical examination was normal. A laboratory work-up revealed AST 880 U/L, ALT 775 U/L, total bilirubin 2.6 mg/dL (normal range 0.2-1) with conjugated bilirubin 0.8 mg/dL; γ-GT, AP, ammonia, α-fetoprotein, electrolytes, hematologic and coagulation parameters, and renal function tests were normal. Serological screening for viral hepatitis (hepatitis A, B, C, E and G virus; cytomegalovirus; herpes simplex; and Epstein–Barr virus) was negative and HBV-DNA and HCV-RNA were not detected in the peripheral blood. Search for autoimmune liver disorders (antinuclear antibodies, anti-smooth-muscle antibodies, and antimitochondrial antibodies) was also negative as were results of iron, copper, ceruloplasmin metabolism and α1-antitrypsin concentrations.

Ultrasonography and contrast-enhanced computed tomography (CT) showed a normal liver and no expanded bile ducts or gallbladder abnormalities; there was no caval or portal thrombosis and no peri-hepatic or perisplenic intraperitoneal fluid. Echocardiography was also normal with no evidence of valvular disease, pericardial effusion, pulmonary hypertension, or left ventricular systolic or diastolic dysfunction.

The patient had an otherwise unremarkable medical record with no previous history of acute or chronic liver disease. He also denied toxic and alcoholic habits or using any other medications, including over-the-counter medications, or herbal remedies.

Rosuvastatin was withdrawn and AST and ALT levels fell to 216 U/L and 198 U/L, respectively, and bilirubin to 1.8 mg/dL on the 3rd day; ammonia and coagulation parameters remained within the normal range. Over the subsequent course symptoms gradually resolved, which

was paralleled by declining levels of liver enzymes and bilirubin; at no time were flapping tremor or other signs or symptoms of liver failure or encephalopathy observed and the patient was discharged on the 6th day with instructions for close follow-up as an outpatient. At that time, AST and ALT were 40 U/L and 32 U/L, respectively; bilirubin, ammonia, and coagulation parameters were normal. The patient refused a liver biopsy and a re-challenge test with rosuvastatin was not done for ethical reasons. At a follow-up visit two weeks later, he was doing well with normal liver function tests and a normal coagulation profile. The clinical and biochemical course of this patient is summarized in Figure 1.

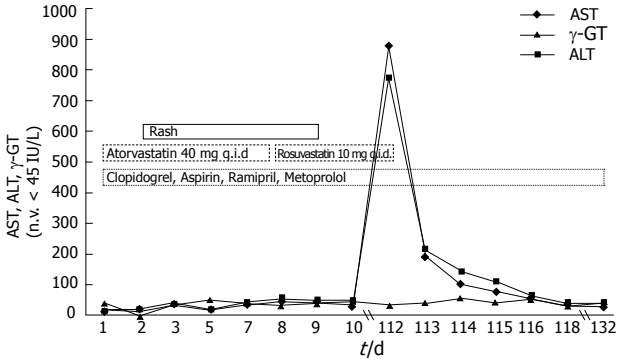

Figure 1 Course of liver enzymes and exposure to statins and other medications.

DISCUSSION

Until today the incidence of elevated serum liver enzymes among patients on treatment with statins generally ranges from 2% to 3%[1] while pre-marketing studies have shown a 20 to 30-fold lower incidence if rosuvastatin is used[2]. We report here a case of clinically significant liver toxicity after a brief course of rosuvastatin, which is the first statin approved by the regulatory authorities since the withdrawal of cerivastatin. According to the Naranjo probability scale our patient had a highly probable rosuvastatin-related adverse event[3]; furthermore, liver function tests were normal before statin treatment was started and we did not find any other plausible alternative cause to explain the onset of such a severe hepatitis in this case. As a matter of fact, we identified a clear temporal relationship between initiation of rosuvastatin therapy and the elevation of liver enzymes and the patient rapidly achieved a complete clinical and biochemical recovery after rosuvastatin was interrupted.

The episode of urticaria accompanied by a modest increase of liver enzymes after the patient was given atorvastatin, which was his first exposure to a statin, raises the view of an idiosyncratic or hypersensitivity cross-reaction between atorvastatin and rosuvastatin as an underlying mechanism of the liver injury that occurred during treatment with rosuvastatin. Even though the true correlation remains to be understood, we might reasonably conceive that allergic cross-reactions between the two statins might have contributed, at least to a certain extent, to the onset of severe liver damage in this case. Our patient had normal eosinophil count and remained apyrexial throughout the entire course. However all these features along with the absence of lymphadenopathy and the negative search for antinuclear and anti-smooth-muscle antibodies do not substantially argue against an allergic or immune-mediate mechanism of liver injury.

The patient was also receiving other medications in combination with rosuvastatin, but this is not a true confounding factor in our opinion. Our Medline search yielded only a few anecdotal reports of hepatitis associated with the use of clopidogrel or ramipril[4-7] and none of liver toxicity linked with metoprolol. In addition, aspirin-related hepatitis has been described, but this is a dose-related phenomenon caused by the intrinsic salicylate hepatotoxicity which generally does occur only when aspirin is given in full anti-inflammatory doses and not with the 75-300 mg used for anti-platelet indications[8]. One important point is that rosuvastatin is neither an inhibitor nor an inducer of cytochrome P450 isoenzymes and rosuvastatin itself is a poor substrate for these isoenzymes. It suggests that fewer drug interactions resulting from cytochrome P450-mediated metabolism should be expected with rosuvastatin compared with other statins[9,10]. There is no available evidence of any clinically significant pharmacokinetic interactions in subjects taking concomitant rosuvastatin and clopidogrel, ramipril, metoprolol, or aspirin.

Whether rosuvastatin has a greater potential compared with other statins to damage the liver is unclear and the involved mechanisms are also unknown. Results from a recent meta-analysis demonstrate that treatment with statins as a class is not associated with a significant risk of liver dysfunction and hepatitis, but studies with rosuvastatin were not included in this meta-analysis[1]. In contrast, a post-marketing analysis has shown that use of rosuvastatin, at least over its first year of marketing, was significantly more likely than atorvastatin, pravastatin, and simvastatin to be associated with a composite end point of adverse events including rhabdomyolysis, proteinuria, nephropathy, and renal failure, and there was also a significant trend in favor of an increased liver toxicity with rosuvastatin compared with the other statins even though this was not targeted as the primary end point of the study[11]. Furthermore, rosuvastatin is taken up by hepatocytes more selectively and more efficiently than other statins, and this may reasonably represent an important variable associated with the hepatotoxic potential of rosuvastatin[12].

Our report supports the view that a clinically significant risk of liver toxicity should be considered even when rosuvastatin is given at the range of doses used in common clinical practice.

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CASE REPORT

Capsule endoscopy retention as a helpful tool in the management of a young patient with suspected small-bowel disease

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Abstract

Capsule endoscopy is an easy and painless procedure permitting visualization of the entire small-bowel during its normal peristalsis. However, important problems exist concerning capsule retention in patients at risk of small bowel obstruction. The present report describes a young patient who had recurrent episodes of overt gastrointestinal bleeding of obscure origin, 18 years after small bowel resection in infancy for ileal atresia. Capsule endoscopy was performed, resulting in capsule retention in the distal small bowel. However, this event contributed to patient management by clearly identifying the site of obstruction and can be used to guide surgical intervention, where an anastomotic ulcer is identified.

Key words: Capsule endoscopy; Retention; Small bowel obstruction; Obscure gastrointestinal bleeding; Surgery

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INTRODUCTION

Capsule endoscopy allows direct visualization of the entire small bowel in a noninvasive manner and has become the gold standard in evaluating suspected disease of the small bowel[1-4]. Capsule retention remains a major concern for physicians performing capsule endoscopy, since it can lead to a need for surgery to remove the capsule in a patient who otherwise might have been treated medically for the same illness. Nevertheless, it has been postulated that capsule endoscopy in certain patients with suspected small bowel obstruction, contributes to patient management by clearly identifying the site of obstruction and can be used to guide surgical intervention[5-7].

CASE REPORT

A 22-year old patient was admitted to our hospital for evaluation of recurrent episodes of overt gastrointestinal (GI) bleeding of obscure origin. The patient presented at birth with ileal atresia and underwent partial small bowel resection (excision of 30 cm of ileal loop sparing ileocaecal valve). He was reoperated three times during the first years of his life, initially due to anastomotic leak and subsequently twice for postoperative repair of fistulae. Since then, the patient had remained free of symptoms and developed normally. At the age of 18 years (in 2001), he had a severe episode of overt GI bleeding requiring blood transfusion, and two new bleeding episodes during the next two years. Upper and lower GI endoscopy, computed tomography of the abdomen, Meckel scintigraphy and enteroclysis, revealed no remarkable findings. In 2004 he had symptoms of incomplete intestinal obstruction, which resolved after conservative treatment. A new episode of GI bleeding with negative GI endoscopy requiring transfusion a few days before admission was the reason for referral to our unit, in order to carry out capsule endoscopy.

Because of his medical history, a patent capsule (Given Imaging Ltd., Yoqneam, Israel) was given prior to Pillcam SB, in order to avoid capsule retention. The patent capsule was impacted in the distal small bowel, causing temporary abdominal pain, which was confirmed by two abdominal X-rays on the second and forth day, as the patent capsule was detected at the lower abdomen to the middle line. On the sixth day, a new abdominal X-ray showed that the patent capsule was dissolved. Considering that the patent capsule stopped in a stenotic lesion, probably on the level of entero-enteric ileal anastomosis, surgical management

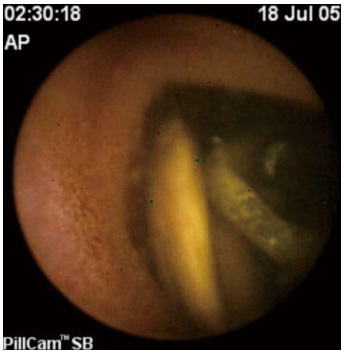

Figure 1 PillCam SB enteroscopy showing the point of capsule retention (Bezoar).

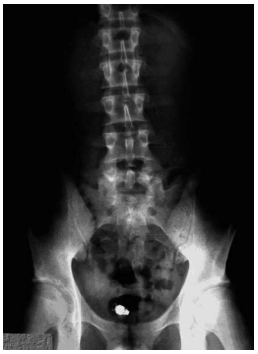

Figure 2 Plain abdominal film on the fifth day after capsule ingestion.

was decided. After discussion with the surgeons and based on data supporting that capsule endoscopy in patients with suspected small bowel obstruction can guide surgical intervention by identifying the site of obstruction[5], capsule endoscopy was carried out after a written consent was obtained from the patient. Four sachets of PEG preparation were given the day before capsule endoscopy procedure was performed.

The capsule passed through the stomach, duodenum and jejunum in 2 h and 30 min, with no evidence of disease and remained impacted in an ileal loop, where a food bezoar was observed (Figure 1). Abdominal X-rays after 24 h and on the fifth day confirmed capsule retention (Figure 2). The patient was operated on the sixth day. Loose adhesions were identified and resected. The capsule was palpated 50 cm proximal to the ileocaecal valve, on the level of the entero-enteric anastomosis and an anastomotic ulcer causing stenosis was found after incision. Intraoperative enteroscopy through the incision point was performed in order to examine the ileal segment from the stenosis to the ileocaecal valve that was found normal. The lesion was resected (Figure 3) and histology demonstrated focal ulceration with chronic inflammation, but no evidence of granulomata, crypt abscesses or malignancy. The patient was discharged on the fifth postoperative day and remained asymptomatic 12 mo post-surgery.

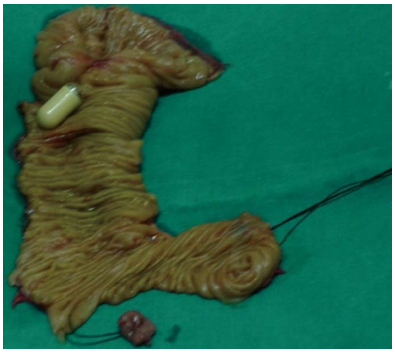

Figure 3 Surgical specimen with ulceration and retained PillCam SB capsule.

DISCUSSION

Atresia and stenosis are common birth defects affecting the small intestine, but few population-based studies have examined the epidemiology of small intestinal atresia and stenosis. It was reported that the rate of small intestinal atresia and stenosis is 2.9 per 10 000 live births (I 95% CI = 2.3-3.6)[8]. Current operative techniques and contemporary neonatal critical care result in a 5% morbidity and mortality rate, with late complications not uncommon[9]. Protein-losing enteropathy and gastrointestinal bleeding due to anastomotic ulcer after 4-12 years have been reported[10]. Actually, gastrointestinal bleeding occurred in our patient due to anastomotic ulcer 18 years after small bowel resection. For these reasons, follow-up of these patients in their adulthood is recommended and physicians must be aware to identify and address these late occurrences[9].

Capsule endoscopy is currently the preferred test for mucosal imaging of the entire small intestine[4]. When integrated into a global approach to the patient, capsule endoscopy can achieve effective decision-making, concerning subsequent investigations and treatments. Capsule retention remains the major concern for physicians performing capsule endoscopy, since retention could lead to a need for an otherwise unnecessary surgery to remove the capsule. As there is no accepted imaging method for completely avoiding capsule retention, it is clear that obtaining a good medical history is the best single method[11].

Our patient had two risk factors for capsule retention: a history of previous small-bowel resection and a distant history of partial small-bowel obstruction. For these reasons, a patent capsule was given initially to the patient. The retention of patent capsule in the small intestine, confirmed our suspicions. Nevertheless, Cheifetz *et al*[5] showed that capsule retention in the small intestine could help a pre-decided operation by guiding the surgeons to clearly indentify the site of obstruction in patients with suspected small-bowel obstruction. Even though capsule endoscopy did not help us to diagnose a definite lesion, as we did not take clear images of the site of capsule retention, palpation of the capsule by the surgeons during operation, could lead them directly to the site of stenosis.

In conclusion, capsule retention is not always an adverse event, as in certain patients with suspected small bowel obstruction, the retained capsule can be used as a "guiding point" for a pre-decided surgical intervention.

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CASE REPORT

Simultaneous development of diabetic ketoacidosis and Hashitoxicosis in a patient treated with pegylated interferon-alpha for chronic hepatitis C

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Abstract

Classical interferon-alpha has been shown to be correlated with the development of a variety of autoimmune disorders. A 38 year-old female patient developed simultaneously diabetic ketoacidosis and hyperthyroidism 5 mo following initiation of treatment with pegylated interferon-α and ribavirin for chronic hepatitis C. High titers of glutamic acid decarboxylase, antinuclear and thyroid (thyroid peroxidase and thyroglobulin) antibodies were detected. Antiviral treatment was withdrawn and the patient was treated with insulin for insulin-dependent diabetes mellitus and propranolol for hyperthyroidism. Twelve months after cessation of pegylated interferon-α therapy the patient was euthyroid without any medication but remained insulin-dependent.

Key words: Autoimmune thyroiditis; Insulin dependent diabetes mellitus; Pegylated interferon-alpha; Chronic hepatitis C

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INTRODUCTION

Interferon-alpha (IFN-α), a natural protein with anti-viral, anti-proliferative and immunomodulatory effects is routinely administered in chronic hepatitis C (CHC). Classical IFN-α has been correlated with the development of a variety of autoimmune disorders including Hashimoto thyroiditis, immune-mediated thrombocytopenia, hemolytic anemia, psoriasis, rheumatoid arthritis, systemic lupus-like syndromes, primary biliary cirrhosis and sarcoidosis. The reported cumulative incidence of all autoimmune disorders ranged from 1% to 3%[1,2].

Clinical thyroid disease has been reported to develop in 10%-15% of patients treated with IFN-α for CHC[3,4]. However, it was not established whether IFN-α treatment is associated with the development of insulin dependent diabetes mellitus (IDDM). The prevalence of diabetes mellitus development in patients receiving classical IFN-α for CHC is very low ranging from 0.08% to 0.7%[1,2]. The prevalence of pancreatic auto-antibodies appeared to rise from 3% to 7% prior to and following initiation of IFN-α treatment, respectively, in a review of 9 relative studies by Fabris *et al*[5]. In those studies different types of IFN-α and variable schedules were used.

Pegylated IFN-α has been recently approved for the treatment of CHC and has been associated with only a few cases of autoimmune thyroiditis[6]. We herein describe the simultaneous development of diabetic ketoacidosis and Hashitoxicosis in a patient treated with pegylated IFN-α for CHC.

CASE REPORT

A 38-year-old female patient presented with a two fold increase of aminotransferases and positive hepatitis C virus (HCV) antibodies. HCV RNA was high (> 1 000 000 copies/mL, genotype 1b) and liver histology revealed an activity grade of 4/18 and a fibrosis score of 3/6 according to Ishak's modified HAI classification system[7]. Treatment with pegylated IFN-α-2α 180 μg/wk, in combination with oral ribavirin 1000 mg/d was initiated in February 2004. During treatment alanine aminotransferase (ALT) flares did not occur. Virological response (negative HCV RNA) was achieved at the fourth week of treatment.

In July 2004 the patient developed weakness and rapid weight loss up to 12 kg within 2 wk. Thyroid function tests revealed hyperthyroidism of autoimmune etiology, i.e. thyroid stimulating hormone (TSH): 0.008 μIU/mL (normal values: 0.15-6.1), free triiodothyronine (FT_3): 6.90 pg/mL (normal values: 2.03-4.6), free thyroxine (FT_4): 1.8 ng/dL (normal values: 0.9-1.7), positive thyroid peroxidase

(anti-TPO > 1300 IU/mL, normal values < 2 IU/mL), thyroglobulin (anti-Tg 18.6 IU/mL, normal values < 2 IU/mL) and thyroid stimulating immunoglobulin antibodies (TSI 96%, normal values: 0.02%-15%). Propranolol was administered. A few days later the patient was admitted with clinical and laboratory features indicating diabetic ketoacidosis (blood glucose: 470 mg/dl, pH: 7.08, HCO_3: 5 mmol/L). The antiviral treatment was withdrawn. Her clinical condition was improved with i.v. fluids and insulin therapy. Following normalization of the acute metabolic profile, intensive insulin therapy was recommended. The patient had no family history of diabetes mellitus. The HLA class Ⅱ typing revealed a genetic predisposition to IDDM as demonstrated by the presence of type 1 diabetes associated DRB1*03 (A*01, A*02/B*08, B*35/Cw*04, Cw*07, DRB1*03, DRB1*03/DQB1*02) haplotype (Diabetes, Pathogenesis of type 1A Diabetes In: http://www-endotext.com). Glutamic acid decarboxylase (GAD) antibodies were strongly elevated (725.52 RU/units) whereas insulin autoantibodies (IA-2) were undetectable at the onset of IDDM (< 5.3 RU/units). Additional immunological profile showed positive antinuclear antibodies (> 1:640). All the other auto-antibodies including anti-smooth muscle, anti-dsDNA, anti-ENA, anti-RNP, anti-SSA, anti-SSB, p-ANCA, c-ANCA, anti-MPO, anti-PR3, anti-LF, anti-mitochondrial were negative. The value of plasma C peptide with test glucagon was 0.61 ng/mL and 0.76 ng/mL at 0 and 6 min, respectively (normal values: 0.5-3.2 ng/mL), indicating an insulin secretion deficiency.

Twelve months after cessation of pegylated IFN-α and ribavirin therapy, the patient remained insulin dependent with a daily requirement of insulin 35 units (C-peptide levels remained low), but no medication was required for the thyroid. Last thyroid evaluation revealed a reversible condition with a decrease in the anti-TPO titers (109.5 IU/mL) and normal TSH. HCV RNA in serum was undetectable.

DISCUSSION

Classical IFN-α has been reported to induce insulin resistance[8-11] although there are also reports suggesting a beneficial effect on glucose metabolism[12-14]. However, the potential of classical IFN-α to induce IDDM has not been well established.

There have been a variety of mechanisms that may account for the effect of IFN-α on pancreatic beta cell dysfunction in patients with CHC. First, it has been reported that viral dsRNA activates the toll-like receptor-3 and the nuclear factor NFnB to induce pancreatic beta cell apoptosis and also the production of IFN-α, which is directly cytotoxic to beta cells of the pancreas. Second, IFN-α activates the oligoadenylate synthase-RnaseL pathway and the protein kinase R pathway thus inducing apoptosis of pancreatic beta cells[15]. Third, IFN-α may stimulate a counter regulatory hormone secretion (growth hormone, glucagon, etc.), thus resulting in impaired glucose tolerance[8]. Regarding IDDM, IFN-α may favour the development of Th1 immune reaction and thereby contribute to the development of autoimmune disease by the activation of CD4 lymphocytes secreting interleukin IL-2, IFN-gamma, and tumor necrosis factor[15]. IFN-α expression has also been associated with over-expression of MHC class Ⅰ antigens in human islets of pancreas[16].

Thirty five cases of IFN-α related IDDM had been reported[17-20] up to 2005. In 2003 Fabris *et al*[5] reviewed 31 cases of classical IFN-α related IDDM. A family history of IDDM was present in 3 cases and HLA haplotypes conferring susceptibility to IDDM were present in 89% of the reviewed cases. A time-period of 10 d to 4 years elapsed between the onset of treatment and the clinical development of IDDM. Fifty percent of the patients were positive for at least one pancreatic autoantibody before therapy. The rate increased to 77% during IFN-α treatment whereas 5 patients initially negative for pancreatic autoimmunity were seroconverted during therapy. Clinical manifestations included polyuria, polydipsia and weight loss in the vast majority of patients. Permanent insulin administration was required in 75% of the cases[5].

To date, the development of IDDM during pegylated IFN-α and ribavirin therapy for CHC was documented in only two cases in the English literature[21,22]. In the case reported by Jabr *et al*[21], pegylated IFN-α and ribavirin were administered in a patient with CHC and human immunodeficiency virus infection. Seven months following initiation of treatment, polyuria, generalized weakness, increased thirst and loss of appetite were manifested. Hyperosmolarity and ketoacidosis eventually developed. The patient required permanent insulin therapy thereafter. Pancreatic autoimmunity markers were not assessed. In the case presented by Cozzolongoa *et al*[22], IDDM developed following a 3-mo treatment with pegylated IFN-α-2b and ribavirin for CHC. An increase in the titers of islet-cell and glutamic acid decarboxylase antibodies before the start of therapy and 2 mo after the diagnosis of diabetes mellitus was documented. HLA class Ⅱ typing showed a predisposition to IDDM. The patient eventually required permanent insulin therapy.

Diabetic ketoacidosis was reported in a few classical IFN-α related cases[23-26], in one pegylated IFN-α related case[21] and the case herein described. The development of diabetic ketoacidosis and the permanent insulin dependency thereafter indicated a severe metabolic disturbance, which may be attributed to a rapidly developing Th1-mediated pathogenic reaction[23].

Co-existence of positive thyroid and pancreatic autoimmunity markers was documented in a few cases in the literature[23,27-29]. In the case presented by Bosi *et al*[23], clinical features of autoimmune hyperthyroidism and IDDM coexisted. In the current case the multiple autoimmune manifestations, ie. IDDM and Hashitoxicosis with highly elevated GAD and anti-TPO antibodies and additional autoimmunity markers, illustrated a vigorous triggering of the immune system by pegylated IFN-α in a genetically predisposed individual.

Gogas *et al*[30] have suggested a predictive model to identify patients with a predisposition to autoimmunity disease before the start of IFN-α therapy for melanoma. Prospective identification of the individual benefit/risk ratio would facilitate personalized treatment strategies

when IFN-α treatment is planned.

In conclusion, it seems that pegylated IFN-α shares common features with classical IFN-α as far as autoimmunity is concerned. A high clinical awareness is recommended in patients with known genetic susceptibility or positive autoimmunity markers prior to or during IFN-α therapy.

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CASE REPORT

Gallstone ileus: Report of two cases and review of the literature

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Abstract

Gallstone ileus is a rare disease and accounts for 1%-4% of all cases of mechanical intestinal obstruction. It usually occurs in the elderly with a female predominance and may result in a high mortality rate. Its diagnosis is difficult and early diagnosis could reduce the mortality. Surgery remains the mainstay of treatment. We report two cases of gallstone ileus. The first was a 78-year old woman who had a 2-d history of vomiting and epigastralgia. Plain abdominal film suggested small bowel obstruction clinically attributed to adhesions. Later on, gallstone ileus was diagnosed by abdominal computed tomography (CT) based on the presence of pneumobilia, bowel obstruction, and an ectopic stone within the jejunum. She underwent emergent laparotomy with a one-stage procedure of enterolithotomy, cholecystectomy and fistula repair. The second case was a 76-year old man with a 1-wk history of epigastralgia. Plain abdominal film showed two round calcified stones in the right upper quadrant. Fistulography confirmed the presence of a cholecystoduodenal fistula and gallstone ileus was also diagnosed by abdominal CT. We attempted to remove the stones endoscopically, but failed leading to an emergent laparotomy and the same one-stage procedure as for the first case. The postoperative courses of the two cases were uneventful. Inspired by these 2 cases we reviewed the literature on the cause, diagnosis and treatment of gallstone ileus.

Key words: Gallstone ileus; Intestinal obstruction; Pneumobilia; One-stage procedure; Enterolithotomy; Cholecystoduodenal fistula

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INTRODUCTION

The cause of mechanical small bowel intestinal obstruction includes gallstones, foreign bodies, bezoars, tumors, adhesions, congenital abnormity, intussusceptions, and volvulus[1]. Among these causes, a gallstone-induced intestinal obstruction is also referred to as a "gallstone ileus". Gallstone ileus is a rare and potentially serious complication of cholelithiasis[2]. It accounts for 1%-4% of all cases of mechanical intestinal obstruction, but for up to 25% of those in patients over 65 years of age with a female to male ratio of 3.5-6.0:1[3-6]. The morbidity and mortality rate of gallstone ileus remain very high, partly because of misdiagnosis and delayed diagnosis[7]. Thus, early diagnosis and prompt treatment could reduce the mortality rate. Here, we report two cases of gallstone ileus and review the literature of this rare disease.

CASE REPORTS

Case 1

A 78-year old woman presented to our emergency department (ED) with a 2-d history of vomiting and epigastric pain. She had a past medical history of hypertension and underwent a hystectomy for a myoma at the age of 38. On physical examination, her abdomen was mildly distended without tenderness or rebound or Murphy's sign. The white blood count was 16 630/μL with 87.6% neutrophils, blood urea nitrogen 67 mg/dL, 2.3 mg/dL creatinine 2.3 mg/dL and k 2.7 mmol/L. The other tests were unremarkable. A plain abdominal film demonstrated mildly dilated bowel loops, but a vague stone was not identified in the left iliac fossa (Figure 1). The diagnostic impression was ileus due to adhesions. Abdominal ultrasound (US) disclosed a shrunken gallbladder with internal air, but without stones. An abdominal computed tomography was performed, demonstrating air in the biliary tree (Figure 2A). Moreover,

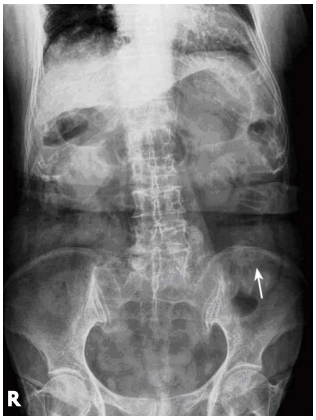

Figure 1 Plain abdominal film showing a vague stone not identified in the left iliac fossa (arrow).

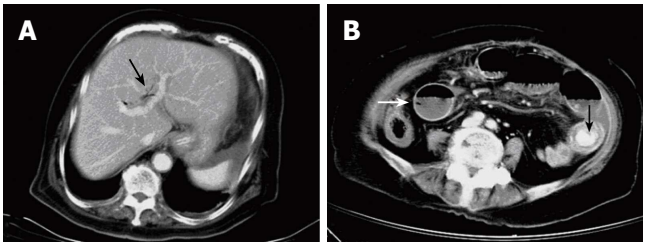

Figure 2 Abdominal computed tomography scan showing air in the biliary tree (arrow) (**A**) and an ectopic stone (arrow) impacting the jejunal lumen accompanying dilatation of proximal small bowel loops (white arrow) (**B**).

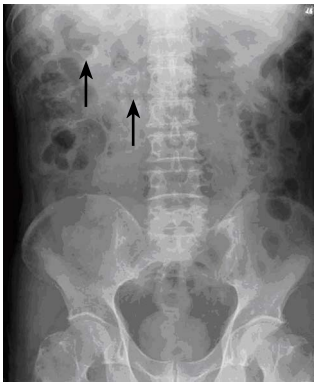

Figure 3 Plain abdominal film showing two round calcified stones in the right upper quadrant of abdomen (arrow).

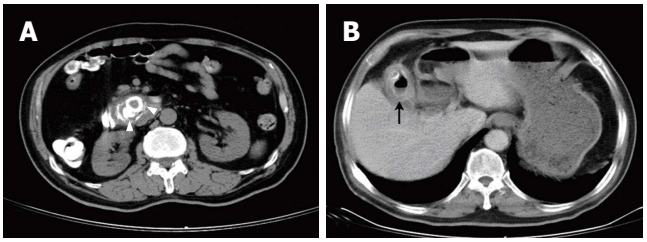

Figure 4 Abdominal computed tomography scan showing two round calcified stones impacting the proximal third portion of the duodenum (arrowhead) (**A**) and a thickened wall gallbladder with internal air and small calcified content (arrow) (**B**).

an ectopic stone impacted in the jejunal lumen was seen in the left lower quadrant along with dilatation of the proximal small bowel (Figure 2B). The diagnosis of gallstone ileus was made and she underwent an emergent laparotomy. At operation a small shrunken gallbladder with dense adhesion to the stomach was found. After lysis of adhesion, a fistula between the gallbladder dome and the lesser curvature of gastric antrum was confirmed. Moreover, a stone was palpated in the jejunum about 140 cm distal to the ligament of Treitz causing total obstruction of the lumen. When the jejunum was opened, the stone was laminated and cylinder shaped, measuring 3 cm × 3 cm × 2 cm in diameter. Enterolithotomy, cholecystectomy and repair of the cholecystogastric fistula were performed. The pathology showed chronic cholecystitis and a fistula walled by granulation tissue. The postoperative course was uneventful except for mild wound infection, and she remained in good health after one-year follow-up.

Case 2

A 76-year old man was brought to our ED because of epigastric pain for one week. He denied any history of abdominal surgery or biliary disease. On physical examination his abdomen was soft, but tender in the epigastric area. Laboratory tests were unremarkable. Abdominal X-ray showed two round calcified stones in the right upper quadrant of the abdomen (Figure 3). An EGD disclosed a deep hole in the posterior wall of the duodenal bulb, but no stone was identified. Fistulography confirmed the presence of a cholecystoduodenal fistula. An abdominal CT was performed, showing two round calcified stones located in the proximal third portion of the duodenum causing dilatation of proximal bowel lumen (Figure 4A). There was a thickened wall gallbladder with internal gas and small calcified content was also discovered (Figure 4B). Duodenoscopy was performed again, showing two stones impacted in the proximal third portion of the duodenum. We attempted to catch the stones with a Dormia basket, but failed because of their larger size. Therefore, he was referred to our department of surgery for emergent laparotomy. Laparotomy displayed that the gallbladder was adhered to the duodenum accompanying a cholecystoduodenal fistula. A large black stone, measuring 5 cm × 4 cm × 3 cm in diameter, impacted the proximal third portion of the duodenum. No other stones were found in the gastrointestinal (GI) tract. Enterolithotomy, cholecystectomy, duodenorrhaphy, feeding jejunostomy, and repair of the cholecystoduodenal fistula were performed. Microscopically, the gallbladder showed cholelithiasis with acute and chronic inflammation and a fistula walled by fibrous and granulation tissue. The postoperative course was complicated by a wound infection, and he was discharged 2 mo later.

DISCUSSION

Gallstone is a common disease with a prevalence in 10% of the adult population in the United States[8]. However, it is only symptomatic in 20%-30% of patients, with biliary colic being the most common symptom[9]. The most common complications of gallstone disease include acute cholecystitis, acute pancreatitis, choledocholithiasis with or without cholangitis, and a gangrenous gallbladder. Other uncommon complications include Mirizzi syndrome,

cholecystocholedochal fistula, and gallstone ileus[2,10].

The term "gallstone ileus" was first coined by Bartolin in 1654 and referred to the mechanical intestinal obstruction due to impaction of one or more large gallstones within the GI tract. Biliary-enteric fistula is the major pathologic mechanism of gallstone ileus[11]. The gallstone enters the GI tract through a fistula between a gangrenous gallbladder and the GI tract. Occasionally a stone may enter the intestine through a fistulous communication between the common bile duct and the GI tract. Although the gallstone can impact anywhere in the GI tract, its size should be at least 2 cm to 2.5 cm in diameter to cause obstruction[6]. Reisner and Cohen[5] reviewed 1001 cases of gallstone ileus and reported that the most common locations of impaction of gallstone are the terminal ileum and the ileocecal valve because of the anatomical small diameter and less active peristalsis. They also found that the less common locations for impaction are the jejunum, the ligament of Treitz, and the stomach, while the duodenum and colon are the rare locations for impaction[5].

The clinical manifestations of gallstone ileus are variable and usually depend on the site of obstruction. The onset may be manifested as acute, intermittent or chronic episodes[12]. The most common symptoms include nausea, vomiting and epigastric pain. Moreover, a small portion of patients may present with hematemesis secondary to duodenal erosions. Laboratory studies may show an obstructive pattern with elevated values of bilirubin and alkaline phosphophatase.

The diagnosis of gallstone ileus is difficult, usually depending on the radiographic findings. In 50% of cases the diagnosis is often only made at laparotomy[5]. The classic Rigler's triad of radiography includes mechanical bowel obstruction, pneumobilia, and an ectopic gallstone within bowel lumen. However, air in the gallbladder is also a frequent finding in gallstone ileus[13]. Plain abdominal films usually show non-specific findings because only 10% of gallstones are sufficiently calcified to be visualized radiographically. For example, in our first case, although the ectopic gallstone appeared on the initial plain abdominal film, it was misdiagnosed as fecal material due to insufficient calcification. But in our second case, two gallstones were definitely identified on a plain abdominal film. Upper or lower GI barium studies occasionally identify the site of obstruction or fistula. Abdominal US is useful to confirm the presence of cholelithiasis and may identify fistula, if present[14]. Abdominal CT becomes the more important modality in diagnosing gallstone ileus because of its better resolution. By comparing with plain abdominal film and abdominal US, it can provide a more rapid and specific diagnosis in emergency use. Lassandro *et al*[15] compared the clinical value of plain abdominal film, abdominal US and abdominal CT in diagnosing 27 cases of gallstone ileus, and found that the Rigler's triad presents 14.81% in plain abdominal film, 11.11% in abdominal US, and 77.78% in abdominal CT, respectively. Additionally, Yu *et al*[16] studied the value of abdominal CT in the diagnosis and management of gallstone ileus and concluded that the abdominal CT offers crucial evidence not only for the diagnosis of gallstone ileus but also for decision making in management strategy[16]. Rarely, laparoscopy is used to diagnose this disease[17].

Gallstone ileus usually requires emergent surgery to relieve intestinal obstruction. Bowel resection is only indicated when there is intestinal perforation or ischemia[18]. There is no uniform surgical procedure for this disease because of its low incidence. Although enterolithotomy alone remains the popular operative method in most reports, the one-stage procedure composed of enterolithotomy, cholecystectomy and repair of fistula is necessary, if indicated[19]. Tan *et al*[20] compared the two surgical strategies of enterolithotomy alone and enterolithotomy with cholecystectomy for the emergent treatment of gallstone ileus, and concluded that both procedures are safe with no mortality, but the better surgical option is enterolithotomy. Doko *et al*[21] agreed that the one-stage procedure should be reserved only for highly selected patients with absolute indications. Recently, laparoscopy-guided enterolithotomy has become the preferred surgical approach in treating gallstone ileus[22]. Additionally, the non-surgical treatment of gallstone ileus has been suggested, including endoscopic removal and shockwave lithotripsy, but this depends on the location of obstruction[23,24]. In our second case, the two stones were detected by radiography preoperatively, but they were not found on the first EGD. The second duodenoscopy revealed two stones impacting the third portion of the duodenum. We attempted to remove them endoscopically, but failed due to their larger size.

The prognosis of gallstone ileus is usually poor and worsens with age. Previous studies reported that the mortality rate is 7.5%-15%[5,6], largely due to delayed diagnosis and concomitant conditions such as cardiorespiratory disease, obesity and diabetes mellitus. The postoperative recurrence rate of gallstone ileus is 4.7% and only 10% of patients require secondary biliary surgery for recurrent biliary symptoms[5,25].

In conclusion, gallstone ileus is a rare cause of intestinal obstruction. It must be considered in intestinal obstruction patients with a past history of gallstone, especially in elderly females. Abdominal CT is the preferred modality because of its rapid diagnosis of gallstone ileus. Surgical treatment is emergent when the radiological finding is highly or even suspicious or confirmed.

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CASE REPORT

Primary intestinal malignant fibrous histiocytoma: Two case reports

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Abstract

Malignant fibrous histiocytoma (MFH) occurs most commonly in the extremities and trunk, but rarely in the intestine. Here we report two cases of primary intestinal MFH. The first case was a 70-year old man admitted for recurrent right lower quadrant abdominal pain. At laparotomy, a tumor was found originating from the cecum, with a suspicious metastatic nodule on the surface of the right lobe of the liver. A right hemicolectomy was performed followed by an ileotransverse end-to-end anastomotic reconstruction. The second case was a 43-year old man with intussusceptions of the small intestine. An emergent laparotomy revealed 4 pedunculated masses in the small bowel and a partial resection of the small intestine was performed. Though the symptoms were not typical, based on histological and immunohistochemical studies, the patients were diagnosed as MFH of the intestine. They were not treated with chemotherapy or radiotherapy and both died within 3 mo. MFH of the intestine is an extremely rare neoplasm with an aggressive biological behavior. The pathogenesis of this disease has not been clarified to date. Complete surgical excision is preferred, adjuvant chemotherapy or radiotherapy may be advisable.

Key words: Malignant fibrous histiocytoma; Intestinal neoplasms; Abdominal pain

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INTRODUCTION

Malignant fibrous histiocytoma (MFH) is a common soft tissue sarcoma, usually occurring in the extremities. Primary intestinal MFH is a very rare neoplasm, with not more than 40 cases reported in international literatures[1-5]. Here we report two cases of primary intestinal MFH and review other reported cases.

CASE REPORTS

Case 1

A 70-year old man was admitted for recurrent right lower quadrant abdominal pain for about 50 d. He had no recent history of fever, vomiting, diarrhea, or constipation. Physical examination revealed a slightly tender fist-size mass in the right lower quadrant of the abdomen. Laboratory examination showed a white blood cell count of 5.02×10^9/L, and a neutrophil count of 68.3%. Abdominal ultrasonography revealed a large hypoechoic mass in the right lower quadrant and an abdominal computed tomography demonstrated a large soft-tissue mass in the right lumbar region and iliac fossa (Figure 1A). Chest X-ray and computed tomography showed a mass on the hilum of the right lung (Figure 1B). Barium enema and colonofiberscopy were not performed owing to the patient's abdominal pain. At laparotomy, a tumor was found originating from the cecum and infiltrating into the right lateral peritoneum. There was a suspicious metastatic nodule located on the surface of the right lobe of the liver. A right hemicolectomy was carried out, and reconstruction was performed by an ileotransverse end-to-end anastomosis. Grossly, an ulcerative annular tumor undergone necrosis was seen in the cecum, measuring 12 cm × 10 cm. There was a tumor nodule on the mesentery, measuring 3 cm × 2 cm. On cut section, the tumor was soft in consistency, solid and grayish-white in color. Microscopic examination revealed that the tumor was pleomorphic and consisted of spindle-shaped cells, oval cells, pleomorphic giant cells, and inflammatory cells. There were involvements of two lymph nodes. Immunohistochemical stains were positive for vimentin (Figure 2), KP-1, α 1-antitrypsin, LYS, P53, Ki67, and

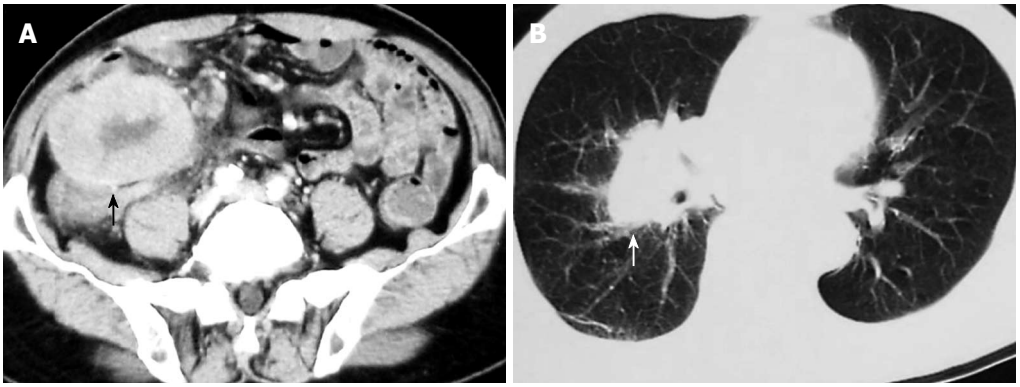

Figure 1 Computed tomography showing a large soft-tissue mass (black arrow) in the right lumbar region and iliac fossa (**A**) and a mass (white arrow) on the hilum of the right lung (**B**).

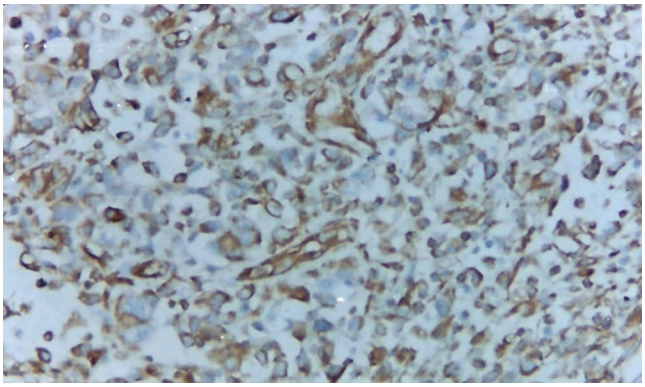

Figure 2 Tumor cells showing positive staining for vimentin (EnVision × 200).

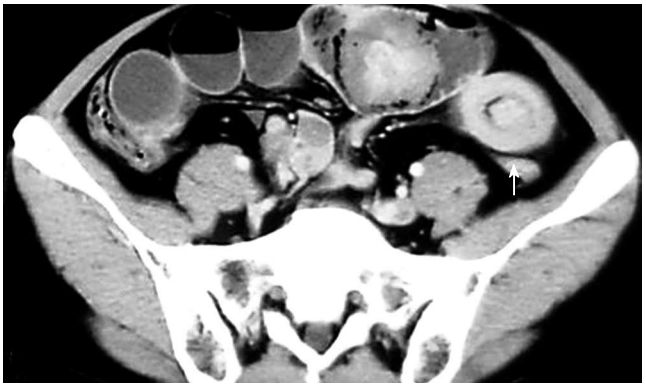

Figure 3 Computed tomography showing features of multiple intussusceptions in the small intestine (white arrow).

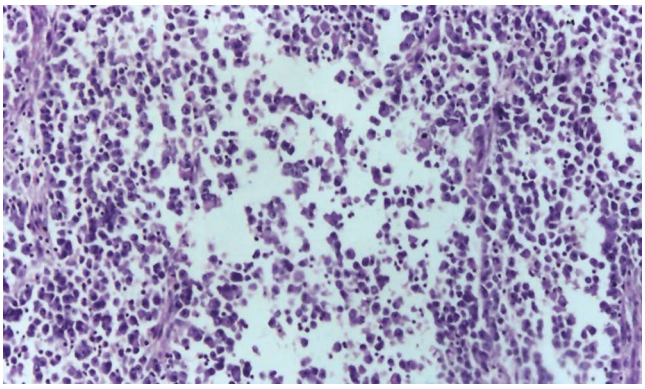

Figure 4 Histology revealing tumor cells consisting of fibroblast-like cells, histiocyte-like cells and pleomorphic giant cells (HE, × 40).

negative for S-100, CD1a, CD34, CD117, HMB45, CK, and SMA. The final histopathological diagnosis was MFH of the cecum. Postoperatively, the patient developed pyrexia for about one week which was subsequently followed by breathlessness as a result of lung metastasis. He was not treated with chemotherapy or radiotherapy, and died a month later.

Case 2

A 43-year old man with a history of melena for 10 d had intermittent abdominal pain, fever, vomiting and diarrhea. Physical examination revealed a slightly distended tender abdomen. Laboratory findings showed a white blood cell count of 23.39 × 10^9/L, and an elevated C-reactive protein level of 193.0 mg/L. Plain abdominal X-ray films showed small bowel obstruction. Computed tomography demonstrated features of multiple intussusceptions, i.e. some portions of the small intestine were seen invaginating into contiguous segments (Figure 3). Chest X-ray and computed tomography showed a mass on the apex of the right lung. The diagnosis of intussusceptions of the small intestine was made, and an emergency operation was performed. At laparotomy, 4 masses measuring 8, 5, 5, 3 cm in diameter were seen in the small bowel and local partial resections of the small intestine were performed. The resected specimens contained pedunculated tumors. Some metastatic nodules were also noted on the mesentery. Microscopic examination demonstrated fibroblast-like cells, histiocyte-like cells and pleomorphic giant cells within the tumor substance (Figure 4). Immunohistochemical stains were positive for vimentin, Ki67 (Figure 5A), P53 (Figure 5B), CK, KP-1 (Figure 5C), LYS, and negative for ER, PR, c-erbB2, MMP2, MMP9, S-100 and HMB45. The pathological findings were compatible with MFH of the small intestine. Postoperatively, the patient had high fever, vomiting and abdominal pain. White blood cell count varied from 26.64 × 10^9/L to 55.5 × 10^9/L, and plain abdominal X-ray films demonstrated partial bowel obstruction. The patient was not treated with chemotherapy or radiotherapy, and died 2 mo later.

DISCUSSION

Malignant fibrous histiocytoma (MFH) was first described

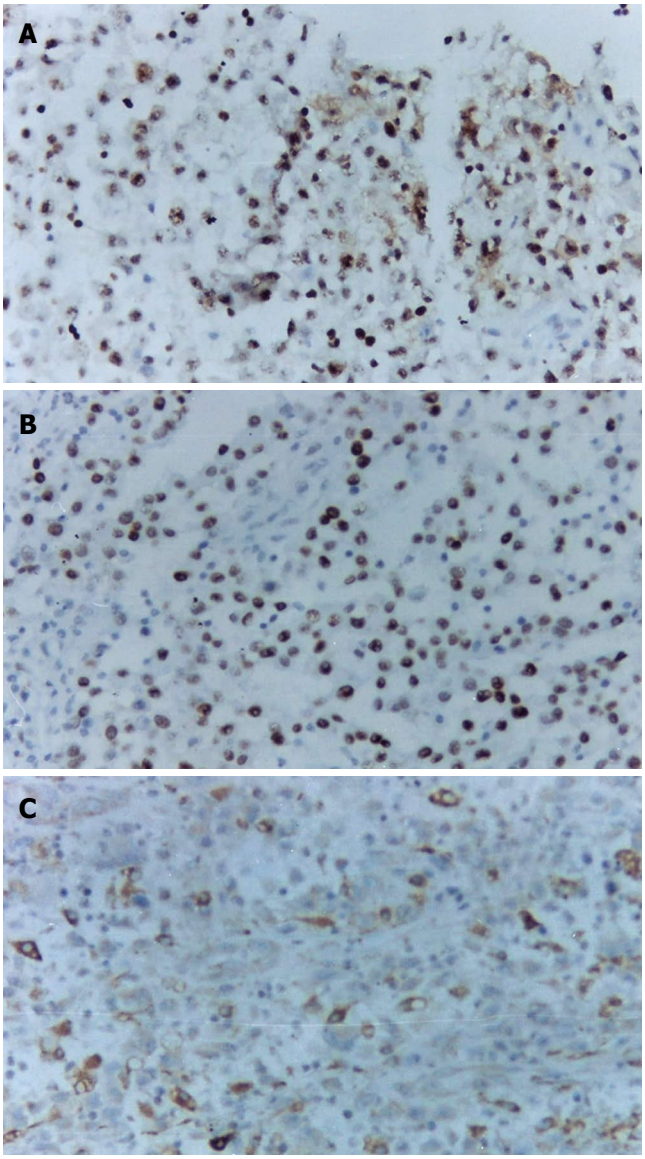

Figure 5 Tumor cells showing positive staining for Ki67 (**A**), P53 (**B**), and KP-1 (**C**) (EnVision × 200).

as malignant histiocytoma and fibrous xanthoma by Ozello *et al* in 1963, and was established by O'Brien and Stout in 1964 to describe soft tissue sarcomas arising from fibroblasts and histiocytes[3,5]. The peak incidence of extraintestinal MFH occurs in the sixth and seventh decades of life. It usually occurs in the extremities, presenting as a painless mass, and less commonly in the retroperitoneal space, associated with weight loss and increased intra-abdominal pressure[6]. Five histological subtypes of MFH have been described: pleomorphic storiform, myxoid, giant cell, inflammatory, and angiomatoid; the most common being pleomorphic storiform and myxoid types. The first two subtypes tend to be high-grade neoplasms, while the others are usually low-grade sarcomas[7]. The karyotypic abnormalities in MFH are usually complex, with multiple numerical and structural rearrangements[8]. Schmidt[9] reported that chromosomes 1, 3, 6, 9, 12, 16, 18, and 20 are involved in structural aberrations, and that the breakpoint regions are most frequently observed in 1p32, 3p25, and in the centromeric region of chromosomes 1 and 16. The pathogenesis of MFH has not been clarified to date. However, it has been recognized as a complication of radiation, resulting from chronic postoperative repair, trauma, surgical incisions or burn scars[10-13].

Primary MFH of the intestine is very rare. Not more than 40 cases have been reported in international literatures[1-4]. We reviewed the total 37 cases of intestinal MFH, including ours, and summarized the findings as follow. The ratio of men to women was 2.4:1, with an average age of 54.2 years and a range of 12-84 years. The tumor originated from the duodenum in 2 patients, the jejunum in 5, the ileum in 6, the jejunum and the ileum in 1, the cecum in 4, the ascending colon in 6, the transverse colon in 5, the descending colon in 2, the sigmoid colon in 2, the rectum in 3, and from the anal canal in 1. In most patients, the tumor was solitary, while in 4 cases multiple tumors were observed. The distribution of the colorectal MFH was different from that of carcinoma. Despite the fact that most of the tumors were large, ranging from 2 to 19 cm in diameter, their early detection was difficult. The symptoms were not typical: most patients complained of abdominal pain, fever, anorexia, bloody stools and diarrhea, but few had constipation. Thirty-five of the patients were treated surgically, 3 received adjuvant chemotherapy, one was given radiotherapy, and one underwent chemoradiation. In some patients, initial laparatomy revealed metastatic masses, especially in the regional lymph nodes, liver and lungs. Local recurrence was the most common pattern of recurrence in MFH of the intestine. Several patients died of local recurrence or metastasis including our reported two cases.

The diagnosis of MFH depends on an accurate differential diagnosis from other sarcomas, observation of karyomorphism and differential figures, and positive results on immunohistological staining. It was reported that MFH frequently expresses vimentin, actin, CD-68, and α 1-antitrypsin and α 1-antichymotrypsin[14]. Thus, the final diagnosis of our patients was MFH. The differential diagnosis of MFH should include pleomorphic liposarcoma and rhabdomyosarcoma. The former lacks the storiform pattern and shows evidence of cellular differentiation, while the latter shows cross striations on histological examination.

Murata[15] reported that colorectal MFH of the inflammatory type produces granulocyte colony-stimulating factor (G-CSF). Liesveld[16] reported that patients with MFH have leukocytosis, leukemoid reaction, and paraneoplastic syndrome because of various cytokines produced by tumor cells. Thus, postoperative recurrent leukocytosis and elevated CRP level might be predictors for recurrence of MFH. The second case presented to our center had leukocytosis and leukemoid reaction postoperatively.

The treatment for MFH is early and complete surgical excision with en-bloc regional lymph node dissection. Postoperatively, our patients were not given chemotherapy due to their poor general condition. Chemotherapy and radiotherapy were used, but without success. Zagars[17] reported that adjuvant chemotherapy cannot minimize the rate of metastasis. Patients with myxoid tumors do

not require systemic therapy. However, patients with nonmyxoid disease exceeding 5 cm are at a significant risk of developing metastases and the development of effective adjuvant treatment is an important research goal. Another report stated that inadequate peritumoral excision is a cause for recurrence[18].

Most of the reports suggest that the prognosis associated with colonic MFH is poor. Weiss and Einzinger's analysis of MFH[6] showed that the 2-year survival rate of patients with pleomorphic/storiform type of MFH is 60% and the rate of metastases is 42%. Tumor site (extremity *vs* nonextremity), location (proximal *vs* distal), size ($\leqslant$ 5 cm *vs* > 5 cm), and histology (myxoid *vs* nonmyxoid) are not significant determinants for final outcome [17]. For metastatic relapse, the major determinants were histology (myxoid *vs* nonmyxoid) and tumor size. Myxoid tumors have a low metastatic propensity (13% 10-year metastatic rate) compared to nonmyxoid tumors (40% 10-year metastatic rate).

In conclusion, primary intestinal MFH is an extremely rare neoplasm with an aggressive biological behavior. Clinicians and pathologists must consider the possibility of this tumor and include it in the differential diagnoses of intestinal lesions. Complete surgical excision is preferred and adjuvant chemotherapy or radiotherapy may be advisable. Due to the high recurrence, life-long surveillance should be carried out.

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CASE REPORT

Eosinophilic gastroenteritis with ascites and hepatic dysfunction

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Abstract

Eosinophilic gastroenteritis is a rare gastrointestinal disorder with eosinophilic infiltration of the gastrointestinal wall and various gastrointestinal dysfunctions. Diagnosis requires a high index of suspicion and exclusion of various disorders that are associated with peripheral eosinophilia. We report a case of eosinophilic gastroenteritis, which had features of the predominant subserosal type presenting with ascites and hepatic dysfunction, and which responded to a course of low-dose steroid.

Key words: Ascites; Eosinophilia; Gastroenteritis

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INTRODUCTION

Eosinophilic gastroenteritis, first reported by Kaijser in 1937, is a rare gastrointestinal disorder that can present with various gastrointestinal manifestations, depending on the specific site of the affected gastrointestinal wall. Klein *et al*[1] have demonstrated that this disorder could be pathologically classified into three major types: predominant mucosal layer, predominant muscle layer, and predominant subserosal layer. However, its clinical features, etiology, and treatment have not yet been definitely established. We report a case of eosinophilic gastroenteritis with features of the predominant subserosal type, including ascites and liver dysfunction.

CASE REPORT

In July 2006, a 33-year-old man presented with mild upper abdominal pain and, three days later, with abdominal distention, frequency of bowel motion and fever. His temperature was 38.5℃. He had no past history of ulcer pain and never had any abdominal operation. There had been no weight loss and neither blood nor mucus was present in the stool. The patient denied taking any drugs or herbal medicine. There was a history of allergy to levofloxacin. Chest examination showed no abnormality. Abdominal examination revealed a soft, mildly tender abdomen, and shifting abdominal dullness.

The patient was admitted to the Second Affiliated Hospital of Zhejiang University in July 2006. The hemoglobin level was 13.0 g/L(normal range, male, 11-16 g/L) and white cell count was 13.6 × 10^9/L, with 41.1% neutrophils, 27.9% lymphocytes, 8% monocytes and 22.5% eosinophils. The platelet count was 138 × 10^9/L, and the sedimentation rate was 8 mm/h. The tests for antinuclear factor, rheumatoid factor, serum hepatitis B surface antigen and serum hepatitis A, C, E antibodies gave negative results. The alanine aminotransferase level was 337 U/L (normal range, 0-50 U/L) and the aspartate aminotransferase was 223 U/L (normal range, 0-40 U/L). The anti-amoebic titer was negative and stool microscopy for ova and cysts was negative. Ultrasonography of the abdomen revealed a moderate amount of ascites (Figure 1). Gastroscopy showed mild antral gastritis. Biopsy of gastric antrum and proximal duodenum revealed inflammation with marked eosinophilic infiltration (Figure 2 and Figure 3). Colonoscopy showed mild proctitis and sigmoid colitis.

After excluding the possibilities of a malignancy, parasitic disease, and autoimmune disease, eosinophilic gastroenteritis was diagnosed. This diagnosis was supported by the presence of peripheral eosinophilia and eosinophilic infiltration of the serosa and subserosa of the gastric antrum and proximal duodenum. A small daily dose of steroid (prednisolone 20 mg) was given. Four days later, the patient recovered from his symptoms. The white cell count was 9.5 × 10^9/L, with 49.1% neutrophils, 27.2% lymphocytes, 7.7% monocytes and 15% eosinophils. Seven days later, the alanine aminotransferase level was 128 U/L and the aspartate aminotransferase was 38 U/L. Ultrasonography of the abdomen revealed no ascites. Steroid treatment was gradually tapered and eventually terminated after 4 wk.

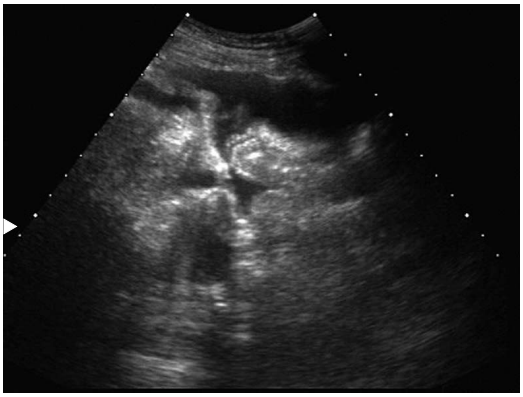

Figure 1 Ultrasonography of the abdomen revealed a moderate amount of ascites.

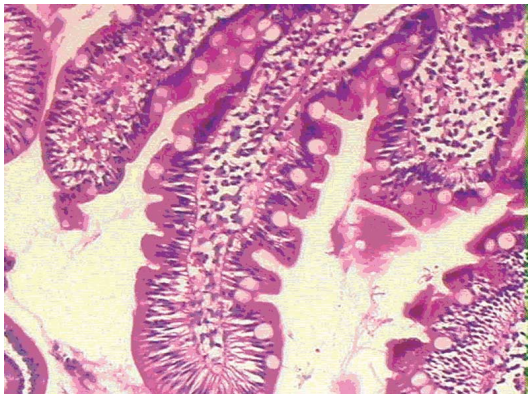

Figure 2 Histologic section of proximal duodenum showed inflammation with marked eosinophilic infiltration (HE, × 100).

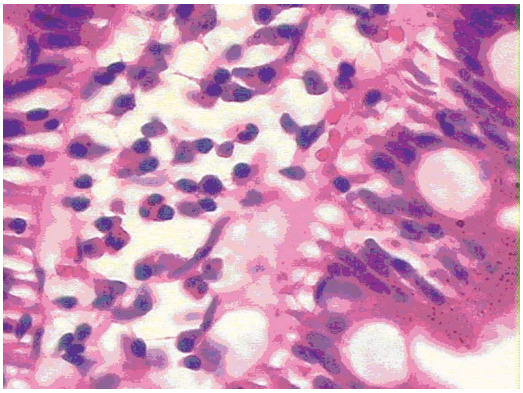

Figure 3 Histologic section of proximal duodenum showed inflammation with marked eosinophilic infiltration (HE, × 400).

DISCUSSION

The important feature in this case of eosinophilic gastroenteritis is the extremely high eosinophil count in the peripheral blood and the serosa and subserosa of the gastric antrum and proximal duodenum. The diagnosis of eosinophilic gastroenteritis was confirmed after the exclusion of other disorders that have similar features, such as gut lymphoma, parasitic infection, carcinoma, inflammatory bowel disease, and allergy.

Eosinophilic gastroenteritis is a rare disease, which was first reported by Kaijser in 1937. In 1970, Klein demonstrated that this disorder could be pathologically classified into three major types: predominant mucosal, predominant muscle layer, and predominant subserosal. The involvement of different layers usually gives rise to different clinical manifestations. Mucosal disease generally presents with bleeding, protein-losing enteropathy, or malabsorption. Involvement of the muscle layer may cause bowel wall thickening and subsequent intestinal obstruction. The subserosal form usually presents with peritonitis and eosinophilic ascites, which was the case in this patient.

Eosinophilic gastroenteritis can involve any part of the gastrointestinal tract from the esophagus down to the rectum. The stomach and duodenum, however, are the most common sites of involvement[2-5]. The etiology of eosinophilic gastroenteritis is currently unknown. Allergies to certain foods or immunological abnormalities have been speculated as its etiology[6-8]. On the other hand, some investigators[9,10]have demonstrated that the etiology of this disorder is not associated with allergic reactions. In our case, there was a past history of levofloxacin allergy. However, the patient denied taking any drugs or herbal medicine. The finding of high eosinophil count in the peripheral blood suggested allergic disorder or parasitic disorders, or abdominal lymphoma. The likelihood of parasitic disorders and abdominal lymphoma was remote because of the history of the patient's illness, the physical findings, and the findings on gastroscopy, colonoscopy and ultrasonography.

In the mucosal form of eosinophilic gastroenteritis, multiple biopsies must be taken during endoscopy, because mucosal involvement is often patchy in nature. When there is a submucosal disease, biopsy of the mucosal layer sometimes fails to expose eosinophilic gastroenteritis. Laparotomy or laparoscopy is often required to make a diagnosis in such cases.

Treatment with a steroid is the mainstay in the management of eosinophilic gastroenteritis. Clinical improvement usually is seen after treatment with a low dose of steroid. Surgical intervention may sometimes be required for patients with complicating obstruction or when performing a full thickness intestinal biopsy to establish the diagnosis.

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LETTERS TO THE EDITOR

What caused the increase of autoimmune and allergic diseases: A decreased or an increased exposure to luminal microbial components?

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TO THE EDITOR

The dramatic increase of allergic and autoimmune diseases such as asthma, atopic dermatitis (eczema), allergic rhinitis, inflammatory bowel disease (IBD, including both Crohn's disease and ulcerative colitis), multiple sclerosis, and insulin-dependent diabetes mellitus (type I diabetes) in the developed countries in the last century[1-3] is a big puzle. "Hygiene Hypothesis" was proposed more than two decades ago and it suggested that the increase in these allergic and autoimmune diseases is caused by the aberrant development and response of the immune system due to a reduced exposure to microorganisms along with the improved hygiene[2-4]. Interestingly, recent studies revealed that these allergic and autoimmune diseases are closely related to the microbes in the gut[5]. For instance, even asthma, an allergic reaction of the lung to inhaled antigens, is closely related to a reduced exposure to foodborne and orofaecal microbes, rather than the amount of allergens in the air or the exposure to airborne microbes[5-7]. It is known that bacteria in the gut could be 10 times in number of the eukaryotic cells of the body[8]. Therefore, it would be not too surprising that microbes in the gut may have a great impact on these autoimmune and allergic diseases.

On the other hand, many studies revealed that an increase in intestinal permeability was shown in patients with these autoimmune and allergic diseases, such as those with IBD[9,10], multiple sclerosis[11], type I [12,13] (but not type Ⅱ[14]) diabetes, asthma[15,16], and atopic eczema[17,18]. The increased intestinal permeability in these patients seems to be a prerequisite rather than a consequence of these diseases, as it could precede the clinical onset of these diseases[12,19,20]. In addition, increased intestinal permeability was also seen not only in healthy relatives[10,21], but also in spouses of these patients[10,22]. An increase in intestinal permeability would result in an increased infiltration of the luminal components. Therefore, it would be reasonable to ask what have caused the increase of these autoimmune and allergic diseases: is it a deceased exposure to luminal microbial components due to the improved hygiene, or an increased exposure to luminal microbial components due to the increased intestinal permeability?

There is a large amount of microbes in the gut. The endotoxin in gut bacteria could be enough to kill the host thousands of times over[23]. Therefore, it would be more likely that the tightness of the gut barrier would have played a more important role in determining the extent of exposure rather than the absolute number of microbes in the gut. Actually, there could be an intrinsic link between the increased intestinal permeability and improved hygiene. An effective inactivation of the digestive proteases depends on gut bacteria[24,25]. The increased intestinal permeability could be a result of the improved hygiene and a reduction in gut bacteria, by a mechanism such as the impaired inactivation of digestive proteases by gut bacteria, and thus an over-digestion of gut barrier. A reduction in gut bacteria not only can be the result of improved sanitary conditions, along with the more clean food and water, it also can be caused by an inhibition of gut bacteria by factors such as the widely used sweetener saccharin[26]. Interestingly, saccharin consumption correlated well with the ups and downs of the IBD, and probably had played a causative role in IBD[27,28]. This would lead us to raise a more general question: Will the improved hygiene and thus a reduction in gut bacteria necessarily result in a decreased exposure to luminal microbial components, the fundamental assumption of the "Hygiene Hypothesis"[29,30]. Further study in this area would be worthwhile. It would help better understand why both Th1 type diseases such as Crohn's diseases and type I diabetes, and Th2 type diseases such as ulcerative colitis and atopic diseases increase with the improved hygiene[29,30].

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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Books

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Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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