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*EDITORIAL*

# Chronic intestinal pseudo-obstruction

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## Abstract

Chronic intestinal pseudo-obstruction (CIPO) is a severe digestive syndrome characterized by derangement of gut propulsive motility which resembles mechanical obstruction, in the absence of any obstructive process. Although uncommon in clinical practice, this syndrome represents one of the main causes of intestinal failure and is characterized by high morbidity and mortality. It may be idiopathic or secondary to a variety of diseases. Most cases are sporadic, even though familial forms with either dominant or recessive autosomal inheritance have been described. Based on histological features intestinal pseudo-obstruction can be classified into three main categories: neuropathies, mesenchymopathies, and myopathies, according on the predominant involvement of enteric neurones, interstitial cells of Cajal or smooth muscle cells, respectively. Treatment of intestinal pseudo-obstruction involves nutritional, pharmacological and surgical therapies, but it is often unsatisfactory and the long-term outcome is generally poor in the majority of cases.



**Key words:** Chronic intestinal pseudo-obstruction; Small bowel manometry; Immunohistochemistry; Prokinetics; Intestinal transplantation

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## INTRODUCTION

Chronic intestinal pseudo-obstruction (CIPO) is a rare, severe disease characterized by the failure of the intestinal tract to propel its contents which results in a clinical picture mimicking mechanical obstruction in the absence of any lesion occluding the gut. CIPO is one of the most important causes of chronic intestinal failure both in pediatric (15%) and adult cases (20%)[1-5], since affected individuals are often unable to maintain normal body weight and/or normal oral nutrition. The severity of clinical picture, generally characterized by disabling digestive symptoms even between sub-occlusive episodes, contributes to deterioration of quality of life of the patients. Furthermore, CIPO often passes unrecognized for long time, so that patients almost invariably undergo repeated, useless and potentially dangerous surgical procedures.

This article is aimed at reviewing the current knowledge on pathophysiology, clinical features and management of patients affected by CIPO.

## ETIOLOGY AND PATHOPHYSIOLOGY

CIPO is idiopathic in the majority of cases. In our experience organic, systemic or metabolic causes of the disease were identified in only 4 patients of 77 CIPO patients consecutively referred in our laboratory (5%)[2]. Nevertheless, it is mandatory to investigate affected individuals by traditional diagnostic procedures (radiology, endoscopy, lab tests, *etc*) in order to exclude every possible cause of secondary CIPO. The main secondary causes of CIPO are specified in Table 1.

In fact, every disease that affects one of the control mechanisms of intestinal functioning, including intrinsic and extrinsic neural supplies as well as muscle cells, can be responsible for secondary and potentially curable forms of CIPO. The extrinsic autonomic nervous system can be affected both centrally (i.e. Parkinson syndrome, Shy-Drager syndrome, stroke, encephalitis, neoplasm and any

**Table 1 Main causes of secondary chronic idiopathic pseudo-obstruction and relative gut tissue that is predominantly involved**

| Underlying disease | Main causes |
|---|---|
| Diseases of central autonomic and enteric nervous systems | Stroke, encephalitis, calcification of basal ganglia, orthostatic hypotension, Von Recklinghausen, Hirschsprung |
| Immune-mediated and collagen diseases | Paraneoplastic (CNS neoplasms, lung microstoma, bronchial carcinoid, leyomyosarcomas), scleroderma, dermatomyositis, amyloidosis, Ehlers-Danlos, LES |
| Endocrine and metabolic diseases | Diabetes, hypothyroidism, hypoparathyroidism, phaeochromocytoma |
| Other | Iatrogenic (radiation enteritis, clonidine, phenothiazines, antidepressants, antiparkinsonians, antineoplastics, bronchodilators, anthraquinones) jejunal diverticulosis, chagas |

other disease that could affect the encephalic autonomous centres), and peripherally (i.e. diabetic neuropathy, or other neuropathies potentially involving the enteric nervous system including Hirschsprung, Chagas, Von Recklinghausen, as well as non-specific diseases, like paraneoplastic syndromes, autoimmune diseases, viral infections). Enteric smooth muscle cells can be markedly damaged in patients affected by myotonic dystrophy or progressive systemic sclerosis. Collagenosis, Ehlers-Danlos syndrome, jejunal diverticulosis and radiation enteritis can be responsible for both a neuronal and myogenic impairment.

Nonetheless, diseases like hypothyroidism, hypoparathyroidism, and celiac disease have been described to be responsible for some cases of secondary CIPO, even if the underlying mechanism remains undetermined[1,4].

CIIPO is generally sporadic, but familial forms have also been described both with autosomal dominant, autosomal recessive and X-linked transmission[1,6,7]. Some genes and loci have been identified in syndromic forms of CIIPO, including the transcription factor *SOX10* on chromosome 22 (22p12), the DNA polymerase gamma gene (*POLG*) on chromosome 21 (21q17) and a locus on chromosome 8[7-9]. In terms of X-linked transmission, recently Gargiulo *et al* have identified a 2-base pair deletion in exon 2 of the *filamin A* gene (encoding for a large cytoskeletal protein involved in the modulation of the cellular response to chemical and mechanical environmental factors) that is present at the heterozygous state in the carrier females of a family with syndromic CIPO[10]. Familial cases are more frequent in mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), which is characterized by subocclusive episodes and lactic acidosis, skeletal muscle abnormalities (i.e. "ragged red fiber") and specific mitochondrial changes at the ultrastructural level[11,12]. Mutations of the gene encoding the thymidine phosphorylase gene (*TP* or endothelial cell growth factor-1, *ECGF1*), mapped to locus 22q13.32qter have a pathogenic role and are responsible for MNGIE[11-15]. The biochemical dysfunctions underlying MNGIE consists of decreased TP activity leading to accumulation of thymidine (dThd) and deoxyuridine (dUrd) in blood and tissues[16,17].

Toxic levels of dThd and dUrd induce nucleotide pool imbalance that, in turn, leads to mitochondrial DNA abnormalities including point mutations, multiple deletions and depletion[16,18].

### Histopathology and putative pathogenic mechanisms

Examination of full-thickness biopsies of the intestinal wall may help in establishing a correct diagnosis, revealing pathological abnormalities underlying the neuromuscular impairment. Histolopathic features of CIPO include neuropathic, mesenchymopathic and myopathic forms based on abnormalities affecting the integrity of nerve pathways supplying the gut (either intrinsic or extrinsic), interstitial cells of Cajal (ICC) and smooth muscle cells, respectively. Neuropathic, mesenchymopathic and myopathic changes may contribute to gut dysmotility either individually or in combination (e.g. neuro-myopathies or neuro-ICC alterations) (Table 2)[1,6,7].

### Enteric neuropathies and enteroglial cell abnormalities

Enteric neurodegenerative abnormalities and immune-mediated changes may occur in gut specimens of patients with neuropathic CIPO. Inflammatory neuropathies are characterized by a dense inflammatory infiltrate characterized by CD3 positive (composed of both CD4 and CD8) lymphocytes almost invariably confined to the myenteric plexus (hence the term of *lymphocytic myenteric ganglionitis*)[7-9,19]. The close apposition of CD3 lymphocytes to myenteric neurons provides the basis to neuro-immune interactions targeting and affecting ganglion cell structure and survival[20,21]. Indeed, experimental evidence indicates that inflammation/immune activation in the gastrointestinal tract can profoundly affect both morphology and function of the enteric nervous system (ENS).

The evidence that patients with inflammatory neuropathy have circulating anti-neuronal auto-antibodies (e.g. anti-Hu anti-neuronal antibodies) also suggests the role of the immune system in neuronal dysfunction[19]. Previous results indicated that these autoantibodies alter ascending reflex pathway of peristalsis in *in vitro* preparations[22] and elicit neuronal hyperexcitability as demonstrated by $Ca^{2+}$-imaging technique[23]. In addition, anti-HuD neuronal antibodies evoked activation of caspase-3 and apaf-1 along with apoptosis when incubated with primary culture of myenteric neurons[24]. Taken together, these experimental data suggest that anti-Hu antibodies may exert either a direct pathogenic role or contribute in association with the lymphocytic infiltrate in ENS dysfunction in patients with CIPO related to an inflammatory neuropathy. Although the etiology of inflammatory neuropathies remains undetermined, the demonstration showing herpes virus DNA in the myenteric plexus of patients with CIPO[25] raises the exciting possibility that infectious agents can be involved in the pathogenic cascade leading to inflammatory damage of the ENS.

**Table 2 Immunohistochemical markers to analyze full thickness biopsy of patients with CIPO**

| Markers | Cell targets and sites | Description |
|---|---|---|
| PGP9.5, NSE, MAP-2, NFs, tubulins, Hu C/D | Neurons: Membrane/Cytoplasmic | Identification of the general structure of the ENS |
| β-S-100, GFAP | Glial cells: Cytoplasmic | Detection of enteroglial cells |
| Kit | Interstitial cells of Cajal: Membrane/Cytoplasmic | Different ICC networks |
| SP, VIP, PACAP, CGRP, NPY, Galanin, 5-HT, NOS, ChAT, somatostatin, Calbindin, NeuN, NK1, NK2 and NK3 | Subclasses of enteric neurons; interstitial cells of Cajal: Membrane/Cytoplasmic | Characterization of neurochemical coding and enteric neuron subclasses; subsets of interstitial cells of Cajal |
| Bcl-2, TUNEL, Caspase-3, Caspase-8, Apaf-1 | Apoptosis and related mechanisms: Nuclear/Cytoplasmic | Assessment of apoptosis and related pathways |
| Actin, myosin, desmin, smoothelin | Smooth muscle cells: Cytoplasmic | Assessment of smooth muscle integrity |
| CD3, CD4, CD8, CD79α, CD68; MIP-1α, TNF-α, IFN-γ | Immune cells, chemokines and cytokines: Membrane/Cytoplasmic | Evaluation of B (CD79α) and T-lymphocytes (CD3), T-helper (CD4), T-suppressor (CD8), macrophages (CD68) in enteric ganglionitis; MIP-1α is a chemokine; TNF-α and IFN-γ are inflammatory cytokines |

Bcl-2: B cell lymphoma-2 protein; ChAT: Choline acetyltransferase; CGRP: Calcitonin gene-related peptide; ENS: Enteric nervous system; GFAP: Glial fibrillary acidic protein; Hu C/D: Hu C/D molecular antigen; IFN-γ: Interferon γ; MAP-2: Microtubule associated protein-2; MIP1-α: Macrophage inflammatory protein-1α; NeuN: Neuronal-specific nuclear protein; NFs: Neurofilaments; NK1, NK2, NK3: Neurokinin1, neurokinin2, neurokinin3; NOS: Nitric oxide synthase; NPY: Neuropeptide Y; NSE: Neuron-specific enolase; PACAP: Pituitary adenylate cyclase activating polypeptide; PGP9.5: Protein gene product 9.5; 5-HT: 5-hydroxytryptamine (serotonin); SP: Substance P; TNF-α: Tumor necrosis factor α; Tubulins: Cytoskeletal proteins; TUNEL: Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling; VIP: Vasoactive intestinal polypeptide.

Further to lymphocytic ganglionitis, Schappi *et al* have reported on eosinophilic ganglionitis characterized by eosinophils infiltrating the myenteric plexus of pediatric patients with CIPO[26]. In contrast to lymphocytic, the eosinophilic ganglionitis does not appear to evoke neuronal degeneration and loss and, therefore, gut dysmotility may be interpreted as a functional impairment of the ENS due to the infiltrate *per se* or humoral messengers released by eosinophils. Recently, mast cell predominant ganglionitis has been described in patients with severe gut dysmotility (including CIPO)[27]. The mast cells detected within myenteric ganglia in these patients were associated with markedly reduced neuronal nitric oxide synthase expression identified at molecular and immunohistochemical level. These findings suggest an impaired enteric inhibitory innervation in these peculiar subsets of CIPO.

Degenerative (or non inflammatory) neuropathies may be regarded as the end result of several putative pathogenic mechanisms, such as altered calcium signaling, mitochondrial dysfunction and production of free radicals, leading to degeneration and loss of the intrinsic neurons of the gut[28]. Degenerative neuropathies can be familial (related to a genetic background-see above) or sporadic and classified into primary (idiopathic) or secondary forms to a variety of causes, such as radiations, vinka alkaloids, myxedema, diabetes mellitus, muscular dystrophy and amyloidosis. Typical neuropathological findings reported in neurodegenerative CIPO include various qualitative (neuronal swelling, intranuclear inclusions, axonal degeneration and other lesions) and quantitative (especially hypoganglionosis) abnormalities of the ENS. Sporadic cases of visceral neuropathies are associated with two major patterns of alterations: (1) A marked reduction of intramural (especially myenteric) neural cells mainly associated with swollen neural cell bodies and processes, fragmentation and loss of axons and proliferation of glial cells; (2) A loss of the normal staining in subsets of enteric neurons, in the absence of dendritic swelling or glial proliferation[6,20,21]. Since no reliable models of degenerative neuropathies exist, the mechanisms through which exogenous noxae or other triggering factors initiate degenerative processes in enteric neurons remain obscure. Enteric neurons of patients with severe forms of idiopathic intrinsic neuropathy display a decreased expression of the protein encoded by *Bcl-2*, a gene related to one of the intracellular pathways leading to programmed cell death[4,29,30]. Indeed, this finding has been associated with an increased number of neurons displaying TUNEL, a marker of apoptosis[31].

Abnormalities of enteric glia may also contribute to intrinsic neuropathy either attracting immune cells to the ENS or resulting in insufficient support/trophism to enteric neurons and thus eliciting neurodegenerative events in the absence of inflammation[32].

### Enteric mesenchymopathies

Abnormalities to ICCs have been detected in gut tissues of patients with CIPO. These include decreased ICC density, loss of processes and damaged intracellular cytoskeleton and organelles as revealed by immunohistochemical analysis and electron microscopy[33-37]. As a result, it has been proposed that the impairment of the major functional subclasses of ICC (i.e. those involved in pacemaker activity and neurotransmission to smooth muscle) may contribute to enteric motility abnormalities detectable in patients with CIPO.

### Enteric myopathies

Histopathological analysis of the enteric muscle layer may reveal the existence of muscular abnormalities (i.e. smooth muscle fibrosis and vacuolization) of the circular and longitudinal layers in patients with primary visceral myopathy[38,39]. A controlled multinational study conducted by Knowles *et al* has proposed that a selective decrease or even absence of α-actin in the circular muscle of the small bowel wall can be regarded as biological markers of

CIPO[40]. Although exciting, the possibility that a defective expression/localization of α-actin may be a biomarker of a heterogeneous disease such as CIPO awaits solid confirmatory evidence.

The histopathological details concerning other segments of the gut as well as extra-digestive systems (i.e. urinary tract, gall-bladder) is poorly characterized and further studies are awaited to elucidate this important aspect.

## CLINICAL FEATURES

Subocclusive episodes can strike in apparently healthy people, but the onset of CIPO is generally insidious, with gastrointestinal symptoms which precede the first acute episode.

The typical clinical manifestation of CIPO is characterized by recurrent episodes of abdominal pain, abdominal distension and inability to defecate (flatus may not be completely suppressed), with or without vomiting, mimicking a mechanical sub-occlusion. During acute episodes radiological evidence of distended bowel loops and air-fluid levels in the upright position is an important diagnostic marker of this pathological condition. Acute episodes can last only a few hours, but in the most severe cases intestinal loops are chronically distended and air-fluid levels are invariably detected. Due to this misleading clinical manifestation, a history of multiple, useless surgeries are typical of the syndrome. Thus, many patients have abdominal adhesions and the concomitant presence of functional and mechanical (secondary to adhesions) obstruction is often impossible to rule out despite extensive investigations.

Between subocclusive episodes patients are very rarely asymptomatic, and almost invariably complain of severe digestive symptoms[1,2] suggestive of delayed transit in the proximal and/or distal portions of the alimentary canal. Nausea, vomiting and weight loss are predominant symptoms when the functional derangement primarily affects the upper gastrointestinal tract, while diffuse abdominal pain, abdominal distension and constipation are suggestive of a more distal involvement of the gut. Dysphagia is present in a low proportion of CIIPO patients although it is relatively frequent in forms secondary to progressive systemic sclerosis.

Diarrhea and steatorrhoea often occur as a consequence to small bowel bacterial overgrowth.

This pathologically accelerated transit is often well accepted by patients since it is associated with partial relief of other digestive symptoms, but it contributes to determine intestinal malabsorption and deteriorate nutritional conditions. Indeed, many patients are afflicted by inability to maintain a normal body weight, despite dietary manipulations, both because of the deranged digestive functions and because food ingestion often exacerbates digestive symptoms and consequently patients tend to avoid a normal oral nutrition.

Urinary symptoms, generally associated with evidence of urinary tract distension, are also frequent.

Depression or other psychological disturbances are often secondary to the disabling digestive problems and the disappointing quality of healthcare received.

## DIAGNOSTIC PROCEDURES

The diagnosis of CIPO is mainly clinical, supported by radiographic documentation of dilated bowel with air-fluid level, after exclusion of organic lesions occluding the gut lumen, as detected by radiologic and/or endoscopic investigations. Thus, diagnostic tests in patients with suspected CIPO are necessary to exclude mechanical occlusion, identify possible causes of secondary forms, explore underlying pathophysiological mechanisms and disclose possible complications.

### *Radiology*

Radiology is one of the most important examinations in the diagnosis of CIPO. Plain abdominal films identify typical signs of intestinal occlusion such as distended bowel loops with air-fluid levels, the latter obtained with the patients in the upright position (as specified above). Contrast studies are necessary to exclude the presence of organic lesions responsible for the occlusion. Entero-CT scan allows simultaneous internal and external views of the gut wall, abdominal CT and MR scans are important in investigating possible causes of gut compression, while MR angiography may non-invasively identify congenital or acquired vascular abnormalities. Excretory urograms should be performed in patients with urinary symptoms.

Symptoms suggestive of a subocclusive state in the absence of dilated bowel with air fluid levels at radiology have been defined by some authors as a "mild forms of CIPO"[41]. Nonetheless, this definition has been criticized[4]. In fact, preliminary studies suggest that patients with extremely severe digestive symptoms and malnutrition, but no radiological evidence of intestinal occlusion, have a significantly reduced probability of undergoing abdominal surgery and present less severe motility disorders[42].

### *Endoscopy*

The main indication of upper gastrointestinal endoscopy is exclusion of mechanical occlusions in the gastro-jejunal and ileo-colonic regions. It allows to exclusion of false positive radiologic diagnoses of mechanical occlusion in the duodenum and proximal small bowel, as in many cases of the so-called "aorto-mesenteric compression syndrome"[43]. Mucosal biopsies of the small bowel should be taken to rule out celiac disease. Colonoscopy also has a therapeutic potential, since it can be used to try to decompress the large bowel[44].

### *Laboratory tests*

Laboratory tests are useful to identify the presence of potentially curable diseases responsible for secondary forms, but also to monitor hydro-electrolyte balance and circulating levels of essential elements in patients on parenteral nutrition or, in general, with a severe malnutrition.

### *Manometry*

Small bowel manometry is invariably abnormal in CIPO patients[2,3,45]; however, the test is not of diagnostic value due to its low specificity. At best, it can play a supportive role in defining the diagnosis, since it can contribute to differentiate mechanical from functional obstruction and

to recognize the underlying pathophysiological mechanism[2,3,45].

Describing in detail small bowel manometric abnormalities of CIPO goes beyond the scope of the present review. They can be summarized as follows: uncoordinated bursts of powerful contractions with variable duration are suggestive of an underlying intrinsic neuropathy[1-4,45-48] conversely, normally coordinated motor patterns with low amplitude have been reported in patients with a myogenic disorder[1-4,45-48]. Nonetheless, low amplitude contractions may merely reflect the inability of the manometric technique to record non-occlusive contractions, such as in the case of dilated bowel loops[1-4,45-48].

Unlike what is observed in pseudo-obstruction, the manometric pattern of mechanical occlusion is characterized by giant contractions (prolonged contractions lasting at least 10 s and can be either propagated or non propagated) or clustered contractions (3-10 regular contractions, occurring 1 per 5 s preceded and followed by $\geqslant$ 1 min of absent motor activity lasting at least 20 min and can be either propagated or non propagated)[1-4,45-49].

Esophageal manometry generally adds very little to the diagnostic work-up of CIPO, but it plays an important diagnostic and prognostic role if the disease is secondary to scleroderma. Ano-rectal manometry is important to rule out Hirschsprung's disease, particularly in patients with intractable constipation and a marked distension of the large intestine.

### Biopsy and pathologic examination

Full thickness biopsies should be obtained from dilated and nor dilated tracts of the alimentary canal in all patients with suspected CIPO who undergo surgery for unexplained occlusive episodes. Biopsies should be processed for in depth pathological evaluation by both traditional staining and immunohistochemistry techniques in dedicated laboratories with a specific interest in this area, as specified above.

## NATURAL HISTORY

Even if clinical experience shows that CIPO is a progressive disease that often leads to death, only few studies have precisely described the natural history of this pathology and its symptoms prognostic values, especially in the adult age. Children generally present the first manifestations of CIPO at birth or during the first years of age[50-53]. The pediatric expression of the disease is often characterized by a particularly severe course, with mortality rates extremely high within the first year of age, mainly due to surgical and parenteral nutrition complications[52,53]. Several predictors of poor outcome have been identified in children including myopathic forms, malrotation, short bowel syndrome, and urinary tract involvement[50,51].

In the adult population, the first sub-occlusive episode is often preceded by a long history of non-specific, progressively more severe digestive symptoms. An acute onset of the disease occurs in only one-fourth of the cases[2].

After diagnosis is established the frequency of sub-occlusive episodes and, consequently, also of surgical procedures tend to decrease. Nevertheless, the clinical course of CIPO is almost invariably severe[1,2,52-54] with progressive deterioration of bowel function and digestive symptoms. In order to control both the body weight and the abdominal pain most patients progressively limit oral nutrition and end up on long-term parenteral nutrition.

The main causes of death are TPN-related complications, surgery-related complications, and post-transplantation complication, together with septic shock of GI origin. A variety of clinical, histological and manometric parameters have been found to be predictive of a poor clinical outcome in adult patients, including myopathy and decreased contractile activity[2,50,52,53,55-60]. MNGIE has a particularly poor prognosis with slowly progressive evolution and death around 40 years of age[11].

## THERAPY

The treatment of CIPO is difficult and often provides unsatisfactory results. Of course, treatment of the underlying disease is mandatory in secondary forms whenever available[57].

### Treatment of the acute phase

During acute phases patients should be treated as those with acute mechanical obstruction. Fluid and electrolytes balance should be maintained *via IV* infusions; abdominal decompression should be attempted by positioning of nasogastric and rectal tubes. The former generally prevents vomiting and *ab ingestis* while the latter is generally ineffective and colonic decompression can be attempted by colonoscopy or cecostomy (see below). In case of prolonged subocclusive episodes systemic or poorly absorbable antibiotics are necessary to prevent bacterial overgrowth. Appropriate caloric support must be provided by *IV* infusion. Erythromycin, somatostatin and neostigmine can be used to promote transit and decrease the duration of acute episodes[61-63].

### Nutritional support

The nutritional status of patients with CIPO is generally poor. Frequent small meals with liquid or homogenized foods, with or without oral nutritional supplements, may help patients with sufficient residual digestive functions. Enteral nutrition is an option for patients whose motility disorder is mainly localized in the stomach and duodenum. It presents fewer complications than parenteral nutrition, but clinical experience suggests that enteral feeding is rarely tolerated by patients. In the most severe cases, when small bowel function is diffusely affected, parenteral nutrition is necessary to satisfy nutritional requirements. The main limitations of this nutritional support include liver insufficiency, pancreatitis, glomerulonephritis and catheter-related complications (i.e. thrombosis and septicemia)[58,59].

### Pharmacological therapy

The pharmacological treatment of CIPO is aimed at controlling symptoms and avoiding complications. Co-

prescription of antiemetics, antisecretory, antispasmodics, laxatives or antidiarrheal and analgesic drugs is often necessary. Prokinetics are often prescribed, with the intention to improve gastrointestinal motility and to control visceral sensitivity[2,54].

Some prokinetics seem to be more effective than others: metoclopramide, domperidone, bethanechol or neostigmine are often used, but with only limited success, while cisapride, that is currently available only in some parts of the world, has been reported to exert positive effects[60,64-67]. Two controlled trials including CIPO patients described positive effects of cisapride in accelerating gastric emptying[60] and improving symptoms[66]. Erythromycin is a macrolide antibiotic with a specific agonist action on the motilin receptors of the proximal gastrointestinal tract. It increases antral contraction and promotes gastric emptying, while its effects on colonic motility are controversial: at low doses it stimulates intestinal contractions, but doses normally used to enhance gastric emptying decrease motility of the small intestine[61]. Octreotide is a long-acting somatostatin analog which increases intestinal motor activity and decreases bacterial overgrowth[62]. Co-prescription of erythromycin and octreotide can be useful to control both the gastric emptying and the intestinal motility[68]. Anticholinesterase drugs have been described as effective in autoimmune gastrointestinal motor disorders[69]. Tegaserod, a more recent 5-HT4 agonist, was also recommended for the treatment of subocclusive episodes in CIPO, but the drug has been withdrawn from the market[70]. A preliminary open study describes encouraging results exerted gastric electrical stimulation on nausea and vomiting in a small number of CIPO patients[71].

Opioids are required in patients with intractable pain, but their constipating effect can further deteriorate digestive functions[72,73].

Antibiotics are often useful to contrast bacterial overgrowth. Poorly absorbable antibiotics such as paramomicine and rifaximine should be preferred, but alternating cycles with metronidazole and tetracycline are necessary to limit resistances[74].

Steroids or other immunosuppressive treatments are recommended when CIPO is related to an underlying inflammatory neuropathy. These cases have to be selected through tissue analysis or at least suspected by the identification of circulating anti-neuronal antibodies[6]. Treatment of MNGIE is largely supportive, being based on parenteral nutrition and/or supplementation with coenzyme Q, riboflavin and other vitamins (vitamin C, vitamin K3, carnitine). Prompt treatment of fever and infections and avoidance of extremes in temperature, over exercise, drugs known to interfere with mitochondrial functions (phenytoin, chloramphenicol, tetracycline, macrolides, and aminoglycosides), are also recommended. Infusion platelets to reduce thymidine level have been reported to exert some positive effect in preliminary study in MNGIE patients[75].

### *Surgical therapy*

Even if CIPO patients often undergo surgical procedures, this kind of approach has only a limited role in the management of the disease and has to be considered only in some carefully selected patients. Specifically, since CIPO generally involves the whole alimentary canal, only rare cases can benefit from surgical resections. Indeed surgery can precipitate deterioration of the clinical conditions and should be performed only if strictly necessary. Full thickness biopsies should be obtained whenever possible for pathological examination as stated above. In particular, surgery can be considered in patients having what appears to be localized involvement of the gastrointestinal tract, but CIPO is often a progressive disease and the benefit is likely temporary[41,76].

Gastrostomies and enterostomies can effectively decrease retching, vomiting and abdominal distension and represent a possible option in patients who can be fed by enteral nutrition. Furthermore, decompression of distended bowel loops can exert a positive effect on the transport capacities of the alimentary canal which, in turn, results in a decreased frequency of further hospital admissions and surgeries.

Small bowel or, when needed, multivisceral transplantation is available only in a few highly specialized centers. The general outcome of this surgical procedure has markedly improved with the use of the immunosuppressive agent tacrolimus associated with steroid and together a number of induction agents such as alemtuzumab, antithymocyte globulins and daclizumab[77]. However, the need for long-term parenteral nutrition, re-laparotomies, organ rejection and, especially, bacterial infections are frequent complications and the procedure still have mortality rates approaching 50% at 5 years. Predictors of post-transplant complications are: concomitant neuromuscular disorders of the urinary tract, chronic use of opioids and technical problems determined by previous multiple laparotomies and/or the need of gastrectomy for gastroparesis. Nonetheless, transplantation should be considered when all other therapeutic options have failed according to the following indications: chronic intestinal failure with a high risk of mortality, life-threatening complications of parenteral nutrition, lack of venous access, disease-related poor quality of life despite optimal parenteral nutrition[58].

## CONCLUSION

CIPO is a rare and often misdiagnosed pathological condition. Even if the acute phases can be hardly differentiated by mechanical occlusions and the inter-crisis digestive symptoms can mimic other severe functional digestive syndromes, the syndrome should be recognized based on the typical combination of clinical features, natural course and radiological signs. The diagnostic suspicion should be then confirmed by more accurate examinations, in order to identify possible causes of secondary forms and underlying pathophysiological mechanisms.

Management of CIPO remains extremely challenging and often disappointing.

A greater awareness of the clinical features of CIPO would help to limit surgical procedures to a minimum and, even more importantly, to collect full-thickness biopsies

for analysis of the gut neuromuscular layer at an early and potentially curable stage of the disease.

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*EDITORIAL*

# Endoscopic submucosal dissection for gastrointestinal neoplasms

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## Abstract

Endoscopic submucosal dissection (ESD) is an advanced technique of therapeutic endoscopy for superficial gastrointestinal neoplasms. Three steps characterize it: injecting fluid into the submucosa to elevate the lesion, cutting the surrounding mucosa of the lesion, and dissecting the submucosa beneath the lesion. The ESD technique has rapidly permeated in Japan for treatment of early gastric cancer, due to its excellent results of en-bloc resection compared to endoscopic mucosal resection (EMR). Although there is still room for improvement to lessen its technical difficulty, ESD has recently been applied to esophageal and colorectal neoplasms. Favorable short-term results have been reported, but the application of ESD should be well considered by three aspects: (1) the possibility of nodal metastases of the lesion, (2) technical difficulty such as location, ulceration and operator's skill, and (3) organ characteristics.



**Key words:** Endoscopic submucosal dissection; Gastric cancer; Esophageal cancer; Colorectal cancer; Endoscopic mucosal resection; Therapeutic endoscopy

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## INTRODUCTION

Application of endoscopic resection (ER) to gastrointestinal (GI) neoplasms is limited to lesions with no risk of nodal metastasis. Either polypectomy or endoscopic mucosal resection (EMR) is beneficial for patients because of its low level of invasiveness. However, to ensure the curative potential of these treatment modalities, accurate histopathologic assessment of the resected specimens is essential because the depth of invasion and lymphovascular infiltration of the tumor is associated with considerable risk for lymph node metastasis. For accurate assessment of the appropriateness of the therapy, en bloc resection is more desirable than piecemeal resection. For a reliable en bloc resection of GI neoplasms, a new method of ER called endoscopic submucosal dissection (ESD) has been developed. In this article, an outline of the current status of ESD will be discussed.

## DEVELOPMENT OF ESD

The ESD technique has developed from one of the EMR techniques, namely endoscopic resection after local injection of a solution of hypertonic saline-epinephrine (ERHSE)[1]. Initially, the ESD technique was called by various names such as cutting EMR, exfoliating EMR, EMR with circumferential incision *etc*. However, a new name was proposed to this technique in 2003, as a treatment positioned between EMR and laparoscopic surgery, since this technique is innovative and enables complete resection of neoplasms that were impossible to resect en bloc by EMR.

At present, numerous electrosurgical knives such as insulation-tipped diathermic knife (IT-knife)[2-6], needle knife[7], hook knife[8], flex knife[9-11], triangle-tipped knife[12], flush knife[13], mucosectomy[14], splash needle[15] and a special device called a small-caliber tip transparent (ST) hood[7] are available for this technique. One or two of these electrosurgical knives are used in combination with a high frequency electrosurgical current (HFEC) generator with an automatically controlled system (Endocut mode, Erbotom ICC200, ICC350, VIO300D, ERBE, Tubingen, Germany) (PSD-60, Olympus, Tokyo, Japan). New types of endoscopes are available for ESD, such as an endoscope with a water jet system (EG-2931, Pentax, Tokyo, Japan, GIF-Q260J, Olympus, Tokyo, Japan), an endoscope with a multi-bending system (M-scope: XGIF-Q240M, R-scope: XGIF-2TQ240R, Olympus, Tokyo, Japan) to facilitate the ESD procedure[16-19]. As another approach to successful

ESD, investigations of submucosal injection solutions have been actively done. It was reported that a hyaluronic acid solution makes a better long-lasting submucosal cushion without tissue damage than other available solutions[7,20-23]. As a further improvement of hyaluronic acid solution, usefulness of a mixture of high-molecular-weight hyaluronic acid, glycerin, and sugar has also been reported[24,25].

ESD is characterized by three steps: injecting fluid into the submucosa to elevate the lesion from the muscle layer, circumferential cutting of the surrounding mucosa of the lesion, and subsequent dissection of the connective tissue of the submucosa beneath the lesion. Major advantages of this technique in comparison with polypectomy or EMR are as follows. The resected size and shape can be controlled, en bloc resection is possible even in a large neoplasm, and neoplasms with submucosal fibrosis are also resectable. So this technique can be applied to the resection of complex neoplasms such as large neoplasms, ulcerative non-lifting neoplasms, and recurrent neoplasms. The disadvantages of this technique are the requirement of two or more assistants, it is time-consuming, there is a higher risk of bleeding and perforation than EMR. In Japan, ESD is now gaining acceptance as the standard endoscopic resection technique for stomach neoplasms in an early stage, especially for large or ulcerative neoplasms. Recently, the ESD technique is applied to esophageal or colorectal neoplasms in some institutions, although it is still controversial considering the technical difficulty, associated risks, and favorable outcomes by EMR.

## INDICATION FOR ENDOSCOPIC RESECTION

### *Gastric cancer*

Early gastric cancer (EGC) is defined to a mucosal or submucosal invasive cancer (T1 cancer) irrespective of the presence of lymph node metastasis. Lesions indicated for ER should be EGC with no risk of nodal metastasis and that can be resected in a single fragment. Using a large database of more than 5000 EGC patients who underwent gastrectomy with D2 lymph node dissection, a criteria of node negative cancer has been defined[26]. At present, lesions with preoperative endoscopic diagnosis of differentiated type intramucosal cancer without ulcer findings, differentiated type intramucosal cancer no larger than 3 cm in diameter with ulcer findings, differentiated type minute invasive submucosal (less than 500 micrometers below muscularis mucosa) cancer no larger than 3 cm in diameter are considered as expanding indication for ER[27]. Undifferentiated type cancer lesions, and preoperative diagnosis of ulcerative findings is difficult, so that ER for these lesions should be carefully considered.

### *Esophageal cancer*

Early esophageal cancer (EEC) involving the epithelium (m1: carcinoma in situ) or the lamina propria (m2) are candidates for ER because no lymph node metastasis have been reported in cancers limited to these two layers[28]. For EEC invading the muscularis mucosa (m3), the lymph node metastasis rate is reported as 9%, and for cancer with minute submucosal invasion (< 200 micrometers below the muscularis mucosa; sm1) the rate is 19%[29]. The lymph node metastasis rate of m3 or sm1 cancer without lymphovascular infiltration of the tumor is reported as 4.7%[29]. Therefore, for patients unwilling for esophagectomy or patients with comorbid diseases not suited for surgery, ER may be a relative indication for m3 or sm1 cancer. Also, for lesions spreading more than three-quarter of circumference of the esophagus are considered as relative indication for ER because post-operative stricture occurs in a high rate.

### *Colorectal cancer*

Early colorectal cancer (ECC) limited to the mucosa or with slight submucosal invasion (< 1000 micrometers below the muscularis mucosa; sm1) are candidates for ER[30]. However, even for lesions that meet the criteria above, laparoscopic or open surgery may be selected in some institutions considering the location and size of the lesion. In institutions actively performing ESD for colorectal lesions, depressed lesions and laterally spreading tumors of non-granular type (LST-NG) are considered as good candidates for ESD because these lesions have a high possibility of submucosal invasion which may be difficult to diagnose preoperatively, and a thorough histopathological assessment of the resected specimen is essential.

### *Preoperative evaluation for candidates of ER*

Endoscopy with chromoendoscopy is essential to define the lesion. To evaluate the depth of the lesion, size, redness, presence or absence of ulceration, superficial structure of the lesion, and deformity of the wall of the organ in compliance with air-flow rate are carefully observed by endoscopy and chromoendoscopy. Magnification endoscopy with narrow band imaging technique (NBI) has been reported as a promising new modality to evaluate the depth of EEC. Magnification endoscopy with NBI is also useful to distinguish the border of EGC in case of lack of utility of chromoendoscopy with indigocarmine. Magnification endoscopy with crystal violet staining or NBI is useful in estimating the depth of colorectal lesions. Endoscopic ultrasonography is often performed to evaluate the depth of invasion, and computed tomography may be performed to detect lymph node metastasis if any, if the diagnosis of node negative cancer is difficult to judge even with multiple diagnostic modalities.

### *Pathological evaluation of the removed specimen*

Whether a lesion may be included into the criteria of node-negative neoplasms is considered before treatment. However, at present, it is impossible to make a definite diagnosis of a neoplasm regarding depth, histological type and lymphatic vessel invasion before treatment. It is often experienced that although a biopsy specimen shows adenoma/dysplasia of a lesion, a diagnosis of cancer is made after total resection of the lesion. Therefore, a precise pathological evaluation of the resected specimen is essential, and an en bloc resection of the lesion is desirable in this respect.

After removal, the specimen should be oriented immediately before it is immersed in formalin. Orientation of the specimen is accomplished by fixing the periphery with thin needles on a plate of rubber or wood. The submucosal side of the specimen is faced to the plate. After fixation, the specimen is sectioned serially at 2 mm intervals parallel to a line that includes the closest part between the margin of the specimen and of the neoplasm, so that both lateral and vertical margins are assessed. The depth of invasion is then evaluated microscopically along with the degree of differentiation and lymphovascular infiltration, if any.

In result of thorough pathological assessment, if the lesion is resected en bloc with negative margins of neoplasm and fulfills the criteria of node-negative neoplasms with no lymphovascular infiltration, the treatment is judged as curative resection. For lesions with piecemeal resection but being judged as node-negative neoplasms, or lesions with histologically non-evaluable areas due to artifact or tissue burning, a periodical endoscopic follow-up should be performed to detect residual neoplasm or local recurrence. On the other hand, for lesions that do not fulfill the criteria of node-negative neoplasms, additional surgery with nodal dissection should be strongly recommended.

## OUTCOMES OF ESD

### *En bloc resection rate*

Recent results of en bloc resection rate and local recurrence of ESD for neoplasms in the stomach, esophagus and colorectum are described in Table 1. For gastric neoplasms larger than 20 mm, en bloc resection rate is extremely low among conventional EMR methods, and local recurrence rates are around 10%[44]. Although ESD was considered as a difficult and complicated technique when it was first described in the stomach, after maturity of the techniques of ESD, en bloc resection rates became greater than 90%, regardless of size, and local recurrence rates became almost zero. Technical feasibility and favorable results of ESD have also been reported in recurrent neoplasms[45-47], neoplasms of the esophago-gastric junction[48], and duodenal neoplasms although the number of cases is small. Few reports of ESD for resection of subepithelial tumors have also been published[49].

**Table 1 Recent outcomes of various endoscopic submucosal dissection methods for stomach, esophagus and colorectum**

| Site | Author | Yr | Method | En bloc resection rate (%) | Local recurrence rate (%) |
|---|---|---|---|---|---|
| Stomach | Yamamoto[33] | 2002 | EMRSH | 76 (53/70) | 3 (2/67) |
| | Ishigooka[34] | 2004 | s-ERHSE | 79 (27/34) | 0 (0/34) |
| | Oda[35] | 2005 | ESD-IT knife | 93[1] (957/1033) | - |
| | Kakushima[32] | 2006 | ESD-Flex knife | 91[1] (347/383) | - |
| | Imagawa[36] | 2006 | ESD-Flex knife | 84[1] (181/195) | 0 (0/164) |
| | Oyama[37] | 2006 | ESD-Hook knife | 94 (104/111) | 0 (0/111) |
| | Onozato[38] | 2006 | ESD-Flex knife | 94[1] (161/171) | 0 (0/99) |
| | Hirasaki[39] | 2007 | ESD-IT knife | 96 | - |
| Esophagus | Oyama[8] | 2005 | ESD-Hook knife | 95 (95/102) | 0 (0/102) |
| | Fujishiro[11] | 2006 | ESD-Flex knife | 100 (58/58) | 2.5 (1/40) |
| Colorectum | Fujishiro[31] | 2007 | ESD-Flex knife | 91.5 (183/200) | 1.8 (2/111) |
| | Saito[40] | 2007 | ESD several knives | 84 (168/200) | 0.5 (1/180) |
| | Tanaka[41] | 2007 | ESD several knives | 80 (56/70) | 0 (0/62) |
| | Tamegai[42] | 2007 | ESD-Hook knife | 98.6 (33/42) | 11 (4/36) |
| | Onozato[43] | 2007 | ESD-Flex knife | 77 (27/35) | 0 (0/23) |

[1]*En bloc* resection + R0 resection rate.

### *Complication*

Complications of ESD include pain, bleeding, perforation, and stricture. Pain after ESD is often mild and lasts one or two days after the procedure although the frequency is low. Patients of esophageal ESD are more likely to develop pain than gastric or colorectal ESD.

Complications of post-operative bleeding and perforation among various ESD methods in the stomach, esophagus and colorectum are described in Table 2. Bleeding is more frequent in the stomach cases, whereas perforation is more frequent in the colorectal cases. To prevent post-procedural bleeding, hemostasis of appearing vessels on the artificial ulcer after removing the specimen is essential. Hemostasis is performed by hemostatic forceps (HDB2422/HDB2418, Pentax), coagrasper (FD-410LR, Olympus), hot biopsy forceps, argon plasma coagulation or endoclips. According to perforation, recent case series suggest that small perforation immediately recognized can be successfully sealed with endoclips and treated conservatively by nasogastric suction, fasting and antibiotics without emergency laparotomy[51,52]. However, there are rare cases of delayed perforation, which requires surgical rescue. Delayed perforation may occur in the esophagus, stomach, duodenum and colorectum[31,53-56], mostly at two or more days after a successful ESD. The reason for delayed perforation is unknown, however patients with uncontrolled diabetes mellitus, patients on permanent hemodialysis, lesions located on surgical anastomosis, and too much coagulation are considered as possible risk factors.

Stricture after ESD may occur in esophageal ESD when the ESD ulcer is larger than two-third of circumference of the esophageal lumen, or in gastric ESD when the ESD ulcer involves more than three quarter of the pylorus or pre-pylorus area. In these cases, early intervention to avoid passage obstruction is required. Dilation using bougie or balloon are often applied one week after ESD and repeated several times until healing of the ESD ulcer[8,11,57].

## MANAGEMENTS AFTER ESD

In Japan, ESD is performed on hospitalized patients. After ESD, eating is usually started on the next or 2 d after ESD if there is no complication, and the patient

**Table 2 Bleeding and perforation rate of various endoscopic submucosal dissection methods for stomach, esophagus and colorectum**

| Site | Author | Year | Method | Total cases | Bleeding (%) | Perforation (%) |
|---|---|---|---|---|---|---|
| Stomach | Yamamoto[33] | 2002 | EMRSH | 70 | 4 | 0 |
| | Ishigooka[34] | 2004 | s-ERHSE | 34 | 0 | 12 |
| | Oda[35] | 2005 | ESD-IT knife | 1033 | 6 | 4 |
| | Kakushima[32] | 2006 | ESD-Flex knife | 383 | 3.4 | 3.9 |
| | Imagawa[36] | 2006 | ESD-Flex knife | 159 | 0 | 6.1 |
| | Oyama[37] | 2006 | ESD-Hook knife | 111 | - | 1 |
| | Onozato[38] | 2006 | ESD-Flex knife | 171 | 7.6 | 3.5 |
| | Hirasaki[39] | 2007 | ESD-IT knife | 112 | 4 | 1 |
| Esophagus | Oyama[8] | 2005 | ESD-Hook knife | 102 | - | 0 |
| | Fujishiro[11] | 2006 | ESD-Flex knife | 58 | 0 | 6.9 |
| Colorectum | Fujishiro[31] | 2007 | ESD-Flex knife | 200 | 1 | 6 |
| | Saito[40] | 2007 | ESD-several knives | 200 | 2 | 5 |
| | Tanaka[41] | 2007 | ESD-several knives | 70 | 1.4 | 10 |
| | Tamegai[42] | 2007 | ESD-Hook knife | 74 | - | 1.4 |
| | Hurlstone[50] | 2007 | ESD-Flex knife | 42 | 12 | 2.4 |
| | Onozato[43] | 2007 | ESD-Flex knife | 35 | 0 | 2.9 |

may be discharged within a few days. Antacids are usually administered to gastric and esophageal ESD patients to relieve pain, prevent postoperative bleeding and promote ulcer healing. A recent study showed that proton pump inhibitors more effectively prevented bleeding from the gastric ulcer created after ESD than did H2-receptor antagonists[58]. Ulcers after ESD are reported to heal within 6 to 8 wk in the esophagus, stomach and colorectum[59-63].

Endoscopic surveillance should be carried out in patients after ESD not only to detect local recurrence but also metachronous cancer especially in the esophagus and stomach. A recent study showed that the average time to detect a first metachronous gastric cancer (MGC) was 3.1 ± 1.7 years after EMR/ESD, and the cumulative 3-year incidence was 5.9%[64]. In order to detect MGC at an early stage to perform a successful ER, annual endoscopic surveillance program may be practical for post-ER patients.

## LONG-TERM OUTCOMES AFTER ESD

Long-term outcomes after ESD for gastric cancers within the expanded indication are currently under investigation. Survival data is still lacking in the literature, however in the 2007 annual meeting of Japanese gastroenterological endoscopy society (JGES), a symposium was held upon long-term outcomes after gastric and esophageal ESD. For gastric ESD, 3-year disease free survival rate was reported as 90%-92%, local recurrence rate was reported as 0.8%-12%. For lesions within the criteria of node negative cancers, there were no reports of distant metastasis. Metachronous gastric cancer detection rate during follow-up was reported as 3.4%-10.2%. In comparison, long-term outcomes after EMR for small differentiated mucosal EGC less than 20 mm in diameter have been reported as comparable to those after gastrectomy. The disease-specific 5- and 10-year survival rates were 99% and 99%[65]. For esophageal ESD, in the 2007 JGES meeting, 3-year survival rate for m1-2 cancer and m3-sm1 cancer were 95.1% and 86.7%, respectively. According to colorectal ESD, there is still no long-term data at present.

## FUTURE PERSPECTIVES

With the development of ESD, more than half of GI cancers in the early stage are removed by ER in advanced institutions in Japan. En bloc retrieval of lesions is essential for detailed histopathologic studies, which form the basis for stratification of treatment outcomes and patient's prognosis. ESD theoretically offers greater histopathological accuracy than conventional EMR methods or piecemeal resection. However, ESD requires highly skilled endoscopists, and a suitable training program is demanded for permeation of this technique. For trainees starting ESD, skills of routine endoscopy and colonoscopy, target biopsy, endoscopic hemostasis techniques and simple EMR techniques should be required. A trainee would gain early proficiency of ESD after 30 cases under supervision of a mentor[32,66]. On the other hand, serious complications such as delayed perforation have been reported, and a thorough patient care before and after ESD is essential. At present, selection of a lesion within the criteria for ER, selection of the patient with adequate general function should be well considered. It is important to share the information and experience among endoscopists to skill up and avoid serious complications. The ESD technique is still not a treatment at ease, and further refinements of the technique is required to popularize ESD as a safe and reliable, less invasive treatment for patients with GI neoplasms.

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*CLINICAL PRACTICE GUIDELINES*

# Pharmacological approach to acute pancreatitis

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## Abstract

The aim of the present review is to summarize the current knowledge regarding pharmacological prevention and treatment of acute pancreatitis (AP) based on experimental animal models and clinical trials. Somatostatin (SS) and octreotide inhibit the exocrine production of pancreatic enzymes and may be useful as prophylaxis against Post Endoscopic retrograde cholangiopancreatography Pancreatitis (PEP). The protease inhibitor Gabexate mesilate (GM) is used routinely as treatment to AP in some countries, but randomized clinical trials and a meta-analysis do not support this practice. Nitroglycerin (NGL) is a nitrogen oxide (NO) donor, which relaxes the sphincter of Oddi. Studies show conflicting results when applied prior to ERCP and a large multicenter randomized study is warranted. Steroids administered as prophylaxis against PEP has been validated without effect in several randomized trials. The non-steroidal anti-inflammatory drugs (NSAID) indomethacin and diclofenac have in randomized studies showed potential as prophylaxis against PEP. Interleukin 10 (IL-10) is a cytokine with anti-inflammatory properties but two trials testing IL-10 as prophylaxis to PEP have returned conflicting results. Antibodies against tumor necrosis factor-alpha (TNF-$\alpha$) have a potential as rescue therapy but no clinical trials are currently being conducted. The antibiotics beta-lactams and quinolones reduce mortality when necrosis is present in pancreas and may also reduce incidence of infected necrosis. Evidence based pharmacological treatment of AP is limited and studies on the effect of potent anti-inflammatory drugs are warranted.



**Key words:** Acute pancreatitis; Diclofenac; Gabexate; Indomethacin; Interleukin-10; Necrotizing pancreatitis; Nitrogen oxides; Octreotide; Protease inhibitors; Somatostatin

## INTRODUCTION

Acute pancreatitis (AP) is a localized inflammatory condition, which may extent to other organs. The etiology is usually excessive consumption of alcohol or gallstone disease, but is in some cases iatrogenic following medication or endoscopic retrograde cholangiopancreatography (ERCP).

Only a few reviews summarizing the available pharmacological options for treating AP have been published despite various experimental and clinical testing of potential drugs[1]. The aim of the present review is to validate the current literature covering the pharmacological treatment of AP. The main focus is on human clinical trials but some animal experimental models have been included as well (Table 1). AP after ERCP [post-ERCP pancreatitis (PEP)] can be regarded as a clinical "experimental" model of AP and hence is subject to preventive measures. The studies of potentially prophylactic drugs to PEP are therefore included (Table 2).

## PATHOPHYSIOLOGY

The pathobiological processes have primarily been investigated in experimental animal models and it is widely accepted that the acinar cells play a central role in the development of AP. The secretory acinar cells (SAC) contain zymogen precursors and the intra-acinar activation of digestive enzymes is a key event in the pathogenesis of AP. The molecular mechanism by which zymogen in AP fails to leave the SAC is unknown. Studies suggest a loss of the terminal actin web or a displacement of one of the SNARE membrane proteins, which regulate exocytosis[2].

The inflammatory response is partly caused by the release of chemokines from SAC, which is followed by recruitment of helper T lymphocytes and macrophages leading to pancreatic edema and accumulation of neutrophils. The systemic release of cytokines including major pro-inflammatory cytokines causes a systemic

**Table 1 Pharmacological treatment of acute pancreatitis: Overview of drugs tested in animal experimental models and clinical trials**

| Name | Mechanism | Effect in animal models | Result in human trials |
|---|---|---|---|
| Somatostatin | Inhibition of pancreatic secretion | No reduced mortality | No reduced mortality |
| Octreotid | Inhibition of pancreatic secretion | No effect (divergent results) | No reduced mortality |
| Gabexate mesilate | Protease inhibitor | Reduced histology score | Maybe reduced mortality |
| N-acetyl-cysteine | Reduction of oxidative stress | Reduced severity | No reduced mortality |
| Nitrogen oxide | Improvement of micro-circulation | Reduced edema | No published trials |
| Steroids | Non-specific anti-inflammatory | Reduced mortality | No published trials |
| Interleukin-10 | Anti-inflammatory | Reduced mortality | No published trials |
| TNF-alpha antibody | Specific anti-inflammatory | Reduced mortality | No published trials |
| PAF inhibitor | Specific anti-inflammatory | No reduced mortality | No reduced mortality |
| Antibiotics | Antibacterial | - | Reduced mortality |
| Probiotics | Prevention of colonization of the gut | - | No reduced mortality |

**Table 2 Pharmacological prevention of PEP: Overview of drugs tested in clinical trials**

| Name | Mechanism | Incidence of PEP |
|---|---|---|
| Somatostatin | Inhibition of pancreatic secretion | No effect |
| Octreotid | Inhibition of pancreatic secretion | Probably reduced |
| Gabexate mesilate | Protease inhibitor | No effect |
| N-acetylcysteine | Reduction of oxidative stress | No effect |
| Nitrogen oxide | Improvement of micro-circulation | No effect |
| Steroids | Non-specific anti-inflammatory | No effect |
| Interleukin-10 | Anti-inflammatory | Probably no effect |
| TNF-alpha | Specific anti-inflammatory | No published trials |
| Antibiotics | Antibacterial | Reduced |

response, which may include remote organs[3]. The inflammatory process is followed by interstitial edema and the disease will in 10%-15% of the cases progress to necrosis in parts of the pancreas and possible bacterial infection. During an attack of AP the microvascular circulation is affected, which compromises oxygenation of the tissue[2].

The clinical manifestations of AP include upper abdominal pain and symptoms related to the systemic inflammatory response. In complicated cases with involvement of remote organ systems mortality is increased to 5% with ranges from 0% to 47% depending on the severity of the disease. The current treatment of AP is mainly supportive care using analgesics and relevant measures when other organs are involved. Enteral nutrition must be initiated as soon as possible and whenever patients are unable to eat, feeding tubes should be used. Supplementary treatment often involves antibiotics when an infection is suspected and surgery or endoscopic ultrasonic (EUS)-guided drainage in case of infected necrotizing pancreatitis[4].

AP after ERCP is a common complication and reported incidences vary from 5% to 15% in larger series. The majority of the cases are mild self-limiting conditions but up to 1% may develop a severe and potentially fatal pancreatitis. Although the pathophysiological mechanism remains to be elucidated several risk factors are identified: known sphincter Oddi dysfunction, sphincterotomy, injection of contrast more than one time and experience of the endoscopists[5-7].

## METHODS

Published trials were identified on PubMed using the MeSH term "AP" in combination with the following MeSH terms: steroids, cortisone, hydrocortisone, corticosteroids, nitroglycerin (NGL), non-steroidal anti-inflammatory drugs (NSAID), celecoxib, COX-Ⅱ, diclofenac, indomethacin, interleukin 10 (IL-10), tumor necrosis factor-alpha (TNF-α), infliximab, Remicade®, etanercept, adalimumab, Humira®, platelet activating-factor (PAF), lexipafant, antibiotics, somatostatin (SS), octreotide, Sandostatin®, probiotics, gabexate, nutrition. Only articles in English were included. Earlier published reviews were manually examined for other relevant studies.

### *SS/octreotide*

SS is a peptide hormone mainly produced in the gastrointestinal tract, where it has an inhibitory effect on gastric emptying, intestinal motility and intestinal blood flow. Furthermore, SS strongly inhibits the production of pancreatic enzymes, which has been the basis for using SS or its analogue octreotide as treatment for AP[8].

Both experimental and clinical trials have been conducted with SS and octreotide, but no effect on the course of the disease has hitherto been demonstrated. Most of the clinical trials comprised only few patients and often patients with interstitial pancreatitis were included although this condition is self-limiting and does not require specific therapy[9]. The largest and best conducted study is a German prospective multicenter study with 302 patients from 32 hospitals with moderate to severe AP randomized to either octreotide or placebo. This study revealed no significant differences between the treatment groups with respect to mortality, rate of complications, surgical interventions or length of hospital stay[10].

Several studies have examined SS or octreotide as prophylaxis to PEP. Various regimes have demonstrated a reduced incidence of PEP compared to placebo: SS administered as a bolus immediately after ERCP (4.4% *vs* 13.3%, $P = 0.01$)[11], SS given as a 12-h continuous infusion starting 30 min before ERCP (1.7% *vs* 9.8%, $P < 0.05$)[12] and octreotide in repeated injections starting 24 h prior to ERCP (2% *vs* 8.9%, $P = 0.03$)[13]. It should be noted that these studies have a fairly high incidence of PEP in the placebo groups. Andriulli *et al* have performed two similar

large, double blind, multicenter, placebo-controlled trials using SS. They used a dosage of 750 micrograms SS as an infusion starting 30 min prior to ERCP, ending 2 h (SS = 183, placebo = 199) or 6 h (SS = 351, placebo = 395) after ERCP. The incidences of PEP in the placebo groups were 6.5% and 4.8% respectively and no advantageous effect of SS was observed[14,15].

The reports published during the years 2002 to 2006 have been summarized in a meta-analysis, which concluded that SS or octreotide have no effect as prophylaxis prior to ERCP[16]. However, this meta-analysis did not include the most recent trial from China with 832 patients. In this study, octreotide was administered as a combination of intravenous infusion and subcutaneous injections and the incidence of PEP in the treatment group ($n$ = 414) and the placebo group ($n$ = 418) was 2.42% and 5.26% respectively ($P$ = 0.046)[17].

Octreotide and SS have thus been investigated in several clinical studies and may have an advantageous effect as prophylaxis prior to ERCP. Optimal dosage and cost-effectiveness still need to be elucidated.

### Protease inhibitor-Gabexate mesilate (GM)

The intracellular activation of proteases is a mandatory step in the development of AP and the protease inhibitors could theoretically have an effect in the treatment of AP or as prophylaxis prior to ERCP. The first protease inhibitor, Aprotinin, was widely used in the 1960's but randomized trials could not demonstrate any beneficial effect[18,19]. GM is a synthetic protease inhibitor, which improve histology score in animal models of AP[20].

In the 1980's several reports with a varying number of patients with AP ($n$ = 42 to 223) have been published but none showed any advantage of GM[21-26]. Conversely Chen *et al* observed a significant improved survival in a randomized trial including 52 patients with severe AP who received GM (mortality 33% *vs* 8%)[27]. A meta-analysis later concluded that GM may reduce the mortality in patients with moderate to severe pancreatitis, but the authors also noted that poor quality of the included randomized trials limits the power of this meta-analysis[28].

Several papers from Japan report a reduced mortality rate in patients with necrotizing AP receiving GM as continuous regional arterial infusion (CRAI). However this conclusion is based merely on clinical observations and not placebo-controlled randomized trials[29,30].

Looking at the effect on PEP two large studies by Andriulli *et al* with in total 1172 patients did not reveal any beneficial effect of GM. These results are in conflict with an earlier study by Cavallini *et al*, who in a study of 418 patients observed a PEP incidence of 6% in the GM group and 14% in the placebo group ($P$ = 0.009)[31]. However, a recent meta-analysis concludes that GM does not have an advantageous effect as prophylaxis to PEP[32].

The question continues to be a matter of debate and based on their trial with 608 patients, Manes *et al* argue that high-risk patients may benefit from GM. They administered GM either before or after ERCP compared to a saline solution. The incidence of PEP was 9.4% in the placebo group, and significantly lower ($P$ < 0.01) in the GM groups regardless of the time GM was administered (before ERCP 3.9%, after ERCP 3.4%)[33].

The FDA does not approve GM and the use of GM is not recommended in published guidelines concerning the treatment of AP[4,2,34]. However, Japanese national guidelines recommend the use of protease inhibitors either applied intravenously or as CRAI[35] and GM is also approved in Italy where it is also used as prophylaxis against PEP[36].

### Antioxidants

Oxidative stress most likely plays a major role in the early development of AP[37] and several experimental animal models show a beneficial effect of anti-oxidative drugs[38-44]. In humans depletion of antioxidants is observed in AP[45,46] correlating to the severity of the disease[47].

Therapy with antioxidants administered intravenously has been investigated in a prospective double-blind placebo controlled randomized trial on patients with predicted severe AP but no effect on mortality could be demonstrated[48]. The prophylactic effect on the incidence of PEP was tested in two randomized prospective randomized trials with 256 and 106 patients, respectively. N-acetylcysteine (NAC) was administered before and after ERCP and both studies concluded that NAC was without any preventive effect[49,50]. Thus, there is no evidence for the use of NAC.

### NGL

NGL is a donor of nitrogen oxide (NO), which causes vasodilatation and reduces cardiac preload. The main indication for using NGL is angina pectoris[51].

Experimental animal models have shown reduced pancreatic edema when administering NO as an infusion[52], but until now no clinical randomized trials using NGL in the treatment of AP have been published. As prophylaxis prior to ERCP three prospective randomized trials have evaluated NGL. NO induces periampullary sphincter relaxation and dilation of the micro vascular vessels, which hypothetically improve pancreatic circulation and nutrition[53].

Sudhindran *et al* observed in a study of 186 patients randomized to either NGL 2 mg sublingual 5 min prior to ERCP or placebo, an incidence of PEP in the NGL group of 8% compared to 18% ($P$ < 0.05) in the placebo group[54]. This finding was supported by Moreto *et al* who randomized 144 patients to either NGL as dermal patch or placebo, and found a significant reduction in the incidence of PEP (4% *vs* 16%, $P$ < 0.05)[55]. Both studies have been criticized for having a surprisingly high incidence of PEP in the placebo groups[56]. In a recently published prospective randomized trial of 318 patients the overall incidence of PEP was 7.5% and no significant difference between the NGL group and the placebo group was revealed[57].

NGL has the optimal qualities as a prophylactic agent as it is cheap and easy to administer. However further trials are needed to determine its potential use as prophylaxis against PEP.

### Corticosteroids

Corticosteroids are potent unspecific anti-inflammatory

drugs utilized in a variety of inflammatory diseases. Several case reports have suspected steroids of being the etiology to iatrogenic AP but a definitive relationship has not been established[58-60].

In rat models of AP hydrocortisone has reduced mortality and blood cytokine levels[61,62]. No human trials using steroids as treatment of AP have been published and attempts to show a beneficial effect of steroids as prophylaxis against PEP in prospective placebo-controlled trials have so far been disappointing. In 1999 De Palma *et al* randomized 539 patients to either placebo ($n$ = 266) or hydrocortisone 100 mg ($n$ = 273) administered intravenously prior to ERCP. The total incidence of PEP was 5.3% ($n$ = 28) and no significant difference between the two groups could be demonstrated[63]. In a Polish trial published in 2001, 300 patients received oral prednisone 40 mg, allopurinol 200 mg or placebo 15 h and 3 h prior to ERCP. The total incidence of PEP was 10.7% and no significant difference among the three groups was displayed[64]. Sherman *et al* have confirmed these negative findings in an even larger prospective trial with 1115 patients[65].

Although steroids have the potential to inhibit the inflammatory cascade there is no evidence for the use of neither hydrocortisone nor prednisone as prophylaxis against PEP.

### NSAID

NSAID have an analgesic as well as an anti-inflammatory effect. Most NSAID act as non-selective inhibitors of the enzyme COX which catalyses the formation of prostaglandins and thromboxane from arachidonic acid. NSAID are used for virtually every known inflammatory disease.

Salicylic acid and indomethacin have in isolated case reports been related to the development of AP[66-68] as has the selective cyclooxygenase (COX)-2 inhibitor celecoxib[69-72].

Experimental animal models studying the effect of NSAID on AP have been contradictory and not revealed any effect on mortality[73-76].

The only randomized human study on the therapeutic effect of NSAID on AP has been conducted by Ebbehøj *et al* who included 30 patients randomized in two groups receiving either indomethacin suppositories 50 mg twice daily for 7 d or placebo. No difference in serum amylase or calcium was observed but patients in the indomethacin group demanded less opiate as analgesics during hospitalization. Mortality was not registered[77].

Two studies testing the prophylactic effect of indomethacin given prior to ERCP to prevent PEP have been published. Montano *et al* included 117 patients, who received either indomethacin suppositories 100 mg or placebo 2 h prior to ERCP. The incidence of PEP was 2.5% and 6.8% respectively but the difference was not significantly different[78]. In a larger study from Iran 490 participants received 100 mg indomethacin suppositories or placebo and an incidence of PEP of 3.2% in the indomethacin group and 6.8% in the placebo group was observed. The difference was only borderline significant different ($P$ = 0.06) and a post hoc analysis showed significant lower incidence of PEP in the subpopulation of patients who underwent pancreatography[79]. However this conclusion was hampered by the fact that the post hoc analysis was conducted on 10 subpopulations, which in general reduces the statistical power considerably[80].

Another NSAID, diclofenac, has been investigated in a study including 220 patients receiving either diclofenac suppositories 100 mg or placebo immediately after ERCP. PEP occurred with lower frequency in the group receiving diclofenac compared to the placebo group (6% *vs* 15%, $P < 0.05$)[81].

The overall impression from placebo-controlled trials suggests a beneficial effect of NSAID used as prophylaxis against PEP. Both diclofenac and indomethacin can be administered easily as suppositories and are inexpensive drugs. Still, placebo controlled randomized trials with a larger sample size are needed to verify this promising effect.

### IL-10

IL-10 is produced and released by the helper T cells and its primary effect is anti-inflammatory. Clinical observations have shown increased levels of IL-10 in the blood during AP but its role in the treatment of AP remains to be determined[82,83]. The effect of IL-10 on AP has been validated in two experimental studies which showed a reduced mortality[84,85].

No human study on the therapeutic effect of IL-10 has been conducted but the prophylactic effect of IL-10 on PEP has been evaluated in two randomized studies. No significant difference among the IL-10 and placebo-treated group was observed in a study with 200 patients receiving either recombinant IL-10 (8 μg/kg) or placebo (9% *vs* 11%, $P$ = 0.65)[86]. A second study randomized 137 patients to placebo or IL-10 (4 μg/kg or 20 μg/kg) administered 30 min prior to ERCP. Overall incidence of PEP was 14% and a significant difference in the incidence among the three groups was noted (24%, 10%, and 7%). However the incidence of PEP in the placebo was remarkable high[87].

The results from these two published studies do not definitively support the use of IL-10 as prophylaxis against PEP.

### TNF-α

During AP the serum level of TNF-α is elevated[88,89]. The synthesis and release of TNF-α takes place in macrophages located in the pancreas. SAC may as well release TNF-α and do also express TNF-α receptors during AP[90-92]. A possible relationship between genetic polymorphism and severity of AP has been established[93].

Blocking the TNF-α mediated inflammation with anti-TNF-α antibodies or pentoxifylline seems to have a beneficial effect on histology score and mortality in experimental animal models[94-98].

No data on humans has hitherto been published apart from a single case-report concerning a patient with interstitial pancreatitis. In this case, a male patient with severe bloody diarrhea due to segmental Crohn's disease also showed signs of AP. Serum amylase was high and ultrasound and abdominal computer tomography (CT) scans revealed an edematous pancreas. Because of these

findings treatment with steroids and azathioprine was abandoned and instead a single infusion with infliximab 5 mg/kg was administered without complications. The patient's overall condition improved and serum amylase levels normalized[99].

Thus experimental data suggest a potential role of specific TNF-α inhibition in the treatment of AP, but high risk of bacterial infection during AP is a matter of concern. Infliximab has been evaluated in alcoholic hepatitis, another condition associated with a high risk of bacterial infection. The administration of prednisolone 40 mg daily and infliximab 10 mg/kg at wk 0, 2 and 4 showed an increased mortality due to infection and the study was terminated prematurely by the monitoring committee[100].

Hence clinical studies on AP must be carefully designed to evaluate the safety of infliximab or other specific TNF-α inhibitor.

### PAF

PAF was discovered in the 1970's and soon recognized to be an important inflammatory mediator[101]. Later studies with experimental pancreatitis revealed that PAF is released during AP[102] and induce AP when infused in arteries supplying the pancreas[103,104]. Experimental studies have shown a benefit from PAF inhibition with various antagonists on pancreatic edema and systemic inflammation as well as a decreased bacterial translocation[105-107].

As a consequence of the promising results with experimental AP different clinical studies have evaluated the effect of PAF inhibition. The first trial consisted of 83 patients with AP receiving lexipafant ($n$ = 42) or placebo ($n$ = 41). Lexipafant was administered intravenously (60 mg/d for 3 d) and follow-up was assessed for 5 d by Organ Failure Score (OFS). The investigators reported a greater reduction in OFS in the lexipafant group (0.905 *vs* 0.341, $P$ = 0.048), but during the 5 d period mortality was unaffected[108]. These findings were confirmed in a second trial including only patients with severe AP. The participants received lexipafant ($n$ = 27), 100 mg/d for 5-7 d or placebo ($n$ = 23). In the treatment group a larger reduction in OFS was registered (1.42 *vs* 0.17, $P$ = 0.003). Overall mortality was 18% with no difference between the groups[109]. The last study was published in 2001 and involved 286 patients with severe AP. Lexipafant (100 mg/d, $n$ = 148) or placebo ($n$ = 138) was administered for 7 d. No positive effect could be shown neither on OFS nor mortality[110]. It has been argued that data on the effect of lexipafant on mortality from experimental AP were warranted before initiation of human trials and the sponsor's communication of the result has been questionable[111]. After the termination of the clinical trials the lack of effect on mortality in experimental AP was acknowledged[112]. Randomized trials on sepsis were also disappointing[113,114] and inhibition of PAF in the treatment of AP has thus been abandoned.

### Antibiotics

Antibiotics is used to prevent or treat infected necrosis in the pancreas and does not have potential to change the pathobiologic course of AP. Infected necrosis in pancreas is a major clinical problem during AP which severely deteriorate the prognosis[115,116]. Hence, administration of antibiotics to prevent infection has been evaluated in several randomized trials.

Sainio *et al* randomized 60 patients with necrotizing pancreatitis. The inclusion criteria were C-reactive protein > 120 mg/L and pancreatic necrosis verified by an abdominal CT scan. The treated group received infusion of cefuroxim 1.5 g × 3 daily, while patients allocated to the control group only received antibiotics in case of clinical signs of infection. A significant higher mortality was registered in the control group compared to the cefuroxim group (23% *vs* 3%, $P$ = 0.03)[117].

Pederzoli *et al* randomized 74 patients with CT verified pancreatic necrosis to receive either imipenem or placebo but no effect on mortality was registered[118]. In another prospective randomized study with 90 patients Nordback *et al* administered imipenem intravenously 1.0 g × 3 daily and found a reduced incidence of multiorgan failure compared to the control group (28% *vs* 76%, $P$ = 0.0003) but no difference in mortality[119].

The studies described above are open-label trials. In 2004 Isenmann *et al* published a controlled double-blind study of 114 patients with CT verified necrotizing AP. The inclusion criteria were C-reactive protein > 150 mg/L and/or CT-verified pancreas necrosis. Placebo was compared to a combination of ciprofloxacin and metronidazole and if any complications occurred the treatment was converted to open conventional treatment. No difference in mortality or incidence of pancreas necrosis could be shown[120].

A Cochrane meta-analysis of 294 patients with CT-verified pancreas necrosis showed a reduced mortality in patients with necrotizing AP when beta-lactams and quinolones were administered intravenously as prophylaxis[121].

The subject continues to be a matter of debate and recommendations from major clinical associations have different approaches to this issue[4,2,34].

Studies on the effect of prophylactic antibiotics given prior to ERCP are limited. In a study by Raty *et al* with 315 patients cephtazidime was administered intravenously prior to ERCP compared with a control group. They found reduced incidence of PEP and cholangitis in the treatment group (3% *vs* 9%, $P$ = 0.009)[122]. However the study was not placebo-controlled and routine administration of antibiotics prior to ERCP cannot be recommended until randomized placebo controlled studies confirm this finding.

### Probiotics

Intestinal permeability is increased during AP, which may facilitate translocation of bacteria from the intestinal lumen. Oral probiotics are living microorganisms that exert health benefits beyond those of inherent basic nutrition[123].

Olah *et al* conducted two prospective placebo controlled double-blinded studies of the therapeutic effect of probiotics to AP. The studies were published in 2002

and 2007 including 45 and 62 patients with interstitial or severe AP. In the latter study reduced mortality in the probiotics groups was observed but the difference was not statistical significant[124,125].

## CONCLUSION

As described in this review we only have limited evidence based pharmacological approaches when treating AP and none of these are curative. Several treatments including animal experimental studies have been tried in order to establish evidence for etiology based medical treatment (Tables 1 and 2). In Italy and Japan gabexate is used routinely with the aim to limit pancreatic auto-digestion, but as reported in this review there is no conclusive evidence for this approach.

Octreotide may be considered as prophylaxis against PEP but high cost of this peptide hormone limits its potential in clinical practice. A much cheaper alternative is NSAID, which may be considered as prophylaxis against PEP.

Antibiotics are the drugs of choice when infection is evident. However, recommendations regarding the prevention of infected pancreatic necrosis are contradictory.

Various problems are encountered when designing clinical studies of AP. The low incidence of severe necrotizing AP constitutes a major problem, which demands multicenter studies. Another clinical challenge is the resemblance of AP to infection and before initialization of any experimental anti-inflammatory therapy bacterial infection must be refuted. This delays the start-up of the experimental protocol and causes bias as the patients in the meantime receive anti-bacterial treatment. A possible solution could be to administer antibiotics to all patients in combination with specific anti-inflammatory treatment or placebo.

In spite of these challenges the search for pharmacological treatment of AP must be sustained. As we present in this review experimental animal models support a potential effect of several anti-inflammatory drugs, which are candidates for randomized trials. The most interesting of these potential drugs is probably steroids, which are standard treatment of numerous inflammatory diseases but have never been investigated in the treatment of AP.

Because the outcome of the disease depends highly on the involvement of other organs, developing methods that inhibit the inflammatory signaling pathways presents a great potential. As new information regarding the inflammatory pathways continues to emerge from animal and clinical trials, specific treatment targeting these inflammatory processes should be considered. It is our opinion that animal models at this time support clinical trials with anti-TNF-α antibodies although a randomized trial must be designed not forgetting the safety issue.

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# Intraductal papillary mucinous neoplasms and other pancreatic cystic lesions

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## Abstract

Pancreatic cystic neoplasms are being increasingly recognized, even in the absence of symptoms, in large part, due to markedly improved imaging modalities such as magnetic resonance imaging (MRI)/magnetic resonance cholangio pancreatography (MRCP) and computer tomography (CT) scanning. During the past 2 decades, better imaging of these cystic lesions has resulted in definition of different types, including pancreatic intraductal papillary mucinous neoplasms (IPMN). While IPMN represent only a distinct minority of all pancreatic cancers, they appear to be a relatively frequent neoplastic form of pancreatic cystic neoplasm. Moreover, IPMN have a much better outcome and prognosis compared to pancreatic ductal adenocarcinomas. Therefore, recognition of this entity is exceedingly important for the clinician involved in diagnosis and further evaluation of a potentially curable form of pancreatic cancer.



**Key words:** Pancreatic cancer; Pancreatic intraductal papillary mucinous neoplasms; Mucinous cystic neoplasm of pancreas; Serous cystadenoma; Pancreatic cystic lesions

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## INTRODUCTION

Due to increasing precision of modern imaging modalities, particularly computer tomography (CT) scanning (with contrast enhancement) and magnetic resonance imaging (MRI)/magnetic resonance cholangio pancreatography (MRCP), pancreatic cystic lesions are commonly being detected. This has been reported in up to 25% of patients, particularly with increasing age[1,2]. From a pathological rather than imaging perspective, however, a cyst is formally defined as a fluid-filled and closed cavity with an epithelial lining. In the pancreas (as opposed to liver and spleen), cyst-like lesions have special significance as different neoplasms in the pancreas are true cysts, or alternatively, may appear cystic either from dilation of a tumor obstructed or stenosed pancreatic duct or from necrotic changes and degeneration within a solid neoplastic lesion, possibly from rapid tumor growth that outstrips its blood supply.

Most commonly, a pancreatic cystic lesion defined by imaging represents a pseudocyst, usually due to alcoholic pancreatitis. Pseudocysts are distributed evenly throughout the pancreas and have no evident risk of malignancy. In a pseudocyst, no epithelial lining is present. As such, the pathological criteria for a true cyst (despite its cystic imaging appearance) are not satisfied. Most other true cysts (with the exception of congenital pancreatic cysts) are neoplastic and, therefore, these represent a potentially significant clinical finding[3,4]. A number of different neoplastic cystic lesions in the pancreas have been identified and labeled as capitalized abbreviations (Table 1)[4]. As prognosis for each type of cystic neoplasm may differ, precise definition of any imaged cystic lesion, even if asymptomatic or incidentally detected, is crucial[5].

## NEOPLASTIC PANCREATIC CYSTIC LESIONS

Most neoplastic cystic lesions of the pancreas occur in young or middle-aged females [serous cystadenoma (SCA), mucinous cystic neoplasia (MCN), and solid pseudopapillary neoplasia (SPN)], however, intraductal papillary mucinous neoplasms (IPMN) are most often detected in elderly males (moreso than females)[4]. Most cystic neoplasms are evenly distributed throughout the pancreas, however, the pancreatic head and uncinate process are

**Table 1 Types of pancreatic cystic neoplasms**

| Types of pancreatic cystic neoplasms |
|---|
| Serous cystadenoma, or SCA |
| Mucinous cystic neoplasm, or MCN |
| Intraductal papillary mucinous neoplasm, or IPMN |
| Solid pseudopapillary neoplasm (with degeneration), or SPN |
| Cystic necrosis (endocrine tumor, ductal adenocarcinoma) |

most common sites for IPMN while the body and tail are most common sites for MCN[4]. For most cystic types, malignant potential appears to be low, except for MCN and rapidly growing pancreatic ductal adenocarcinomas or even more rare endocrine neoplasms[4]. In contrast, most IPMN are slow growing and have low malignant potential, with a much better prognosis, especially if compared to pancreatic ductal adenocarcinoma[4]. Thus, their recognition is significant because an opportunity may be present at the time of recognition to resect surgically this type of pancreatic cystic neoplasm.

## INTRADUCTAL PAPILLARY MUCINOUS NEOPLASMS

IPMN appears to be quite a unique type of neoplasm, representing a broad spectrum of mucin-producing lesions of the exocrine pancreas classified as benign adenomas to invasive carcinoma. IPMN appear to arise from the epithelium of the main duct or its branches, often with variable duct dilation and IPMN are also believed by some to follow the so-called "adenoma-carcinoma" sequence. Neoplastic cells are most often papillary, although flat epithelium may also be defined.

IPMN of pancreas represents up to one-third of all pancreatic cystic neoplasms, but only about 1% of all pancreatic cancers[3,6]. Of particular note, IPMN also represents about 25% of all surgically resected pancreatic neoplasms, again likely emphasizing the critical significance of early recognition[6]. IPMN was first described by Ohhashi and colleagues in 1982 followed by a notation by the same group of a very prolonged survival (over a quarter century) of a case of IPMN[7]. The first series of North American patients were described only in 1990[8]. Since then, interest in this neoplasm appears to have increased exponentially as reflected in the annual number of publications in Medline from 1994 to 2006 related to IPMN noted elsewhere[6]. Interestingly, IPMN has also been documented to be multifocal and there is a higher occurrence rate of synchronous extrapancreatic malignancies (compared to pancreatic ductal adenocarcinoma)[9]. Finally, independent synchronous or metachronous pancreatic ductal adenocarcinomas[10] as well as endocrine tumors[11] with IPMN have also been described.

Based on anatomic involvement of the pancreatic duct, IPMN of the pancreas may predominantly involve the main pancreatic duct ("main duct type"), secondary ducts ("branch duct type") or both ("mixed type"). Branch duct type IPMN are usually smaller, less papillary and tend to occur in the periphery of the pancreatic head or in the distal pancreas. Malignant transformations occur less often in the branch duct type. Evidence suggests that asymptomatic branch duct IPMN less than 3 cm in size without mural nodules, thickened septa or high grade dysplasia have a relatively benign biological behavior and should be considered optimal candidates for surgical treatment[6].

Diagnosis may be enhanced by MRCP combined with dynamic imaging since this improves localization, staging and, most important, the potential for surgical resectability. A contrast-enhanced CT scan may also yield added information regarding invasion, but also the relationship of the lesion to contiguous organs and vessels. Finally, endoscopic visualization of a patulous or so-called "fish-eye" papilla of Vater , especially at the time of ERCP may be helpful, if not pathognomonic. ERCP also permits an opportunity for added sampling of ductal content for cytology. Endoscopic ultrasonograph (EUS) may also provide added imaging information and permit fine needle aspiration biopsy although some concerns have been raised regarding the risk of seeding and dissemination of malignant cells[4]. Positron emission tomographic scanning may be helpful but its role needs to be better defined. Finally, serological studies including CA 19-9 and CEA may serve some value but it is limited since these are reported to be elevated in less than 20% of IPMN[4]. For pre-operative evaluation, all of these investigations may provide useful information, but are most important if surgical resection is being contemplated. Unfortunately, some patients are older with other concomitant health issues. In these, surgical treatment and resection may not lead to a significant positive long-term result and, as in these patients, even a more judicious approach to invasive evaluative investigations may be reasonable. If surgical excision is complete, recurrence may still occur, usually at a distant site, but this rate of recurrence is limited, and the overall 5-year survival rate has been reported to exceed 80% for noninvasive IPMN and approximately 50% for malignant invasive IPMN. Thus, accurate evaluation of IPMN is exceedingly important as a recent analysis[4] has suggested that this is one of the few surgically curable pancreatic neoplasms.

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*REVIEW*

# Proton pump inhibitors in cirrhosis: Tradition or evidence based practice?

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## Abstract

Proton Pump Inhibitors (PPI) are very effective in inhibiting acid secretion and are extensively used in many acid related diseases. They are also often used in patients with cirrhosis sometimes in the absence of a specific acid related disease, with the aim of preventing peptic complications in patients with variceal or hypertensive gastropathic bleeding receiving multidrug treatment. Contradicting reports support their use in cirrhosis and evidence of their efficacy in this condition is poor. Moreover there are convincing papers suggesting that acid secretion is reduced in patients with liver cirrhosis. With regard to Helicobacter pylori (*H pylori*) infection, its prevalence in patients with cirrhosis is largely variable among different studies, and it seems that *H pylori* eradication does not prevent gastro-duodenal ulcer formation and bleeding. With regard to the prevention and treatment of oesophageal complications after banding or sclerotherapy of oesophageal varices, there is little evidence for a protective role of PPI. Moreover, due to liver metabolism of PPI, the dose of most available PPIs should be reduced in cirrhotics. In conclusion, the use of this class of drugs seems more habit related than evidence-based eventually leading to an increase in health costs.



**Key words:** Proton pump inhibitors; Cirrhosis; *Helicobacter pylori* ; Peptic ulcer; CYP P450

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## INTRODUCTION

Proton Pump Inhibitors (PPI) are extensively used in different acid related diseases. Their efficacy in inhibiting acid secretion is well known[1-4], and the use of this class of drugs has increased worldwide. They act through inhibition of the $H^+/K^+$ ATPase of parietal cells producing the so called "inhibitory complex" and blocking HCl secretion[5]. They are metabolized in the liver by the CYP450 cytochrome[6].

PPI are also often used in patients with liver cirrhosis sometimes in the absence of a specific acid related disease, with the aim of preventing peptic complications in patients with variceal or hypertensive gastropathic bleeding receiving multidrug treatment.

The aim of this editorial is to revise the efficacy and safety profile of PPI in patients with liver cirrhosis.

## GASTRIC ACID SECRETION AND LIVER CIRRHOSIS

The role of gastric secretion in cirrhosis is controversial. Some studies report reduced acid production[7-10] while others reported normal production[11-15]. The evaluation of 24-h acidity by gastric ph-metry in 49 patients with cirrhosis showed a marked hypoacidity in patients with cirrhosis compared to controls, mainly during the night hours[16]. This may depend on hemodynamic alterations consequent to portal hypertension and is supported by experimental studies showing reduced gastric acid secretion in animals with portal hypertension[17,18]. These observations rule out the relevance of gastric acid in the pathogenesis of ulcers in cirrhotics.

Gastrin, the gastric hormone whose secretion is regulated by intragastric pH, and that regulates the production of HCl and pepsin, is partially metabolized

**Table 1 Prevalence of peptic ulcer in patients with liver cirrhosis**

| Investigator | Number of patients | Gastric ulcer prevalence (%) |
|---|---|---|
| Siringo, 1995 | 324 | 4.6 |
| Chen, 1996 | 245 | 20.8 |
| Tsai, 1998 | 130 | 16.1 |
| Kirk, 1980 | 163 | 14.7 |
| Rabinovitz, 1990 | 216 | 7.8 |

**Table 2 Gastric and duodenal ulcer in patients with liver cirrhosis according to the severity of portal hypertension (from Wu *et al* 1995)**

| | Controls ($n$ = 60) | | Compensated cirrhosis ($n$ = 60) | | Decompensated cirrhosis ($n$ = 60) | | $P$ |
|---|---|---|---|---|---|---|---|
| | $n$ | % | $n$ | % | $n$ | % | |
| Duodenal ulcer | 2 | 3.3 | 10 | 16.7 | 8 | 13.3 | 0.046 |
| Gastric ulcer | 1 | 1.7 | 2 | 3.3 | 9 | 15.0 | 0.006 |
| All ulcers | 3 | 5.0 | 12 | 20.0 | 17 | 28.3 | 0.003 |

by the liver and mainly by the kidneys. Gastrin is elevated in serum of patients with Helicobacter pylori (*H pylori*) infection or atrophic gastritis. Few studies have evaluated gastrin levels in cirrhosis, and their contribution towards understanding the pathophysiology of gastric acid secretion is very limited. Avgerinos *et al*[19] evaluated the urinary gastrin output in patients with cirrhosis with and without hepato-renal syndrome. Serum gastrin levels were higher in cirrhotics compared to controls; and in cirrhotics with hepato-renal syndrome the difference was greater suggesting that impaired urinary gastrin secretion may contribute to their hypergastrinemia. The same results were found by Lo *et al*[11] who also showed a significantly lower maximal pepsin output in cirrhotics compared to controls.

Progastrin and gastrin serum levels have been reported to be significantly higher in patients with cirrhosis of any Child-Pugh class compared to controls while there are no differences between controls and patients with chronic hepatitis B or C[20]. Indeed, it is important to note that in this study, the prevalence of *H pylori* infection in cirrhotic patients was 83% *versus* 50% in controls. Therefore, it is not clear whether the difference in progastrin and gastrin level was due to reduced liver metabolism, to *H pylori* infection, or both. In summary, gastrin increase in patients with liver cirrhosis could be related to: (1) impaired hepatic gastrin catabolism; (2) impaired renal function, at least in those with HRS; (3) gastric mucosal alteration due to gastropathy-related cirrhosis.

## PEPTIC ULCERS AND LIVER CIRRHOSIS

Many authors reported an increased prevalence of peptic ulcers in patients with cirrhosis[21,22] and it was shown that cirrhotics have an increased risk of developing gastric or duodenal ulcers during an interval of one year compared to non cirrhotics[23]. The prevalence of peptic ulcers ranges between 4.6% and 21% in patients with cirrhosis[21,22,24-26,39] (Table 1). However, the pathogenesis of this finding is far from being elucidated and different factors have been proposed in relation to increased ulcer prevalence in patients with cirrhosis. Furthermore the prevalence of duodenal and gastric ulcers in patients with liver cirrhosis increases with disease progression[27] (Table 2). Several theories have been postulated. It has been demonstrated that the gastric mucosa in rats with portal hypertension is more susceptible to aggressive agents such as bile acids, aspirin and alcohol[28]. Some investigators have attributed to portal hypertension itself the increased risk of peptic ulcer[29], nevertheless no study has clarified the pathogenesis of peptic ulceration in cirrhosis.

## *H PYLORI* IN PATIENTS WITH LIVER CIRRHOSIS

The prevalence of *H pylori* in patients with cirrhosis has been investigated in many epidemiological studies with values ranging from 27% to 89%[24,27,30-33]. This large variability may be due to the test used to evaluate *H pylori* infection. In the study with the largest prevalence of *H pylori* infection, values were been obtained by titration of serum IgG, against *H pylori*. The tests usually used for evaluating the presence of *H pylori* should be revised since haemodynamic alterations in cirrhosis could impair the results of urea 13C BT, and hypergammaglobulinemia typical of cirrhosis, might produce a false positive test[34-38]. Italian studies generally and sometimes significantly showed a higher prevalence than in non cirrhotic patients, while studies from Taiwan failed to show a similar trend. When evaluating the prevalence of *H pylori* infection in cirrhotics there seems to be no relationship between the aetiology of cirrhosis and the prevalence of *H pylori* evaluated by determination of serum IgG[24]. The role of *H pylori* in determining peptic ulceration in cirrhosis is controversial: some authors conclude that the increased risk of gastroduodenal ulcer is not related to *H pylori* infection, whilst others conclude that peptic disease and non-ulcer dyspepsia are firmly linked to *H pylori* infection[32,39-41]. A meta-analysis showed an increased risk of ulcers developing in patients with *H pylori* infection and cirrhosis[42].

If *H pylori* infection were an etiopathological factor implicated in digestive bleeding in cirrhosis, eradication of infection would decrease the risk of ulcer recurrence. However a study aiming to investigate the role of *H pylori* eradication in cirrhotics demonstrated a similar recurrence rate between cirrhotics with successful *H pylori* eradication and those with active *H pylori* infection[43]. In conclusion, the role of *H pylori* infection in the occurrence of gastric or duodenal ulcers or in determining digestive bleeding in the setting of liver cirrhosis is still unclear.

## ESOPHAGEAL DISORDERS AND LIVER CIRRHOSIS

It has been postulated in the past, that gastro-esophageal reflux may contribute to oesophagitis and variceal

bleeding in cirrhotic patients[44], and acid reflux could be exacerbated by the presence of ascites and water retention[45]. More recent papers do not confirm these hypotheses[46,47] and report a high incidence of gastro-esophageal reflux only in patients with alcoholic cirrhosis, though the presence of reflux did not correlate with disease severity or bleeding episodes[48]. Functional studies showed decreased lower esophageal sphincter function with low amplitude of primary peristalsis and acid clearance in patients with large varices[49-51]. These phenomenon could also be due to a mechanical effect of the presence of varices. In conclusion, it is unclear whether the presence of cirrhosis itself could predispose to the onset of gastro-esophageal reflux. It seems that the presence of varices is related to reflux episodes, although it is not clear whether these might contribute to bleeding from varices.

Another more studied point is the fact that endoscopic treatment for variceal bleeding or prevention of bleeding varices, may produce oesophageal motility dysfunction. Several studies evaluated the effect of endoscopic variceal sclerotherapy (EVS) on gastro-oesophageal reflux. Some authors suggest that endoscopic treatment produces an acute impairment of oesophageal motility which is partially restored after days or weeks[52-54], others suggest that sclerotherapy produce a chemical esophagitis that impairs oesophageal motility and in turn may favour acid related reflux esophagitis[55]. It seems that endoscopic variceal ligation (EVL) is safer in terms of oesophageal dysmotility induction when compared to EVS[56-59]. The reason for this finding is unclear. Autoptical studies after EVS show the presence of obliteration of the submucosal vascular channels, fibrosis and oesophagitis[60] reflecting the necrosis induced by the sclerosing agent. The inflammation caused by EVS may justify motor dysfunctions and acid reflux. Avgerinos *et al*[61] showed that EVL produces a higher early increase in lower oesophageal sphincter pressure, and this might prevent gastro-oesophageal reflux.

Apart from the pathogenesis of motor dysfunction following EVS and EVL, these procedures are related to local complications such as oesophageal ulcerations, strictures and perforations[62,63]; although from this point of view, EVL seem to be safer than EVS[64,65]. Uncontrolled non randomized studies, showed that PPI may have a role in the prevention and healing of post-EVS ulcerations[66-69] although this was not confirmed by other authors[70]. With regard to post EVL ulcers, the incidence is between 2% to 5%[71,72]. Pantoprazole has been shown to reduce the size of ulcers in patients undergoing elective band ligation, but not the rate of occurrence or the symptoms[73]. Given the relatively benign nature of the intervention, the authors conclude that PPI treatment is advisable in patients undergoing elective EVL.

In summary, expert opinion based on evidence of scarce value, advise PPI use in cirrhotic patients undergoing endoscopic treatment for varices, especially when treatment is performed by EVS, to prevent gastro-esophageal reflux which may worsen the procedure related inflammation or ulceration.

## PPI SAFETY IN CIRRHOTIC PATIENTS

Acute hepatitis due to PPI use is described in the literature for most PPIs available on the market[74-79]. All PPIs are metabolized in the liver by cytochrome CYP450; two isoenzymes are involved in PPI metabolism (CYP2C19 and CYP3A4)[6]. CYP2C19 is the main metabolic pathway while CYP3A4 is activated only when the other enzyme is saturated[80]. Nevertheless, the affinity of each isoenzyme for different PPIs is different and rabeprazole is metabolized mainly by a non enzymatic pathway. There are two CYP2C19 phenotypes: extensive and poor metabolisers[81-83]. The poor phenotype is present in 2%-6% of Caucasians and 20% of the Asian population. Poor metabolisers have higher plasma levels of PPI, which could lead to higher efficacy but also to potential adverse events. The effects of these genotypes varies according to the specific PPI used and in general is greater when using omeprazole decreasing progressively to lansoprazole, esomeprazole, pantoprazole and finally rabeprazole[6,83].

PPI are metabolized in the liver and secreted by the kidney. Renal impairment has minimal effect on PPI clearance, and therefore there is no need to reduce PPI dosage in patients with renal diseases[80,84]. This is not the case for liver impairment in which the Area under the Curve (AUC) of PPIs increases and their half-life becomes 4 h to 8 h greater[80] with increasing risk of accumulation. This effect was also seen with rabeprazole[85] although a dose reduction seems to be unnecessary with a 20 mg, once daily dose in patients with mild to moderate liver cirrhosis. When using other PPIs or rabeprazole at 40 mg/d dose, dose reduction in patients with cirrhosis is advisable.

## CONCLUSION

PPI drugs are extensively used in clinical practice in cirrhotic patients. Besides habit, the evidence that PPI are necessary in most indications is very weak. First of all, there is convincing evidence that acid secretion is reduced in patients with liver cirrhosis. This is mainly due to the presence of hypertensive gastropathy for which there is no evidence of any efficacy of PPI. With regard to *H pylori* infection, its prevalence in patients with cirrhosis is largely variable among different studies, probably as a result of different diagnostic tests used. We believe that the condition of hypochloridemia of cirrhotics makes it more probable that its prevalence is lower than in the general population. Nevertheless, it seems that *H pylori* eradication does not prevent from gastro-duodenal ulcer formation and bleeding.

It is probable that the main reason for PPI use in cirrhosis might be the prevention and treatment of oesophageal complications after banding or sclerotherapy of oesophageal varices. However even in this case evidence for a protective role of PPI are scarce. When using PPI in cirrhotic patients, the dose should be reduced in consideration of the increased half-life of these drugs in this group of patients. Dose adjustment does not seem necessary when using rabeprazole at a 20 mg, once daily

dose. The use of this class of drugs seems more habit-related than evidence- based, eventually leading to an increase in health costs.

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*TOPIC HIGHLIGHT*

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# Mechanisms of biliary carcinogenesis and growth

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## Abstract

Cholangiocarcinoma is a rare cancer originating from the neoplastic transformation of the epithelial cells (i.e. cholangiocytes) that line the biliary tract. The prognosis for patients with cholangiocarcinoma is grim due to lack of viable treatment options. The increase in world-wide incidence and mortality from cholangiocarcinoma highlights the importance of understanding the intracellular mechanisms that trigger the neoplastic transformation of cholangiocytes and the growth of biliary cancers. The purpose of the following review is to address what has been learned over the past decade concerning the molecular basis of cholangiocarcinogenesis. The material presented is divided into two sections: (1) mechanisms regulating neoplastic transformation of cholangiocytes; and (2) factors regulating cholangiocarcinoma growth. An understanding of the growth regulatory mechanisms of cholangiocarcinoma will lead to the identification of therapeutic targets for this devastating cancer.



**Key words:** Biliary carcinogenesis; Cholangiocarcinomas; Primary biliary cirrhosis; Primary sclerosing cholangitis

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## INTRODUCTION

Cholangiocytes are simple epithelial cells that line the intrahepatic biliary tract, which is a three-dimensional network of interconnecting ducts. The primary physiological function of cholangiocytes is the modification bile of canalicular origin and drainage of bile from the liver[1]. In addition to their role in the modification of ductal bile, cholangiocytes also participate in the detoxification of xenobiotics[1]. In recent years, interest in the study of cholangiocytes has increased dramatically due to a rise in the incidence of cholestatic liver diseases and biliary tract cancers (i.e. cholangiocarcinoma) in patients worldwide[2-5].

In diseases of the biliary tree (e.g. primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), liver allograft rejection and graft-*versus*-host disease), cholangiocytes are the primary target cells[1]. These cholangiopathies cause morbidity and mortality and are a major indication for liver transplantation[1]. In fact, these diseases contribute to 20% of the liver transplants in adults and 50% of those in pediatric patients[6]. Proliferation of cholangiocytes is critical for the maintenance of biliary mass and secretory function during the pathogenesis of chronic cholestatic liver diseases, such as primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC). Previous studies have demonstrated that proliferating cholangiocytes serve as a neuroendocrine compartment during liver disease pathogenesis and as such secrete and respond to a number of hormones and neuropeptides contributing to the autocrine and paracrine pathways that modulate liver inflammation and fibrosis, which are predicted to play key roles in cholangiocarcinogenesis[7].

Cholangiocarcinomas are adenocarcinomas that arise from the neoplastic transformation of cholangiocytes. Cholangiocarcinoma occurs in approximately 2 per 100 000 people and account for approximately 13% of primary liver cancers[4]. Cholangiocarcinomas can be divided into three categories based upon anatomic location: (1) intrahepatic

cholangiocarcinoma, occurring in the bile ducts residing within the liver; (2) hilar cholangiocarcinoma, occurring at the confluence of the right and left hepatic ducts; and (3) distal extrahepatic bile duct cancers[8]. The prognosis for cholangiocarcinoma is grim due to lack of early diagnostic modalities and effective treatment paradigms. Cholangiocarcinomas are slow growing, metastasize late during the cancer's progression, and present with symptoms of cholestasis due to the blockage of the bile duct by tumor growth[8]. In most cases, the tumors are well advanced at the time of diagnosis, which results in limited treatment options[9]. Many of these tumors are too advanced to be removed surgically and chemotherapy and radiation therapy usually are not effective, which indicates the dire need to understand the mechanisms that activate the neoplastic transformation of cholangiocytes and control the growth of cholangiocarcinomas.

## RISK FACTORS FOR CHOLANGIOCARCINOMA

Several recent studies have shown that there is an increasing incidence of cholangiocarcinoma world-wide and in particular in Western countries, such as the United States, the United Kingdom, and Australia[4,10-12]. Although most patients do not have identifiable risk factors for the disease, several risk factor have been established for cholangiocarcinoma[13]. The list of risk factors includes: gallstones or gallbladder inflammation, chronic ulcerative colitis, chronic infection of liver flukes, *Clonorchis sinensis* and *Opisthorchis viverrini*, and primary sclerosing cholangitis (PSC)[14]. In addition, a recent study revealed several novel risk factors for intrahepatic cholangiocarcinoma[15]. Hepatitis C virus infection, non-alcoholic fatty liver disease, obesity and smoking are all associated with intrahepatic cholangiocarcinoma[15]. The majority of these risk factors have the common features of chronic liver inflammation, cholestasis, and increased cholangiocyte turnover[16,17].

## MOLECULAR MECHANISMS CONTRIBUTING TO CHOLANGIOCARCINOGENESIS

A common and important contributor to the malignant transformation of cholangiocytes is chronic inflammation of the liver. This inflammation is often coupled with the injury of bile duct epithelium and obstruction of bile flow, which increases cholangiocyte turnover (i.e. dysregulation in the balance of cholangiocyte proliferation and apoptosis)[9,18]. Persistent inflammation is thought to promote carcinogenesis by causing DNA damage, activating tissue reparative proliferation, and by creating a local environment that is enriched with cytokines and other growth factors[19]. Thereby, chronic inflammation creates local environmental conditions that are primed for cells to develop autonomous proliferative signaling by constitutive activation of pro-proliferative intracellular signaling pathways and enhanced production of mitogenic factors. Indeed recent studies have demonstrated that cholangiocytes release cytokines, such as interleukin 6 (IL-6), transforming growth factor-β (TGF-β), IL-8, tumor necrosis factor-α (TNF-α) and platelet–derived growth factor (PDGF). These factors can interact with cholangiocytes in an autocrine/paracrine fashion to regulate cholangiocyte intracellular signaling responses, which are thought to be altered during cholangiocarcinoge nesis[20].

Cytokines activate inducible nitric oxide synthase (iNOS) in cholangiocytes. This results in the generation of nitric oxide, which along with other reactive oxygen species, may alter DNA bases, result in direct DNA damage and trigger the downregulation of DNA repair mechanisms[18]. Nitric oxide can directly or through the formation of peroxynitrite species can lead to the deamination of guanine and DNA adduct formation thereby promoting DNA mutations[18,21]. The resultant DNA damage leads to an increased mutation rate and alteration of genes critical to cell proliferation control. Consistent with this line of thought, activating mutations and the overexpression of EGFR, erb-2, K-ras, BRAF and hepatocyte growth factor/c-met (HGF) have been reported for cholangiocarcinoma. In addition, the proto-oncogene c-erbB-2 is activated in patients with cholangiocarcinoma[22,23]. Mutations affecting the promoter of the tumor suppressor $p16^{INK4a}$ that result in loss of transcription occur in both PSC and PSC-associated cholangiocarcinoma[24]. Alterations of p53, APC, and DPC4 tumor suppressor genes by a combination of chromosomal deletion, mutation, or methylation; and infrequently microsatellite instability have also been linked to cholangiocarcinoma[25]. In addition to increased ability of cholangiocytes to escape from senescence, activation of iNOS promotes the upregulation of COX-2 in immortalized mouse cholangiocytes suggesting that COX-2 and COX-2 derived prostanoids might play a key role in cholangiocarcinogenesis[26,27]. COX-2 also upregulated the expression of Notch, which has been implicated in other cancer types[27].

Cytokines, such as IL-6, appear to play an important role in cholangiocyte evasion of apoptosis. During the pathogenesis of cancer, the activation of evasion of apoptosis pathways helps to prevent cells with accumulating DNA damage from undergoing cell death pathways that normally eliminate such dysfunctional cells. Cholangiocarcinoma cells have been shown to secrete IL-6 and in an autocrine fashion IL-6 activates the pro-survival p38 mitogen activated protein kinase[28]. In fact, IL-6 upregulated the expression of myeloid cell leukemia-1 (Mcl-1) expression through STAT3 and AKT signaling pathways[29,30]. Mcl-1 is an anti-apoptotic protein in the Bcl-2 family of apoptotic proteins. Recently, the cellular expression of Mcl-1 has been shown to be controlled by the small endogenous RNA molecule, mir-29, which is down regulated in malignant cells consistent with Mcl-1 overexpression[31]. Enforced expression of mir-29 reduced Mcl-1 expression the malignant human cholangiocytes, KMCH[31]. Modulation of expression small endogenous RNAs such as mir-29 might represent a therapeutic paradigm for cholangiocarcinomas.

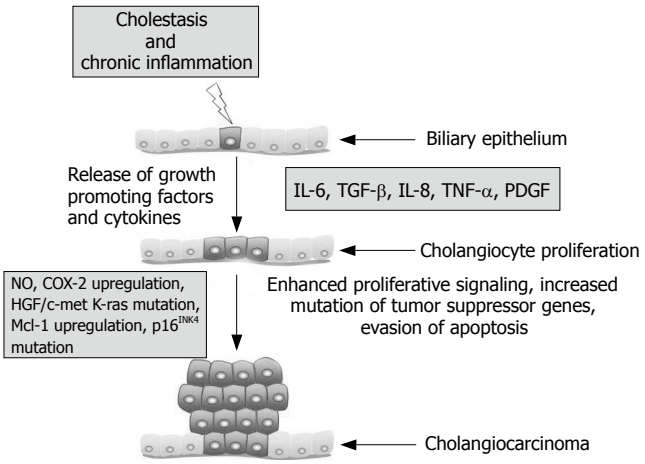


**Figure 1** Summary of key mechanisms regulating cholangiocarcinogenesis.

## FACTORS REGULATING CHOLANGIOCARCINOMA GROWTH

A number of recent studies have shown that the nervous system, neuropeptides and neuroendocrine hormones play a key role in the modulation of cholangiocarcinoma growth[32]. We have demonstrated that proliferating cholangiocytes serve as a neuroendocrine compartment during liver disease pathogenesis and as such secrete and respond to a number of hormones and neuropeptides contributing to the autocrine and paracrine pathways that modulate liver inflammation and fibrosis[7]. The sympathetic nervous system has been shown to have a role in the negative regulation of cholangiocarcinoma growth[33]. The cholangiocarcinoma cell lines, Mz-ChA-1 and TFK-1, express the $\alpha_{2A}$-, $\alpha_{2B}$-, and $\alpha_{2C}$-adrenergic receptor subtypes[33]. Stimulation of the $\alpha_2$ receptors induced an upregulation of intracellular cAMP, which inhibited EGF-induced MAPK activity through an increase in Raf-1 and the sustained activation of B-raf, which resulted in the subsequent reduction of cholangiocarcinoma proliferation[33]. Although muscarinic acetylcholine receptor are expressed by cholangiocytes and play a role in regulating secretin-induced bile flow in rodents, the role of the parasympathetic nervous system has not been evaluated in cholangiocarcinoma and warrants consideration as a factor regulating cholangiocarcinoma growth[34,35].

Other neuroendocrine hormones and neurotransmitters have also been shown to play a role in the regulation of cholangiocarcinoma growth. The cholangiocarcinoma cell lines, Mz-ChA-1, HuH-28 and TFK-1, express the gastrin/CCK-B receptor and gastrin inhibits the proliferation and induces the activation of apoptosis in these cholangiocarcinomas through the activation of $Ca^{2+}$-dependent PKC-α signaling. Most recently, Fava *et al* have shown that γ-aminobutyric acid (GABA) inhibits cholangiocarcinoma cell proliferation and migration[36]. This effect was also evident *in vivo* with GABA significantly decreasing the growth of cholangiocarcinoma tumor xenografts in nude mice[36].

Female steroid hormones, such as estrogens, have also been shown to play a role in the promoting of cholangiocarcinoma cell growth. 17-β estradiol stimulated the proliferation of human cholangiocarcinoma cells *in vitro*, which was blocked by tamoxifen[37]. Alvaro *et al* have demonstrated that human intrahepatic cholangiocarcinomas express the receptors for both estrogens and insulin-like growth factor (IGF-1)[38]. Their study indicates that estrogens and IGF-1 coordinately regulate cholangiocarcinoma growth and apoptosis[38].

Modulation of the endocannabinoid system is currently being targeted to develop possible therapeutic strategies for other cancer types. We recently demonstrated the novel finding that the two major endocannabinoids, anandamide and 2-arachidonylglycerol, have opposing actions on cholangiocarcinoma growth[39]. Interestingly, anandamide was found to be antiproliferative and to promote apoptosis, while, in contrast, 2-arachidonylglycerol stimulated cholangiocarcinoma cell growth[39]. Anandamide was shown to recruited Fas and Fas ligand into lipid rafts resulting in the activation of death receptor pathways and apoptosis in the cholangiocarcinoma cells[39].

## CONCLUSION

Cholangiocarcinoma is a devastating neoplasm of the biliary tract that is increasing in incidence. While treatment options are limited, our knowledge base of the factors controlling cholangiocarcinogenesis and cholangiocarcinoma growth has greatly expanded in the past decade. These studies have clearly demonstrated that, during the course of chronic cholestasis and associated liver inflammation, a number of factors are released into the local environment that set into motion a series of events compound genomic damage leading to autonomous proliferation and escape from apoptosis (Figure 1). Several potential areas seem promising for the development of prevention and treatment strategies for cholangiocarcinoma. In particular, inhibition of the COX-2 pathway during PSC warrants further investigation. Also, modulation of cholangiocarcinoma growth by the regulating neural imput or the endocannabinoid system might prove fruitful. Understanding the molecular mechanisms triggering biliary tract cancers will be key for the development of new treatments and diagnostic tools for cholangiocarcinoma.

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*TOPIC HIGHLIGHT*

**Gianfranco D Alpini, PhD, Professor; Sharon DeMorrow, Assistant Professor,** *Series Editor*

# c-Met targeted therapy of cholangiocarcinoma

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## Abstract

Cholangiocarcinoma continues to be a challenging disease to treat. Systemic therapy is used in unresectable disease, disease progression after surgery, and in the palliative setting. Unfortunately, results of multiple phase Ⅱ trials have rarely yielded positive results. As data on the molecular carcinogenesis of cholangiocarcinoma is developing, we are more able to understand the disease process and can use this understanding to create unique targeted therapies. We reviewed the role of c-Met/hepatocyte growth factor (HGF) in the development of cholangiocarcinoma. Furthermore, we explored the use of the c-Met guided cascade as a target to treat cholangiocarcinoma. We reviewed the current use and options for future development of c-Met agents to treat this disease.



**Key words:** Cholangiocarcinoma; c-Met; Chemotherapy; Target therapy

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## CHOLANGIOCARCINOMA

Cholangiocarcinoma continues to be a challenging disease to treat. The only curative option remains surgical resection. Recent trends have allowed previously inoperable patients to undergo potentially curative surgery. Most recently, liver transplantation has been used in locally unresectable tumors with variable results[1-3]. Becker *et al*[4] reported outcome analysis for 280 patients treated at multiple centers over an 18-year period. Their data shows 5 and 10 years survivals of 74% and 38% respectively. Unfortunately, relatively few patients are diagnosed with limited stage disease that is amenable to surgical intervention (either resection or transplantation). Systemic therapy has been used in unresectable disease, disease progression after surgery, and in the palliative setting. Results of multiple phase Ⅱ trials have rarely yielded positive results[5]. Average median survival remains less than one year and response rates are generally under 30%. Although the benefit is minimal, the most efficacious and clinically utilized chemotherapy regimens have been either gemcitabine or 5-fluorouracil (5FU)-based. Alberts *et al*[6] conducted a Phase Ⅱ trial of gemcitabine, 5FU, and Leucovorin in advanced biliary disease. This study delivered 4 wk cycles of gemcitabine/5FU/Leucovorin on d 1, 8 and 15. The study enrolled carcinoma of the gallbladder and cholangiocarcinoma. For our scope, we will focus on their cholangiocarcinoma data. The study enrolled 28 patients with biliary cancer at multiple centers. Using the Response Evaluation Criteria in Solid Tumors (RECIST)[7] criteria two patients with biliary tract cancer achieved a partial response. The median time to disease progression was 4.6 mo and median survival was 9.9 mo. The primary endpoint of the study was successfully achieved, namely, to determine 6 mo survival. The overall data is fairly consistent with the previously reported data for single agent 5FU or gemcitabine. Although it is clear that 5FU and gemcitabine are active in cholangiocarcinoma, this study showed that the combination of the two most potent agents failed to produce a greater response rate or duration of response than either agent alone. This study and others like it increases suspicion that traditional chemotherapy is unlikely to make tangible progress in this devastating disease.

## MOLECULAR BIOLOGY OF CHOLANGIOCARCINOMA

Like many other malignancies, cholangiocarcinoma cells over-express epidermal growth factor receptors (EGFR)[8-10]. This observation led several investigators to postulate that Erlotinib (an oral inhibitor of EGFR/

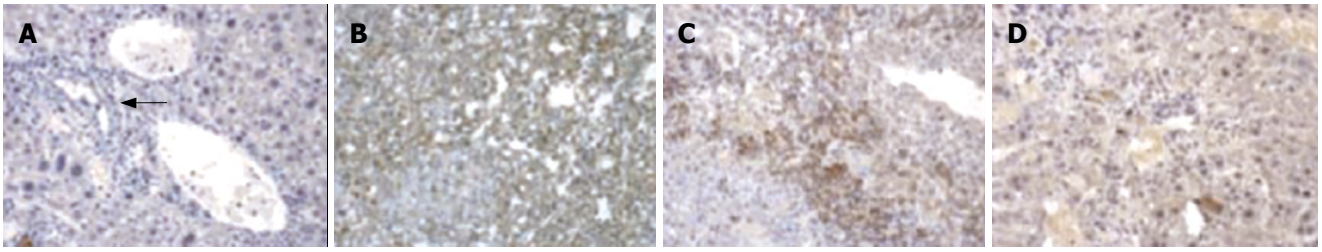


**Figure 1** c-Met immunohistochemistry performed on: **A**: Normal liver; **B**: Cholangiocarcinoma; **C**: Early stage cholangiocarcinoma; **D**: Bile duct hyperplasia reproduced from Fazari (19) with permission.

HER1 tyrosine kinase) would show activity against cholangiocarcinoma. This interest intensified when activity was demonstrated against other malignancies such as lung[11] and pancreatic cancer[12]. Philip *et al*[13] reported a Phase Ⅱ trial in 42 patients with advanced biliary cancer. Using the Response Evaluation Criteria in Solid Tumors (RECIST) criteria three patients achieved a partial response (7%). Median time to disease progression was only 2.6 mo, and median overall survival was 7.5 mo. Interestingly, there was a certain subgroup (17%) that seemed to achieve prolonged (greater than 24 wk) disease stability. There did not appear to be a correlation between *EGFR/HER1* gene over-expression and response. This was not unexpected since *EGFR* mutations, *K-Ras* mutations, *p-AKT* levels, and proteomic signatures are also important predictors of erlotinib response and signal pathway dependence is difficult to predict from gene expression alone[11,14,15]. Although these results are promising, clinicians are left searching for better treatment options. Further advancement in the treatment of cholangiocarcinoma begins with a better understanding of the molecular mechanisms of carcinogenesis.

Data on the molecular carcinogenesis of cholangiocarcinoma is developing rapidly[16,17]. As in most cancers, multiple genes have been implicated in the molecular transformation of normally functioning tissue to malignant cells. These genetic changes cause a cascade of effects that include activation of oncogenes, inactivation of tumor suppressor genes, alterations in cell signaling, resistance to apoptosis, and direct induction of DNA damage. These genetic alterations affect all phases of the cell cycle and work in concert to transform bile secreting cells into an aggressive carcinoma. A detailed description of all of these mutations and their specific role in cholangiocarcinogenesis is beyond the scope of this publication. Here, we focus on the role of c-Met/hepatocyte growth factor (HGF) and its possible therapeutic implications. It has been reported that c-Met is over-expressed in more than half of biliary carcinomas[18]. As shown in Figure 1, Farazi *et al*[19] demonstrated c-met over-expression in 80% of humanoid murine intrahepatic cholangiocarcinoma. Radaeva *et al*[20] confirmed that cholangiocarcinoma expressed strong cell-surface immunoreactivity for c-Met. *c-Met* is a proto-oncogene located on chromosome 7q that codes for a tyrosine kinase growth factor receptor called HGF receptor[21]. HGF (also known as scatter factor) binds to c-Met and initiates autophosphorylation of an intracellular tyrosine kinase on the beta-subunit of the receptor. This activation allows the binding and ultimate activation of

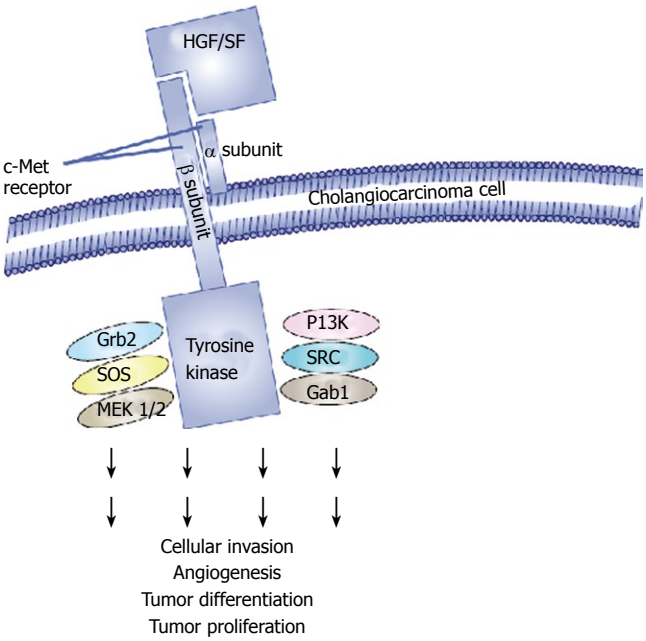


**Figure 2** Schema of *c-Met* signaling pathways.

multiple signaling molecules such as Src, P13K, Gab1, SOS, Grb2, and MEK1/2 (Figure 2). The interaction of this multi-faceted activation system ultimately results in cellular alterations that contribute to carcinogenesis. It has been suggested in multiple studies that over-expression of c-Met is linked to cell invasion, angiogenesis, and tumor differentiation/proliferation[22-24] (www.vai.org/met). Although the data is not conclusive, several researchers have suggested that c-Met behaves differently in intrahepatic and extrahepatic cholangiocarcinoma[25,26]. Leelawat *et al*[27] demonstrated that stimulated over-expression of the *c-Met* gene in cholangiocarcinoma cells resulted in increased cell migration and invasion. Conversely, inhibition of *c-Met* expression decreased cellular phosphorylation and ultimately reduced cellular invasiveness. The presence of the *c-Met* oncogene and its unique cell signaling pathway provides one of many avenues by which specific cell targeting can be used to achieve better tumor control in cholangiocarcinoma[28].

## C-MET THERAPIES

There are multiple focal points for interrupting c-Met activity with clinical compounds[29]. The earliest target in the cascade focuses on inhibition of the interaction

between HGF and the c-met receptor. Blocking the binding of the HGF to the transmembranous c-Met receptor works to halt c-Met signaling at the earliest point. Ultimately, c-Met fails to dimerize and tyrosine kinase activation does not occur. The alteration of this HGF/c-Met interaction can occur via multiple modalities including small interference RNAs (siRNA) which block c-Met expression, monoclonal antibodies against c-Met or HGF, and soluble c-Met fragment which can block HGF binding. Another target in the c-Met system is the direct tyrosine kinase inhibition. Similar to the tyrosine kinase inhibitors in chronicmyeloidleukemia (CML) and other tumors, designer compounds that are specific to the *c-Met* gene tyrosine kinase are administered. Although the interaction between HGF and the c-Met receptor is preserved, the cascade is halted by the selective binding of the inhibitor to the tyrosine kinase. Theoretically, all of these mechanisms would function to reduce cellular invasion, migration, angiogenesis, and ultimately, halt the process of carcinogenesis.

## C-MET THERAPIES FOR CHOLANGIOCARCINOMA

To date, only one study has reported c-Met targeted therapy in an animal model of cholangiocarcinoma[27]. This study showed that inhibition in c-Met expression or its downstream target MEK1/2 through specific targeted therapy is effective in halting disease progression *in vivo*. Inhibition was achieved through two molecular strategies. First, c-Met expression was altered through a *c-Met* specific small interfering RNA (siRNA) binding to the c-Met coded receptor. Second, siRNA specific binding to the *c-Met* downhill cascade product, MEK1/2, resulted in blunting of the cellular invasiveness of cholangiocarcinoma cells.

A number of c-Met and HGF antibody directed therapies receptor interaction have been shown biological activity in non-biliary cancer animal models[30,31] and human studies[32]. AMG102 is a fully human IgG2 monoclonal antibody against HGF. This compound has completed both preclinical trials and phase I dosing trials[33]. Although, the dose-escalating trials were performed on a variety of solid tumors, there is not current data on cholangiocarcinoma. A one-armed c-Met antibody has shown activity in preclinical studies[34]. Again, patients with cholangiocarcinoma have not been treated. Decoy met[35] is a soluble met receptor that interferes with HGF binding. It has been shown *in vivo* to have multiple anti-malignant properties including inhibiting angiogenesis, suppressing metastasis, and halting cellular proliferation. Decoy met functions to block the c-Met receptor as well as altering met dimerization. Decoy met has several properties that may make it more desirable than standard antibody directed therapies. For example, decoy met has a logarithmically greater affinity for the c-met receptor.

A series of c-Met tyrosine kinase inhibitors have been examined. XL880 is an oral c-Met tyrosine kinase inhibitor that is completing Phase I trials and beginning Phase Ⅱ trials in humans. XL880 is a multi-kinase inhibitor that affects both the HGF/c-Met receptor family and the VEGF receptor family. The most common side effect of XL880 is hypertension. Although XL880-induced hypertension is very common, in phase I testing, it was manageable with anti-hypertensive medications[36]. XL880 is currently undergoing phase Ⅱ clinical trials in a number of cancers. Early Phase Ⅱ data on renal cell cancer has been positive[37]. It has shown activity in lung cancer (both small cell and non-small cell) xenografts in immunocompromised mice[38]. Additional tyrosine kinase inhibitors that are specific to the c-Met receptor have been developed[39-41]. ARQ 197 is a c-Met specific receptor tyrosine kinase inhibitor. This compound has completed Phase I dose escalation and has reached the recommended phase Ⅱ dose. Partial responses and durable long term disease control have been achieved in several malignancies[42]. PHA665752 is a selective small molecule tyrosine kinase inhibitor of c-Met[38,43]. It has been shown to inhibit angiogenesis and induce apoptosis and cell cycle arrest. Interestingly, PHA665752 has been shown to have a cooperative effect when administered with rapamycin[43]. No current data on PHA665752 in humans is available.

**Table 1 Target sites of c-Met therapies**

| Target | Example | Current phase |
|---|---|---|
| HGF/c-met monoclonal antibody | AMG102[33] | Phase Ⅱ |
| Soluble c-met receptor | Decoy met[35] | Phase Ⅰ |
| Tyrosine kinase inhibition | ARQ 197[42] | Phase Ⅱ |
| | XL880[36,37] | Phase Ⅱ |
| | PHA665752[38] | Animal testing |

## CLINICAL TRIALS WITH C-MET THERAPIES

There is scant data for any of the compounds in patients with cholangiocarcinomas (Table 1). The previously mentioned XL880 Phase I trial included 1 patient with cholangiocarcinoma. The slides presented at the 2007 ASCO meeting indicated that there was as 5 mo duration of response in Phase I testing. Unfortunately, the XL880 trial did not select for tumors over-expressing c-Met. Progress is rapidly being made through inhibition of the c-Met cascade. Hopefully, this will result in treatment advances in cholangiocarcinoma. Furthermore, other molecular mechanisms exist for using c-Met to target cellular death in cholangiocarcinoma. The possibility of using HGF or a monoclonal antibody to c-Met for immunotoxin construction should also be explored[45]. This would result in preferential introduction of deadly toxins into the cholangiocarcinoma cellular environment sparing normal cells (non c-Met expressing). As treatments directed against aggressive incurable cancers develop, their success will likely depend on their ability to deliver tumor selective, highly toxic treatments to carcinoma cells while sparing normal tissue. The c-Met cascade and others like it provide such an opportunity. Through these rapid developments, researchers, clinicians, and patients have hope of better treatments in the future.

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*TOPIC HIGHLIGHT*

**Gianfranco D Alpini, PhD, Professor; Sharon DeMorrow, Assistant Professor,** *Series Editor*

# Review of endoscopic techniques in the diagnosis and management of cholangiocarcinoma

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## Abstract

Cholangiocarcinoma is a rare malignancy of the biliary tract. Key factors in determining therapeutic options include knowledge of tumor extent, anatomy and obtaining tissue diagnosis. Endoscopically, there are three modalities available to make the diagnosis of cholangiocarcinoma. These include endoscopic retrograde cholangiopancreatography, endoscopic ultrasound with fine needle aspiration and cholangioscopy. Management of cholangiocarcinoma endoscopically is typically confined to stent placement for palliative purposes or as a bridge to surgery. In this article, we will review the endoscopic techniques available for the diagnosis and management of cholangiocarcinoma.



**Key words:** Cholangiocarcinoma; Endoscopic ultrasound; Endoscopic cholangiopancreatography; Cholangioscopy; Diagnosis; Management

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## INTRODUCTION

Cholangiocarcinomas are rare malignancies involving the biliary tract. They can be divided into three anatomic groups: intrahepatic, perihilar, and distal extrahepatic. Perihilar tumors, also known as Klastkin tumors, involve the hepatic duct bifurcation. They are the most common, accounting for about 60%-80% of cholangiocarcinomas. Intrahepatic tumors are least common[1]. The Bismuth classification is used to describe the biliary tract involvement and is helpful in planning surgical intervention. Type I tumors are found below the bifurcation of the left and right hepatic ducts. Type Ⅱ tumors involve the bifurcation. Type Ⅲa and Ⅲb tumors occlude the common hepatic duct and either the right or left hepatic duct, respectively. Type Ⅳ tumors are multicentric, or they involve the bifurcation and both the right and left hepatic ducts (Figure 1). The incidence rates for cholangiocarcinomas vary depending on geographic location with the highest rates found in Southeast Asia. In the United States, between 4000 and 5000 cases are found annually. For unknown reason, the incidence of and mortality rates for intrahepatic cholangiocarcinomas have been increasing in recent years while the incidence of extrahepatic cholangiocarcinomas have been decreasing[2]. Accurate knowledge of tumor extent and anatomy as well as obtaining a tissue diagnosis is important in determining therapeutic options. In this article, we will review the endoscopic modalities available in the diagnosis and management of cholangiocarcinomas.

## DIAGNOSIS

The etiology of biliary strictures can often be difficult to establish. The differential diagnosis of biliary strictures is extensive and includes, but is not limited to, primary sclerosing cholangitis, gallbladder carcinoma, pancreatic carcinoma, intraductal papillary mucinous tumor, or benign biliary strictures from causes such as pancreatitis.

Cholangiocarcinomas often pose a diagnostic challenge due to difficulties in obtaining an adequate specimen for cytology. Tissue diagnosis is important in certain subgroups of patients such as those who are borderline surgical candidates, those with indeterminate strictures (e.g. patients with primary sclerosing cholangitis), or before chemotherapy and radiation therapy.

### *Magnetic resonance cholangiopancreatography*

Magnetic resonance cholangiopancreatography (MRCP) can be a useful noninvasive adjunct to current techniques. It has the ability to define the proximal and distal extent of strictures as well as to evaluate for any intrahepatic mass lesion. One series evaluated the role of MRCP in patients with bile duct obstruction. Of 126 patients, 14 had bile duct malignancy. Of those 14, 12 patients were diagnosed by MRCP, with a sensitivity of 86% and specificity of 98%[3]. Another study by Rosch *et al*[4] had lower specificity for malignant obstructions. This study compared endoscopic retrograde cholangiopancreatography

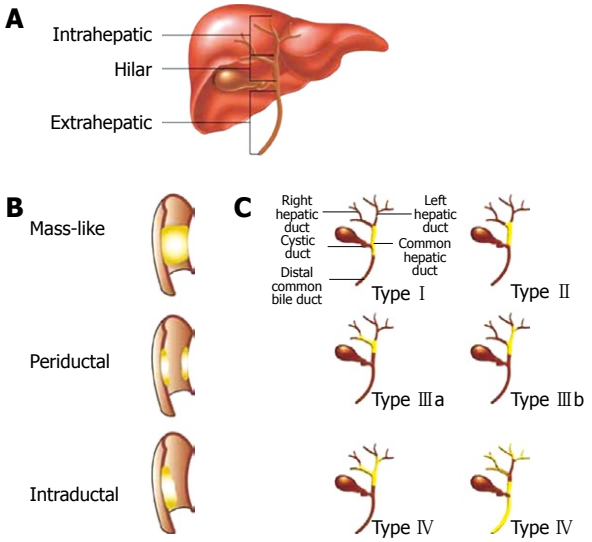


**Figure 1** Classification of cholangiocarcinoma. **A**: The classification of cholangiocarcinoma can be based on anatomic location, intrahepatic, hilar or extrahepatic; **B**: Nonhilar lesions can be described as mass-like, periductal or intraductal; **C**: Bismuth classification for hilar lesions.

(ERCP), MRCP, computed tomography (CT) and endoscopic ultrasound (EUS) in the evaluation of biliary strictures. The specificity and sensitivity for MRCP to detect malignancy was 77% and 63%, respectively. Although MRCP provides the same imaging information as ERCP, many times ERCP is still required to provide a tissue diagnosis.

### ERCP

ERCP is useful in both the diagnosis and management of cholangiocarcinomas. It can delineate the anatomy of the biliary system and determine the extent of bile duct involvement which is important in determining resectability and surgical management (Figure 2). On cholangiography, the appearance of a stricture can suggest malignancy, but is not conclusive. Some characteristics suggestive of malignancy include a length greater than 10 mm, irregular margins, and an abrupt transition from normal duct to stricture, also known as shouldering[5]. Hilar strictures should also raise the suspicion for malignancy. Although, the appearance and location of a biliary stricture can suggest malignancy, tissue confirmation is usually needed in the majority of patients. Tissue for cytology may be obtained during ERCP by brushing, biopsy, bile aspiration or a combination of these. When necessary, therapeutic procedures can be performed, such as the placement of a biliary stent for treatment of obstructive jaundice.

Brush cytology has a high specificity of nearly 100%, but sensitivity is much lower, ranging from 18%-60% in most series[6,7]. The low sensitivity is likely related to low cellularity of these tumors and the desmoplastic reaction that is present.

Stricture manipulation by dilation theoretically should increase the availability of malignant cells for cytological examination. However, studies by deBellis *et al*[8] did not show a statistically significant difference in sensitivity before and after dilation. Patients underwent dilation with either a graduated dilating catheter or a dilating balloon. However, when the results of the pre- and post-dilation brushings were combined, the diagnostic yield increased from 35% to 44% ($P = 0.001$). This indicates that repeated brushing, not necessarily the stricture manipulation, should increase the diagnostic yield.

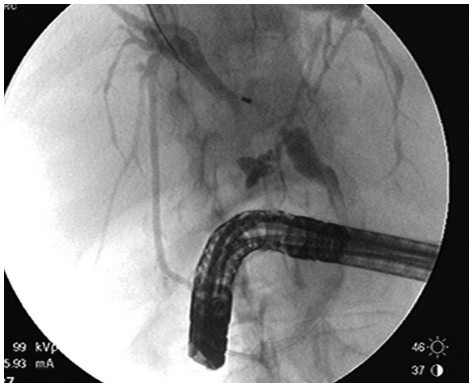

**Figure 2** Hilar lesion causing bilateral strictures at the bifurcation of the left and right hepatic ducts with proximal dilation.

Further studies compared different brush lengths and stiffness[6]. A standard cytology brush, 1.5 cm long with soft bristles, was compared to the Cytolong brush, 5 cm long with rigid bristles. Detection rates were not increased with usage of the longer cytology brush.

Advances in diagnostic methods have increased the diagnostic yield of brush cytology. Digital image analysis (DIA) is useful in specimens with limited cellularity as it looks at the DNA content of individual cells. DIA uses spectrophotometric methods to quantify DNA content, chromatin distribution, and nuclear morphology. Aneuploidy, or the presence of increased amounts of DNA, is quantitated and, if present, suggests malignancy. DIA increases the sensitivity of routine brush cytology from 18%-40%, but decreased the specificity from 98%-77%[9].

Fluorescence *in-situ* hybridization (FISH) uses a commercial probe set to assess for polysomy of chromosomes 3, 7, 17, and 9p21. FISH increased the sensitivity from 15%-34%, and increased the specificity from 91%-98%[10].

Of special note, when performing ERCP in patients with biliary strictures, there is a risk of cholangitis due to the injection of contrast and possible bacteria into an obstructed biliary system. Therefore, it is important to obtain adequate drainage across the biliary obstruction with placement of a stent to decrease this risk. In patients with a stent that had been previously placed, the removed biliary stent can be sent for cytology in order to increase diagnostic yield.

### EUS

Another modality that has become useful in diagnosing hilar cholangiocarcinomas is EUS with fine needle aspiration (FNA). CT or percutaneous ultrasound guided fine needle aspiration are not routinely used because these tumors are small and isoechoic to the liver, making them more difficult to assess. EUS has high-resolution imaging and can visualize lesions of 3 mm or greater. Although ERCP is the conventional test for evaluating biliary strictures, as we have discussed, the sensitivity remains low. In patients with ERCPs that are indeterminate or non-diagnostic for ma-

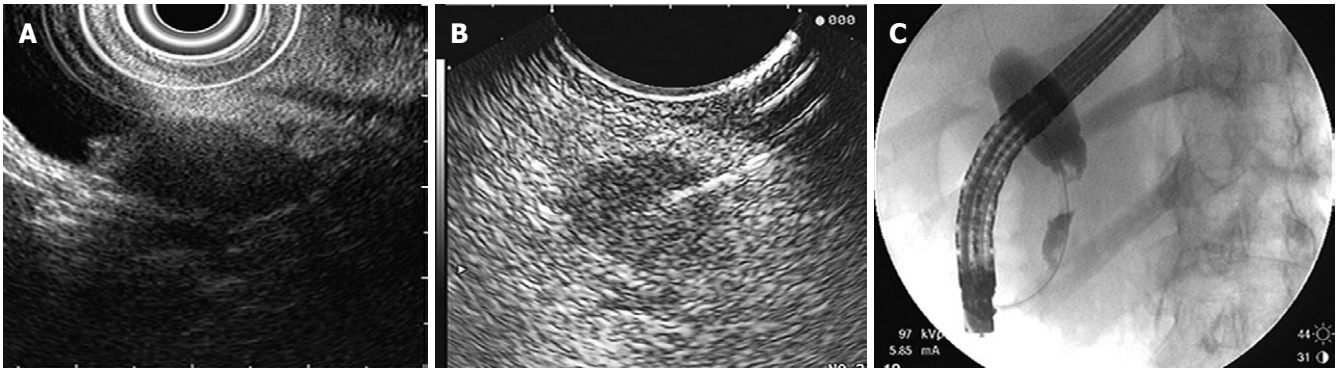


**Figure 3** **A**: Distal common bile duct lesion with proximal biliary dilation; **B**: Fine needle aspiration of common bile duct lesion; **C**: ERCP shows distal common bile duct stricture consistent with findings on EUS.

lignancy, EUS with fine needle aspiration is a useful tool (Figure 3).

EUS images, alone without FNA, are not reliable in evaluating hilar lesions. Criteria such as echotexture, size of mass, contour abnormalities, and the shape and borders of the stenosis do not reliably differentiate malignant from benign lesions. EUS also provides visualization of hilar, celiax axis and para-aortic lymph nodes to determine local and distant metastasis. Fine needle aspiration of these lymph nodes is the most accurate way to diagnose cholangiocarcinoma and also allows for staging. In addition, EUS can evaluate the pancreas for causes of biliary strictures such as pancreatic masses or changes of chronic pancreatitis.

In a study by Fritscher-Ravens *et al*[11], patients with hilar strictures and inconclusive tissue diagnosis by ERCP, underwent EUS with fine needle aspiration. Of 44 patients, lesions at the hilum were noted in all the patients, and adequate material was obtained in 43 patients. Cytology revealed hilar cholangiocarcinoma in 59% of patients, with an accuracy of 91%, sensitivity of 89% and specificity of 100%. Accurate diagnosis changed the management in more than half of these patients that previously had non-diagnostic ERCPs.

In 2004, Eloubeidi *et al*[12] evaluated 28 patients in a prospective study to assess how EUS-FNA impacted patient management. Of the 28 patients, 3 were excluded because the lesion could not be identified by EUS. The sensitivity, specificity, and accuracy were 86%, 100%, and 88%, respectively, with numbers similar to the study by Fritscher-Ravens *et al*. A positive impact was made in 84% of patients. In 10 patients, surgery was prevented in patients with inoperable disease, 8 patients had surgery facilitated as they had unidentifiable cancer by other modalities, and 4 patients with benign disease avoided surgery. Prior studies have shown that 13%-24% of patients with suspected cholangiocarcinomas had benign disease at the time of surgery. By performing EUS with FNA in these patients with indeterminate strictures, surgical treatment could be tailored and appropriate management decisions be made.

A further modification of ultrasound technology allows the placement of a high frequency intraductal ultrasound probe (IDUS). Although several features such as irregular wall thickening can be highly suggestive of malignancy, IDUS as yet has no associated capability for tissue acquisition.

### *Peroral cholangioscopy and spyglass*

During ERCP, miniature cholangioscopes can be used to directly visualize the bile ducts and any strictures or filling defects seen during ERCP. Directed tissue biopsies can also be obtained with miniature cholangioscopic biopsy forceps. Shah *et al*[13] in 2006 evaluated 62 patients with suspected pancreatic or biliary malignancy that had prior nondiagnostic studies. Cholangioscopy with either cholangioscopic-directed or assisted biopsies performed when applicable. Sixty-two patients underwent 72 examinations and 53 lesions were seen on cholangioscopy. Twenty-nine patients had either cholangioscopy-directed or assisted biopsies and 24 had both. Cholangiocarcinoma was identified in 14 patients. Two patients with intrahepatic cholangiocarcinomas were missed by cholangioscopy. In this study, the sensitivity and specificity for cholangioscopy to detect malignancy was 89% and 96%, respectively.

More recently, a single-operator peroral cholangiopancreatoscopy system known as Spyglass has been developed[14,15]. Older cholangioscopes were fragile, had limited tip deflection, and had limited ability to clean the lens and visual field. In addition, they required two endoscopists, one to operate the duodenoscope and another to operate the cholangioscpe. With the Spyglass system, a single operator can control both scopes, there is 4-way deflected steering, and there are separate irrigation channels. A single operator system allows tight coordination of the duodenoscope and cholangioscpe. Mastering the use of the system does require experience and advanced skills. The increased maneuverability of the Spyglass system allows for 4 quadrant biopsies. In bench stimulations, the Spyglass system had 100% success rates in obtaining target quadrant biopsies compared to 50% in conventional choledochoduodenoscopes. A feasibility study was performed with 35 patients, 22 of whom had indeterminate strictures. The procedure was successful in 91% of patients. Spyglass-directed biopsies were performed in 20 patients, and 19 had adequate tissue for examination. The preliminary sensitivity and specificity of Spyglass to detect malignancy were 71% and 100%, respectively. In this study, 2 patients

(6%) developed complications; one developed ascending cholangitis and the other intrahepatic abscess. Both patients recovered without sequelae. Currently, prospective multicenter clinical trials are ongoing.

## MANAGEMENT

Cholangiocarcinomas have a very poor prognosis with an average five-year survival of only 5%-10%. The only curative therapy for cholangiocarcinomas is surgical resection. If patients are not candidates for surgical resection, their median survival is 6.7-11.6 mo compared to 37.4-42.9 mo for patients who undergo surgical resection[16,17]. Distal cholangiocarcinomas have the highest resectability rates of about 91% while perihilar tumors have the lowest at 56%. Based on the experience at Johns Hopkins Hospital over 23 years, distal, intrahepatic, perihilar cholangiocarcinomas after resection have five-year survival rates of 28%, 44%, and 11%, respectively[18].

Biliary decompression by placing a stent prior to surgery is a controversial issue. A biliary stent may make it difficult to assess the proximal extent of the tumor intraoperatively and may increase the risk of infections postoperatively. However, elevated bilirubin levels and liver dysfunction are factors that adversely affect postoperative morbidity. Indications for biliary stent placement preoperatively include cholangitis or prevention of cholangitis after a diagnostic ERCP is performed or if surgery is to be delayed for an extended amount of time[19,20].

Only about 10%-20% of patients are candidates for surgery at the time of diagnosis secondary to advanced disease or overall poor medical health. In these patients with unresectable disease, the survival is very poor and there is rapid progression with biliary obstruction. Biliary decompression for palliative purposes can be accomplished surgically, radiologically or endoscopically.

### *Unilateral versus bilateral stents*

In order to provide palliation and relieve jaundice, only 25% of the liver needs to be adequately drained. Therefore, unilateral stents of either the left or the right system are typically sufficient. In a randomized controlled prospective trial, De Palma *et al*[21] evaluated 157 patients with malignant hilar biliary obstruction due to cholangiocarcinoma, gallbladder cancer or periportal metastatic lymphadenopathy. In patients with unilateral stenting, there was a higher success rate for stent insertion (89% *vs* 77%) and drainage (81% *vs* 73%) and, therefore, a lower early complication rate (19% *vs* 27%) when compared to bilateral stenting of both hepatic lobes. Early complications included cholangitis and stent occlusion. No differences were found in survival or procedure-related mortality.

In order to decrease the risk of cholangitis during an ERCP, it is important not to inject contrast above the level of a stricture unless adequate drainage can be ensured. Selective cannulization with a guidewire above the level of the stricture should be performed. Following that, the catheter should be passed above the stricture before injecting contrast. With the guidewire in place, a stent can be placed in the proper position ensuring that the contaminated segment will be properly drained (Figure 4).

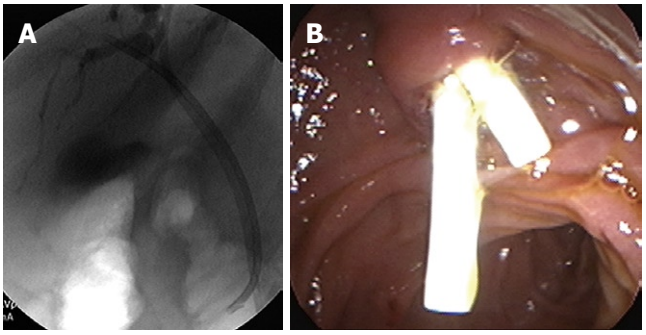


**Figure 4** **A**: In this patient with a hilar mass, double stents were placed within the right and left hepatic systems to allow adequate drainage of contrast after cholangiogram was performed to decrease the risk of cholangitis; **B**: Endoscopic view of bilateral stents placed.

### *Plastic versus metal stents*

Both plastic and metal biliary stents are available. Numerous studies have compared plastic *versus* metal stents with regards to cost, complication rates, and survival[22-24]. There are no differences in survival with the use of either stents. Plastic stents have a higher risk of occlusion, with 30% occlusion rates after 3 mo and 70% after 6 mo[23]. In order to prevent problems with occlusion and cholangitis, they need to be exchanged every 3 mo. Metal stents have a longer patency of approximately 12 mo due to the fact that they have larger diameters compared to plastic stents (10 mm *vs* 3.8 mm). However, once placed they are very difficult to manipulate or remove. As far as cost effectiveness, the initial cost of a metal biliary stent is higher. However, with plastic stents, there are subsequent costs due to the need for repeat procedures for stent exchange and hospitalization for complications. Overall, there is no significant difference in the cost between metal and plastic stents. The decision to place a plastic versus metal stent should take into consideration the patients' overall health, expected length of survival, quality of life and local expertise. Often, a plastic stent is placed initially while further diagnostic workup is underway. Once the diagnosis is made and the patient has unresectable disease and a life expectancy of more than 6 mo, then the plastic stent can be replaced with a metal stent. Placement of a metal stent eliminates the need for repeated procedures and their associated risks.

## CONCLUSION

The diagnosis of cholangiocarcinomas is often challenging. Multiple endoscopic modalities are available to evaluate strictures or masses of indeterminate origin. ERCP with brush cytology using FISH or DIA technology along with EUS with FNA and cholangioscopy are available. Oftentimes, repeated procedures and a combination of these different techniques are necessary to achieve a tissue diagnosis. Having a cytologic diagnosis as well as knowing the stage of the disease plays an important role in decisions regarding management. Surgery is curative if the disease is detected at an early stage. When there is metastatic or advanced disease, endoscopic drainage plays a central role in providing palliation and improving quality of life. Placement of a unilateral stent is sufficient in providing adequate drainage and has lower morbidity than bilateral stents. In patients who require short-

term drainage, plastic stents are a good option. Because long term survival is so poor, metal stents should be considered if patients are not surgical candidates.

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*TOPIC HIGHLIGHT*

**Gianfranco D Alpini, PhD, Professor; Sharon DeMorrow, Assistant Professor,** *Series Editor*

# Diagnosis and initial management of cholangiocarcinoma with obstructive jaundice

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## Abstract

Cholangiocarcinoma is the second most common primary hepatic cancer. Despite advances in diagnostic techniques during the past decade, cholangiocarcinoma is usually encountered at an advanced stage. In this review, we describe the classification, diagnosis, and initial management of cholangiocarcinoma with obstructive jaundice.



**Key words:** Cholangiocarcinoma; Obstructive jaundice; Diagnosis; Treatment; Initial management

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## INTRODUCTION

Cholangiocarcinoma is the second most common primary hepatic cancer. Despite advances in diagnostic techniques during the past decade, cholangiocarcinoma is usually encountered at an advanced stage. In this review, we describe the classification, diagnosis, and initial management of cholangiocarcinoma with obstructive jaundice.

## CLASSIFICATION

Cholangiocarcinomas are epithelial neoplasms that originate from cholangiocytes and can occur at any level of the biliary tree. These lesions are broadly classified into intrahepatic cholangiocarcinoma, hilar cholangiocarcinoma, and distal extrahepatic bile duct tumors. Histologically, most cholangiocarcinomas (> 95%) are adenocarcinomas. They are pathologically classified into sclerosing, nodular, and papillary intraductal cancers[1]. A recent pathological classification applicable to both intrahepatic and extrahepatic cholangiocarcinomas divides these lesions into mass-forming (nodular), periductal-infiltrating (sclerosing), and intraductal-growing (papillary) cholangiocarcinomas[2].

## DIAGNOSIS

### *Laboratory data*

Liver test abnormalities reflecting obstruction of the bile duct are usually observed. Strikingly elevated CA19-9 values in symptomatic patients usually signify advanced disease. Carcinoembryonic antigen (CEA) is also elevated in patients with cholangiocarcinoma, but is not diagnostic because of low sensitivity and specificity. Cholangitis and hepatolithiasis commonly lead to increased levels of tumor markers. Cholangiocarcinoma should not be diagnosed on the basis of laboratory data alone.

### *Ultrasonography*

Ultrasonography is the imaging technique of choice for the diagnosis of cholangiocarcinoma with obstructive jaundice. Visualization allows adequate diagnosis and staging in more than 90% of cases. The presence of dilated ducts without clear communications within a liver lobe indicates the extension of tumor into the segmental bile ducts. Ultrasonography is useful for evaluating the local extent of disease, but is of limited value for staging distant metastases. Intrahepatic cholangiocarcinomas may be identified as mass lesions, sometimes associated with bile duct dilatation proximal to the obstructing lesion. Tumor vascularity is an important characteristic that can be assessed by color Doppler ultrasonography. An abnormal pulsed Doppler signal obtained from the portal venous system due to severe narrowing or occlusion

strongly suggests major involvement and unresectable tumor. However, a normal pulsed Doppler signal does not exclude such involvement, if the tumor is contiguous with vessels showing interruption of the hyperechoic tumor-vessel interface[3,4].

Endoscopic ultrasound (EUS) is useful for assessing the extent of disease and performing fine needle aspiration. Eloubeidi *et al*[5] reported that EUS-guided fine needle aspiration biopsy is useful for the diagnosis of suspected cholangiocarcinoma. The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were 86%, 100%, 100%, 57%, and 88%, respectively. EUS-guided fine needle aspiration of lymph nodes facilitates staging of disease in addition to visualization of the biliary tree[6].

### Computed tomography (CT)

CT permits the identification of bile duct dilatation and assessment of the hepatic parenchyma and lymph nodes. However, the evaluation of horizontal spread by diagnostic imaging via the bile duct remains challenging in patients with cholangiocarcinoma, especially on conventional CT examination. Recently, the development of multidetector row CT scanners has permitted a reduction in the voxel size and facilitated rapid image reconstruction, enhancing the value of CT as an interactive diagnostic tool. Moreover, innovative methods for CT image reconstruction, including multiplanar reconstruction and three-dimensional images, were recently introduced for the visualization of biliary structures[7]. CT angiography has been demonstrated to be useful for the detection and assessment of vascular encasement[8-10].

### Magnetic resonance imaging (MRI)

MRI with concurrent magnetic resonance cholangiopancreatography (MRCP) is the radiologic technique of choice for assessing the extent of disease[11,12]. The limitations of conventional imaging techniques have led to the increased use of MRCP, which is a noninvasive and highly accurate technique for the evaluation of patients with biliary obstruction. MRCP is optimally suited for the visualization of both intrahepatic and extrahepatic cholangiocarcinomas, which appear as hypointense lesions on T1-weighted images and hyperintense lesions on T2-weighted images. Images can be enhanced with the use of superparamagnetic iron or by delayed gadolinium enhancement[13,14]. The overall diagnostic accuracy for assessment of the level and cause of obstruction was 96.3% and 89.7%, respectively[12]. MR angiography can be used to evaluate vascular involvement[15].

### Cholangiography

Before the development of MRCP, direct cholangiography was only technique for assessment of the biliary system. Direct cholangiography can be performed by either percutaneous transhepatic cholangiography or endoscopic retrograde cholangiography, and samples of the bile duct can be obtained[16,17]. Brushings are analyzed cytologically. In one study, the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of brush cytology were 75%, 100%, 100%, 12.5%, and 75.9%, respectively. Biopsy specimens of the bile duct are examined histologically[18]. The diagnostic performance of transluminal forceps biopsy for malignant biliary obstructions was as follows: sensitivity, 78.4%; specificity, 100%; and accuracy, 79.2%[19]. Savader *et al*[20] compared the diagnostic accuracy of three different techniques for percutaneous transhepatic intraductal biopsy: brush cytology, clamshell forceps under choledochoscopic guidance, and clamshell forceps under fluoroscopic guidance. The choledochoscope-directed biopsy technique had the highest sensitivity and specificity among the three techniques, but was not significantly better than either the brush or fluoroscopic clamshell techniques ($P > 0.10$). Multiple biopsies did not increase the overall sensitivity of intraductal biliary biopsy as a diagnostic technique. All three techniques were safe and easy to perform. In patients with malignant biliary obstruction, brush cytology was more sensitive for the diagnosis of cholangiocarcinoma than for the diagnosis of non-cholangiocarcinoma ($P < 0.05$). The site of stenosis was unrelated to sensitivity and technical success ($P > 0.05$)[18,21].

Rotational cine cholangiography is used to diagnose bile duct carcinoma. Rotational cine cholangiography is a reliable technique for detecting the confluence of the bile ducts, as well as for diagnosing the longitudinal extent of cancer spread along the bile duct wall[22]. Furukawa *et al*[23] evaluated the usefulness of three-dimensional cholangiography and rotating cine cholangiography for depicting the anatomy of the hilar bile duct and tumor extension, and for planning surgical procedures for hilar cholangiocarcinoma. Three-dimensional and cine cholangiography allowed accurate assessment of the biliary system in patients with hilar cholangiocarcinoma, facilitating the planning of surgery.

### Angiography

Angiography reveals the anatomy of the hepatic and biliary arteries. Angiography is a superb technique for the detection of vascular encasement. It is also useful for planning surgical procedures.

### Scintigraphy

**Technetium-99m galactosyl human serum albumin scintigraphy:** Technetium-99m-diethylenetriaminepentaacetic acid-galactosyl-human serum albumin (99mTc-GSA) is an analog ligand of asialoglycoprotein that binds specifically to asialoglycoprotein receptors (ASGP-R) residing in mammalian hepatocytes[24-26]. The hepatic uptake of 99mTc-GSA at 15 min or later reflects the receptor population or functional hepatocyte mass[27].

Nanashima *et al*[28] studied the relation between morphological measurements of hepatic volume on CT and functional volume on 99mTc-GSA scintigraphy. There were no significant differences in the volume measurements between these two volumetric techniques. Volumetric measurement by 99mTc-GSA scintigraphy is useful for detecting changes in the functional volume of individual lobes of the liver and is a more dynamic method than the assessment of morphological changes on CT scanning.

We confirmed hemodynamic changes in the distribution of splenic venous flow in the liver, especially in the cirrhotic liver, and demonstrated the participation

of splenic venous flow in the regeneration or enlargement of the hepatic lobe by means of scintiphotosplenopor tography after percutaneous intrasplenic injection of 99mTc-GSA. We concluded that splenic venous blood flow promotes liver fibrosis in the right lobe of the liver exposed to continuous damage, with gradually increasing flow into the left lobe, showing milder fibrosis[29].

**Positron emission tomography (PET):** PET with [18F]-fluorodeoxyglucose can be used to rule out metastatic disease, although the findings should be interpreted cautiously because of false positive results in inflammatory lesions; moreover a normal PET scan does not exclude cancer[30].

## INITIAL MANAGEMENT

### Biliary drainage

In patients with obstructive jaundice who have cholangiocarcinoma, especially hilar cholangiocarcinoma, preoperative biliary drainage has been recommended to improve liver function before surgery and to reduce postoperative complications. Percutaneous transhepatic biliary drainage (PTBD) with multiple drains was previously the preferred method for the preoperative relief of obstructive jaundice. In patients with hilar cholangiocarcinoma, drainage is currently performed only for liver lobes that will remain after resection and for areas of segmental cholangitis. Endoscopic biliary drainage (EBD) is less invasive than PTBD. However, EBD has to be converted to PTBD in patients with segmental cholangitis, those requiring prolonged drainage, or those in whom the extent of longitudinal tumor extension is poorly defined[16].

Kamiya *et al*[31] reported that impaired intestinal barrier function does not respond to external biliary drainage without bile replacement. Bile replacement during external biliary drainage can restore intestinal barrier function in patients with biliary obstruction, primarily by promoting the repair of physical damage to the intestinal mucosa. Koivukangas *et al*[32] reported that cell protein synthesis is disturbed earlier than cell dynamics in obstructive jaundice. Decreased baseline skin-collagen synthesis is partly restored by the resolution of jaundice[33].

We previously reported that elevated serum collagen IV is a feature of malignant obstructive jaundice commonly associated with prolonged bilirubin clearance, and a useful indicator of clinical course, postoperative morbidity, and mortality in patients with malignant obstructive jaundice[34].

The procedure of choice for biliary drainage before major hepatectomy in patients with obstructive jaundice remains controversial, i.e. selective biliary drainage of only the future remnant liver or total biliary drainage. Ishizawa *et al*[35] reported that selective biliary drainage is superior to total biliary drainage for promoting hypertrophy of the future remnant liver in patients undergoing portal vein embolization and for guaranteeing good liver function before major hepatectomy. Hochwald *et al*[36] showed that preoperative biliary stenting in proximal cholangiocarcinoma increases the incidence of contaminated bile and postoperative infectious complications. Cherqui *et al*[37] found that major liver resection without preoperative biliary drainage is a safe procedure in most patients with obstructive jaundice. Recovery of hepatic synthetic function is identical to that of patients without jaundice. Transfusion requirements and the incidence of postoperative complications, especially bile leaks and subphrenic collections, are higher in jaundiced patients. Pitt *et al*[38] concluded that preoperative PTBD does not reduce operative risk but does increase hospital costs and, therefore, discouraged routine use. The indication for preoperative biliary stenting in patients with obstructive jaundice remains controversial.

### Portal vein embolization (PVE)

PVE before hepatectomy is designed to induce atrophy of the embolized lobe scheduled to be resected, while inducing compensatory hypertrophy of preserved lobe[39,40]. PVE with compensatory contralateral hypertrophy of the future liver remnant has been performed to enable extended hepatectomy (resection of $\geqslant$ 5 hepatic segments)[41,42]. We have reported on combined embolization of the hepatic artery and portal vein[43].

### Biliary ablation

Selective biliary infusion of ethanol can be performed safely without serious complications, inducing lobar ablation with contralateral hypertrophy of the liver[44,45].

### Operation

Surgical resection has been the mainstay of curative treatment for cholangiocarcinoma[46]. Major hepatectomy with systematic nodal dissection is associated with a good chance of prolonged survival in patients with carcinoma involving the hepatic hilus, including those with advanced disease[47,48]. Extended hemihepatectomy, with or without pancreatoduodenectomy, plus extrahepatic bile duct resection and regional lymphadenectomy has recently been recognized as the standard curative treatment for hilar bile duct cancer. Pancreatoduodenectomy is the choice of treatment for middle and distal bile duct cancer. Major hepatectomy with pancreatoduodenectomy (hepatopancre atoduodenectomy) has been performed in selected patients with widespread disease. Miyazaki *et al*[49,50] reported that parenchyma-preserving hepatectomy could result in curative resection and improve the outcomes of patients with hilar cholangiocarcinoma localized to the hepatic duct confluence who do not require vascular resection. Less-extensive procedures were also beneficial for less-advanced disease if the resection margins were free of tumor. Even with carefully selected treatment with curative intent, the 5-year survival of patients with cholangiocarcinoma ranges from 30% to 40%. A tumor-free surgical margin is the best predictor of survival. Several staging schemes have been proposed, but none correlates with resectability. Lymph node involvement is also a predictor of survival[48,51].

### Adjuvant therapy

Neoadjuvant therapy with several types of treatment, including radiation, photodynamic therapy and chemotherapy, provides no clear benefit[52,53].

### Palliative therapy

Previously, plastic endoprostheses were placed for the

palliative treatment of malignant biliary obstruction[54-57]. An expandable metal stent (EMS) is used to provide palliation in patients with malignant obstructive jaundice[58]. EMSs have been compared with plastic endoprostheses for the palliative treatment of malignant obstructive jaundice[59,60]. EMSs are inserted percutaneously[61-63] or endoscopically[59,64].

Biliary stent placement combined with local tumor therapy, such as brachytherapy, extra-radiation therapy, or arterial infusion chemotherapy, can prolong the survival time of patients with malignant biliary obstruction[65-68]. Mezawa *et al*[69] developed a new PTBD tube coated with carboplatin.

Intrahepatic cholangiojejunostomy has been performed in patients with unresectable malignant biliary obstruction[70-72]. Endoscopic stenting for the management of this condition costs significantly less than surgical treatment[73]. Recently, EUS-guided hepaticogastrostomy has been performed[74].

### *Transplantation*

Although early survival after transplantation for cholangiocarcinoma is excellent, high recurrence rates have generally discouraged liver replacement. Recent findings, however, have lead to a resurgence in orthotopic liver transplantation for unresectable, albeit locally contained cholangiocarcinoma. Becker *et al*[75] reported a series of 280 patients with cholangiocarcinoma who received orthotopic liver transplantation. After a median follow-up of 452 d, the survival rates at 1 and 5 years were 74% and 38%, respectively. Heimbach *et al*[76] reported on 56 patients who were treated for unresectable, stage I and Ⅱ perihilar cholangiocarcinoma. Disease-free survival at 5 years was excellent (82%) in carefully selected patients who underwent neoadjuvant external-beam radiation therapy, transcatheter intrabiliary radiation, chemotherapy, and pretransplant-staging exploratory laparotomy. Neoadjuvant chemoradiotherapy with liver transplantation produces excellent results for selected patients with localized, regional node negative, hilar cholangiocarcinoma[76,77].

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*GASTRIC CANCER*

# Investigation of transcriptional gene silencing and mechanism induced by shRNAs targeted to *RUNX3 in vitro*

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## Abstract

**AIM:** To investigate transcriptional gene silencing induced by short hairpin RNAs (shRNAs) that target gene prompter regions of *RUNX3* gene, and whether shRNAs homologous to DNA sequences may serve as initiators for methylation.

**METHODS:** According to the principle of RNAi design, pSilencer3.1-H1-shRNA/*RUNX3* expression vector was constructed, The recombinant plasmid shRNA was transfected into human stomach carcinoma cell line SGC7901 with Lipofectamine 2000. Then, the positive cell clones were screened by G418. The mRNA and protein expression level of RUNX3 in the stable transfected cell line SGC7901 were determined by RT-PCR, Western blotting and immunocytochemistry. Characteristics of the cell lines including SGC7901, pSilencer3.1-H1/SGC7901 and pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 were analyzed with growth curves, clone formation rate and cell-cycle distribution. The activated level of RUNX3 was examined after treatment with the different density of 5'-aza-2'-deoxycytidine (5-Aza-CdR) by using semi-quantitative RT-PCR and Western blotting.

**RESULTS:** In the cell line SGC7901 transfected with pSilencer3.1-H1-shRNA/*RUNX3*, mRNA and protein expression of the *RUNX3* gene was lost identified by RT-PCR, Western blotting and immunocytochemistry assay. The growth of pSilencer3.1-H1-shRNA/ *RUNX3*/SGC7901 cells without expression of *RUNX3* was the fastest ($P < 0.05$), its rate of clone formation was the highest ($P < 0.01$), and the cell distribution in $G_0/G_1$ and S/M phases was lowest and highest, respectively ($P < 0.05$), compared with that of the transfected pSilencer3.1-H1 and non-transfected cells. Through RT-PCR and Western blot assay, inactivated *RUNX3* could not be reactivated by 5-Aza-CdR.

**CONCLUSION:** We found that, although shRNAs targeted to gene prompter regions of *RUNX3* could effectively induce transcriptional repression with chromatic changes characteristic of inaction promoters, this was independent of DNA methylation, and the presence of RNA-dependent transcriptional silencing showed that RNA-directed DNA methylation might be an existing gene regulatory mechanism relative to the methylated in humans.



**Key words:** RNA interference; Short hairpin RNAs; Promoter; DNA methylation; RUNX3; Stomach carcinoma

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## INTRODUCTION

The human runt-related transcription factor 3 gene (*RUNX3*), a novel tumor suppressor, was originally cloned as *AML2* in human leukocythemia by Levanon in 1994 and is located on human chromosome 1p36.1[1]. *RUNX3* contains two promoters, p1 and p2, one at the beginning of exon 1 and the other in front of exon 2. The mRNA expression of *RUNX3* comes from transcription by p2, which has a high GC content due to a large conserved

CpG island around it and contains Sp1 sites and a CCAAT box without a TATA box. Recently, *RUNX3* has been considered to be a vital gene in the occurrence and development of gastric carcinoma[2].

Multiple genes participate in the occurrence and development of gastric carcinoma, which is at the top list of malignant tumors in China. Deactivation of anti-oncogenes is more frequent than activation of proto-oncogenes in the gastric carcinogenesis process. Anti-oncogenes are devitalized by chromosomal absence, gene mutation, and aberrant CpG island hypermethylation of gene promoters. Moreover, the data have suggested that, due to transcription termination of *RUNX3* by aberrant CpG island hypermethylation at the promoter's 5'-terminal CpG island area, the active level of *RUNX3* decreased by 40% and 90%, respectively, in early and advanced gastric cancer. However, the inactivation mechanism of the aberrant CpG island hypermethylation is still unclear[3-6].

The phenomenon by which small interference RNA molecules that target gene prompter regions can induce transcriptional gene silencing in a DNA cytosine methylation-dependent manner (RNA-directed DNA methylation, RdDM) was initially discovered by Wassenegger *et al* in 1994 in plants infected with epiviruses[7]. This suggested that RdDM might have an important role in the loss of expression of tumor suppressor genes, and *RUNX3*. We constructed a recombinant plasmid, which expressed short hairpin RNAs (shRNAs) complementary to the CpG island, including the promoter of the human *RUNX3* gene, to investigate transcriptional gene silencing and the mechanism mediated by shRNAs that target gene prompter regions of *RUNX3* in human stomach carcinoma cell lines.

## MATERIALS AND METHODS

### Design and synthesis of shRNAs

shRNAs that target the nucleotide sequence (GCCACTT-GATTCTGGAGGA) in the promter of human *RUNX3* (NCBI, accession number AL023096) were designed by Ambion using restriction enzyme sites *Bam*H I and *Hin*dⅢ, and synthesized by Shanghai Biological Engineering Limited Company (China). The oligonucleotide sequence was as follows: 5'-GATCCGCCACTTGATTCTG GAGGATTCAAGAGATCCTCCAGAATCAAGTGGCG GTTTTTTGGAAA-3' and 5'-AGCTTTTCCAAAAAACC-GCCACTTGATTCTGGAGGATCTCTTGAATCCTC-CAGAATCAAGTGGCG-3'.

### Construction of recombinant plasmid

The eukaryotic expression vector pSilencer3.1-H1 (Ambion), which was kindly provided by Doctor Lei XiaoYong (Institute of Pharmacologic Research, Nanhua University), was digested by *Bam*H I and *Hin*dⅢ restriction enzymes (MBI). The digested products, which were reclaimed using Gel-reclamation Kit (Omega), and the oligonucleotide sequence were ligated by $T_4$ DNA ligase (MBI) at 4℃ overnight to generate the recombinant pSilencer3.1-H1-shRNA/RUNX3. Then, the recombinant plasmid was validated by restriction enzymes digestion and sequencing.

### Cell line and cell culture

Human gastric cancer SGC-7901, MKN-28, SGC7901, MGC803 and BGC823 cell lines, which were first cultured by Academia Sinica (Shanghai, China), were purchased from the Central South University (Hunan, China). All cells were cultured with RPMI 1640 (Gibco) medium and supplemented with 100 mL/L calf serum (Sijiqing, Hangzhou, China) at 37℃ in a humidified atmosphere containing 50 mL/L $CO_2$.

### Transfection of plasmids

Fifty thousand SGC-7901 cells were seeded into each well in six-well plates and grown overnight. The medium was replaced with complete medium without fetal bovine serum (FBS). The recombinant pSilencer3.1-H1-shRNA/*RUNX3* and the empty pSilencer3.1-H1 were transfected into SGC7901 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The medium was replaced with a fresh medium of calf serum (150 mL/L) after 5 h transfection. Two days later, the transfected cells were selected by G418 (200 μg/mL) (Huamei Biotechnology Company, Beijing, China) until positive clones were discovered after 10 d. The cells were cultured and finally selected by G418 (100 μg/mL) for a further 10 d. Single clones were selected to build a stable transfected cell line.

### Chemical reagents

5'-Aza-2'-deoxycytidine (5-Aza-CdR), which is a DNA methyltransferase inhibitor, was purchased from Sigma (USA). It was diluted with PBS (pH 6.8), and finally prepared as 10 μmol/L mother liquor by filtrated sterilization. The mother liquor was stored at -70℃.

### Total RNA extraction and cDNA preparation

Total RNA was extracted from each sample using the Total RNA Extract Kit (Omega) following the manufacturer's instructions. The concentration of RNA was measured by spectrophotometry. Total RNA was reverse-transcribed to cDNA with reverse transcriptase (RT) reagents (Promega) according to the manufacturer's protocol. Briefly, the RT reaction was carried out in a final volume of 20 μL that contained 4 μL 25 mmol/L $MgCl_2$, 2 μL 10 × RT buffer, 2 μL 10 mmol/L dNTP mixed liquor, 1 μL RNase inhibitor, 1 μL Oligo (dT) 15 primers, 15 U AMV retroviridase, 8.37 μL water free from enzyme, and 2 μL total RNA. The mixture was incubated at 70℃ for 10 min and 42℃ for 60 min, and RT was inactivated by heating at 95℃ for 5 min, followed by incubation at 4℃ for 5 min.

### Determination of growth curve

SGC-7901, pSilencer3.1-H1/SGC7901 and pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 cells were suspended in RPMI1640 medium and seeded in a 24-well plate at a density of $2.0 \times 10^4$ cells/well. Culture medium was changed every 2 d. The number of cells was counted consecutively for 8 d. Each experiment was done in triplicate.

### Colony formation

Single cells were mixed with the semi-solid agar (3 g/L) with growth medium and plated on a basal agar layer

(6 g/L) in three six-well plates. The cells were cultured at 37℃ in a humidified atmosphere that contained 50 mL/L $CO_2$ for 10 d. Then, colonies that contained > 100 cells in soft agar were counted under an inverted microscope and the rate of colony formation was calculated by the mean percentage of colonies.

### Flow cytometry (FCM) with propidium iodide (PI) staining

The cells were passaged at a density of $5.0 \times 10^5$/mL. After 4 h, they were treated with serum-free medium for 12 h, followed by treatment with medium that contained calf serum (100 mL/L) for 24 h. Cells were collected by trypsinization and prepared as a single cell suspension by mechanical blowing with PBS, washed with cold PBS twice, and fixed with 700 mL/L alcohol at 4℃ for 24 h. Fixed cells were washed with PBS and stained with PI (50 μg/mL in PBS) for 30 min at room temperature in the dark. DNA content in PI-stained cells was detected by FCM.

### Primers and real-time RT-PCR

As shown in Table 1, specific primers for *RUNX3* and β-*actin* genes were designed based on sequence data from the GenBank database. The primers were purchased from Shanghai Biological Engineering. Conditions for all PCRs were optimized on Cycler iQ (Bio-Rad, USA) and the optimum annealing temperature was 55℃. The following cycler running protocol was used: denaturation (95℃, 2 min), amplification and quantification repeated 35 times (94℃ for 30 s, 55℃ for 30 s, and 72℃ for 1 min). In addition, a non-template control (dd$H_2O$ control) was analyzed for each template. All samples were amplified simultaneously in triplicate.

### Western blot analysis

Cells were collected, washed three times with PBS, lysed in cell lysate that contained 0.1 mol/L NaCl, 0.01 mol/L Tris-HCl (pH 7.6), 0.001 mol/L EDTA (pH 8.0), 1 μg/mL aprotinin and 100 μg/mL PMSF, and then centrifuged at 13000 × *g* for 10 min at 4℃. The extracted protein sample (50 μg total protein/lane) was added to the same volume of sample buffer and subjected to denaturation at 100℃ for 10 min. The samples were electrophoresed on 100 g/L SDS PAGE at 28 mA for 30 min until they reached the bottom of the spacer gel, separated on the separation gel at 120 V for 1.5 h, and finally transferred onto PVDF membrane at 105 mA for 3.5 h at 4℃. The PVDF membrane was treated with TBST that contained 50 g/L skimmed milk at room temperature for 2 h, followed by incubation with the primary antibody RUNX3 (1:100 dilution) (Boaosen Biotechnology Company, Beijing China; bs-0378R) at 37℃ for 2 h or 4℃ overnight. After being washed with TBST for 30 min, the corresponding secondary antibody (1:2000 dilution) (Zhongsan Jinqiao Biotechnology Company, Beijing, China) was added and incubated at room temperature for 1 h. The membrane was then washed three times for 15 min each with TBST. Fluorescence was produced from solution A and B that contained a chemiluminescence generator. Both RUNX3 and β-actin protein expression were quantitatively estimated by densitometric scanning performed with Imaginer 2200. RUNX3 protein concentration was normalized to β-actin level and expressed as a densitometric ratio.

**Table 1 Sequence of primers and amplified length of genes**

| Gene | Sequence | Amplified length (bp) |
|---|---|---|
| *RUNX3* | 5′-GAGTTTCACCCTGACCA TCACTGTG-3′ | 870 |
| | 5′-GCCCATCACTGGTCTTGAAGGTTGT-3′ | |
| β-*actin* | 5′-CTACAATGAGCTGCGTGTGC-3′ | 500 |
| | 5′-AGGAGGACTTCTTCTCGAT-3′ | |

### Immunohistochemistry

Cells were seeded on the axenic cover glass in six-well plates at $1 \times 10^5$ cells/well, and were grown overnight. The cover glasses with adhered cells were washed three times with PBS, and fixed in 95% alcohol for 10 min. After being rinsed three times in PBS, the endogenous peroxidase activity was suppressed by 30 mg/L hydrogen peroxide for 15 min, followed by rinsing three times in PBS. Antigen repair was performed by immersing the sections in 10 mmol/L sodium citrate buffer (pH 6.0) and heating for 15 min in a microwave oven. Non-specific binding was blocked by incubation with 30 mL/L bovine serum albumin (BSA) for 40 min. The cells were treated for 16 h with goat anti-human polyclonal IgG antibodies of RUNX3 (Boaosen Biotechnology Company; bs-0378R) at 37℃ for 2 h or 4℃ overnight, according to the manufacturer's recommended concentration (1:200 dilution). PBS was used as a negative control. After washing three times in PBS, the cover glasses were treated with biotinylated rabbit anti-goat immunoglobulin (Zhongsan Jinqiao Biotechnology Company) for 1 h at room temperature and then by horseradish peroxidase-streptavidin complex (Man Xin Biotechnology Company, Huzhou, China) for 30 min. The cover glasses were then washed three times in PBS and incubated in DAB for 2 min. Next, the cover glasses were rinsed gently with distilled water and counterstained with hematoxylin for 30 s, and dehydrated in alcohol prior to mounting. Images were collected by Olympus DD70 BX51 (Olympus, Japan) and analyzed by IMAGE-PRO plus 4.1 software (Media Cybernetics, USA). Eight visual fields in each section were randomly selected and the mean value of relative OD was measured and calculated by taking the OD of background as 1. The extent of immunohistochemical staining was categorized as positive (1-1.5) and strongly positive (> 1.5).

### Statistical analysis

Experimental data in each group were expressed as mean ± SD. Analysis of variance (ANOVA) was performed with the Statistical Package for the Social Sciences (SPSS 13.0) for Windows by using one way ANOVA and pairwise comparison with Student's *t* test. $P < 0.05$ was considered statistically significantly.

## RESULTS

### RUNX3 protein expression in human gastric carcinoma cell lines

Western blot analysis showed that the relative densities

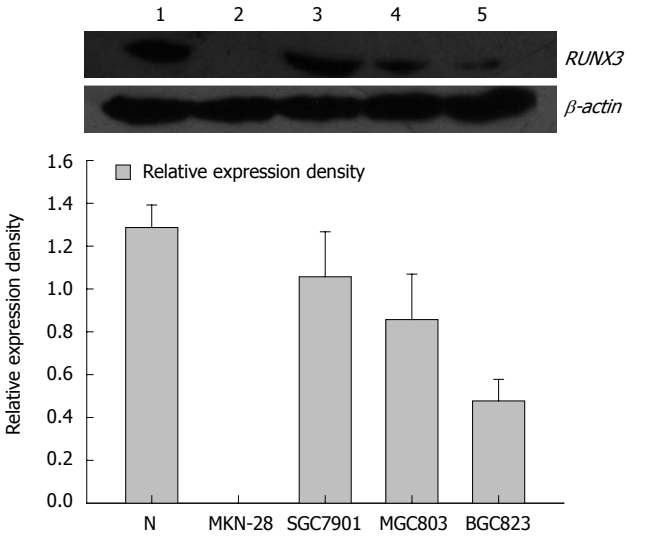



**Figure 1** RUNX3 expression in the gastric cancer cells detected by Western blot. 1: Positive control: Normal gastric tissue; 2: MKN-28 cells; 3: SGC-7901 cells; 4: MGC-803 cells; 5: BGC-823 cells.

of RUNX3 protein bands of normal gastric mucosa, well-differentiated gastric carcinoma cell line MKN-28, moderately differentiated gastric carcinoma cell line SGC7901, poorly differentiated gastric carcinoma cell lines MGC803 and BGC823 were: 1.279 ± 0.105, 0.003 ± 0.001, 1.057 ± 0.610, 0.857 ± 0.212, 0.477 ± 0.321 respectively (Figure 1). The moderately differentiated gastric carcinoma cell lines SGC7901 was selected for further experiments.

### *Identification of the recombinant plasmids*

The recombinant eukaryotic expressing vector-pSilencer3.1-H1-shRNA/*RUNX3* was cut by the double restriction enzyme *Bam*H I and *Hin*dⅢ. Then the enzyme products were electrophoresis using the agar gel (27 g/L). And the objective fragment 65 bp was obtained (Figure 2).

### *mRNA and protein expression of RUNX3 in transfected SGC-7901 cells*

In SGC-7901, pSilencer3.1-H1/SGC7901 and pSilencer3.1-H1-shRNA/ *RUNX3*/SGC790 cells, RT-PCR showed that the relative densities of *RUNX3* mRNA bands were 1.116 ± 0.217, 1.057 ± 0.187 and 0.002 ± 0.001, respectively (Figure 3), and Western blot analysis indicated that the relative densities of RUNX3 protein bands were 0.812 ± 0.091, 0.786 ± 0.103 and 0.021 ± 0.002, respectively (Figure 4). Immunocytochemistry disclosed that RUNX3 protein was located in the endochylema and the nucleus, and loss of expression of *RUNX3* in SGC790 cells transfected with recombinant plasmid-pSilencer3.1-H1-shRNA/*RUNX3* (Figure 5).

### *Effect of RUNX3 on proliferation of SGC-7901 cell line*

The growth curves showed that the pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 cells grew more quickly than the control cells, SGC7901 and pSilencer3.1-H1/SGC7901 cells ($P < 0.05$, Figure 6), but that the rate of cell growth

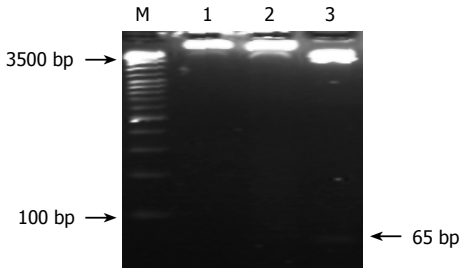



**Figure 2** Identification of recombinant by restrict endonucleases digestion. M: DNA Ladder; 1: pSilencer3.1-H1/SGC7901; 2: pSilencer3.1-H1-shRNA/*RUNX3*/SGC-7901; 3: pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 cut with *Bam*H Ⅰ/*Hin*dⅢ.

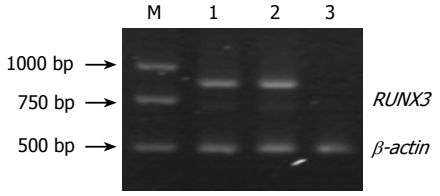



**Figure 3** *RUNX3* expression in the three groups of cells detected by RT-PCR. M: DL2000 marker; 1: SGC7901 cells; 2: pSilencer3.1-H1 /SGC7901 cells cells; 3: pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 cells.

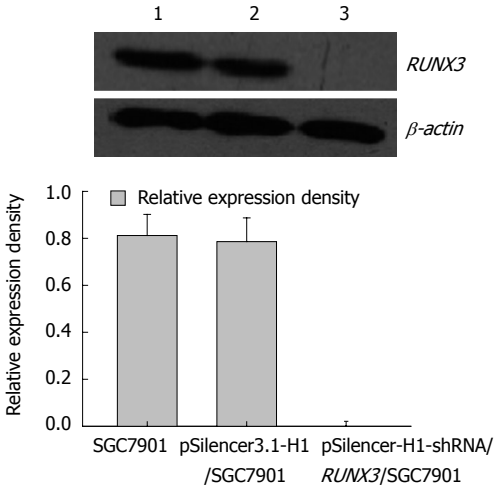



**Figure 4** RUNX3 expression detected by Western blot. 1: SGC7901 cells; 2: pSilencer3.1-H1/c SGC7901 cells; 3: pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 cells.

was similar in the two control groups. The results of the soft-agar colony-formation experiment indicated that a significant increase in the colony-formation rate in the pSilencer3.1-H1-shRNA/*RUNX3*/SGC790 cells (17.4 ± 0.31)% was found compared with that in the control SGC-79011 (9.9 ± 0.3)% and pSilencer3.1-H1/SGC7901 (9.7 ± 0.6)% cells ($P < 0.01$, Figure 7). The colony-formation rates of the two control cells were similar. FCM with PI staining suggested that the proportion of cells in $G_0/G_1$ and S phases in pSilencer3.1-H1-shRNA/RUNX3/SGC7901 cells significantly decreased and increased, respectively ($P < 0.05$, Figure 8 and Table 2), compared with the control cells.

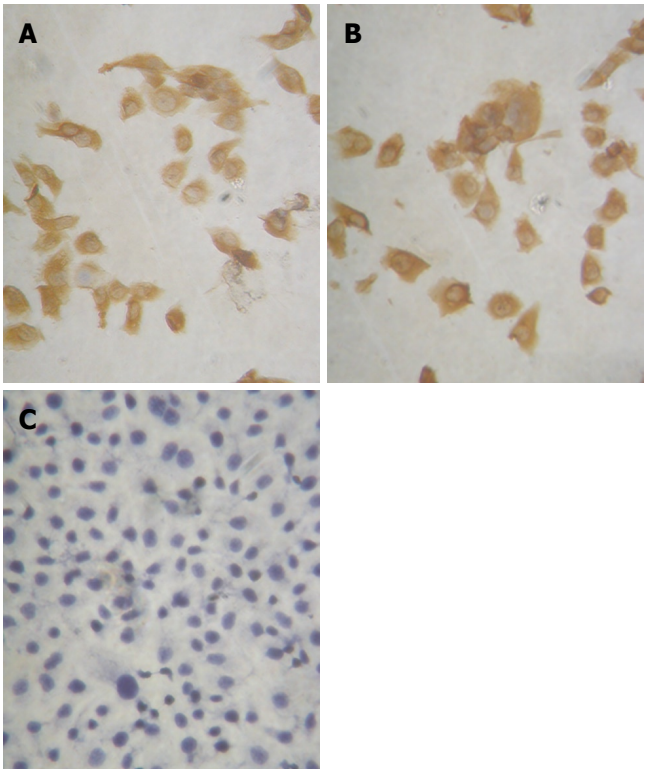



**Figure 5** RUNX3 expression in SGC7901 cells by immunohistochemistry (× 400). **A**: SGC7901 cells, the protein of RUNX3 expressed and located in the endochylema and the nucleus in the SGC7901 cell without transfection; **B**: pSilencer3.1-H1/SGC7901 cells, the protein of RUNX3 expressed and located in the endochylema and the nucleus in the SGC7901 cell with the plasmid DNA of pSilencer3.1-H1; **C**: pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 cells, the protein of RUNX3 lost expression in the SGC790 cells transfected with the recombinated plasmid-pSilencer 3.1-H1-shRNA/RUNX3.

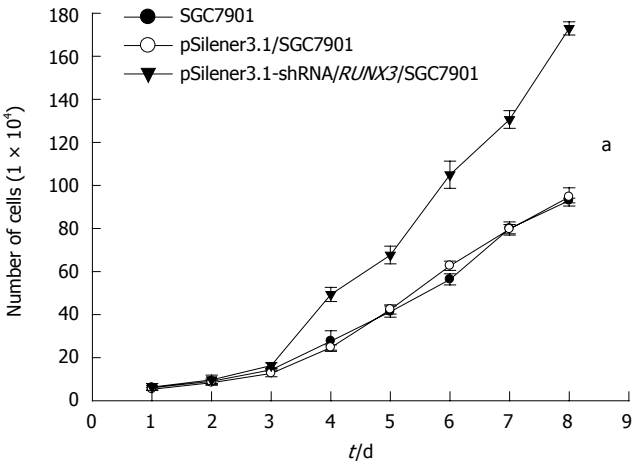
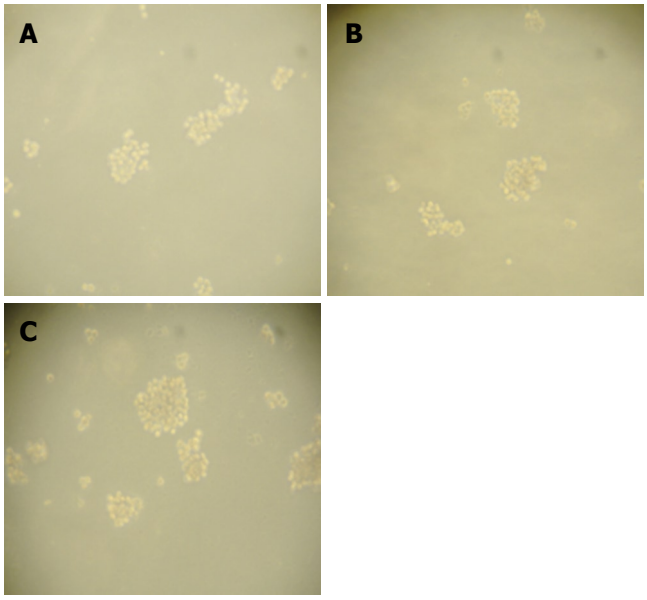



**Figure 6** The growth effect of SGC7901 cell by silencing *RUNX3* ($^aP < 0.05$).

### *Expression of RUNX3 in transfected cells treated with 5-Aza-CdR*

The pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 cells ($1 \times 10^6$/100 mL) were treated with $5 \times 10^{-6}$ mol/L and $1 \times 10^{-5}$ mol/L 5-Aza-CdR. Cells were collected after 3 d treatment. The cells treated with $5 \times 10^{-6}$ mol/L 5-Aza-CdR were named experimental group 1, and those treated with $1 \times 10^{-5}$ mol/L 5-Aza-CdR were experimental group 2.



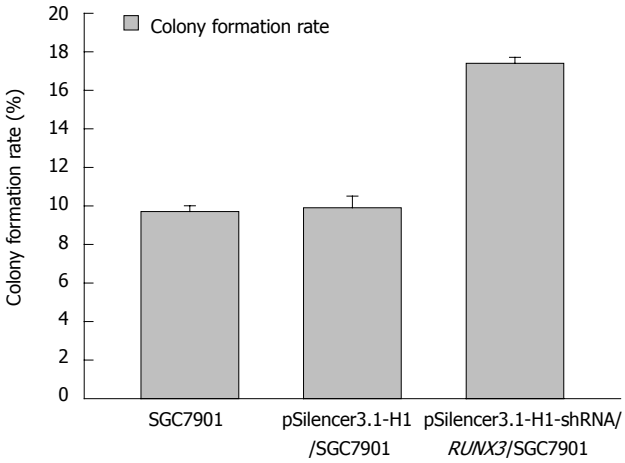




**Figure 7** The colony formation assay of SGC7901 in the soft agar (×100). **A**: SGC7901 cells, the cloning efficiency was 9.9% ± 0.3% in the SGC7901 cell without transfection; **B**: pSilencer3.1-H1/SGC7901 cells, the cloning efficiency was 9.7% ± 0.6% in the SGC7901 cell with the plasmid DNA of pSilencer3.1-H1; **C**: pSilencer3.1-H1-shRNA/*RUNX3*/SGC 7901 cells, the cloning efficiency was 17.4% ± 0.31% in the SGC790 cells transfected with the recombinated plasmid-pSilencer3.1-H1-shRNA/RUNX3. A significant increase of the colony formation rate in the pSilencer3.1-H1-shRNA/*RUNX3*/SGC790 cells was discovered compared with the controls-the SGC-79011cells and pSilencer3.1-H1/SGC7901cells ($P < 0.01$).

**Table 2 Cell cycle distribution of three groups by FCM ($n$ = 3, mean ± SD, %)**

| Groups | $G_0/G_1$ | S | $G_2/M$ |
|---|---|---|---|
| SGC7901 | 43.2 ± 1.2 | 47.7 ± 1.1 | 9.0 ± 1.5 |
| pSilencer3.1-H1-shRNA/SGC7901 | 40.3 ± 2.0 | 49.3 ± 0.9 | 8.1 ± 0.3 |
| pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 | 37.2 ± 1.9 | 60.5 ± 0.8 | 7.3 ± 0.9 |

RT-PCR showed that the relative densities of *RUNX3* mRNA bands were 0.861 ± 0.167, 0.004 ± 0.001, 0.002 ± 0.001 and 0.002 ± 0.001, respectively (Figure 9), and Western blot analysis that the band densities of RUNX3 were 1.013 ± 0.138, 0.003 ± 0.001, 0.002 ± 0.001 and 0.005 ± 0.001, respectively (Figure 10).

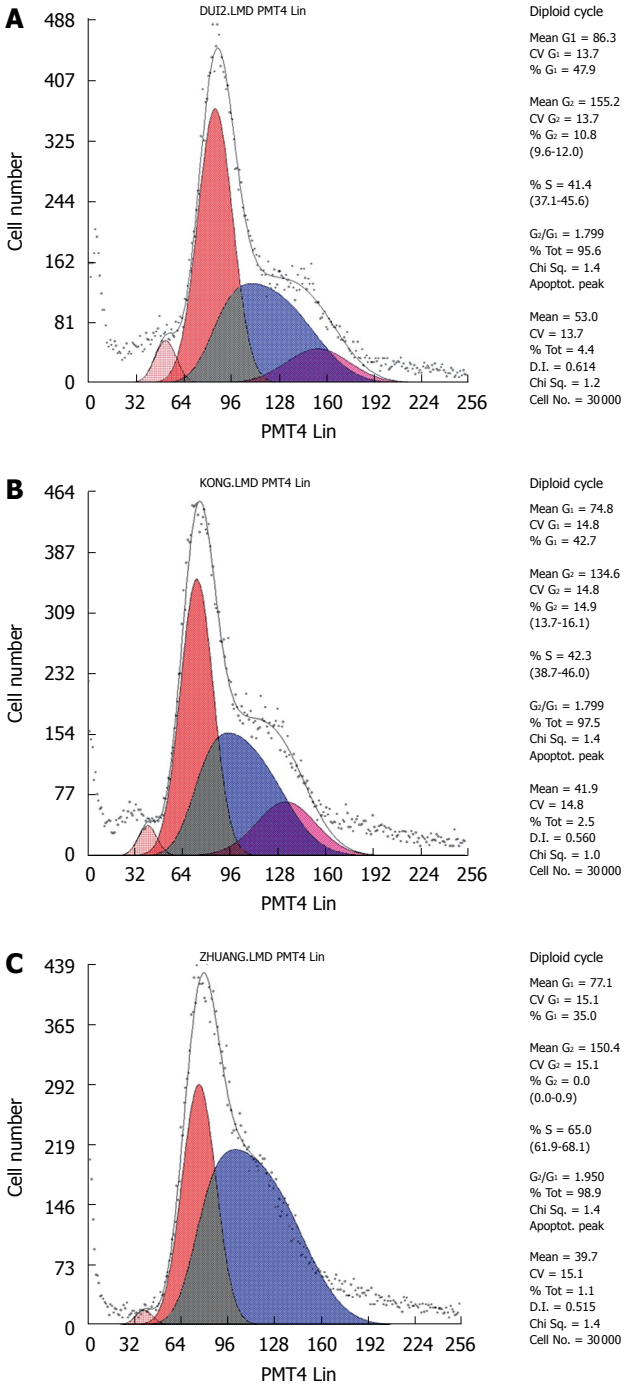



**Figure 8** The cell cycle analysis by FCM. **A**: SGC790 cells, the cell proportions of $G_0/G_1$ and S stages were 43.2% ± 1.2% and 47.7% ± 1.1% in the SGC7901cell without transfection, respectively; **B**: pSilencer3.1-H1/SGC7901 cells, the cell proportions of $G_0/G_1$ and S stages were 40.3% ± 2.0% and 49.3% ± 0.9% in the SGC7901 cell with the plasmid DNA of pSilencer3.1-H1 respectively; **C**: pSilencer3.1-shRNA/*RUNX3*SGC7901 cells, the cell proportions of $G_0/G_1$ and S stages were 37.2% ± 1.9% and 60.5% ± 0.8% in the SGC790 cells transfected with the recombinated plasmid-pSilencer3.1-H1-shRNA/RUNX3 respectively. Compared with that of $G_0/G_1$ and S stages of two controls, the cell proportions of $G_0/G_1$ and S stages in the pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 cells decreased and increased obviously, respectively ($P < 0.05$).

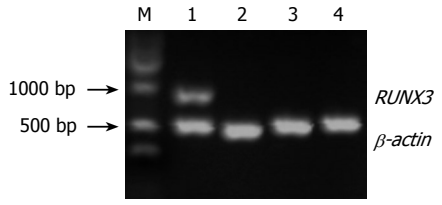



**Figure 9** *RUNX3* expresion detected by RT-PCR in the four groups of SGC7901 cells. M: Marker; 1: Positive control, SGC7901 cells untreated with 5-Aza-CdR; 2: Negative control, pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901cells untreated with 5-Aza-CdR; 3: Experimental group 1, pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901cells treated with $5 \times 10^{-6}$ mol/L 5-Aza-CdR; 4: Experimental group 2, pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 cells treated with 1 x $10^{-5}$mol/L 5-Aza-CdR.

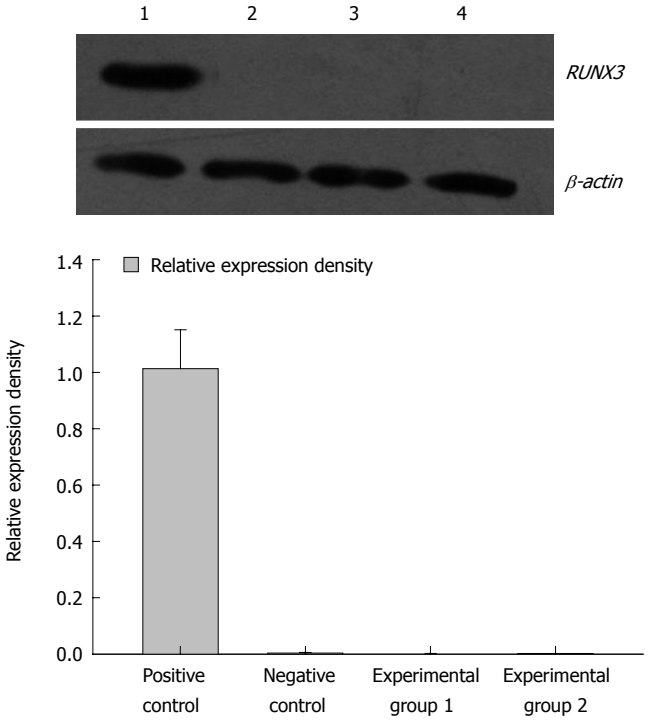



**Figure 10** RUNX3 protein detected by Western blotting in the four groups of SGC7901 cells. 1: Positive control, SGC7901 cells untreated with 5-Aza-CdR; 2: Negative control, pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901cells untreated with 5-Aza-CdR; 3: Experimental group 1, pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901cells treated with $5 \times 10^{-6}$ mol/L 5-Aza-CdR; 4: Experimental group 2, pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 cells treated with $1 \times 10^{-5}$ mol/L 5-Aza-CdR.

## DISCUSSION

In the occurrence and development of gastric carcinoma, the mechanism of abnormal silencing of anti-oncogenes mediated by epigenomics in the un-first-class structure levels of nucleotide sequence such as DNA methylation and histone modification, is still unclear. Many researchers have assumed that aberrant CpG island hypermethylation of the promoters of anti-oncogenes exists frequently in tumors. However, it is still not known how the aberrant CpG island hypermethylation takes place. Recently, the phenomenon by which siRNA molecules that target gene prompter regions can induce transcriptional gene silencing in a DNA cytosine methylation-dependent manner has attracted much attention.

siRNA can induce post-transcriptional gene silencing through RNA-RNA binding and transcriptional gene silencing through RNA-DNA binding. Transcriptional gene silencing refers to siRNA molecules that hinder production of mRNA from DNA before gene transcription, by

modifying chromosomal DNA and histones. It include three patterns; RNA-directed DNA methylation, RNA-directed heterochromatinization and RNA-directed DNA ablation[8-10]. The idea of RNA-directed DNA methylation was obtained from a propagation study in *Arabidopsis thaliana*[11]. It is not known whether a similar mechanism exists in mammalian systems.

Some elements, such as the required constituents of RNA-directed DNA methylation in plants, have been discovered in mammals. They contain three DNA methyltransferases (DNMT) 1, which play a role in maintaining methyltransferases. DNMT3A and DNMT3B can be let in the new methylated sites. The two processes including the methylated maintenance and repeated methylation might exist to affect genome DNA in mammals as in plants[12]. DNMT3A and DNMT3B have been observed to participate in the orientation of siRNA that target gene promoters[13]. As to human HeLa cells, the percentage of the mature-type miR-21 located in the cytoplasm and the nucleus were 80% and 20%, respectively. After transfection with fluorescently-labeled siRNA/miR-21, the fluorescence which siRNA/miR-21 binds the complementary sequences was observed in the cytoplasm and the nucleus. A identical consequence has appeared in cells transfected with fluorescently-labeled siRNA/let-7a[14].

Some previous investigations have reported that siRNA molecules that target gene prompter regions can induce transcriptional gene silencing and DNA cytosine methylation around the promoter region. In the study by Morris *et al*, elongation factor 1 α (*EF1*α) was knocked down by the prompter-directed siRNA in HEK293 cells by transfection. After treatment with 5-Aza-CdR (4 μmol/L) and trichostatin A (TSA, 0.05 mmol/L), deactivation of EF1α was reversed and detected through nuclear run-on assays and RT-PCR. According to the above data, the transcriptional silencing of EF1a might be associated with DNA methylation of the targeted sequence[15]. According to research by Castanotto *et al*, shRNAs, molecules homologous to DNA sequences in the prompter and early transcribed regions of RASSF1, can direct the partial gene silencing and low levels of *de novo* DNA methylation. They used a methylation-specific polymerase chain reaction (MSP) and bisulfite sequencing in HeLa cells[16]. Moreover, Weinberg's investigation showed that transcriptional silencing and DNA methylation of *EF1a* can be directed by antisense interference alone in human 293T cells transfected with *EF1*α siRNA. This targets the promoter of it using the peptide MPG, which transports siRNAs to the nucleus. This silencing is accompanied by increased methylation level in histone 3 lysine 9 (H3K9) and histone 3 lysine 27 (H3K27). Furthermore, siRNAs *EF52* is associated with the transient expression of Flag-tagged DNMT3A, the targeted *EF1*α promoter, and trimethylated H3K27[17]. Previous studies have indicated that, in mammalian cells, the unique RNA endonuclease Dicer, which cuts long dsRNA to form siRNA, is situated in the cytoplasm[18-20]. RNA-induced silencing complex (RISC), which binds to siRNAs in the cell-substance, may gain access to the nucleus when caryotheca vanishes during cell division, or it may be transported into the nucleus, as in plants. Furthermore, siRNA can integrate the homologous DNA sequences (promoters) and induce transcriptional gene silencing in a DNA cytosine methylation-dependent manner[11,21].

We confirmed that aberrant CpG island hypermethylation of the promoter of *RUNX3* is a critical pathway to cause down-regulation or loss of expression of the gene. How may the pathway be connected with the mechanism of RNA-directed DNA methylation? In our experiment, on the basis of the principle of RNAi design, pSilencer3.1-H1-shRNA/*RUNX3* expression vector was constructed, and transfected in SGC7901 cells by liposomes. RT-PCR, western blot and immunocytochemistry showed that mRNA and protein expression of RUNX3 were absent in the stable cell line SGC7901 transfected with the recombinant plasmid. Moreover, compared with those transfected with pSilencer3.1-H1 and non-transfected cells, cells transfected with pSilencer3.1-H1-shRNA/*RUNX3* grew most quickly. Both the clone number and size were largest following soft-agar colony-formation assay ($P < 0.01$) and the number of cells in $G_0/G_1$ and S/M phases was lowest and highest following FCM ($P < 0.05$). The above results indicated that, through RNA-dependent transcriptional silencing (RdTS), knockdown of the gene at the transcriptional stage was feasible and effective. After loss of expression of RUNX3 protein, the proliferation rate of SGC7901 cells increased. These results, like the previous description[3-6], suggested that RUNX3, a putative tumor suppressor, might have an important role in stomach tumorigenesis. However, after the deal with the different density of 5-Aza-CdR, which may reactivate anti-oncogenes silenced by *de novo* methylation, inactivated RUNX3 was not reactivated, as shown by RT-PCR and the silenced. The phenomenon was different from the RdDM described in plants. At the same time, similar results have been reported. In two human glioblastoma cell lines, U-87 and U-118, siRNA homologous to the promoter region of *huntingtin* gene can repress transcriptional expression of the target gene. However, no CpG methylation has been observed on the target sequences by bisulfite-mediated genomic sequencing[21,22]. In the research of Ting *et al*, the human colorectal cancer cells, HCT116, were transfected with two 21-nucleotide-long dsRNAs (dsCDH1-1 and dsCDH1-2). The two sequences were homologous to the CpG island of the endogenous gene promoter of *CDH1* but did not overlap any known transcribed sequences. The findings suggested that promoter-targeting dsRNAs could effectively silence *CDH1* transcription, which results in a net decrease in mRNA and protein production, as demonstrated by RT-PCR and Western blot. Absence of DNA methylation at the targeted sequences was reviewed by MSP and bisulfite sequencing. Transfection of the human breast cancer cells, MCF-7, used a silencing strategy virtually identical to that above for *CDH1*. The transcriptional silencing without DNA methylation was reviewed and the results were identical[23].

What accounts for these opposing results? We believe the answer may lie in the types of DNA methylation assays performed. In the work by Morris *et al*, the DNA methylation data remain to be verified because DNA

methylation was not as extensive, and there was indirect evidence for 5-Aza-CdR and TSA, a histone-deacetylase inhibitor. Co-administration alleviating the silencing was difficult to interpret as the reason of the *EF1α* silencing. In other investigations, MSP distinguishes between non-methylated and methylated alleles by using two sets of primers to amplify either non-methylated or methylated sequences after bisulfite treatment, which specifically converts non-methylated cytosines to uracils. The shortcoming of MSP analysis is that it examines a few CpG sites in the sequences that are recognized by the primers, and the design of primers is very difficult. Mismatch sequencing was used to verify the overall target region by unbiased primers that did not contain CpG dinucleotides to amplify the bisulfite-converted promoter region. Due to the existence of incomplete mismatch conversions, unconverted cytosine residues in both CpG and CpG contexts remained as cytosine and created a false negative result. Furthermore, the PCR primers that were used previously to obtain the initial mismatch sequencing template that contains CpG sites may bias the amplification step and produce problematic mismatch sequencing results[5]. Such non-conversions may partly explain the different results in the mismatch sequencing. In the current study, the level of DNA methylation was testified preliminary and indirect. The finding that inactivation of *RUNX3* was not reactivated with 5-Aza-CdR could not confirm sufficiently that the transcriptional silencing of *RUNX3* was independent of DNA methylation. Therefore, further study is needed.

Taken together, the presence of RdTS indicated that RdDM might be an existing gene regulatory mechanism relevant to methylation in humans, even though we are currently unable to verify this. A recent study has shown that, in *A. thaliana,* RdDM is studied in the progeny of plants being silenced, and DNA methylation may still be involved in a prolonged silencing event in human cells[24]. Therefore, further experiments, which include a thorough examination of the long-term and full-scale outcomes of RdTS, are needed to research RNA-directed DNA methylation in mammalian systems.

## COMMENTS

### Background

Aberrant CpG island hypermethylation of the promoter of *RUNX3*, a tumor suppressor, is associated with its transcriptional silencing and the loss of gene functions in stomach cancer. However, the mechanism of the aberrant CpG island hypermethylation is still unclear.

### Research frontiers

In plants, siRNA molecules that target gene prompter regions can induce transcriptional gene silencing in a DNA cytosine methylation-dependent manner (RdDM). Whether a similar mechanism exists in mammalian systems is a vital and controversial issue. Recently, the RdDM theory on gene regulation has attracted close attention from researchers and has become a highlight in tumor studies.

### Innovations and breakthroughs

This article focuses on the relationship between siRNA molecules and aberrant CpG island hypermethylation in the promoter domain of *RUNX3*, through the RNA interference principle and gene transfection technology in human gastric cancer cell lines.

### Applications

*RUNX3* is an important anti-oncogene. Its mechanism of methylation may contribute to the study of the etiology of gastric cancer, and offer a theoretical basis for gene therapy of gastric cancer

### Terminology

RNA interference is an evolutionarily conserved mechanism of gene silencing. It can affect transcriptional gene silencing induced by siRNA that target gene prompter regions in a DNA cytosine methylation-dependent manner.

### Peer review

This is a good study in which the authors demonstrated that, shRNAs that targeted gene prompter regions of *RUNX3* could effectively induce transcriptional repression with chromatic changes characteristic of inaction promoters, but it was independent of DNA methylation. The study was well designed and the results are convincing.

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# Transplanted bone marrow stromal cells are not cellular origin of hepatocellular carcinomas in a mouse model of carcinogenesis

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## Abstract

**AIM:** To investigate the malignant potential of hepatic stem cells derived from the bone marrow stromal cells (BMSCs) in a mouse model of chemical hepatocarcinogenesis.

**METHODS:** BMSCs from male BALB/c mice were harvested and cultured, then transplanted into female syngenic BALB/c mice *via* portal vein. Hepato-carcinogenesis was induced by 6 mo of treatment with diethylnitrosamine (DEN). Six months later, the liver was removed from each treated mouse and evaluated by immunohistochemistry and fluorescence *in situ* hybridization (FISH).

**RESULTS:** Twenty-six percent of recipient mice survived and developed multiple hepatocellular carcinomas (HCCs). Immunohistochemically, HCC expressed placental form of glutathione-S-transferase (GST-P) and α-fetoprotein, but did not express cytokeratin 19. Y chromosome positive hepatocytes were detected by fluorescent *in situ* hybridization (FISH) in the liver of mice treated with DEN after BMSCs transplantation while no such hepatocytes were identified in the liver of mice not treated with DEN. No HCC was positive for the Y chromosome by FISH.

**CONCLUSION:** Hepatic stem cells derived from the bone marrow stromal cells have a low malignant potential in our mouse model of chemical hepatocarcinogenesis.



**Key words:** Bone marrow stromal cell; Stem cell; Hepatocarcinogenesis; Animal study

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world[1]. Hepatitis B or C virus can induce chronic hepatitis and potentially result in liver cirrhosis and HCC, and these viral infections are frequently seen among HCC patients[2]. However, there is no clear evidence as to which cell is directly involved in the development of HCCs[3-5]. Two cell lineages have been considered as candidates: the first is hepatic stem cell, and the second is mature hepatocyte.

Oval cells are small, oval shaped epithelial cells identified as hepatic stem cells in the adult liver only following severe, repetitive liver injury[6]. There are increasing evidences that oval cells are the cellular targets for transformation in the development of HCC[7-8]. Oval cells might give rise to HCC as a result of the arrest of stem cell maturation[9]. Previous studies indicated that bone marrow cells can differentiate into oval cells in rodents and that a similar process could possibly take place in humans[10,11]. The incidence of plasticity has been shown to be very variable, from extremely rare to a range from 20% to 40%[12,13]. Although there is still controversy about which part of bone marrow cells can differentiate into hepatocytes, the present study clearly shows that transplanted bone marrow cells may help restore the hepatic degenerative diseases and reduce $CCl_4$-induced liver fibrosis[14]. Some studies readily demonstrated bone marrow stromal cells (BMSCs) differentiated into hepatocyte-like cells in culture

after HGF treatment *in vitro*[15]. Therefore, BMSCs could be a valuable strategy for future replacement therapy of damaged or malfunctioned hepatocytes, because getting autologous BMSCs is easier than obtaining other tissue-specific stem cells. However, the safety and efficacy of hepatic stem cells derived from bone marrow cells should be adequately confirmed before any such therapies are tested in humans.

Our aim was to study the malignant potential of hepatic stem cells derived from BMSCs *in vivo*. To identify hepatic stem cells, BMSCs of male mice were transplanted into recipient female mice. After BMSCs transplantation, HCC was induced in the recipients by chemical hepatocarcinogenic compounds and the presence of the Y chromosome was evaluated in HCC.

## MATERIALS AND METHODS

### Animals

Six to eight week old BALB/c mice were purchased from the Animal Breeding Center of Sun Yat-Sen University (Guangzhou, China). Mice were bred and maintained in an air-conditioned animal house with specific pathogen-free conditions, using an alternate 12 h cycle of daylight and darkness, and unlimited access to chow and water. All animal experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of Sun Yat-Sen University.

### Isolation and culture of bone marrow stromal cells (BMSCs)

BMSCs were harvested from bone marrow of the femurs and tibias of male mice by inserting a 21-gauge needle into the shaft of the bone and flushing it with DMEM medium supplemented with heparin[16]. The cell suspension was centrifuged over a Ficoll step gradient (density 1.077 g/mL) (Sigma, St. Louis, MO) at 1500 r/min for 10 min. The interface fraction was then collected and cultured in DMEM medium, supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Isolated cells were grown at 37℃ and 5% $CO_2$ for 3 d. After removing the suspended cells, the adherent BMSCs were grown to 90% confluence and used between passages 3 and 4. After serum starvation for 4 h, BMSCs were treated with human recombinant HGF (Sigma-Aldrich, USA) at a concentration of 50 μg/L. Cultures were maintained by media exchange every 3 d. On d 21, all cells were detached for the next experiment.

The above detached cells were coated on the glass slides and fixed with 4% paraformaldehyde for 10 min at room temperature, followed by methanol for 2 min at -20℃, and permeabilized with 0.1% Triton X-100 for 10 min. Slides were blocked for 30 min using blocking and diluent solution, then incubated with rabbit anti-α-fetoprotein antibody or goat anti-albumin antibody at 4℃ overnight. The cells were reincubated sequentially for 30 min with FITC-conjugated secondary antibody or PE-conjugated secondary antibody. The slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma) before mounting and observed under fluorescence microscope.

### Transplantation of BMSCs

BMSCs were harvested after cultured for passages 3 or 4 and suspended in DMEM medium supplemented with penicillin/streptomycin. BMSCs were washed twice in DMEM medium before intraportal injection. Cell viability (> 95%) was measured by trypan blue dye exclusion.

Anesthesia was performed with ether and partial hepatectomy used the standard method for two-thirds resection[17]. Briefly, after ligation of the pedicule and resection of the two largest lobes (median and left), the remaining liver was composed of the caudate and epiploic lobes. BMSCs were injected into the female liver *via* the superior mesenteric vein using insulin syringes after hepatectomy[18]. A total of $10^6$ cells were injected per mouse.

### Diethylnitrosamine(DEN)-induced hepatocarcinogenesis

After partial hepatectomy and BMSCs injection, mice were allowed to recover for one week. Thereafter, DEN (Sigma) was continuously administered for 12 wk through drinking water at a final concentration of 100 μg/L to induce hepatocarcinogenesis[3].

Sixty female BALB/c mice were randomly assigned to three groups. Ten mice in the normal control group were given BMSCs and non-supplemented drinking water. Twenty-five mice in the model group were continuously administered DEN in the drinking water. Twenty-five mice in the experimental group received BMSCs and DEN. The animals were sacrificed at 6 mo after the carcinogen regimen and the livers were fixed in 10% formalin for 24 h and embedded with paraffin. Routine histology was performed with haematoxylin-eosin staining. Serial sections were cut from liver samples with the macroscopically visible liver tumors and the right lobe for pathologic examination.

### Liver histopathology

To identify characteristics of tumors in the liver after BMSCs transplantation and DNE administration, placental form of glutathione-S-transferase (GST-P), α-fetoprotein, and cytokeratin 19 were assayed immunohistochemically for these tumor nodules as previously described[19]. Briefly, after being deparaffinized with xylene, quenched with hydrogen peroxide and blocked with normal serum, the liver tissue sections were incubated for 1 h with rabbit anti-α-fetoprotein polyclonal antibody (dilution 1:100; Santa Cruz, USA), goat anti-cytokeratin 19 monoclonal antibody (dilution 1:100; Santa Cruz), or goat anti-GST-P polyclonal antibody (dilution 1:1000; Stressgen, Canada). FITC-conjugated secondary antibody or PE-conjugated secondary antibody was added. Counterstaining of nuclei was performed with DAPI for fluorescence staining.

### Fluorescent *in situ* hybridization (FISH)

Because BMSCs transplantation was performed from male donor mice to female recipient mice, the transplanted bone marrow derived cells could be recognized in the recipient by the presence of the Y chromosome in the nucleus. Therefore, FISH for the mouse Y chromosome was conducted to detect the transplanted bone marrow derived cells according to the Cambio protocol (http://www.

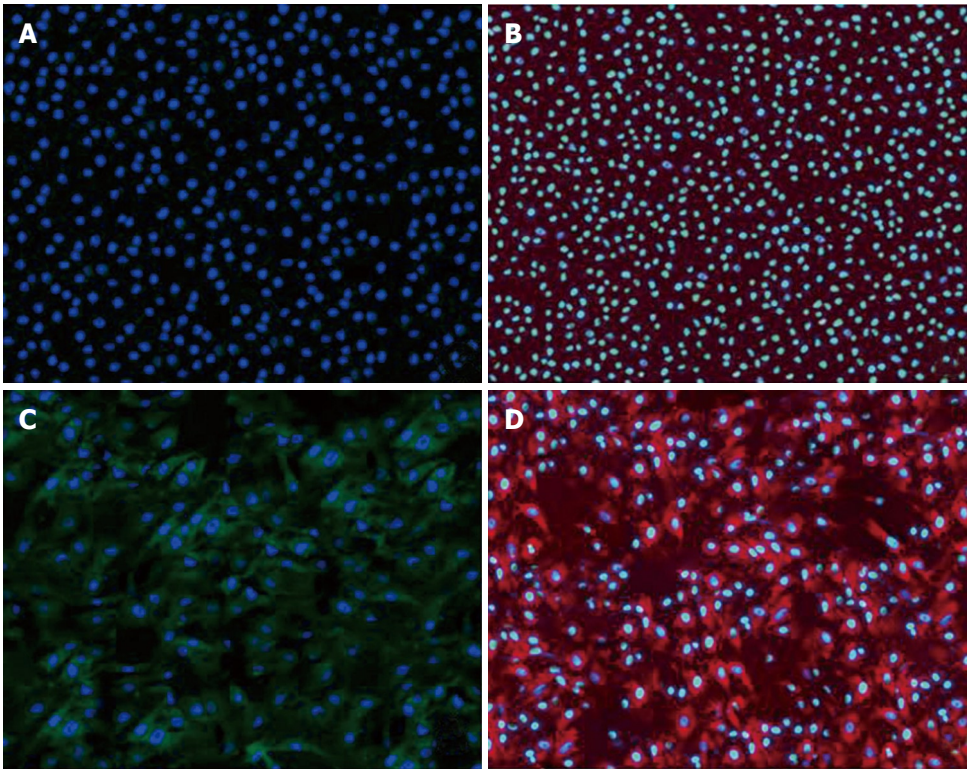


**Figure 1** Immunofluorescence of α-fetoprotein and albumin in bone marrow stromal cells, with or without treatment of HGF in culture (× 400). **A**: α-fetoprotein expression was negative in the absence of HGF; **B**: Albumin expression was negative in the absence of HGF; **C**: α-fetoprotein expression localized at the cytoplasm in hepatocyte-like BMSCs; **D**: Albumin expression localized at the cytoplasm in hepatocyte-like BMSCs.

cambio.co.uk/). Paraffin-embedded slides were deparaffinized by baking in an oven overnight at 37℃ and cleared in xylene three times for 10 min each; and they were then dehydrated and air-dried. Sections were incubated in 1 mol/L sodium thiocyanate for 10 min at 80℃, washed in PBS, and digested in pepsin (0.4% w/v) in 0.1 mol/L HCl at 37℃ for 10 min. The protease was quenched in glycine (0.2% v/w) in 2 × PBS, post-fixed in paraformaldehyde (4% w/v) in PBS, dehydrated through graded alcohols and air-dried. A fluorescein isothiocyanate (FITC)-labeled Y-chromosome paint (Cambio, Cambridge, UK) was added to the sections, sealed under glass with rubber cement, heated to 80℃ for 10 min, and incubated overnight at 37℃. The slides were washed in formamide (50% w/v)/2 × saline sodium citrate (SSC) at 37℃, washed with 2 × SSC and 4 × SSC/Tween-20 (0.05% w/v) at 37℃. The slides were rinsed in 0.5 × SSC at 37℃. FITC amplification kit (Cambio) was used to amplify fluorescence signal. The slides were counterstained with DAPI before mounting and observed under confocal microscope (Zeiss, German).

### *Statistical analysis*

Data were presented as mean ± SD. Significant differences were determined using ANOVA in SPSS10.0. $P < 0.05$ was considered statistically significant.

## RESULTS

### *Differentiation of BMSCs into hepatocytes in vitro*

To confirm the differentiation of BMSCs into hepatocytes, we selected cultural BMSCs with or without treatment of HGF for 21 d in culture and examined the expression of α-fetoprotein and albumin by immunofluorescence. We found that cultural BMSCs without treatment of HGF could not express α-fetoprotein and albumin (Figure 1A and B), while differentiated hepatocyte-like BMSCs with treatment of HGF expressed α-fetoprotein and albumin (Figure 1C and D).

### *Survival rate*

We evaluated the survival rate of mice that underwent BMSCs transplantation and/or DEN treatment. All mice that underwent BMSCs transplantation were still alive at the end of the study. Thirteen (26.0%) of 50 mice induced with DEN survived at the end of the 6-month study period, including six mice in the model group and seven in the experimental group. The survival rates were similar between the model group and the experimental group ($P > 0.05$).

### *Tumor development in the livers of recipient mice*

All of the survived recipient mice developed multiple HCCs. Thirteen mice developed HCCs including six mice in the model group and seven mice in the experiment group. These tumors were evenly distributed among the liver lobes of mice. The average sizes of hepatic tumors were not different between the two groups (4.8 ± 1.5 mm *vs* 4.4 ± 1.1 mm; $P > 0.05$).

HE stained sections of these tumors confirmed to be HCCs (Figure 2A) expressed GST-P and α-fetoprotein (Figure 2B and C), but not Cytokeratin 19 (Figure 2D). No other types of liver tumors, such as hepatoblastoma or cholangiocellular carcinoma, were noted in our experiment.

### *Repopulation and carcinogenesis of transplanted BMSCs*

To follow the repopulation and differentiation of BMSCs, we transplanted male BMSCs into the liver of normal and

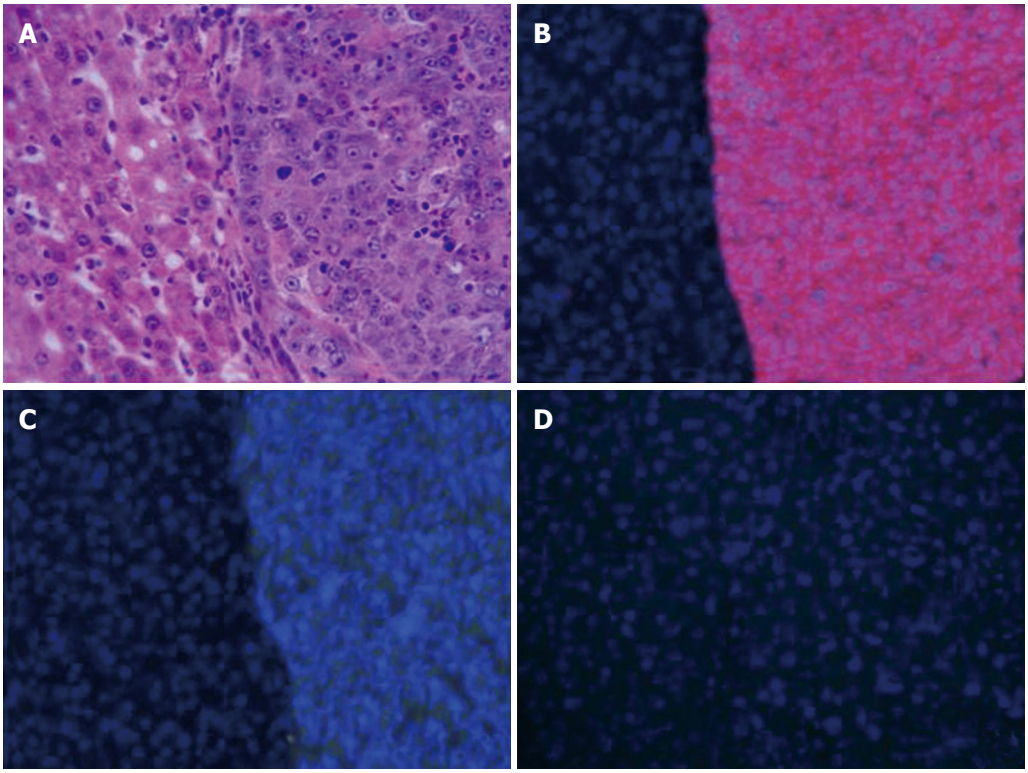


**Figure 2** Histopathological analysis of the tumors in the liver serial sections of recipient mice after 6 mo of DEN treatment (× 400). **A**: HCC nodules development at 6 mo stained with haematoxylin-eosin; **B**: HCC expressing GST-P appeared as red fluorescence by immunofluorescence; **C**: HCC expressing α-fetoprotein appeared as green fluorescence by immunofluorescence; **D**: HCC was negative for cytokeratin 19 by immunofluorescence.

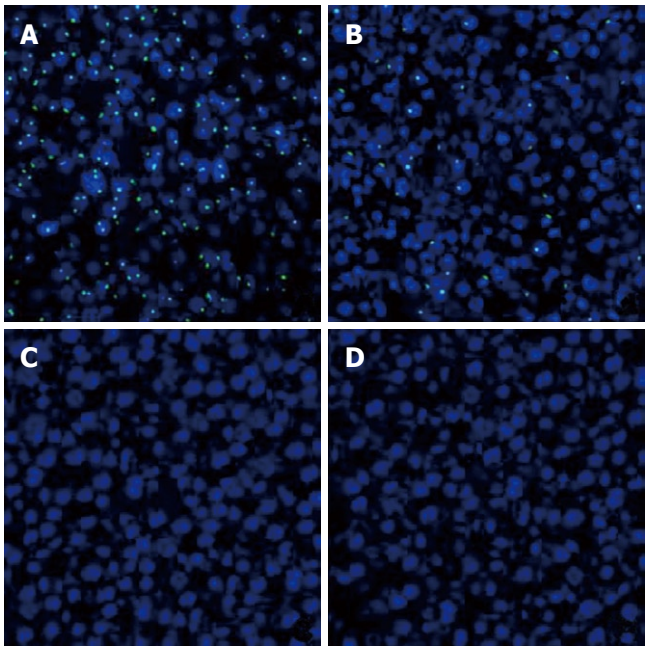


**Figure 3** Repopulation and carcinogenesis of male bone marrow-derived cells in female recipient liver tissues by FISH for Y chromosome (× 400). **A**: normal male liver, positive Y-chromosome signals appeared as green dots in the nuclei stained with DAPI, a chromosomal marker that appears as blue fluorescence; **B**: Six months after BMSCs transplantation and DEN treatment, some of hepatocyte nuclei were positive for the Y chromosome in the liver of female recipients; **C**: Six months after BMSCs transplantation without DEN treatment, none of hepatocyte nuclei was positive for Y chromosome in the liver of female recipients; **D**: HCC was negative for Y chromosome in nucleus.

DEN-treated female mice. FISH was performed to detect Y chromosome in the female recipients. A positive FISH signal was detected in the nucleus which was confirmed by counterstaining with DAPI. In male mouse liver, which served as positive control, most of the cells stained positive for Y-chromosome with fluorescein signal in the nuclei (Figure 3A).

We found that male BMSCs infused *via* portal vein into female syngeneic mouse liver could engraft and differentiate into hepatocytes after induction with DEN using FISH for Y chromosome. Six months after BMSCs transplantation and DEN challenge, FISH revealed that 15% of hepatocyte nuclei were positive for the Y chromosome in the liver of female recipients (Figure 3B). In addition, no nucleus showed two or more signals. However, donor-derived cells were not detected when BMSCs were transplanted to normal recipients without DEN treatment (Figure 3C). Moreover, no HCC was positive for the Y chromosome by FISH (Figure 3D).

## DISCUSSION

The liver is classified as a conditionally renewing tissue and hepatocytes proliferate quiescently and hepatic stem cells are not needed under normal circumstances. Oval cells reside within or adjacent to the canals of Hering and comprise a quiescent compartment of dormant stem cells in adult livers[6]. They can be activated to proliferate and differentiate into hepatocytes or bile duct epithelial cells when there is severe hepatic liver damage and coexistent impaired hepatocyte regeneration.

Accumulated evidence indicates that bone marrow cells can differentiate into specific cell types[20]. It has been reported that 30%-50% liver regeneration with bone marrow-derived cells in the FAH mouse model offers a selective proliferative advantage in the transplanted cells[21]. Bone marrow-derived hepatocytes may originate from the

mesenchymal compartment, rather than the hematopoietic compartment[22]. However, other data demonstrate that bone marrow-derived hepatocyte is only a possible but rare event, even in the presence of very strong selection pressure[23]. Several reports have demonstrated that cell fusion is the principal source of bone marrow-derived hepatocytes[24], and bone marrow-derived hepatocytes are primarily of mature myelomonocytic cells which fuse spontaneously with host hepatocytes producing functional liver repopulation[14].

The identity of the specific cell types that differentiate to express hepatocyte characteristics remains undetermined. BMSCs comprise marrow stromal stem cells, sharing characteristics with other multi-potent stem cells such as neural stem cells and hematopoietic stem cells, because they possess the capability of self-renewal and progeny differentiation potentials[25]. Our study demonstrates that cultured BMSCs differentiated hepatocyte-like cells which expressed α-fetoprotein and albumin with the treatment of HGF *in vitro*. After BMSCs transplantation, Y chromosome positive cells appeared only in mice treated with DEN and not in mice who did not receive DEN. These results suggest that in our model, BMSCs can differentiate into hepatocytes under limited conditions. Bone marrow-derived hepatic stem cells seem not to be required for normal hepatocyte substitution. Indeed, in the present study, we found that in positive hepatocytes, no nucleus had two or more Y chromosomes by FISH. This finding indicates that transdifferentiation, rather than cell fusion, was the main process in our model.

DEN is a DNA alkylating agent that is rapidly metabolized to reactive metabolites. These metabolites interact with DNA to form various DNA adducts, leading to genetic alterations[26]. GST-P is a highly expressed cytoplasmic protein during early and late steps of carcinogenesis and GST-P sensitivity is higher than that of other enzymes for the detection of malignant transformation[27]. Seventy percent of HCCs were stained positively for α-fetoprotein in clinical cases. In our study, chronic exposure to DEN caused multiple HCCs. These HCCs express GST-P and α-fetoprotein, but not cytokeratin 19 which was expressed in cholangiocellular carcinoma.

In this study, we focused our interest on the original cell lineage of carcinogenesis. There are two major nonexclusive hypotheses of the cellular origin of cancer: from stem cells due to maturation arrest or from dedifferentiation of mature cells. Debate has centered on whether hepatocytes are responsible for HCCs through dedifferentiation, or whether oval cells are the prime target for malignant changes after a differential "block"[3,5]. Oval cells are possibly involved in hepatocarcinogenesis based on the followings: (1) massive existence of oval cells in an animal rodent hepatocarcinogenic model[28]; (2) development of HCC after transformation of oval cells[8]; and (3) occurrence of mixed hepatocellular and cholangiocarcinomatous tumors (oval cell exhibits bipotential developmental ability)[29]. However, the relationship between oval cells and cancer is only circumstantial. In this study, no HCC was positive for Y chromosome after long-term carcinogenic induction. However, as all hepatic stem cells might not be labeled by our method as mentioned above, we cannot completely exclude the stem cell theory. Although our results may be limited to BMSCs transplanted mice treated with DEN, we can state that the malignant potential of the hepatic stem cell derived from bone marrow seems to be low. Further studies are needed to clarify the precise interaction of bone marrow cells with hepatic regeneration and carcinogenesis using other animal models or human studies.

In conclusion, our study demonstrates that cultured BMSCs could differentiate hepatocyte-like cells with HGF treatment *in vitro*. BMSCs can differentiate into hepatocytes in our model. Hepatic stem cells derived from bone marrow stromal cells are not cellular origin of hepatocellular carcinomas in the DEN model of carcinogenesis. Bone marrow cells may potentially be used in cell based replacement therapy or gene delivery systems. Under these circumstances, our results indicate that hepatic stem cell therapy derived from bone marrow is safe.

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## COMMENTS

### *Background*

Bone marrow stromal cells can differentiate into hepatic stem cells in rodents and in humans. Bone marrow stromal cells could be a valuable strategy for future replacement therapy of damaged or malfunctioned hepatocytes, because getting autologous bone marrow cells is easier than obtaining other tissue-specific stem cells. However, the safety and efficacy of hepatic stem cells derived from bone marrow stromal cells should be adequately confirmed before any such therapies are tested in humans.

### *Research frontiers*

There are two major nonexclusive hypotheses of the cellular origin of cancer: from stem cells due to maturation arrest or from dedifferentiation of mature cells. Debate has centered on whether hepatocytes are responsible for hepatocellular carcinoma (HCC) through a process of dedifferentiation, or whether oval cells are the prime target for malignant changes after a differential "block". There are increasing evidences that oval cells are the cellular targets for transformation in the development of HCC. Accumulating evidences indicate that bone marrow cells can differentiate into specific cell types. It has been reported that 30%-50% liver regeneration with bone marrow-derived cells in the FAH mouse model offers a selective proliferative advantage in the transplanted cells. Bone marrow-derived hepatocytes may originate from the mesenchymal compartment, rather than the hematopoietic compartment.

### *Innovations and breakthroughs*

This study demonstrates that cultured bone marrow stromal cells could differentiate hepatocyte-like cells *in vitro*. Bone marrow stromal cells can differentiate into hepatocytes. Hepatic stem cells derived from bone marrow stromal cells are not cellular origin of hepatocellular carcinomas in a mouse model of carcinogenesis. Bone marrow cells may potentially be used in cell based replacement therapy or gene delivery systems. The results in this study indicate that hepatic stem cell therapy derived from bone marrow is safe.

### *Applications*

Bone marrow stromal cells might be applicable for future replacement therapy of damaged or malfunctioned hepatocytes.

### *Peer review*

This paper is interesting and the study appears well conducted. The conclusions, although of a limited scope given the design of the study, are in agreement with the results.

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# Cost effectiveness analysis of population-based serology screening and $^{13}$C-Urea breath test for *Helicobacter pylori* to prevent gastric cancer: A markov model

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## Abstract

**AIM:** To compare the costs and effectiveness of no screening and no eradication therapy, the population-based *Helicobacter pylori* (*H pylori*) serology screening with eradication therapy and $^{13}$C-Urea breath test (UBT) with eradication therapy.

**METHODS:** A Markov model simulation was carried out in all 237900 Chinese males with age between 35 and 44 from the perspective of the public healthcare provider in Singapore. The main outcome measures were the costs, number of gastric cancer cases prevented, life years saved, and quality-adjusted life years (QALYs) gained from screening age to death. The uncertainty surrounding the cost-effectiveness ratio was addressed by one-way sensitivity analyses.

**RESULTS:** Compared to no screening, the incremental cost-effectiveness ratio (ICER) was $16166 per life year saved or $13571 per QALY gained for the serology screening, and $38792 per life year saved and $32525 per QALY gained for the UBT. The ICER was $477079 per life year saved or $390337 per QALY gained for the UBT compared to the serology screening. The cost-effectiveness of serology screening over the UBT was robust to most parameters in the model.

**CONCLUSION:** The population-based serology screening for *H pylori* was more cost-effective than the UBT in prevention of gastric cancer in Singapore Chinese males.



**Key words:** Cost-effectiveness analysis; Gastric cancer; *Helicobacter pylori*; $^{13}$C-Urea breath test; Serology

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## INTRODUCTION

Gastric cancer is the second leading cause of cancer death worldwide, which leads to a substantial burden of morbidity, mortality, and health care costs[1,2]. *H pylori* infection has been recognized as an important risk factor for cancer of gastric body and antrum (distal cancers)[3,4]. Approximately 50% of the world population has been affected by *H pylori*[5]. Although less than 1% of the infected will develop cancer, population-based *H pylori* screening in high-risk population has been proposed as a cost-effective strategy in the long term in Western countries[6-8].

The East Asian countries such as China and Japan have the highest incidence of distal gastric cancer, which is twice as common in males as in females[1]. *H pylori* infection was also found to be strongly linked to increased risk of gastric cancer in ethnic Chinese and Japanese[9]. Early detection and eradication of *H pylori* infection might be a useful way to reduce the risk of gastric cancer in Asian populations where prevalence of *H pylori* infection and gastric cancer are significantly higher than in the West[1]. However, it is unknown whether it is cost-effective to implement population-based *H pylori* screening

in high-risk Asian populations. Moreover, two widely used screening programs demonstrated good sensitivity and specificity in detection of *H pylori* infection in Chinese[10,11], therefore the question arises which screening program is more cost effective?

This study was aimed to evaluate the clinical and economic effects associated with no screening, population-based *H pylori* serology screening, and population-based $^{13}$C-Urea breath test (UBT) in Singapore Chinese males using a Markov model.

## MATERIALS AND METHODS

### Model structure

The decision analytical model compared three strategies: strategy 1, no screening and no eradication therapy; strategy 2, single serology screening for *H pylori* and treating those tested positive with eradication therapy; and strategy 3, single screening for *H pylori* using the UBT and treating those tested positive with the same eradication therapy as used in strategy 2. After the screening and treatment, both costs and outcomes of the strategies were evaluated using a Markov model (Figure 1)[12,13], which, from the public healthcare provider's perspective, estimated the costs, number of gastric cancer cases prevented, life years saved, and quality-adjusted life years (QALYs) gained from screening age to death (either died of gastric cancer or other causes, or achieved full life expectancy[14]). The distribution of people in the Markov states before the simulation started (i.e. cycle 0) was determined by the sensitivity and specificity of the screening strategies and prevalence of *H pylori* infection. The transition probabilities and corresponding plausible ranges in the model were obtained from a critical review of the published literature on target population where available (Table 1). Probabilities were converted from available rates using the formula recommended[13].

### Sensitivity analyses

One-way sensitivity analyses were conducted by altering individual variables within the aforementioned ranges. Based on the one-way sensitivity analyses, we additionally performed the best-case and the worst-case analyses, which included the most optimistic and pessimistic values for selected key variables.

### Incidence and prevalence rates

We evaluated all Singapore Chinese males aged from 35 to 44 as the prevalence of *H pylori* infection at this age group increased substantially compared to the younger age[10,15]. Age-specific *H pylori* infection rate, gastric cancer incidence, and mortality were applied when the cohort aged in the model[10,16,17]. The relative risk in developing gastric cancer in *H pylori* infected persons compared to the uninfected was obtained from published literature[3,18]. Proportion of gastric cancer death among deaths from all causes was derived from local reports[17]. The 1- to 5-year survival rates were estimated from a large prospective cohort study in Chinese[19]. Persons who survived for more than 5 years after diagnosis of gastric cancer were assumed to be cured and therefore achieved full life expectancy as the 5-year survival rate adequately reflected the curative success of gastric cancer treatment[7,20].

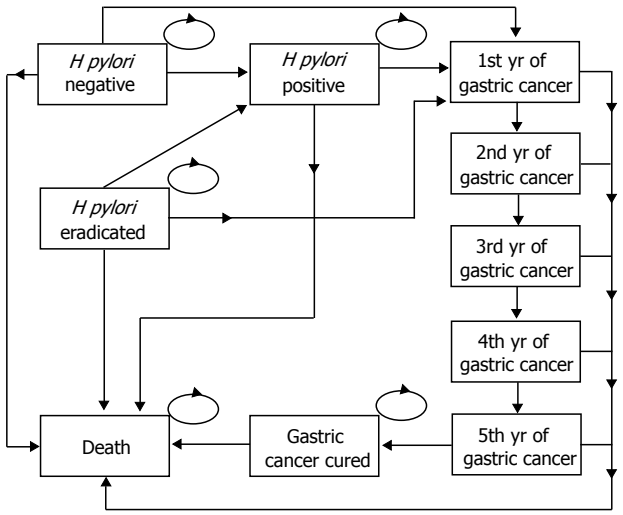


**Figure 1** Markov model schematic. *H pylori* eradicated referred to the state of persons with positive screening test and the infection was successfully eradicated by the triple therapy.

### Screening and eradication therapy

The screening strategies included 1 single serology screening by using enzyme-linked immunosorbent assay (ELISA) with a sensitivity and specificity of 93% and 79%, respectively (strategy 2)[10] and 1 single UBT using simple gas chromatograph-mass selective detector with a sensitivity and specificity of 97.9% and 95.8%, respectively (strategy 3)[11]. In both strategies, persons with positive test for *H pylori* (including both true and false positive) were treated with a triple therapy (i.e. rabeprazole 20 mg, amoxicillin 1000 mg, clarithromycin 500 mg, all twice a day for 4 d) with an eradication rate of 91%[21,22]. This regimen was specifically chosen because it is safe and effective with less resistance rate in patients and is recommended by the Asia-Pacific consensus conference[23-25]. Persons who stopped the triple therapy due to side effects or did not comply with the regimen were considered as treatment failure and thus remained infected. Persons who remained infected despite attempts at eradication had life expectancies and other outcomes identical to the infected who did not undergo treatment. The reinfection rate of the persons whose infection had been successfully eradicated was assumed to be identical to the persons who had never been infected (i.e. 1% annually in the base-case analysis)[6,26]. Once the reinfection occurred, an individual's gastric cancer risk was considered the same as that of an untreated, infected person of the same age.

An underlying assumption of the present study is that eradication of *H pylori* infection can reduce only the certain level of excess risk of distal gastric cancer (60% of all gastric cancers)[4,27]. We conservatively assumed that persons cured of *H pylori* infection will

**Table 1 Parameter estimates in the base-case analysis**

| Input variable | Base-case analysis | Range | Ref. |
|---|---|---|---|
| Incidence and prevalence rates | | | |
| Age-specific prevalence of *H pylori* (%) | 20.0-43.3 | - | [10] |
| Age-specific prevalence of gastric cancer per 100 000 | 3-342 | - | [17] |
| Gastric cancer in distal stomach (%) | 60 | 50-80 | [7] |
| Relative risk of gastric cancer in persons with *H pylori* infection | 3.6 | 2-12 | [7] |
| Age-specific mortality from age of 25, per 1000 | 0.5-50.6 | - | [16] |
| Gastric cancer death in deaths from all causes (%) | 2.27 | 2.20-2.33 | [14,17] |
| Survival rate of gastric cancer after treatment (%) | | | [19] |
| 1-yr | 54.2 | 51-58 | |
| 2-yr | 41.8 | 38-45 | |
| 3-yr | 37.9 | 34-42 | |
| 4-yr | 34.0 | 30-38 | |
| 5-yr | 30.5 | 27-35 | |
| Screening and treatment variables (%) | | | |
| *H pylori* serology screening sensitivity | 93 | 82-95 | [10] |
| *H pylori* serology screening specificity | 79 | 70-92 | [10] |
| *H pylori* $^{13}$C-Urea breath test sensitivity | 97.9 | 90-100 | [11] |
| *H pylori* $^{13}$C-Urea breath test specificity | 95.8 | 90-100 | [11] |
| Effectiveness of *H pylori* eradication | 92.0 | 87-98 | [21] |
| Probability of adverse effects related to eradication therapy necessitating medical intervention | 2.5 | 2-5 | [6] |
| Annual *H pylori* infection rate | 1.0 | 1-3 | [6,26] |
| Excess gastric cancer risk reduction attributable to *H pylori* eradication | 30 | 0-100 | [6] |
| Cost variables (2006USD)[1] | | | |
| *H pylori* serology screening | 26 | 10-50 | |
| *H pylori* $^{13}$C-urea breath test | 83 | 60-100 | |
| *H pylori* eradication (triple therapy) | 30 | 20-50 | |
| Gastric cancer treatment per annum | 4358 | 328-59 000 | |
| Eradication-related adverse effects | 50 | 5-100 | |
| Other variables | | | |
| Annual discount rate for costs and effectiveness (%) | 3 | 0-7 | [19,29] |
| Life expectancy, years | 77 | 76-80 | [14] |
| Utility | | | |
| *H pylori* non-infected | 1.00 | 0.95-1.00 | [26] |
| *H pylori* infected | 0.90 | 0.80-1.00 | [26] |
| Gastric cancer | 0.38 | 0.13-0.65 | [26] |

Triple therapy: Rabeprazole 20 mg, amoxicillin 1000 mg, and clarithromycin 500 mg, twice a day for 4 d. [1]All costs were estimated from the records of local public hospitals.

have 30% of excess risk reduction compared to those *H pylori* infected persons in the base-case analysis, while a wide range of excess risk reduction from 10% to 100% was tested in one-way sensitivity analysis.

### Costs

The present study was done from the public healthcare provider's perspective. Thus, the model included direct medical costs of serology screening, the UBT, and triple therapy. Adverse effects associated with the triple therapy that necessitated medical intervention were also included (Table 1). Annual direct medical costs associated with treatment of gastric cancer were estimated at the average level across different stages of the cancer[28]. Nonmedical direct costs and indirect costs were not included. The costs were accrued from the time of screening until death. All costs were reported in 2006 US dollars and annually discounted at 3% in all analyses[29].

### Effectiveness

Three health outcomes evaluated in this model included number of gastric cancer cases prevented, life years saved, and QALYs gained. All outcomes were also annually discounted at 3% in the base-case analysis[29].

### Incremental cost-effectiveness ratio (ICER)

ICER was expressed as US dollars per life year saved and US dollars per QALY gained, which were calculated for the two screening strategies compared to no screening strategy, as well as the UBT compared to the serology screening. The cost-effectiveness threshold was estimated at $28 000 per QALY gained in local context, which was derived from the conventional threshold of $50 000 per QALY gained used in the United States by comparing the gross national income per capita between the United States and Singapore[28,30].

## RESULTS

There were a total of 237 900 Chinese males aged between 35 and 44 in Singapore[31]. In the base-case scenario, compared to no screening and no eradication therapy, strategy 2 that implemented the serology screening on all cohort members with treatment for those with positive test

**Table 2 Incremental cost-effectiveness ratios of screening strategies at age 40 yr (compared to no screening strategy unless stated)**

| US$/QALY | Base-case | | Best-case[1] | | Worst-case[1] | |
|---|---|---|---|---|---|---|
| | Serology | UBT | Serology | UBT | Serology | UBT |
| ICER per life year saved | 16166 | 38792 | Dominant | Dominant | 389728 | 640000 |
| | | 477079[2] | | Dominant[2] | | 5645449[2] |
| ICER per QALY gained | 13571 | 32525 | Dominant | Dominant | 324773 | 560000 |
| | | 390337[2] | | Dominant[2] | | Dominated[2] |

ICER: Incremental cost-effectiveness ratio; QALY: Quality-adjusted life year; UBT: $^{13}$C-Urea breath test. [1]Variables modified in best and worst-case analyses were, gastric cancer risk reduction by eradication (100% and 10%, respectively), relative risk (12 and 2), cost of annual gastric cancer treatment ($59000 and $328), cost of the serology screening ($10 and $50), cost of the UBT ($60 and $100), and annual discount rate (0% and 7%); [2]The ICER was calculated by comparing the UBT with the serology screening.

cost $9.8 million, which saved 523 life years or gained 623 QALYs by preventing 272 gastric cancer cases. Strategy 3 that implemented the UBT on this cohort with treatment for those with positive test cost $23.0 million, which saved 550 life years or gained 656 QALYs by preventing 281 gastric cancer cases. A total of 875 and 847 persons were screened for each case of gastric cancer prevented in strategy 2 and 3, respectively. The serology screening avoided $1.4 million of discounted expenditures on treatment of gastric cancer, while the UBT avoided $1.5 million. The ICER were $16166 per life year saved and $13571 per QALY gained for the serology screening, and $38792 per life year saved and $32525 per QALY gained for the UBT (Table 2). When compared to serology screening, the ICER was $477079 per life year saved or $390337 per QALY gained for the UBT.

In the one-way sensitivity analyses, the level of excess gastric cancer risk reduction attributable to *H pylori* eradication varied from 10% to 100%[6,7]. Using a $28000 per QALY gained as a threshold, the serology screening would be cost-effective if *H pylori* eradication reduced more than 15% of excess gastric cancer risk. In contrast, the UBT could be cost-effective only when the excess gastric cancer risk was reduced by 35% or more (Figure 2A).

The ICER was sensitive to age at which population-based screening was carried out as shown in Figure 3. When screening age was more than 32 years, the ICER was less than $28000 per QALY gained for the serology screening. The UBT appeared cost-effective when the screening age was more than 45 years (Figure 2B).

Relative risk of gastric cancer for *H pylori* infected population had a significant impact on the ICER. When *H pylori* eradication was assumed to reduce 30% of excess gastric cancer risk (as in the base-case analysis), the serology screening appeared cost-effective over the full range of the relative risk (i.e. from 2 to 12). In contrast, the UBT appeared cost-effective only with the relative risk above 5 (Figure 2C).

Cost of annual gastric cancer treatment imposed a substantial impact on the cost-effectiveness of the strategies. For both strategies, the cost had an approximately linear relation with the ICER that decreased dramatically with the increase in annual cost of the cancer treatment (Figure 2D). When the annual cost was $30075, the one-time expenditure on serology screening and treatment of those with positive test would be fully offset by the savings in preventing gastric cancers (Figure 2D). Cost of the serology screening and the UBT also had a moderate impact on the ICER. Each $5 increment in cost of the serology screening and the UBT augmented the ICER by $2000 and $1800, respectively (data not shown).

The ICER was also sensitive to the annual discount rate. With the increase in the annual discount rate, the ICER appeared less favorable for both strategies.

Other variables had little impact on the cost-effectiveness within the ranges listed in Table 1, which included sensitivity and specificity of the serology screening and the UBT, effectiveness of *H pylori* eradication, probability and costs of adverse effects related to eradication therapy necessitating medical intervention, and utilities of each health state.

In all these sensitivity analyses, the ICER was extremely less favorable for the UBT compared to the serology screening.

In the best-case and worst-case analyses, the most critical variables, including level of excess gastric cancer risk reduction, relative risk of gastric cancer in *H pylori* infected population, annual cost of gastric cancer treatment, cost of the serology screening and the UBT, and annual discount rate, were simultaneously varied. Both strategies achieved more health benefits (i.e. life years gained or QALYs) at a lower cost compared to no screening, and the UBT also received more health benefit at a lower cost compared to the serology screening in the best-case scenario (i.e. dominant) (Table 2). In contrast, the ICER was more than $300000 for all comparisons in the worse-case scenario. The UBT achieved the same gaining in QALYs but at an extra cost of $11290897 compared to the serology screening in the worst case analysis (Table 2).

## DISCUSSION

The present study modeled the life-time cost and effectiveness associated with population-based *H pylori* screening and treatment for those with positive test in Chinese males. Compared to no screening and no eradication therapy strategy, the serology screening was cost-effective, while the UBT was not cost-effective based on the threshold of $28000 per QALY gained. The UBT gained very little extra health benefits in terms of either

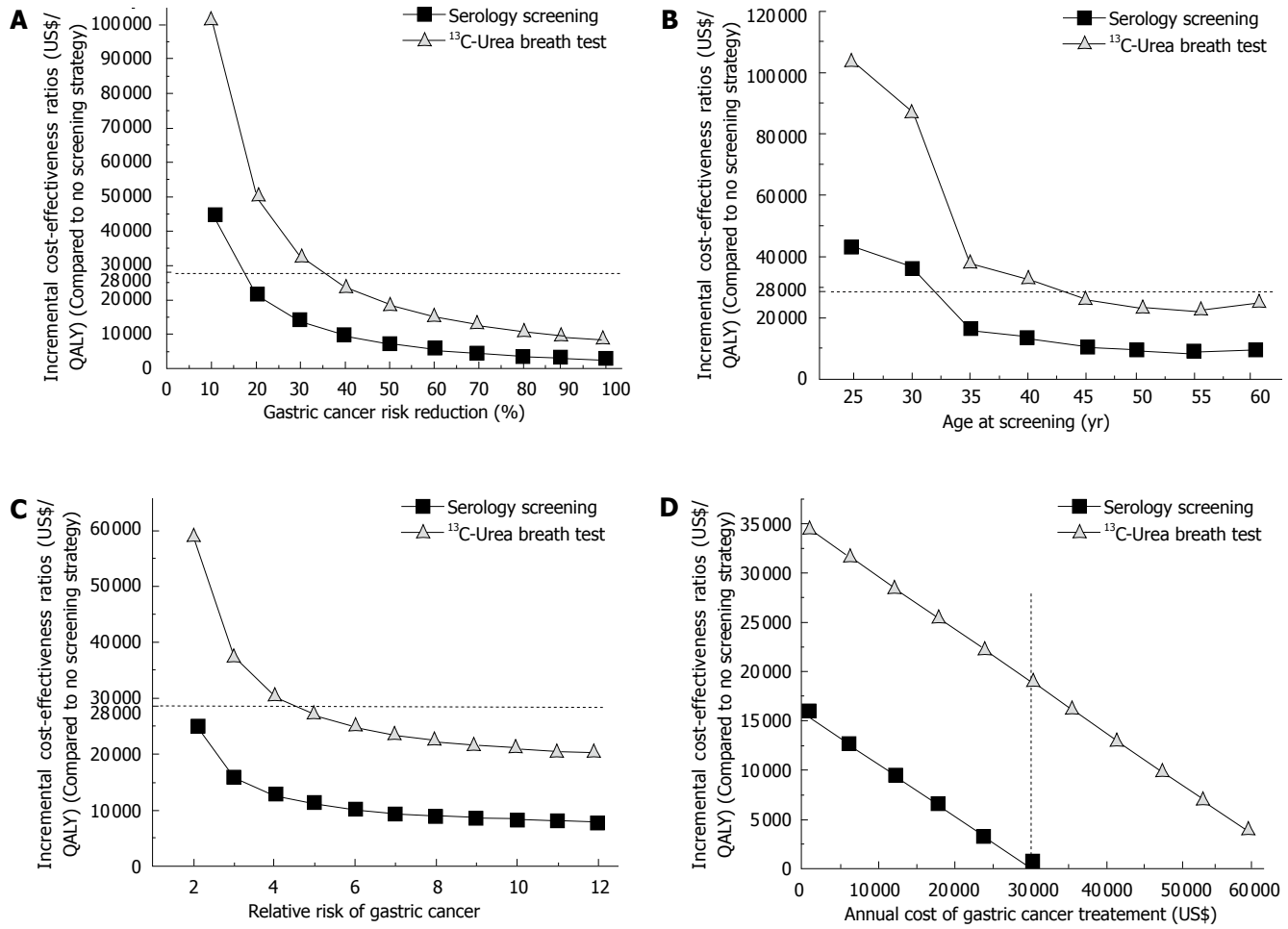


**Figure 2** **A:** Sensitivity analysis on excess gastric cancer risk reduction attributable to *H pylori* eradication; **B:** Sensitivity analysis on age at time of screening; **C:** Sensitivity analysis on relative risk of gastric cancer in *H pylori* infected people with 30% gastric cancer risk reduction attributable to eradication; **D:** Sensitivity analysis on annual cost of gastric cancer treatment.

life years saved or QALYs gained but at a substantially higher cost compared to the serology screening. This suggests that the population-based serology screening for *H pylori* infection be adopted in this specific population, especially under the circumstances that the cost of gastric cancer treatment keeps arising due to the advances in new technologies. Also with this model, future clinical advances on the efficacy of *H pylori* eradication in prevention of gastric cancer can be easily translated into the cost-effectiveness ratio, which is now playing an increasingly important role in informing medical decision making.

The serology screening was found to be cost-effective in the present study, which is similar to the published studies using the similar model to estimate the economic and clinical effects of *H pylori* screening[6,7]. Nevertheless, the model used in the present study had several improvements which are worth noting. First, we have a health state to identify the persons who were *H pylori* positive and successfully eradicated by the triple therapy (i.e. '*H pylori* eradicated' in Figure 1). This is a health state in the Markov model which can allow for successful capturing of the economic and health benefits resulted from the screening strategies. Second, in line with the important assumption that the persons who survived more than 5 years after diagnosis of gastric cancer were assumed to be cured[7,20], we used five tunnel states, instead of a single gastric cancer health state, to represent the status for each of the first five years since diagnosed with gastric cancer. The mortalities for these tunnel states were different from each other based on the epidemiological evidence[19]. This refinement may better simulate the real progress of gastric cancer and thus obtain more accurate estimations in cost and effectiveness. Third, this model is life-time estimation and every person remained in the model until death. Thus some parameters are time-sensitive including *H pylori* incidence, gastric cancer incidence, and mortality (Table 1). Instead of fixed point estimates, age-specific estimates may be more appropriate and accurate to reflect the changes in these important parameters with the aging of the cohort in the model.

Besides, some differences between these two studies and the present study are notable. The cost and effectiveness of the screening strategies essentially stemmed from the actual number of gastric cancer cases prevented by the strategies. Therefore, given the certain level of excess gastric cancer risk reduction by the eradication, cost of gastric cancer treatment and relative risk of gastric cancer in *H pylori* infected persons are deemed to have a very important and significant impact on

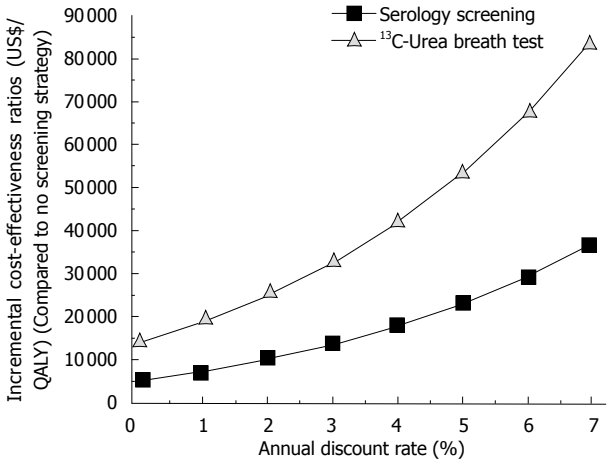


**Figure 3** Sensitivity analysis on annual discount rate.

the estimated ICER. The screening strategies would save more money if the cost needed to treat a gastric cancer case increased and prevent more gastric cancer cases if relative risk of gastric cancer in *H pylori* infected persons increased. Furthermore, the economic and health benefits of prevention of gastric cancer cases may only occur in the future rather than in the present, which highlights the important role of discount rate used: the larger the discount rate used the less the benefits obtained (Figure 3). However, these parameters were not examined in some previous study[6], or only little impact of these parameters was reported[7].

The cost-effectiveness of the serology screening over the UBT study was robust to most of the parameters through the one-way sensitivity analyses. Nevertheless, some findings are worth attention. As shown in Figure 3, the screening strategies would be more cost-effective if the starting age increased, which might be explicitly explained by the fact that both *H pylori* infection rate and gastric cancer incidence would increase with age. However, a recent large randomized controlled trial in Chinese revealed that persons with precancerous lesions (gastric atrophy, intestinal metaplasia, and dysplasia) significantly reduced the efficacy of *H pylori* eradication in prevention of gastric cancer compared to those without the lesions[32]. As the precancerous lesions increased significantly with age in Chinese[33], this could be important evidence to support the younger screening age. Thus, we suggested that the optimal screening age could be 35 years where there would be a substantial improvement on the ICER compared to younger age but only slight improvement compared to older age. Otherwise, if an older screening age was chosen, despite the increase in *H pylori* infection rate and gastric cancer incidence, the level of excess gastric cancer risk reduction (i.e. the efficacy of the eradication) would remain at the far lower end of the spectrum, favoring no screening against the serology screening (Figure 2).

Prevention of gastric cancer will save the medical expenditures for treatment of cancer and increase the life years and QALYs. However, this health benefit could be associated with additional medical expenditures (even the expenditures on daily living for extended life years) incurred during the extended life years, which will not occur in case of premature death. As including this cost component remains controversial, we did not take it into consideration in the present study. We also acknowledged that some parameters used in the model (e.g. survival rate of gastric cancer) were not available for Chinese males in Singapore, which may limit the accuracy of point estimates for cost and effectiveness. Finally, the threshold for ICER used in the present study was estimated from the US threshold using the ratio of gross national income between two countries, which is relative arbitrary and warrants further empirical local studies on this important topic.

In summary, the population-based serology screening for *H pylori* infection was more cost-effective than the UBT in prevention of gastric cancer in Singapore Chinese males.

## COMMENTS

### Background

*H pylori* infection has been recognized as an important risk factor for gastric cancer. Screening for *H pylori* has been proposed as a cost-effective strategy in prevention of gastric cancer.

### Research frontiers

A number of screening strategies are currently available. However, it is unknown which screening strategy is more cost-effective in high-risk populations, especially in Asian populations.

### Innovations and breakthroughs

A separate health state was used to identify the persons who were *H pylori* positive and successfully eradicated by the triple therapy. This state can allow for successful capturing of the economic and health benefits resulted from the screening strategies. Five tunnel states, instead of a single gastric cancer health state, were used in line with the important assumption that the persons who survived more than 5 years after diagnosis of gastric cancer were assumed to be cured.

### Applications

The findings in this study will be useful and important for decision makers to efficiently allocate scarce health resources for population-based *H pylori* screening.

### Peer review

The authors studied a clinically relevant issue. The manuscript is well written and is worth of publication in the Journal as is. This study has a substantial element of novelty. There is no data in literature concerning cost-effectiveness of serology-based screening strategy, particularly in countries with high prevalence of the infection, where the gastric cancer is a problem of special importance.

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*CLINICAL RESEARCH*

# Endoscopic ultrasound: It's accuracy in evaluating mediastinal lymphadenopathy? A meta-analysis and systematic review

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## Abstract

**AIM:** To evaluate the accuracy of endoscopic ultrasound (EUS), EUS-fine needle aspiration (FNA) in evaluating mediastinal lymphadenopathy.

**METHODS:** Only EUS and EUS-FNA studies confirmed by surgery or with appropriate follow-up were selected. Articles were searched in Medline, Pubmed, and Cochrane control trial registry. Only studies from which a 2 × 2 table could be constructed for true positive, false negative, false positive and true negative values were included. Two reviewers independently searched and extracted data. The differences were resolved by mutual agreement. Meta-analysis for the accuracy of EUS was analyzed by calculating pooled estimates of sensitivity, specificity, likelihood ratios, and diagnostic odds ratios. Pooling was conducted by both Mantel-Haenszel method (fixed effects model) and DerSimonian Laird method (random effects model). The heterogeneity of studies was tested using Cochran's *Q* test based upon inverse variance weights.

**RESULTS:** Data was extracted from 76 studies ($n$ = 9310) which met the inclusion criteria. Of these, 44 studies used EUS alone and 32 studies used EUS-FNA. FNA improved the sensitivity of EUS from 84.7% (95% CI: 82.9-86.4) to 88.0% (95% CI: 85.8-90.0). With FNA, the specificity of EUS improved from 84.6% (95% CI: 83.2-85.9) to 96.4% (95% CI: 95.3-97.4). The $P$ for chi-squared heterogeneity for all the pooled accuracy estimates was > 0.10.

**CONCLUSION:** EUS is highly sensitive and specific for the evaluation of mediastinal lymphadenopathy and FNA substantially improves this. EUS with FNA should be the diagnostic test of choice for evaluating mediastinal lymphadenopathy.



**Key words:** Endoscopic ultrasound; EUS-fine needle aspiration; Mediastinal lymphadenopathy

**Peer reviewer:** Damian Casadesus Rodriguez, MD, PhD, Calixto Garcia University Hospital, J and University, Vedado, Havana City, Cuba

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## INTRODUCTION

Management of patients with mediastinal lymphadenopathy depends on the etiology of lymphadenopathy. Differentiating inflammatory from neoplastic processes in the mediastinal lymph nodes is not only important from the treatment standpoint, but also vital in predicting survival. Multiple diagnostic modalities are available to evaluate mediastinal lymphadenopathy. Computer tomography (CT) of the chest does not clearly image the aortopulmonary, subcarinal, and paraesophageal areas due to the lowering of image resolution because of the movement and partial volume effect of pulmonary vessels, aortic arch, and left atrium[1]. Also, for lesions smaller than 1 cm, the sensitivity of CT is low[2-5], and the size-based criteria to diagnose metastatic involvement of the lymph nodes have lower accuracy[6]. Therefore, other methods were introduced, including transbronchial biopsy, CT-guided transthoracic fine-needle aspiration (FNA), mediastinoscopy, or thoracoscopic biopsy.

In the transbronchial technique, the FNA needle

is advanced blindly, reducing the yield of diagnosing subcarinal and paraesophageal nodes to approximately 50%[7,8]. Due to the potential danger of inadvertent vascular puncture, transthoracic biopsy is avoided when the mass is close to major vessels. This procedure is also associated with significant complications, including bleeding and pneumothorax in up to 25%-35% of cases[9,10]. Extended cervical mediastinoscopy or anterior mediastinoscopy can be used to access level 5 (aortopulmonary window) mediastinal nodes, which is not inspected by the standard methods[11-13]. Extended cervical mediastinoscopy has a sensitivity of 83% in examining the paraaortic and subaortic lymph node chains, but the subcarinal group is inaccessible[11]. Thoracoscopy can visualize the inferior mediastinum effectively, but it is limited only to accessing the level of major bronchi, leaving the superior mediastinum non-visualized[14]. Both procedures are invasive, require hospitalization and general anesthesia, and both have limitations.

With the introduction of endoscopic ultrasonography (EUS), it is now possible to visualize not only the gastrointestinal tract but also surrounding structures. However, EUS is limited in its ability to distinguish an inflammatory/reactive process from a malignancy, particularly within lymph nodes[15,16]. The accuracy of EUS in diagnosing mediastinal lymphadenopathy has been varied[17-21]. FNA during EUS may be performed safely in a short outpatient procedure setting without general anesthesia. It is not clear to what extent, if any, FNA adds in improving the accuracy of EUS to diagnose mediastinal lymphadenopathy[22-25].

The goal of this meta-analysis was to evaluate the accuracy of EUS alone and EUS with FNA in correctly diagnosing mediastinal lymphadenopathy. Due to multiple studies scattered in the literature and no published meta-analysis in this area, this meta-analysis was performed in an attempt to answer this essential clinical question. This meta-analysis and systematic review was written in accordance with the proposal for reporting by the QUOROM (Quality of Reporting of Meta-analyses) statement[26]. Since this manuscript looks at diagnostic accuracy of a test, the study design for this meta-analysis and systematic review conformed to the guidelines of Standards for Reporting of Diagnostic Accuracy (STARD) initiative[27].

## MATERIALS AND METHODS

### Study selection criteria

Only EUS-FNA studies confirmed by surgery or appropriate follow-up were selected. From this pool, only studies from which a 2 × 2 table could be constructed for true positive, false negative, false positive and true negative values were included.

### Data collection and extraction

Articles were searched in Medline, Pubmed, Ovid journals, Cumulative Index for Nursing & Allied Health Literature, ACP journal club, DARE, International Pharmaceutical Abstracts, old Medline, Medline non-indexed citations, OVID Healthstar, and Cochrane Control Trial Registry. The search terms used were endoscopic ultrasound, EUS, ultrasound, mediastinal lymphadenopathy, nodal invasion, fine needle aspiration, FNA, staging, surgery, sensitivity, specificity, positive predictive value, and negative predictive value. 2 × 2 tables were constructed with the data extracted from each study. To give validity to the data, two authors (SP and JR) independently searched and extracted the data into an abstraction form. Any differences were resolved by mutual agreement.

### Quality of studies

Clinical trial with a control arm can be assessed for the quality of the study. A number of criteria have been used to assess this quality of a study (e.g. randomization, selection bias of the arms in the study, concealment of allocation, and blinding of outcome)[28,29]. There is no consensus on how to assess studies without a control arm. Hence, these criteria do no apply to studies without a control arm[29]. Therefore, for this meta-analysis and systematic review, studies were selected based on completeness of data and inclusion criteria.

### Statistical analysis

Meta-analysis for the accuracy of EUS in diagnosing the etiology of mediastinal lymphadenopathy was performed by calculating pooled estimates of sensitivity, specificity, likelihood ratios, and diagnostic odds ratios. EUS studies were grouped into time periods to standardize the change in EUS technology and EUS criteria for lymph node involvement[30]. These time periods were 1988 to 1994, 1995 to 1999, and 2000 to 2006. Pooling was conducted using both Mantel-Haenszel method (fixed effects model) and DerSimonian Laird method (random effects model). The confidence intervals were calculated using the F distribution method[31]. The width of the point estimates in the Forrest plots indicates the assigned weight to that study. For 0 value cells, a 0.5 was added as described by Cox[32]. The heterogeneity of the sensitivities and specificities was tested by applying the likelihood ratio test[33]. The heterogeneity of likelihood ratios and diagnostic odds ratios were tested using Cochran's $Q$ test based upon inverse variance weights[34]. Heterogeneity among studies was also tested by using summary receiver operating characteristic (SROC) curves. SROC curves were used to calculate the area under the curve (AUC). The effect of publication and selection bias on the summary estimates was tested by Harbord-Egger bias indicator[35] and Begg-Mazumdar indicator[36]. Also, funnel plots were constructed to evaluate potential publication bias using the standard error and diagnostic odds ratio[37,38].

## RESULTS

The initial search using the search terms identified 4310 reference articles. Among these, 460 relevant articles were selected and reviewed by two authors independently. Data was extracted from 76 studies ($n$ = 9310) which met the inclusion criteria. Of these, 44 studies used EUS alone[17,18,39-80] and 32 studies used EUS-FNA[19-25,81-107]. Figure 1 shows the search results. Table 1 shows the characteristics for EUS studies without FNA and Table 2

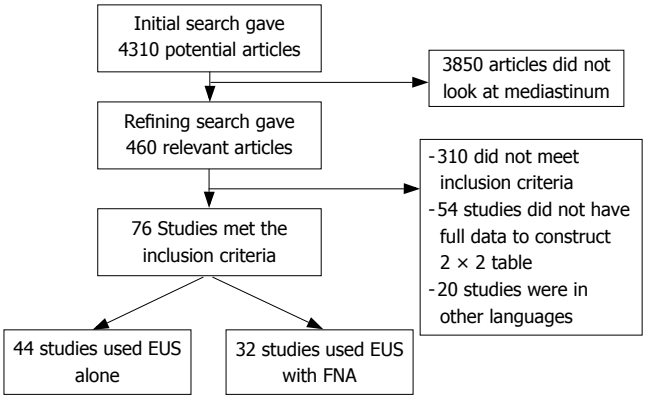


**Figure 1** The search results.

**Table 1 Characteristics of studies included in this meta-analysis for EUS without FNA**

| Author | Year of publication | No. of patients | Type of recruitment | Confirmatory procedure |
|---|---|---|---|---|
| Tio *et al*[71] | 1986 | 26 | Prospective | Surgery |
| Murata *et al*[57] | 1988 | 173 | Consecutive | Surgery |
| Tio *et al*[69] | 1989 | 75 | Prospective | Surgery |
| Vilgrain *et al*[75] | 1990 | 51 | Consecutive | Surgery |
| Tio *et al*[68] | 1990 | 102 | Consecutive | Surgery |
| Rice *et al*[63] | 1991 | 22 | Consecutive | Surgery |
| Heintz *et al*[52] | 1991 | 40 | Consecutive | Surgery |
| Botet *et al*[40] | 1991 | 50 | Consecutive | Surgery |
| Tio *et al*[70] | 1989 | 74 | Prospective | Surgery |
| Ziegler *et al*[80] | 1991 | 52 | Consecutive | Surgery |
| Rosch *et al*[64] | 1992 | 44 | Consecutive | Surgery |
| Fok *et al*[46] | 1992 | 54 | Consecutive | Surgery |
| Yoshikane *et al*[79] | 1993 | 28 | Consecutive | Surgery |
| Grimm *et al*[49] | 1993 | 63 | Prospective | Surgery |
| Dittler *et al*[45] | 1993 | 167 | Consecutive | Surgery |
| Peters *et al*[61] | 1994 | 42 | Consecutive | Surgery |
| Catalano *et al*[43] | 1994 | 100 | Consecutive | Surgery |
| McLoughlin *et al*[18] | 1995 | 15 | Consecutive | Surgery |
| Binmoeller *et al*[39] | 1995 | 87 | Prospective | Surgery |
| HunerBein *et al*[53] | 1996 | 19 | Consecutive | Surgery |
| Hasegawa *et al*[50] | 1996 | 22 | Consecutive | Surgery |
| Francois *et al*[47] | 1996 | 29 | Consecutive | Surgery |
| Natsugoe *et al*[58] | 1996 | 37 | Consecutive | Surgery |
| Milena *et al*[54] | 1997 | 40 | Prospective | Surgery |
| Vikers *et al*[73] | 1997 | 50 | Consecutive | Surgery |
| Shimizu *et al*[67] | 1997 | 431 | Consecutive | Surgery |
| Pham *et al*[62] | 1998 | 28 | Consecutive | Surgery |
| Vikers *et al*[74] | 1998 | 50 | Prospective | Surgery |
| Salminen *et al*[65] | 1999 | 32 | Consecutive | Surgery |
| Krasna *et al*[56] | 1999 | 88 | Consecutive | Surgery |
| Browrey *et al*[41] | 1999 | 98 | Prospective | Surgery |
| Catalano *et al*[42] | 1999 | 149 | Prospective | Surgery |
| Giovannini *et al*[48] | 1999 | 198 | Prospective | Surgery |
| Nishimaki *et al*[60] | 1999 | 224 | Consecutive | Surgery |
| Heidemann *et al*[51] | 2000 | 68 | Consecutive | Surgery |
| Nesje *et al*[59] | 2000 | 68 | Prospective | Surgery |
| Vazquez-Sequeiros *et al*[105] | 2001 | 37 | Consecutive | Surgery |
| Wiersema *et al*[77] | 2001 | 82 | Prospective | Surgery |
| Wakelin *et al*[76] | 2002 | 36 | Consecutive | Surgery |
| Kienle *et al*[55] | 2002 | 117 | Prospective | Surgery |
| Schwartz *et al*[66] | 2002 | 188 | Consecutive | Surgery |
| Wu *et al*[78] | 2003 | 31 | Prospective | Surgery |
| Arima *et al*[17] | 2003 | 58 | Consecutive | Surgery |
| DeWitt *et al*[44] | 2005 | 102 | Prospective | Surgery |

depicts characteristics of EUS studies with FNA. All the

**Table 2 Characteristics of studies included in this meta-analysis for EUS with FNA**

| Author | Year of publication | No. of patients | Type of recruitment | Confirmatory procedure |
|---|---|---|---|---|
| Kondo *et al*[6] | 1990 | 503 | Consecutive | Surgery |
| Schuder *et al*[25] | 1991 | 32 | Consecutive | Surgery |
| Silvestri *et al*[83] | 1995 | 27 | Prospective | Surgery |
| Giovannini *et al*[82] | 1995 | 141 | Prospective | Surgery or appropriate follow-up |
| Pedersen *et al*[21] | 1996 | 9 | Consecutive | FNA and appropriate follow-up |
| HunerBein *et al*[90] | 1996 | 19 | Consecutive | Surgery |
| Gress *et al*[19] | 1997 | 52 | Prospective | Surgery |
| Wiersema *et al*[104] | 1997 | 60 | Consecutive | FNA and appropriate follow-up |
| HunerBein *et al*[91] | 1998 | 15 | Consecutive | Surgery |
| HunerBein *et al*[98] | 1998 | 16 | Consecutive | Surgery |
| Fritscher-Ravens *et al*[101] | 1999 | 16 | Consecutive | FNA and appropriate follow-up |
| Mishra *et al*[102] | 1999 | 111 | Consecutive | FNA and appropriate follow-up |
| Giovannini *et al*[81] | 1999 | 198 | Prospective | Surgery or appropriate follow-up |
| Williams *et al*[89] | 1999 | 333 | Prospective | Surgery or appropriate follow-up |
| Fritscher-Ravens *et al*[84] | 2000 | 35 | Prospective | Surgery |
| Fritscher-Ravens *et al*[98] | 2000 | 35 | Consecutive | FNA and appropriate follow-up |
| Savides *et al*[100] | 2000 | 54 | Consecutive | FNA and appropriate follow-up |
| Fritscher-Ravens *et al*[103] | 2000 | 153 | Consecutive | FNA and appropriate follow-up |
| Vazquez-Sequeiros *et al*[105] | 2001 | 37 | Consecutive | Surgery |
| Wallace *et al*[91] | 2001 | 43 | Consecutive | FNA and appropriate follow-up |
| Wiersema *et al*[85] | 2001 | 82 | Prospective | Surgery |
| Chhieng *et al*[96] | 2001 | 103 | Consecutive | Surgery |
| Devereaux *et al*[22] | 2002 | 49 | Consecutive | Surgery |
| Catalano *et al*[92] | 2002 | 62 | Consecutive | Surgery |
| Schwartz *et al*[66] | 2002 | 188 | Consecutive | Surgery |
| Arima *et al*[95] | 2003 | 58 | Consecutive | Surgery |
| Pellise *et al*[23] | 2004 | 11 | Consecutive | Surgery |
| Kramer *et al*[86] | 2004 | 81 | Prospective | Surgery |
| Walsh *et al*[97] | 2005 | 27 | Consecutive | Surgery or appropriate follow-up |
| Tournoy *et al*[88] | 2005 | 67 | Prospective | Surgery |
| Khoo *et al*[93] | 2006 | 20 | Prospective | Surgery |
| Beek *et al*[87] | 2006 | 43 | Prospective | Surgery |

76 selected studies were published as full-text articles in peer review journals. The pooled estimates given are estimates calculated by the fixed effect model.

### *Accuracy of EUS with and without FNA*

Pooled sensitivity to diagnose the cause for mediastinal lymphadenopathy was 84.7% (95% CI: 82.9-86.4) for

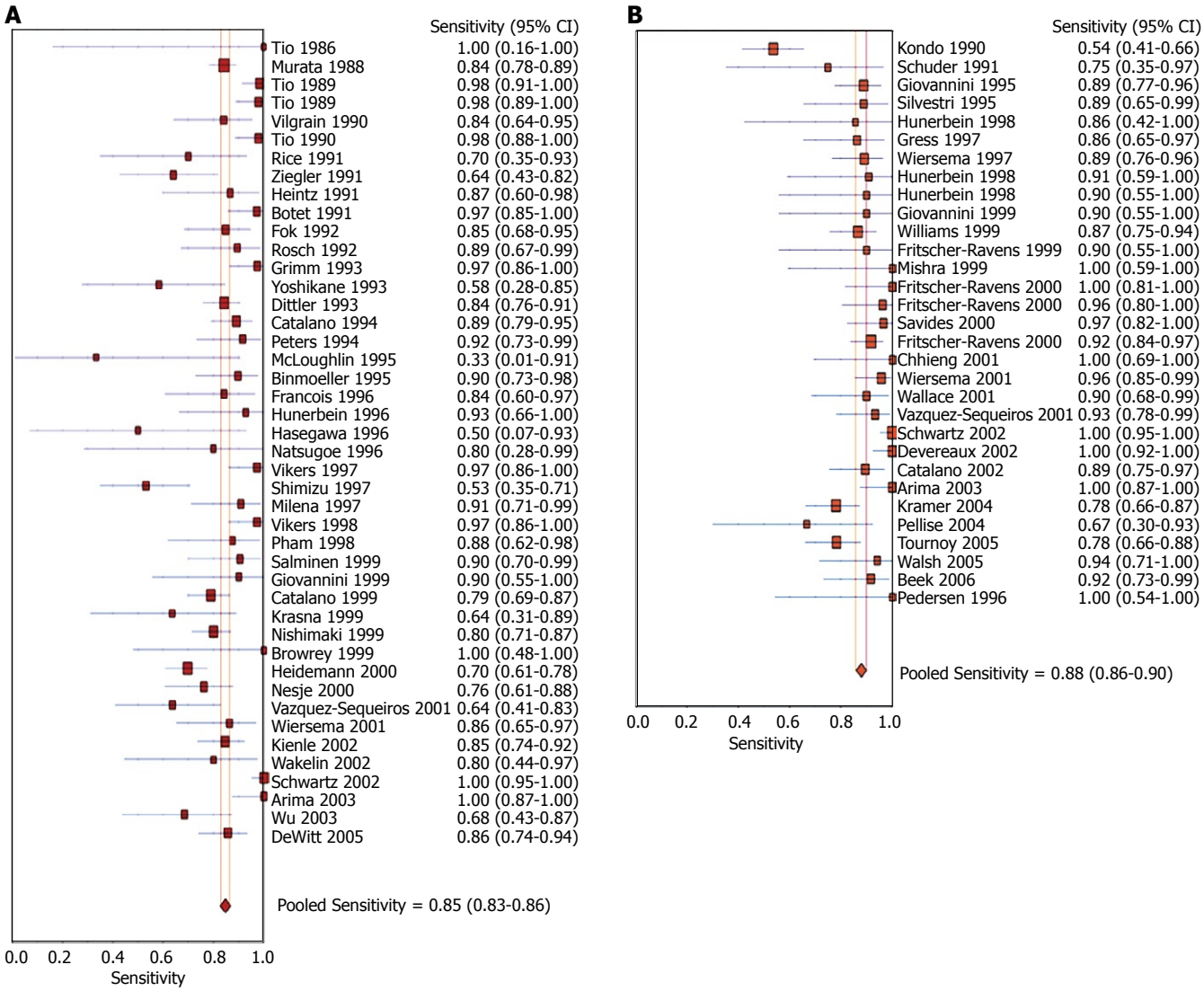


**Figure 2** Forrest plots. **A**: Sensitivity of EUS alone in diagnosing mediastinal lymphadenopathy; **B**: Sensitivity of EUS-FNA in diagnosing mediastinal lymphadenopathy.

EUS alone *versus* 88.0% (95% CI: 85.8-90.0) for EUS with FNA. The Forrest plot showing the sensitivity of EUS with and without FNA in various studies is shown in Figure 2A and B, respectively. EUS without FNA had a pooled specificity of 84.6% (95% CI: 83.2-85.9) and with FNA was 96.4% (95% CI: 95.3-97.4). Forrest plots showing specificity from various studies with and without FNA is depicted in Figure 3A and B, respectively.

The pooled positive likelihood ratio of EUS without FNA was 3.3 (95% CI: 2.6-4.3) and with FNA was 11.2 (95% CI: 5.9-21.2). The pooled negative likelihood ratio was 0.24 (95% CI: 0.1-0.3) for EUS without FNA and 0.13 (95% CI: 0.1-0.2) for EUS with FNA. The diagnostic odds ratio, the odds of having nodal metastasis in positive as compared to negative EUS studies, was 19.1 (95% CI: 12.7-28.5) for EUS without FNA and 106.9 (95% CI: 54.4-210.3) for EUS with FNA. Figure 4 shows a Forrest plot of various studies with FNA and their DOR. All the pooled estimates calculated by random effect models were similar to the estimates of fixed effect model.

SROC curves for EUS without FNA showed an area under the curve (AUC) of 0.91. EUS with FNA showed an AUC of 0.97. Figure 5 shows the SROC curve. The *P* for Chi-squared heterogeneity for all the pooled accuracy estimates was > 0.10. Table 3 shows the accuracy estimates of EUS alone and EUS-FNA.

### *Effect of technology over time*

To standardize the criteria for lymph node involvement and change in technology, the studies were grouped into three time periods[30]. These time periods were 1988 to 1994, 1995 to 1999, and 2000 to 2006. During these time periods, the number of studies that met the inclusion criteria for EUS alone were 17, 17, and 10, respectively. Studies that met inclusion criteria for EUS-FNA were 4, 10, and 18, respectively. For the most recent time period, EUS alone had a sensitivity of 81.6% (95% CI: 77.8-85.1) and specificity of 82.4% (95% CI: 78.2-86.1). During the same time period, EUS-FNA had a sensitivity of 91.7% (95% CI: 89.3-93.7) and specificity of 96.8% (95% CI: 94.9-98.2). All pooled estimates during the three time periods are given in Table 4. The *P* for chi-squared heterogeneity for all the pooled accuracy estimates was > 0.1.

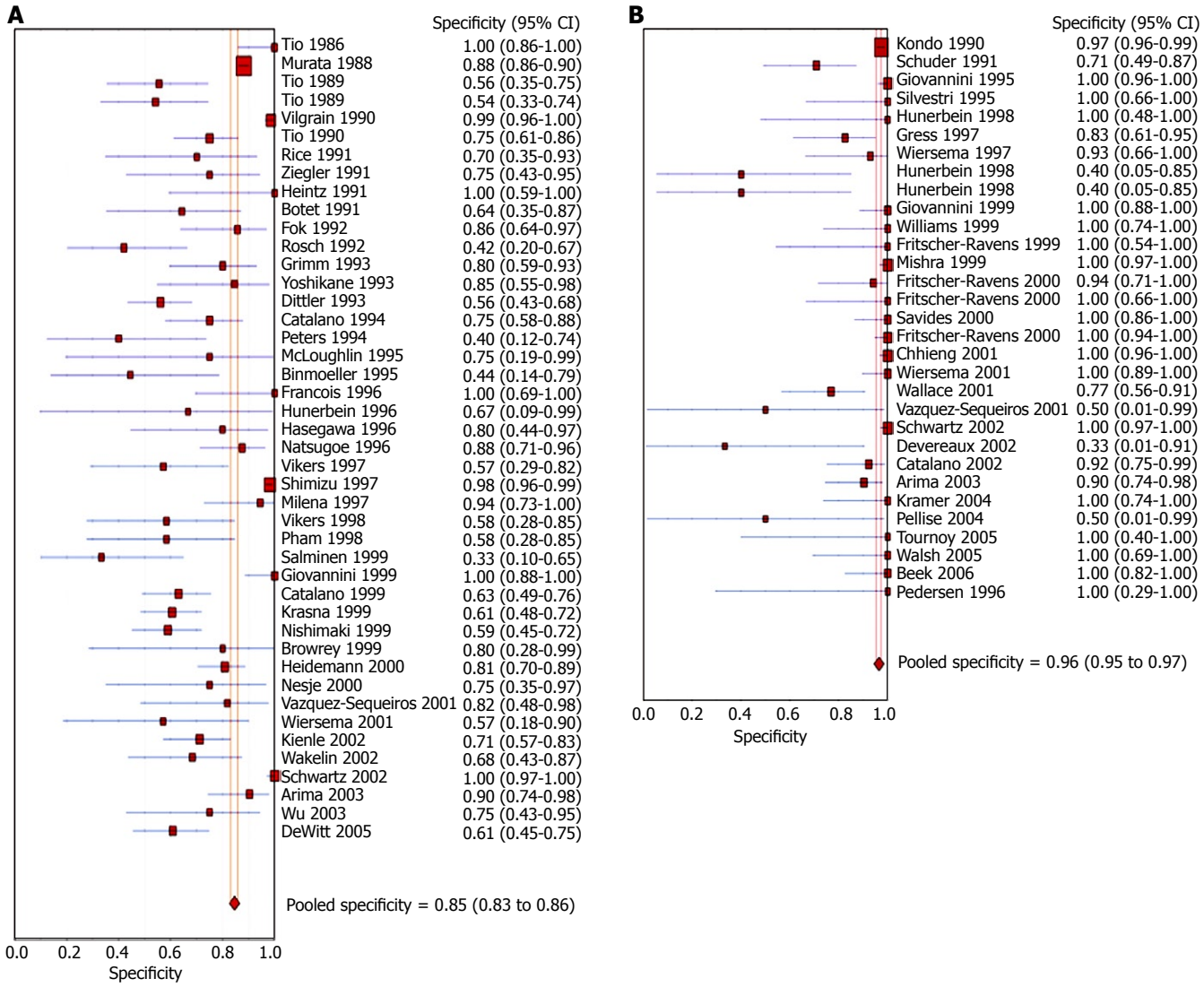


**Figure 3** Forrest plots. **A**: Specificity of EUS alone in diagnosing mediastinal lymphadenopathy. **B**: Specificity of EUS-FNA alone in diagnosing mediastinal lymphadenopathy.

### *Bias estimates*

The bias calculations using Harbord-Egger bias indicator gave a value of 1.08 (95% CI: -0.79-2.95, $P = 0.29$) for EUS studies without FNA and 2.02 (95% CI: 0.29-3.74, $P = 0.04$) for studies with FNA. The Begg-Mazumdar indicator for bias gave a Kendall's tau $b$ value of 0.13 ($P = 0.36$) for studies without FNA and -0.19 ($P = 0.07$) for studies with FNA. The funnel plots for the studies without and with FNA are shown in Figure 6A and B.

## DISCUSSION

Diagnosing the correct etiology for mediastinal lymphadenopathy helps direct precise therapy and prognosis. Thoracoscopic procedures for tissue biopsy carry a risk of complications in 25%-35% of cases[9,10]. The advantage of EUS is the ability to perform FNA during the procedure for tissue diagnosis. The procedure is, in comparison with other alternative options, safe, less invasive, and does not require general anesthesia or hospitalization[107]. The complication rate is extremely low (0.5%-2.3%) with several studies reporting no complications[48,77,83,107]. Modalities using FNA, such as transbronchial, computed tomography, or thoracoscopic procedure, cannot be used for the entire mediastinum[2-13]. EUS has the ability to image the aortopulmonary window, the subcarinal nodes, inferior mediastinum, and entire posterior part of the mediastinum.

This meta-analysis and systematic review was written in accordance with the proposal for reporting by the QUOROM (Quality of Reporting of Meta-analyses) statement[7]. This meta-analysis and systematic review shows that the pooled sensitivity of EUS for mediastinal lymphadenopathy is high and use of FNA during the procedure, further increases such sensitivity. The pooled specificity for diagnosing mediastinal lymphadenopathy is also high with substantial improvement if FNA is performed during the procedure (from 84.6% to 96.4%). Diagnostic odds ratio is defined as the odds of having a positive test in patients with true anatomic disease when compared to patients who do not have the disease. EUS has a very high diagnostic odds ratio for mediastinal lymphadenopathy. For example, if EUS indicates mediastinal lymphadenopathy and if FNA is performed on the enlarged nodes, the patient has odds of 106 times to have the correct etiology for lymph node enlargement. If EUS shows mediastinal lymphadenopathy, then the nodes

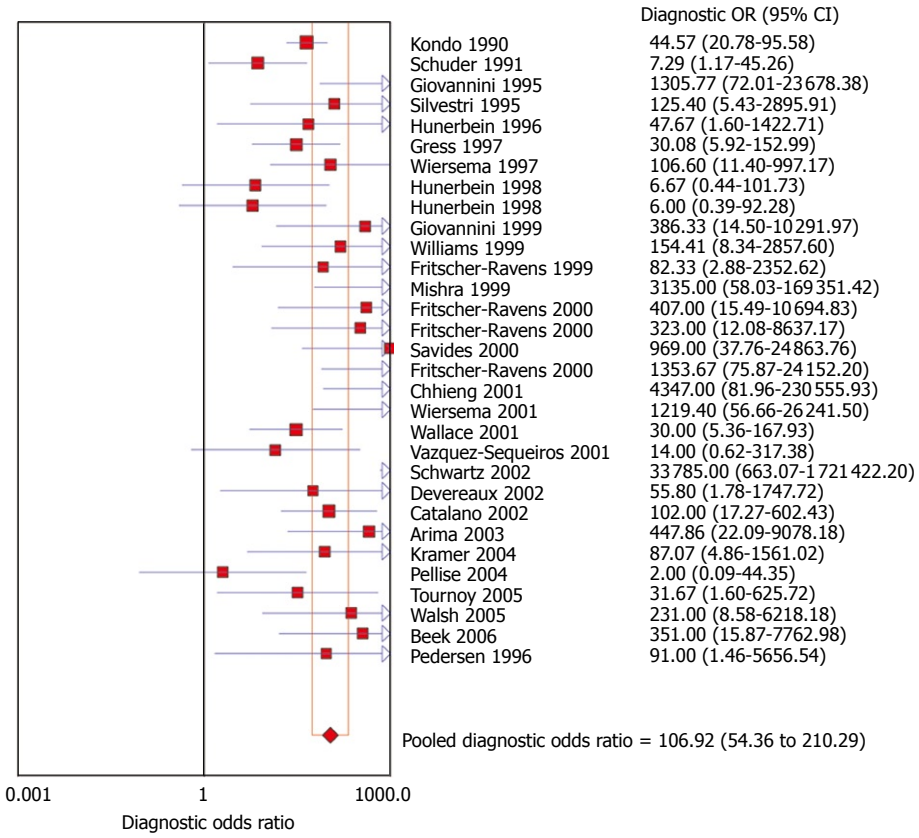


**Figure 4** Forrest plot showing diagnostic odds ratio of EUS-FNA in identifying mediastinal lymphadenopathy.

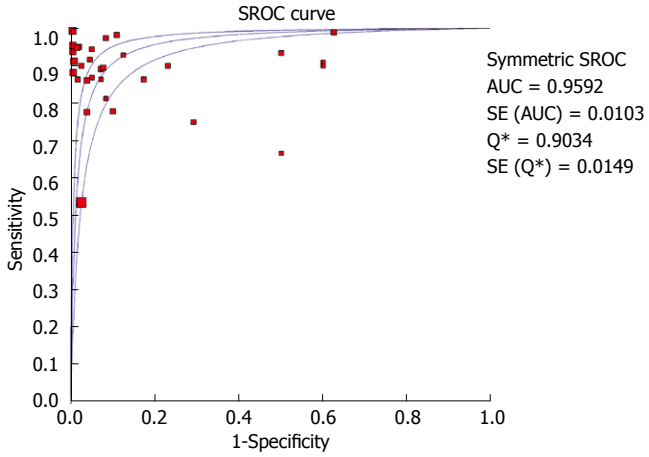


**Figure 5** SROC for EUS to diagnose mediastinal lymphadenopathy.

**Table 3 Pooled diagnostic accuracy estimates of EUS alone and EUS-FNA**

| | EUS | EUS-FNA |
|---|---|---|
| Studies | 44 | 32 |
| Pooled sensitivity | 84.7% (82.9-86.4) | 88.0% (85.8-90.0) |
| Pooled specificity | 84.6% (83.2-85.9) | 96.4% (95.3-97.4) |
| Positive likelihood ratio | 3.3 (2.6-4.3) | 11.2 (5.9-21.2) |
| Negative likelihood ratio | 0.24 (0.1-0.3) | 0.13 (0.1-0.2) |
| Diagnostic odds ratio | 19.1 (12.7-28.5) | 106.9 (54.4-210.3) |
| Area under the curve | 0.91 | 0.97 |

should be biopsied by FNA to improve the diagnostic accuracy.

The positive likelihood ratio measures how well a test identifies a disease state. The higher the positive likelihood ratio, the better the test performs in identifying the correct disease state. The negative likelihood ratio of the same test measures how well the test performs in excluding a disease state. The lower the negative likelihood ratio, the better the test performs in excluding a disease. For mediastinal lymphadenopathy, EUS has a high positive likelihood ratio and low negative likelihood ratio. This indicates that EUS performs better in diagnosing and excluding mediastinal lymphadenopathy. For mediastinal lymphadenopathy, all the pooled accuracy estimates of EUS are higher if FNA is performed during the procedure. Also, these pooled estimates give a baseline for future study comparisons.

The EUS studies with FNA were grouped into time periods and analyzed to standardize the criteria and the technology of EUS over the past two decades. Over the last two decades, the sensitivity and specificity of EUS with FNA has substantially improved.

Due to the possibility of different studies using slightly different criteria for diagnosis, heterogeneity among the studies was tested by drawing SROC curves and finding the AUC. An AUC of 1 for any test indicates that the test is excellent. SROC curves for EUS showed that the value for AUC was very close to 1, indicating that EUS is an excellent test to diagnose mediastinal lymphadenopathy. Publication bias and selection bias may affect the summary estimates. Studies with statistically significant results tend to be published and cited. Smaller studies may show larger treatment effects due to fewer case-mix differences (e.g. patients with only early or late disease) than larger trials. This bias can be estimated by bias indicators and construction of

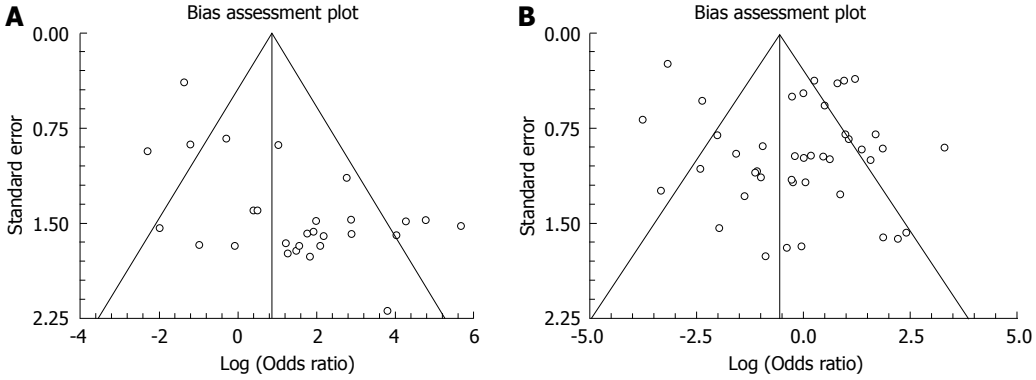


**Figure 6** Funnel plots. **A**: Bias assessment for EUS studies without FNA in examining mediastinal lymphadenopathy; **B**: Bias assessment for EUS-FNA studies in examining mediastinal lymphadenopathy.

**Table 4 Pooled diagnostic accuracy estimates of EUS alone and EUS-FNA for different time periods with 95% CI**

| Time period | No. of studies | Pooled sensitivity | Pooled specificity | Pooled LR+ 1 | Pooled LR-2 | Pooled DOR3 |
|---|---|---|---|---|---|---|
| EUS without FNA | | | | | | |
| 1988 to 1994 | 17 | 88.0% (85.4-90.2) | 85.2% (83.4-86.9) | 3.6 (2.4-5.4) | 0.2 (0.1-0.3) | 27.5 (14.5-52.4) |
| 1995 to 1999 | 17 | 82.6% (78.8-85.9) | 84.4% (81.6-86.9) | 3.0 (2.0-4.5) | 0.3 (0.2-0.4) | 14.8 (7.5-29.3) |
| 2000 to 2005 | 10 | 81.6% (77.8-85.1) | 82.4% (78.2-86.1) | 3.4 (2.2-5.3) | 0.3 (0.2-0.4) | 14.9 (6.7-33.1) |
| EUS-FNA | | | | | | |
| 1988 to 1994 | 4 | 71.8% (63.9-78.9) | 96.8% (94.9-98.1) | 15.5 (2.4-101.2) | 0.3 (0.1-0.6) | 61.8 (10.5-63.8) |
| 1995 to 1999 | 10 | 88.9% (83.5-93.0) | 94.7% (90.7-97.3) | 8.1 (2.8-23.3) | 0.1 (0.1-0.2) | 57.0 (20.7-57.1) |
| 2000 to 2005 | 18 | 91.7% (89.3-93.7) | 96.8% (94.9-98.2) | 12.5 (5.2-29.8) | 0.1 (0.1-0.2) | 17.7 (5.0-62.8) |

[1]LR+: Positive likelihood ratio; [2]LR-: Negative likelihood ratio; [3]DOR: Diagnostic odds ratio.

funnel plots. Bias among studies can affect the shape of the funnel plot. In this meta-analysis and systematic review, bias calculations using Harbord-Egger indicator[36] and Begg-Mazumdar indicator[37] showed no statistically significant bias for EUS studies without FNA. Furthermore, funnel plot analyses showed no significant bias for EUS without FNA and EUS-FNA studies (Figure 6B).

In conclusion, EUS has high sensitivity and specificity to evaluate mediastinal lymphadenopathy. This meta-analysis demonstrates that FNA substantially improves the specificity of EUS in evaluating mediastinal lymphadenopathy. EUS with FNA should be the diagnostic test of choice for evaluating mediastinal lymphadenopathy.

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*RAPID COMMUNICATION*

# Short-term intravenous interferon therapy for chronic hepatitis B

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## Abstract

**AIM:** To investigate the therapeutic efficacy of short-term, multiple daily dosing of intravenous interferon (IFN) in patients with hepatitis B e antigen (HBeAg)-positive chronic hepatitis B.

**METHODS:** IFN-β was intravenously administered at a total dose of 102 million international units (MIU) over a period of 28 d in 26 patients positive for HBeAg and HBV-DNA. IFN-beta was administered at doses of 2 MIU and 1 MIU on d 1, 3 MIU twice daily from d 2 to d 7, and 1 MIU thrice daily from d 8 to d 28. Patients were followed up for 24 wk after the end of treatment.

**RESULTS:** Six months after the end of the treatment, loss of HBV-DNA occurred in 13 (50.0%) of the 26 patients, loss of HBeAg in 9 (34.6%), development of anti-HBe in 10 (38.5%), HBeAg seroconversion in 8 (30.8%), and normalization of alanine aminotransferase (ALT) levels in 11 (42.0%).

**CONCLUSION:** This 4-wk long IFN-β therapy, which was much shorter than conventional therapy lasting 12 wk or even more than 1 year, produced therapeutic effects similar to those achieved by IFN-α or pegylated-IFN-α (peg-IFN). Fewer adverse effects, greater efficacy, and a shorter treatment period led to an improvement in patients' quality of life. IFN-β is administered intravenously, whereas IFN-α is administered intramuscularly or subcutaneously. Because both interferons are known to bind to an identical receptor and exert antiviral effects through intracellular signal transduction, the excellent results of IFN-β found in this study may be attributed to the multiple doses allowed by the intravenous route.



**Key words:** Chronic hepatitis B; Hepatitis B e antigen; Hepatitis B virus; Interferon beta; Multiple daily dosing; Short-term treatment; Intravenous injection

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## INTRODUCTION

The increasing prevalence of chronic hepatitis caused by hepatitis B or C virus infection represents a concern in many regions worldwide. Interferons (IFN) are widely used in the treatment of the disease. With the recent launch of lamivudine, adefovir, and entecavir, the number of treatment options for chronic hepatitis B has increased. Treatment with these oral nucleoside analogues has serious drawbacks, such as the development of resistant HBV strains[1,2] and the need for years of treatment[3,4] or even a lifetime therapy. Thus, a large number of patients still require IFN therapy, which is effective in a relatively short period of time. Recently, however, in some patients, the treatment with IFN is often prolonged up to 24-48 wk to improve efficacy[5-7]. IFN-α is administered intramuscularly or subcutaneously and may be associated with such adverse effects as fatigue, insomnia, anorexia, and alopecia[7,8]. These effects presumably result from prolonged elevation of blood IFN levels. Prolonged exposure to higher levels of the circulating drug may produce a greater therapeutic effect while inducing greater adverse effects[9,10]. Treatment for a higher therapeutic effect without consideration of the burden on patients is not a good therapeutic strategy.

In Japan, IFN preparations for the treatment of hepatitis B include IFN-α for intramuscular or subcutaneous administration and IFN-β for intravenous administration[11]. Both IFN-α and IFN-β bind to the an identical IFN receptor and induce PKR and other antiviral proteins via intracellular signal transduction systems represented by JAK/STAT[12]. Because of the intravenous route, the blood concentration of IFN-β reaches its peak immediately after infusion and then decreases

rapidly[13]. Decrease or loss of efficacy by receptor down-regulation[14-16] and adverse effects with IFN therapy are less likely to occur because blood level of IFN-β does not maintain after signal transduction *via* the IFN receptor. The receptor function is maintained, and thus frequent dosing of IFN-β is likely to produce greater efficacy. Indeed, in patients with hepatitis C, we found that IFN-β in divided doses administered in the morning and evening was more effective than that administered once daily at the same total dose[17].

For the development of short-term IFN therapy for hepatitis B, in the present study we investigated a 4-wk, multiple daily dosing of intravenous IFN-β, a new regimen that produced therapeutic effects similar to those achieved by 24-wk or 1-year treatment with IFN-α.

## MATERIALS AND METHODS

### Patients

Among Japanese adult patients with chronic hepatitis B who were positive for HBeAg and HBV-DNA and presented at our hospital from 1996 to 2002, 26 patients were enrolled in this open-label study. The study was conducted in accordance with the Declaration of Helsinki, and the patients consented to the experimental treatment of hepatitis B. Inclusion criteria were: age of 20 years or older, blood HBeAg positivity, blood HBV-DNA positivity, and persistent abnormal elevation of ALT levels. Exclusion criteria included: coinfection with hepatitis C virus or HIV, presence of hepatocellular carcinoma, symptoms caused by decompensated cirrhosis, alcoholic, autoimmune, drug-induced, or other non-viral liver disorders, and hypersensitivity to IFN-β. Any herbal medicines were discontinued during the treatment with IFN-β.

### Treatment methods

Human fibroblast-derived natural IFN-β (FERON®, Toray Industries Inc., Japan) was used; 1 to 3 MIU was dissolved in 100 mL of 5% glucose or isotonic saline solution for injection and infused intravenously for about 10 minutes. The dosing schedule comprised 2 MIU in the morning and 1 MIU in the evening (twice daily) at d 1 of treatment, 3 MIU in the morning and evening (twice daily) from d 2 through d 7, and 1 MIU each in the morning, in the afternoon, and at bedtime (thrice daily) from d 8 to d 28, with a total dose of 102 MIU administered over a treatment period of 28 d. Patients were followed up for 24 wk after the end of the IFN-β therapy.

### Laboratory methods

Blood samples were collected immediately before the start of treatment, weekly during the treatment, and monthly during the follow-up period. Biochemical and hematological tests were performed each time. HBsAg was measured by reversed passive hemagglutination (R-PHA), anti-HBs was measured by passive hemagglutination (PHA), HBeAg and anti-HBe were measured by radioimmunoassay (RIA). HBV-DNA polymerase activity was measured by radioassay. Serum HBV-DNA was measured by branched DNA probe assay (Chiron Corp, USA) with a detection sensitivity of 0.70 megaequivalents (Meq) per milliliter. Anti-hepatitis C virus antibodies were measured by enzyme immunoassay (EIA). In addition, 2'-,5'-oligoadenylate synthetase (2-5AS), an indicator of IFN activity, was quantitatively measured by RIA.

### Statistical analysis

Values are given as either mean ± SD or median and range. For comparison, Student's *t*-test or the Chi-square test were used. Statistical tests were two-sided, and a *P* value of less than 0.05 was considered as statistically significant.

## RESULTS

### Patient population

Clinical characteristics of the 26 patients with HBeAg positive chronic hepatitis B at beginning of the treatment are shown in Table 1. All patients received a total dose of 102 MIU of IFN-β over a period of 28 d, and none of them dropped out because of adverse effects or other reasons.

### Clinical outcomes

Six months after the end of IFN administration, loss of HBV-DNA occurred in 13 (50.0%) patients, loss of HBeAg in 9 (34.6%), loss of HBV-DNA and HBeAg in 9 (34.6%), development of anti-HBe in 10 (38.5%), and HBe seroconversion in 8 (30.8%). The last parameter is a measure of the therapeutic effect, defined by the loss of HBeAg and the subsequent development of anti-HBe. ALT levels normalized in 11 (42.0%) of the 26 patients. The percentage of patients, which became negative for HBV-DNA, HBeAg, and the change in ALT levels during/after the treatment are shown in Table 2 and Figure 1, respectively.

### Baseline HBV DNA polymerase activity and virological response

Patients were stratified according to baseline DNA polymerase activity (less than 1000 cpm *vs* 1000 cpm or more), and virological responses were recorded. Among 15 patients with an activity lower than 1000 cpm, 11 (73.3%) had a complete virological response, and 4 (26.7%) had no response. Among the 11 patients with an activity of 1000 cpm or more, 2 (18.2%) had a complete virological response, and 9 (81.8%) had no response.

### 2-5AS

Figure 2 shows the change in 2-5AS levels. The level of 2-5AS at baseline was 114.8 ± 102.1 (mean ± SD). The levels at wk 1, 2, and at the end of treatment were 389.9 ± 205.3, 333.3 ± 133.4, and 344.3 ± 181.2, respectively.

### Adverse effects

No patients discontinued treatment because of adverse effects, with a treatment completion rate of 100%. Fever was mild because antipyretic loxoprofen sodium was administered before intravenous infusion to suppress IFN-

**Table 1 Clinical characteristics at the beginning of the treatment**

| Characteristics | Baseline |
|---|---|
| Age (yr) | 31.8 ± 7.0[1] |
| Sex (male/female) | 19/7 |
| ALT (U/L) | 246.9 ± 154.2[1] |
| HBV DNA (≥ 10/< 10 Meq/mL) | 16/10 |
| HBV DNA polymerase (cpm) | 750.5 (10-10710)[2] |
| PLT (× $10^4$/$mm^3$) | 19.3 ± 10.7[1] |

[1]mean ± SD; [2]Median (range).

**Table 2 Response rate in patients with HBeAg positive chronic hepatitis B by interferon-β treatment (%)**

| | wk 1 | wk 2 | End of the treatment | 6 mo after treatment |
|---|---|---|---|---|
| HBV-DNA negative | 5/26 (19.2) | 5/26 (19.2) | 10/26 (38.5) | 13/26 (50.0) |
| HBeAg and HBV-DNA negative | | | 4/26 (15.4) | 9/26 (34.6) |

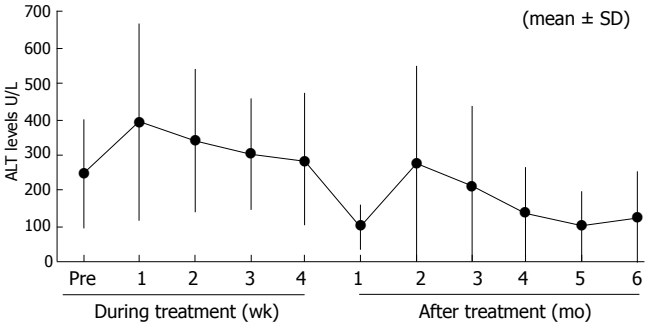


**Figure 1** Change in ALT levels during treatment with interferon-β and during the follow-up.

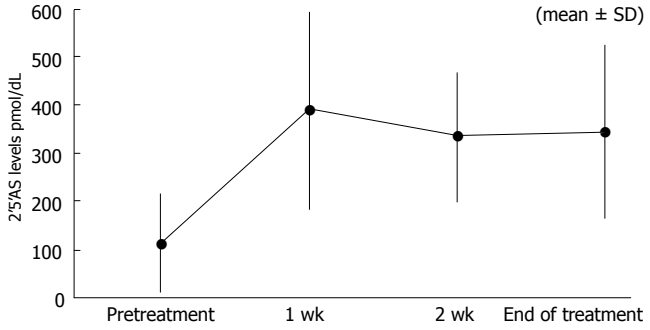


**Figure 2** Change in 2'5'AS levels during treatment.

induced fever. During treatment with IFN, no patients experienced depression. There was no proteinuria, severe thrombocytopenia or leukopenia (as shown in Figure 3).

## DISCUSSION

Approximately 10 years ago, the IFN therapy for chronic hepatitis B was administered for up to 4 wk in Japan. However, a 24-wk regimen has been recently used because

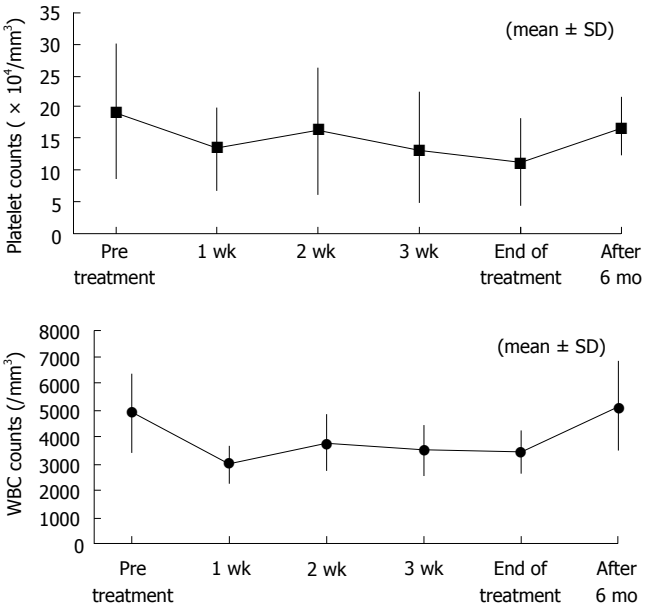


**Figure 3** Changes in platelet and WBC counts.

**Table 3 Comparison of response rates in patients with HBeAg positive chronic hepatitis B at 6 mo after the treatment (%)**

| | INF-β (iv) 4 wk | INF-α (*sc* or im) 12-24 wk | Lamivudine 1 yr | Adefovir dipivoxil 48 wk | Pegylated interferon-α 48 wk |
|---|---|---|---|---|---|
| Loss of serum HBV DNA | 50 | 37 | 44 | 21 | 32 |
| Loss of HBeAg | 35 | 33 | 17-32 | 24 | 34 |
| HBeAg seroconversion | 31 | Difference of 18 | 16-18 | 12 | 32 |
| Loss of HBsAg | | 8 | < 1 | 0 | 3 |
| Normalization of ALT | 42 | Difference of 23 | 41-72 | 48 | 41 |
| Histological improvement | | | 49-56 | 53 | 38 |
| Durability of the response | | 80-90 | 50-80 | | 82 |

a longer treatment seems to improve the efficacy. In the present study, we used a short-term, intravenous therapy of 4 wk, which seems to be against the recent recommendations for long-term regimens. However, 4-wk multiple daily dosing of intravenous IFN-β used in our study produced therapeutic effects similar to those achieved by 12-wk or 24-wk IFN-α or 48-wk peg-IFN-α, which are indicated by the American Association for the Study of Liver Diseases[18,19]. The HBe seroconversion rate with IFN-β in this study was 31%, which was higher than the reported 12-18% with IFN-α[18,20], lamivudine[18,21-23], or adefovir[19,24], and which was almost equal to that achieved by a 48-wk therapy with peg-IFN-α[25] (Table 3). In the United States, the distribution of HBV genotypes was reported as genotypes A (33%), B (21%), C (34%), D (9%), E (1%), F (1%), and G (1%)[26]. Given that the majority (about 80%) of Japanese patients infected with HBV has IFN-resistant genotype C[27], the multiple daily dosing of intravenous IFN-β used in this study appears to be a beneficial treatment.

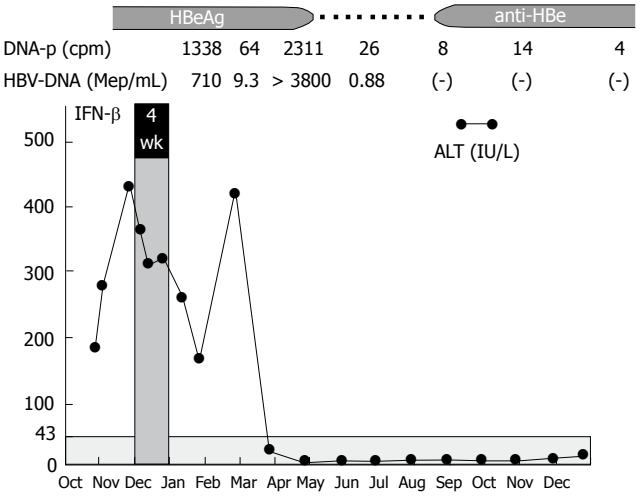


**Figure 4** Typical pattern of clinical course with transient increase in ALT level after treatment with interferon-β.

Our results suggest that HBV DNA polymerase activity at baseline before the treatment may be used to predict the therapeutic effect of IFN to some degree. Multiple daily dosing of IFN-β may be the regimen of first-line choice in patients with baseline HBV DNA polymerase activity less than 1000 cpm because 73.3% of those patients had a complete virological response. We believe that the direct antiviral effect of IFN on HBV is enough to achieve a complete response in those patients, whereas an appropriate host immune response are also needed in patients with a polymerase activity of 1000 cpm or more indicating rapid proliferation of HBV. A typical example is shown in Figure 4. The patient had an HBV DNA polymerase activity of 1338 cpm and an HBV-DNA level of 710 Meq/mL before the IFN therapy. After the end of IFN-β administration, an increase in HBV-DNA and subsequent rapid increase in ALT levels (so-called Schub) occurred, followed by the loss of HBeAg, HBV-DNA, and DNA polymerase, normalization of ALT levels, and development of anti-HB. The rapid increase in ALT levels probably resulted from the host's immune response to the rapid increase in the HBV proliferation following the regimen and the subsequent rapid elimination of infected hepatocytes in an appropriate manner.

Our dosing regimen had a good safety profile with a low incidence of mild adverse effects and no serious adverse effects. This may be attributed to lower daily doses of 3 MIU from d 8 onward and a short treatment period of 1 mo. Although platelet and leukocyte counts decreased at wk 1 compared with baseline levels, the counts remained unchanged thereafter until the end of treatment and almost returned to baseline levels after completion of therapy. Our previous experience suggested that thrombocytopenia and proteinuria should be closely monitored during treatment with IFN-β at doses of 3 MIU twice daily. However, cytopenia did not worsen because of switching to 1 MIU thrice daily from d 8. The levels of 2-5AS in blood (mean ± SD) at baseline and wk 1, 2, and 4 of treatment were 133.9 ± 122.2, 445.0 ± 209.7, 335.0 ± 139.9, and 387.8 ± 200.7, respectively, and remained elevated during treatment, suggesting that the dose regimen produced a potent and durable antiviral effect despite a modest cytopenia.

In general, the pharmacokinetics of an intravenously administered drug are characterized by a higher blood elimination rate, higher peak blood concentration, and greater tissue distribution than an intramuscularly administered drug, and these are also true of IFN. Different types of IFN formulations are available for therapy, and human fibroblast IFN-β is applicable to intravenous administration for the treatment of hepatitis in Japan.

We chose intravenous administration and multiple daily dosing because of the following three reasons. First, intravenously administered IFN-β is rapidly eliminated from the blood and below the detection limit shortly after administration[13]. Compared with intramuscularly or subcutaneously administered IFN-α, IFN-β accumulates to a lesser degree and is likely to have less adverse effects[28]. Second, blood concentrations of IFN administered intravenously in multiple daily doses fluctuate with high blood levels and rapid elimination rates. Accordingly, this regimen is likely to avoid persistently elevated blood IFN levels and resultant downregulation of the IFN receptor[14-16], which is likely to occur after intramuscular or subcutaneous administration. The avoidance of the receptor downregulation allows effective binding of IFN and its receptor, and triggers the host defense mechanisms a few times a day to eliminate the virus. Third, the drug administered intravenously is more extensively distributed into organs than that administered intramuscularly. For elimination of HBV present in hepatocytes, intravenous dosing is considered as an effective route of administration, which allows extensive delivery of IFN to the liver. When IFN-α, which was induced by treating human leukocytes with the Sendai virus, was administered intravenously or intramuscularly to rats, IFN-α was detectable in the liver at 10 and 30 min but not at 1 h after intravenous administration whereas IFN levels remained below the detection limit for 4 h in rats receiving an intramuscular administration[29]. In patients with hepatitis, a transient increase in ALT levels is often observed after intravenous administration of IFN[30]. Because IFN distributes in the liver at high concentrations after intravenous administration, extensive loss of infected hepatocytes may occur, resulting in an increase in ALT levels.

When IFN or any other cytokine that exerts a pharmacological effect *via* receptor binding is administered, it is important to choose an appropriate route of administration that ensures effective delivery of the drug to the target-cells. An ideal pharmacokinetic profile should include a rapid increase to effective blood concentrations and a rapid elimination after receptor binding to avoid downregulation of the receptor. We believe that intravenous IFN therapy can also be used effectively for the treatment of other diseases including cancer, infection with HIV, and SARS. However, intravenous IFN is now available only in Japan. For further promotion of research on the establishment of intravenous IFN therapy as a convenient, general way of treating these diseases,

intravenous IFN should preferably be available in other countries.

Oral nucleoside analogues, such as lamivudine, adefovir, and entecavir, have a potent effect in suppressing hepatitis B virus; however, most patients relapse and become positive for the virus after discontinuation of treatment. Thus, these drugs should be taken for a few years or the rest of patients' lives. These agents also cause problems including development of resistant strains and fetotoxicity, which discourages physicians from administering these agents in pregnant, parturient, and nursing women. Meanwhile, IFN therapy tends to continue for more than 6 months, and increased adverse effects associated with prolonged therapy have become a significant problem. In Japan, both physicians and patients have great difficulty coping with these problems and they are waiting for new effective treatments that ensure improvement in the quality of life for patients.

Short-term treatment with multiple daily dosing of IFN-β used in the present pilot study has fewer adverse effects, good therapeutic effects, and reproducibility to some degree. Further studies and randomized clinical trials are required to confirm our promising results.

## COMMENTS

### Background

Hepatitis B virus (HBV) is a major cause of liver disease worldwide, ranging from acute and chronic hepatitis to cirrhosis and hepatocellular carcinoma. Therefore, in order to improve the hepatitis and cirrhosis, and decrease the risk of hepatocellular carcinoma on the patients of chronic hepatitis B, it is extremely important to achieve sustained suppression of HBV replication, normalize serum alanine aminotransferase (ATL) level, and induce seroconversion by therapies. Recently, interferon (IFN)-α (conventional and pegylated) with or without nucleoside analogues or nucleoside analogues only are used for therapy. However, available therapies are suboptimal.

### Research frontiers

Therapies using IFN-α and/or nucleotide analogues are needed a long period to treat. Furthermore, those therapies are associated with some side effects. So, the authors tried to establish the new therapeutic protocol using IFN-β, because IFN-β belongs to type Ⅰ IFN family like IFN-α and there were some reports that indicated the treatment of IFN-β twice a day was more effective than that of IFN-α or IFN-β once a day in chronic hepatitis C patients.

### Innovations and breakthroughs

In this study, the author's have evaluated the efficacy of a short term (4 wk), multiple daily dosing therapeutic protocol using IFN-β for chronic hepatitis B patients. As a result, the therapeutic efficacy of that regimen is similar to that of PEG-IFN-α treatment for 24 wk or 1 year. Furthermore, the side effects of IFN-β treatment in this study were less than those of PEG-IFN-α or IFN-α treatment for 24 wk or 1 year. Therefore, this treatment method of IFN-β few times a day is more effective than standard therapeutic protocols on chronic hepatitis B patients for the first time.

### Applications

In the present pilot study, the authors indicated that the treatment protocol of IFN-β in this study could improve a rate of side effects compare with the standard IFN-α or PEG-IFN-α treatment protocol without loss of therapeutic effects. Further studies and randomized clinical trials are required to confirm the indication of short term therapy for chronic hepatitis B.

### Terminology

It has reported that the treatment of IFN-β twice a day is more effective than that of IFN-α or IFN-β once a day in chronic hepatitis C patients. However, there is no investigation that described the efficacy of treatment of IFN-β twice a day on chronic hepatitis B patients.

### Peer review

The authors may want to provide end-of-treatment data as well, in addition to the SVR data that they have provided. Overall, I feel this is a novel approach that needs wider consideration.

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*RAPID COMMUNICATION*

# Discrepancies between the responses to skin prick test to food and respiratory antigens in two subtypes of patients with irritable bowel syndrome

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## Abstract

**AIM:** To compare the response to skin prick tests (SPTs) to food antigens (FAs) and inhalant allergens (IAs) in patients with two subtypes of irritable bowel syndrome (IBS) and healthy controls.

**METHODS:** We compared the results of SPTs for IAs and FAs in 87 volunteers divided into three groups: diarrhea predominant IBS (D-IBS) Group Ⅰ ($n$ = 19), constipation predominant IBS (C-IBS) Group Ⅱ ($n$ = 17), and normal controls Group Ⅲ ($n$ = 51).

**RESULTS:** Of the 285 tests (171 for FAs and 114 for IAs) performed in Group Ⅰ we obtained 45 (26.3%) positive responses for FA and 23 (20.1%) for IA. Of the 153 tests for FA in Group Ⅱ, we obtained 66 (20.1%) positive responses, and of the 102 tests for IA, we obtained 20 (19.6%) positive responses. Of the 459 tests for FA performed in Group Ⅲ, we obtained 39 (84%) positive responses, and of the 306 for IA, we obtained 52 (16.9%) positive responses. The numbers of positive responses were not significantly different between the three groups, but in the D-IBS group, the number of SPT FA responses differed significantly from those for the other two groups ($P$ < 0.01).

**CONCLUSION:** Despite the small number of cases studied, the higher reactivity to FAs in Group Ⅰ compared to Groups Ⅱ and Ⅲ adds new information, and suggests the presence of a possible alteration in intestinal epithelial function.



**Key words:** Atopy; Constipation; Diarrhea; Food intolerance; Irritable bowel syndrome; Skin prick test

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## INTRODUCTION

Irritable bowel syndrome (IBS) is an extremely common disorder that affects about one in every 5-10 persons. Estimates of prevalence range from 9% to 22% depending upon population group studied[1-7].

The exact pathophysiology of IBS remains unknown, although various mechanisms including gastrointestinal dismotility and visceral hypersensitivity have been well studied in IBS[8-10]. Recent interest has also been directed to the possible participation of the mucosa in the pathophysiology of IBS[11-13]. Inflammatory mediators cause intestinal dysfunction and a consequent increase in permeability[14-17]. However the role and interaction of inflammatory mediators with IBS remains to be determined[17].

IBS is defined by symptomatic criteria rather than biological markers. No diagnostic tests are available, clinical subtypes of IBS are based on the predominant symptom:

IBS diarrhea predominant (D-IBS), IBS constipation predominant (C-IBS), and mixed (m-IBS), and treatment is selected based on the predominant symptom[3,18,19].

Clinically, the frequency of IBS is associated with psychological stress, food intolerance (adverse reaction to a specific food or ingredient that is not immune mediated or associated with psychological phenomena), intestinal infections, and even previous abdominal surgery[3,7,12,20-25].

Dunlop *et al*[16] have reported that patients with subtype D-IBS (pos-infectious and non-infectious origin) have a more pronounced permeability increase in the proximal intestine compared with controls and those with C-IBS. Another aspect of that study was the detection of a significant correlation between atopy and increased intestinal permeability, which suggests that at least a subset of IBS patients may have a systemic immunological disorder. Other studies have reported a correlation between asthma, food allergy and IBS[26-29], but the role of allergic reactions in the pathophysiology of IBS remains controversial[7,13,27,29-31].

We have previously observed that volunteers with a diagnosis of IBS have reported higher cutaneous reactivity to food antigens than to inhalant allergens, when compared to patients with functional dyspepsia and normal controls[30]. The association between food hypersensitivity and IBS symptoms is still open to question[7,17,20,22-25]. New information is useful for a better understanding of the relationship between increased intestinal permeability, mucosal barrier defects, and intestinal inflammation in IBS patients.

The aim of this study was to compare the response to skin prick tests (SPTs) with food and inhalant antigens in two subtypes of IBS and healthy controls.

## MATERIALS AND METHODS

We studied the response to SPTs with inhalant and food extracts[32] in 87 volunteers, 36 patients with IBS (evaluated by a pre-designed questionnaire based on the Rome Ⅲ criteria[18] for functional gastrointestinal diseases), and 51 normal volunteers (school employees and medical students at Antonio Pedro University Hospital). They were evaluated between September 2006 and January 2007. The volunteers were first evaluated in outpatient clinics for functional gastrointestinal diseases at Antonio Pedro University Hospital. Subjects completed a questionnaire which included Rome Ⅲ criteria for IBS, and were submitted to a clinical evaluation that included a careful history (age, duration of symptoms, psychosocial factors, alarm symptoms, personal history of atopy, family history of gastrointestinal disease), examination, and stool examination for ova and parasites (Brazil is an endemic area for parasitic infections). The inclusion criteria were age > 18 years old and a diagnosis of IBS, or volunteers from the general population. The exclusion criteria were clinical suspicion or diagnosis of organic disease of the gastrointestinal tract (including positives stool examination for ova and parasites) at least 12 mo prior to the study after clinical evaluation.

The subjects were divided into three groups. D-IBS, Group I ($n$ = 19; 14 female, five male; mean age 32.6 years), with IBS ROMA Ⅲ Criteria for recurrent abdominal discomfort or pain at least 3 d per month in the last 3 mo, associated with two or more of the following: (1) improvement with defecation; (2) onset associated with a change in stool frequency; or (3) onset associated with a change in stool form (appearance). C-IBS, Group Ⅱ ($n$ = 17 subjects; 12 female, five male; mean age 31.8 years. Controls, Group Ⅲ ($n$ = 51 subjects; 31 female, 20 male; mean age 26.3 years) without previous or current significant gastrointestinal symptoms. The three groups, after informed consent (approved by the local Ethical Committee: number CAAE 009025800007) were submitted to SPTs with nine food extracts (ovalbumin, egg yolk, nuts, peanuts, wheat flour, cow's milk, soya, crustaceans and chocolate), and six inhalant extracts (*Dermatophagoides* spp., *Blomia tropicalis*, air dust, *Dermatophagoides pteronissimus*, house dust and *Dermatophagoides farinaceous*)[14].

The contents of glycerinated food extracts (1:20), the positive control substance, histamine, and the negative control substance, saline, were commercially available (M Queiroz Laboratory, Rio de Janeiro, Brazil). They were applied by the prick technique (percutaneous) puncture trough the standardized punter (discarded after use to avoid cross reaction), which allowed allergen absorption at multiple points in the skin. The test reading, done at the 20 min after the beginning were made by the measures of the wheel diameter eliciting by the test, obtained in millimeters. A wheal that was 3 mm greater than that of the negative control was considered positive. Anything less was considered negative[14].

### *Statistical analysis*

Data were analyzed using Pearson's $\chi^2$ test. $P < 0.05$ was considered significant.

## RESULTS

A total of 1305 SPTs (783 for the FAs and 522 for the IAs) (nine FAs and six IAs for each volunteer) were accomplished in the three groups. In the D-IBS Group I , we obtained 45 positive responses for food extracts, which corresponded to 26.3% of the 171 tests performed and 23 positive responses for IAs, which corresponded to 20.1% of the 114 tests performed. In the C-IBS Group Ⅱ, we obtained 21 positives responses (13.7% of the 153 tests) for FAs and 20 positive responses (19.6%) for IAs. In the control Group Ⅲ, 39 (8.4%) positive responses were obtained in 459 tests performed for FAs, and 52 (16.9%) for IAs.

The positive responses were not concentrated in one or two subjects, but dispersed throughout the populations examined. The numbers of positive responses to SPT for each antigen did not differ significantly between the three groups ($P > 0.05$).

Nine (52.9%) C-IBS and 11 (57.8%) D-IBS patients reported intolerance to several foods, but we did not find any correlation between positive SPTs and specific food intolerance in these subjects. Ten (58.8%) C-IBS and 12 (63.1%) D-IBS patients reported a personal history of

**Table 1 Personal history of allergies and food intolerance, and SPT response to FAs and IAs in IBS patients and normal controls (%)**

| | D-IBS ($n$ = 19) | C-IBS ($n$ = 17) | Without gastrintestinal symptoms ($n$ =51) |
|---|---|---|---|
| Reported food intolerance | 11 (57.8) | 9 (52.9) | 3 (5.8) |
| Personal history of allergies | 12 (63.1) | 10 (58.8) | 26 (50.9) |
| No. of tests for FAs | 171 | 153 | 459 |
| Positive SPT for FAs | 45 (26.3)[b] | 21 (13.7) | 39 (8.4) |
| No. of tests for IAs | 114 | 102 | 306 |
| Positive SPT for IAs | 23 (20.1) | 20 (19.6) | 52 (16.9) |

Papule 3 mm larger than the negative control was considered to be a positive response. [b]$P < 0.01$ compared to the other groups ($\chi^2$).

allergies. Three (5.8%) volunteers without gastrointestinal symptoms reported food intolerance and 26 (50.9%) had a personal history of allergies. A personal history of allergies and the number of positive SPTs to IAs did not differ significantly between the three groups ($P > 0.05$). However, the number of positive SPTs to FAs in the D-IBS group differed significantly from that in the other two groups ($P < 0.01$) (Table 1).

## DISCUSSION

In the present study, we observed that patients with a diagnosis of D-IBS had higher cutaneous reactivity to FAs than to IAs, when compared with those with C-IBS and healthy controls. An association between IBS and sensitivity to several foods was identified in two groups, but the SPT response was not specific for any type of food. None of the volunteers with IBS reported intolerance to an isolated food, and positive SPT responses also were not correlated significantly with a history of intolerance to a specific food. A positive SPT response for a specific food was not associated with the crises of IBS in any of the patients in Groups Ⅰ and Ⅱ.

IBS is a common disorder worldwide, but its exact pathophysiology remains unknown[3]. Various mechanisms, including gastrointestinal dismotility and visceral hypersensitivity, have been extensively studied in IBS[8-10], but recent interest has also been associated with, or directed to the possible participation of the intestinal mucosa in the pathophysiology of IBS[11-13,17]. Several lines of evidence suggest that IBS may be associated with inflammation in the ileal or colonic mucosa, and at least in a subset of patients with IBS, the mucosal immune system seems to be activated[11,15-17]. Mucosal inflammation and immune system activation in IBS can be caused by many factors, including gastrointestinal infections, changes in the resident microflora, bile salts and FAs[15-17].

FAs can activate the mucosal system when there is disruption of the gut barrier[31,33-36]. It is hypothesized that mucosal immune activation by FAs may contribute to the development of food allergy and IBS[9,11,13,15-17,21,25]. Clinically, the role of food-intolerance-induced symptoms in IBS frequently contrasts with that in food allergy[20,22-25,29,37-42], and dietary elimination may be associated with symptom improvement[31]. However, the interaction of food with the gastrointestinal system is not completely understood[13,17,20,22,23,25,29,33-37,39].

Our results demonstrate that patients with IBS symptoms have non-specific intolerance to foods, probably associated with generalized hypersensitivity. The lack of specificity suggests that people with IBS symptoms, associated or not with food intolerance, have difficulties with food in general and specific foods may not be involved in the pathogenesis of this condition. In agreement with other studies, we suspect that IBS causes food sensitivity rather than vice-versa[3,22,37,38,41].

The mechanisms underlying these inflammatory responses are unclear, but recent studies have suggested that an alteration in the mucosal barrier function and a consequent increase in intestinal permeability are the basis for the increased inflammation in IBS[14-17]. Dunlop *et al*[16] have reported that patients with D-IBS have a pronounced permeability increase in the proximal intestine compared with controls, and those without a history of infection onset have a more severe defect. The increase in intestinal permeability could conceivably activate the release of neurotransmitters that stimulate afferent neurons[13].

Scientific evidence of the functional interface between the immune and sensory motor systems of the gut and respiratory systems has been reported[34,35,39]. Recent studies have reported that the prevalence of asthma and respiratory bronchial hyper-responsiveness are more common in IBS patients than in controls, and have suggested that at least a subset of IBS patients may have a systemic immunological disorder[27,28]. We have previously noticed that IBS patients have greater cutaneous reactivity to FAs than to IAs, when compared to patients with functional dyspepsia or normal controls. An association between IBS and sensitivity to multiple foods and non-specific response to SPTs has also been identified[30,42].

In the current study, the presence of diarrhea in IBS was a significant contributor to the greater cutaneous reactivity response to FAs. No patients with IBS, with or without diarrhea, presented with gastrointestinal infections over the 12 mo that preceded the study, including positive stool examination for ova and parasites. We did not find a significant association between personal history of allergies, IBS sub-type, food intolerance and SPT response. The discrepancies between the response to SPTs to FAs and IAs in the two subtypes of patients with IBS suggest disruption of the gut barrier in patients with D-IBS. Our findings are in agreement with other studies[13,16,17,37,38,41].

We conclude that the lack of specificity to food SPT response and the greater cutaneous reactivity to FAs than to IAs may be associated with altered epithelial function and increase in intestinal permeability in D-IBS. Further studies are needed to clarify the potential pathogenic mechanisms underlying the association between IBS and allergy, and to determine if IBS is one or several disorders.

## COMMENTS

### *Background*

Irritable bowel syndrome (IBS) symptoms are frequently associated with the reporting of many food sensitivities. The role of food-intolerance-induced symptoms in IBS frequently contrasts with that in food allergy, but the pathogenesis

of this association is not completely understood. Food antigens (FAs) can activate the mucosal system when there is disruption of the gut barrier. The report of a significant correlation among atopy and increased intestinal permeability suggests that at least a subset of IBS patients may have a systemic immunological disorder.

### Research frontiers

New information is useful for a better understanding of the relationship between increased intestinal permeability, mucosal barrier defects, and intestinal inflammation in IBS patients. Studies are needed to clarify the potential pathogenic mechanisms underlying the association between IBS and allergy, and to determine if IBS is one or several disorders.

### Innovations and breakthroughs

The results of skin prick tests (SPTs) for IAs and FAs in two sub-types of IBS patients were compared. They confirmed a functional interface between the immune and sensory motor systems in the gut and suggest that in D-IBS, the epithelial function (intestinal permeability) in particular can be altered. Few studies regarding the subject are available in literature. This study provides valuable information about clinical and epidemiological aspects of IBS in Brazil.

### Applications

The underlying cause of the pathophysiological changes encountered in IBS remains unclear. In clinical practice, the type and severity of symptoms determines the treatment of IBS. Our results add new information in answer to the question. Is IBS one or several disorders? Future clinical investigations will be useful for a better understanding of the results obtained here.

### Peer review

The article gives a clear delineation of the research background and provides important data about pathophysiological changes in IBS. The references are appropriate and updated.

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*RAPID COMMUNICATION*

# Managing injuries of hepatic duct confluence variants after major hepatobiliary surgery: An algorithmic approach

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## Abstract

**AIM:** To investigate injuries of anatomy variants of hepatic duct confluence during hepatobiliary surgery and their impact on morbidity and mortality of these procedures. An algorithmic approach for the management of these injuries is proposed.

**METHODS:** During a 6-year period 234 patients who had undergone major hepatobiliary surgery were retrospectively reviewed in order to study postoperative bile leakage. Diagnostic workup included endoscopic and magnetic retrograde cholangiopancreatography (E/MRCP), scintigraphy and fistulography.

**RESULTS:** Thirty (12.8%) patients who developed postoperative bile leaks were identified. Endoscopic stenting and percutaneous drainage were successful in 23 patients with bile leaks from the liver cut surface. In the rest seven patients with injuries of hepatic duct confluence, biliary variations were recognized and a stepwise therapeutic approach was considered. Conservative management was successful only in 2 patients. Volume of the liver remnant and functional liver reserve as well as local sepsis were used as criteria for either resection of the corresponding liver segment or construction of a biliary-enteric anastomosis. Two deaths occurred in this group of patients with hepatic duct confluence variants (mortality rate 28.5%).

**CONCLUSION:** Management of major biliary fistulae that are disconnected from the mainstream of the biliary tree and related to injury of variants of the hepatic duct confluence is extremely challenging. These patients have a grave prognosis and an early surgical procedure has to be considered.



**Key words:** Biliary aberrations; Bile duct injury; Postoperative bile leakage; Hepatic duct confluence; Hepatectomy

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## INTRODUCTION

Dissection of the biliary tract constitutes the most crucial step in liver resections and every effort should aim to secure integrity and normal bile flow of the liver remnant. However, up to 40% of the patients are lacking the conventional biliary branching pattern and are more often exposed to sectorial bile duct transection during liver resection[1].

The most frequent biliary variants are found in the right liver. The right posterior sectorial duct (RPSD) and the right anterior sectorial duct (RASD) may be joining the left hepatic duct (LHD), the common hepatic duct (CHD) or even the cystic duct[2-10]. Although biliary complications in liver resections occur approximately in 10%, they are responsible for one third of the postoperative mortality[11,12]. Fortunately, the majority is amenable to non surgical treatment, but when reoperation is necessitated mortality rate may reach up to 70%[13,14]. This high

mortality rate is due to the fact that accurate diagnosis is delayed and surgical treatment is influenced by the ongoing intra-abdominal septic process. Our study aims to present our experience in managing injuries of hepatic duct confluence variants proposing an algorithmic approach.

## MATERIALS AND METHODS

During a 6-year period (Jan, 2001-Dec, 2006) 234 patients who underwent major hepatobiliary surgery were retrospectively reviewed, in order to evaluate postoperative biliary complications due to anatomic variations of hepatic duct confluence. The hepatectomy procedures performed included segmentectomy, lobectomy and extended lobectomy (trisegmentectomy). Our technique of hepatectomy has been described previously[15], using the Pringle maneuver, vascular outflow obstruction and sharp parenchymal dissection. For major hepatectomies (lobectomy-extended lobectomy) hilar dissection was performed to divide the correspondent vessels[16]. The diagnosis of a biliary complication was based upon the presence of a persistent bile leakage *via* the drain, the surgical incision, or the development of an intra-abdominal biloma confirmed by imaging studies. In our department, we do not perform routine preoperative imaging studies of the biliary tree anatomy. Postoperative diagnostic imaging studies included endoscopic retrograde and magnetic resonance cholangiopancreatography (ERCP-MRCP) and occasionally scintigraphy (Tc99m-HIDA) or fistulography.

Thirty patients (12.8%) who developed postoperative bile leaks were identified and conservative treatment (endoscopic stenting and percutaneous drainage) resolved the problem in 23 patients. In the remaining 7 patients, a biliary variant injury of hepatic duct confluence was diagnosed. One of these seven patients underwent an initial laparoscopic cholecystectomy and was also included in this group.

Clinical characteristics, type of operation performed, type of biliary variant, treatment and outcome of these patients with variant injuries are presented in Table 1. Classification of bile duct injuries was based on that used for hepatic duct confluence by Ayuso *et al*[17]. Treatment involved a conservative (percutaneous drainage of bilomas and perihepatic abscesses, antibiotics, *etc*) and a surgical (liver resection, biliary-enteric anastomosis) approach. Timing of surgical intervention was based upon criteria of non-responsiveness to external drainage and/or persistence of intra-abdominal sepsis. The type of procedure was based upon the estimated volume of the liver remnant and the functional reserve of the liver (Child-Pugh classification) and intraoperative factors (local inflammatory process, aberrant bile duct features, *etc*).

## RESULTS

Injuries of variants of the hepatic duct confluence were retrospectively found in 7 patients (three males and four females) with an age range from 36 to 76 years old. Six patients had undergone initially major liver resections (2 for hydatid cyst and 4 for carcinoma) and in one patient a laparoscopic cholecystectomy was performed. All patients developed a major biliary fistula postoperatively that was disconnected from the mainstream of the biliary tree. According to the classification and imaging workup previously mentioned, 1 type C, 3 type D, 2 type E and 1 type F injuries were recognized. This simply means that the most common injury involved the right posterior sectorial duct (RPSD) in four patients (cases 1, 2, 6 and 7), while injuries of the right anterior sectorial duct (RASD) were recognized in two patients (cases 3 and 4; Figure 1). In one patient (case 5), during an extended right lobectomy, the sectorial bile duct draining liver segment I , joining separately the common bile duct (type E injury), was transected with a consequent biliary fistula (Figure 2).

All patients were initially treated conservatively, but only two (cases 3 and 4) had an uneventful outcome with resolution of the bile leak 2 mo and 4 mo, respectively, after the initial operation. One patient (case 2) refused surgical therapy and died from septic shock. The remaining four patients were approached surgically; two underwent a delayed biliary-enteric (B-E) anastomosis (cases 1 and 7) while the other two had a resection of the compromised liver segments (cases 5 and 6).

In case 1, 14 mo after the initial procedure (left lateral sectionectomy) exploratory laparotomy revealed a complex situation; liver segment's Ⅳ duct was found transected and draining in the abdomen and the RPSD was ligated near its junction with the LHD. Resection of segment Ⅳ and B-E anastomosis of the dilated RPSD with a Roux-en-Y intestinal loop were carried out and the patient had an uneventful postoperative course. In contrast, the delayed B-E anastomosis in case 7, performed 10 mo after the initial procedure (laparoscopic cholecystectomy), failed and resection of liver segments Ⅵ & Ⅶ was carried out. Unfortunately, the postoperative course was complicated by overwhelming sepsis due to accidental injury of the duodenum and the patient died.

The last 2 patients (cases 5 and 6) underwent liver resections of the compromised liver segments, 6 mo and 8 mo, respectively, after initial operation with an uneventful postoperative course. An algorithmic approach for these injuries is depicted in Figure 3.

Overall, hospital stay ranged from 20-150 d (mean, 63 d) and the mortality rate in this group of patients with injuries of variants of the hepatic duct confluence was 28.5% (2/7).

## DISCUSSION

Prevailing strategy in hepatobiliary surgery should always be the ascertainment of the integrity of normal bile flow from the liver remnant; otherwise, life-threatening complications may occur. Biliary complications in hepatobiliary surgery vary between 3%-15% and share a significant portion of the postoperative morbidity and mortality[11-14,18]. The cause of biliary leakage is usually due to unsutured collateral biliary branches of the cut surface and a non-surgical treatment settles the problem in the majority of the patients[19]. However, aberrant biliary anatomy is frequently encountered during hepatobiliary surgery and represents a totally different problem from that aforementioned.

**Table 1 Characteristics of patients with injury of aberrant bile ducts**

| No | Sex | Age | Initial operation | Biliary variant injury[1] | Treatment | Outcome |
|---|---|---|---|---|---|---|
| 1 | F | 51 | Left lateral sectionectomy (Hydatid cyst) | RPSD (type D) | Resection of segment Ⅳ and biliary-enteric (B-E) anastomosis | Uneventful |
| 2 | F | 76 | Left Hepatectomy (CHD carcinoma) | RPSD (type C) | Denied liver resection | Died |
| 3 | M | 65 | Left hepatectomy (Liver carcinoma) | RASD (type D) | Conservative (external drainage) | Resolved after 2 mo |
| 4 | M | 71 | Left hepatectomy (Liver carcinoma) | RASD (type D) | Conservative (external drainage) | Resolved after 4 mo |
| 5 | M | 51 | Right extended lobectomy (cholangiocarcinoma) | Segment's Ⅰ duct (type E) | Resection of segment Ⅰ | Uneventful |
| 6 | F | 49 | Resection of segment Ⅴ (Hydatid cyst) | RPSD (type E) | Liver resection (Ⅵ, Ⅶ) | Uneventful |
| 7 | F | 36 | Laparoscopic cholecystectomy | RPSD (type F) | 1. B-E anastomosis failed<br>2. Liver resection (Ⅵ, Ⅶ) | Died |

[1]Types of biliary variants[17]: Type A: Right hepatic duct (RHD) joins the left hepatic duct (LHD); Type B: Triple confluence of right posterior sectorial duct (RPSD) and right anterior sectorial duct (RASD) and LHD; Type C: RASD or the RPSD joins the common bile duct (CBD); Type D: RASD or RPSD joins separately the LHD; Type E: Absence of confluence; sectorial ducts join separately at the common hepatic duct (CHD); Type F: RPSD joins the cystic duct.

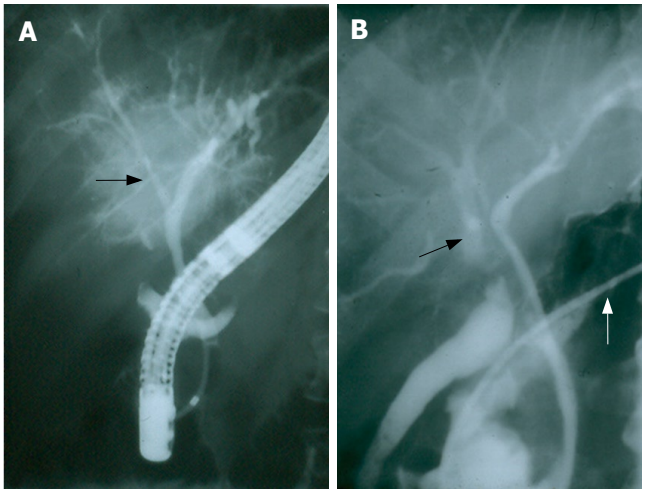


**Figure 1 A:** Retrograde cholangiogram demonstrating the left hepatic duct and its confluence with the right anterior sectorial duct (arrow, case 7); **B:** Fistulogram via the drain tube resulted in a retrograde cholangiogram through the transected right posterior sectorial duct (black arrow). Presence of a nasobiliary tube (white arrow) draining the left hepatic duct (case 7).

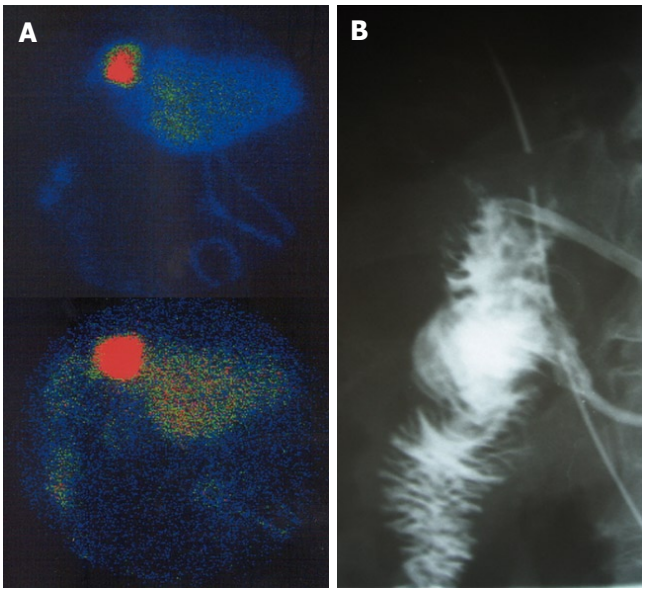


**Figure 2 A:** HIDA scan demonstrating bile leakage after right extended hepatectomy (case 5); **B:** Stentogram with intact hepatico-jejunal anastomosis in the same patient (case 5).

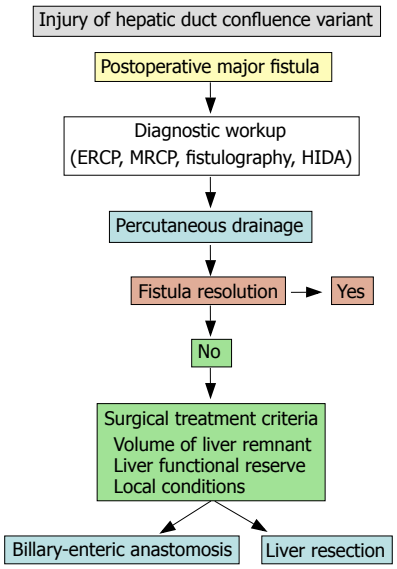


**Figure 3** An algorithmic approach for the management of patients with injuries of hepatic duct confluence variants.

Preoperative assessment of biliary anatomy and possible variations in order to prevent intraoperative injury is currently performed by means of three dimensional helical computed tomography cholangiography[20-23] and various magnetic resonance imaging techniques[24-27]. If a biliary variant is assumed to be injured intraoperatively, the surgeon should perform an intraoperative cholangiography through the injured bile duct in order to estimate the type and extent of injury. In case of a disconnected from the biliary tree sectorial bile duct the decision of the surgical approach should be based on criteria of the volume of the liver remnant and liver functional reserve in case of additional hepatectomy; otherwise a Roux-en-Y biliary-enteric anastomosis must be carried out in order to drain bile to the gut.

Several efforts have been made in order to reduce or manage postoperative bile leaks. A randomized trial using an intraoperative leakage test, injecting isotonic sodium chloride solution *via* the cystic duct had no advantage on reducing postoperative bile leak[28], while the application of a fibrin glue sealant on the cut surface of the liver seems not to be justified[29]. A case report describing infusion

of pure ethanol in an injured sectorial duct resulting in atrophy of the corresponding liver segment and cessation of postoperative bile leak seems to be a minimally invasive approach to this devastating complication needing, however, further evaluation[30]. Postoperative persistent major biliary fistula that has been attributed from diagnostic workup to an injury of a variant of the hepatic duct confluence is initially treated conservatively by means of percutaneous drainage and management of ensuing sepsis usually in a critical care environment. This approach was effective in two of our seven patients and the bile leak resolved 2 mo and 4 mo after initial operation.

Unfortunately, conservative treatment may not settle the problem and ongoing intra-abdominal sepsis fuelled by the major bile leak is associated with high morbidity and mortality. Despite adequate biliary drainage and critical care support, surgical treatment should be instituted in order to manage this problem. A planned approach based upon patient's general status, volume of future liver remnant and liver functional reserve, type and extent of injury and the volume of the corresponding liver segment draining through the injured sectorial bile duct are crucial for decision making. Surgical treatment includes either a resection of the corresponding liver segment or a biliary-enteric anastomosis with a Roux-en-Y limb. In our series, two patients underwent a biliary-enteric anastomosis, which was not successful in one of them and resection of the corresponding liver segment was additionally carried out. Resection of the liver segment, drained by the injured sectorial bile duct, was carried out successfully in two more patients. Therefore, in the case of injury of a variant of the hepatic duct confluence an algorithmic approach is proposed and depicted schematically in Figure 3.

In conclusion, variants of hepatic duct confluence are frequently involved and injured during major hepatic surgery and seriously complicate postoperatively all patients due to delay of diagnosis and ongoing intra-abdominal sepsis. Preoperative imaging of the biliary branching pattern (ERCP, MRCP) remains the only way to recognize and address properly the problem posed by the variant of biliary anatomy. MRCP offers a reliable and non-invasive visualization of the biliary tree in a manner for the surgical approach to be planned and adapted to prevent an injury of a variant of the hepatic duct confluence. However, if this occurs, conservative treatment is the initial approach in managing these patients. Failure to resolve the problem conservatively leads to a planned re-operation which includes either a biliary-enteric anastomosis or a resection of the corresponding to the injury liver segment.

## COMMENTS

### Background

Hepatobiliary surgery is frequently encountered with variations in biliary anatomy. Injuries of variants of the hepatic duct confluence add significant morbidity and mortality after liver resections, due to the development of major bile leakage and ensuing septic sequelae.

### Research frontiers

Preoperative evaluation of anatomical variants seems to be critical in avoiding inadvertent injury. Conservative treatment by means of minimally invasive techniques of injuries of variants of hilar biliary anatomy requires further evaluation. Surgical treatment is still debatable whether to resect the compromised liver segment or to restore bile drainage to the gut by performing a biliary-enteric anastomosis.

### Innovations and breakthroughs

The proposal of an algorithmic approach to manage postoperatively the injuries of the variants of hepatic duct confluence.

### Applications

The implications of this study are for further evaluation of newer conservative therapeutic techniques and/or decision-making regarding surgical management.

### Peer review

The author's proposed an algorithm to manage the injury of the biliary tract after hepatobiliary surgery. Their recommendation is of clinical value for the patients who may have biliary anomaly. However, it is most important for avoiding bile duct injuries during hepatic resection to evaluate accurate anatomy of bile duct preoperatively.

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*RAPID COMMUNICATION*

# Inhibitory effect of dimeric β peptide on the recurrence and metastasis of hepatocellular carcinoma *in vitro* and in mice

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## Abstract

**AIM:** To block the adhesion of tumor cells to the extracellular matrix, and prevent tumor metastasis and recurrence, the dimer of the β peptide (DLYYLMDLSYSMKGGDLYYLMDLSYSMK, β2) was designed and synthesized and its anti-adhesion and anti-invasion effects on hepatocellular carcinoma cells were assessed. Additionally, its influence on the metastasis and recurrence of mouse hepatocellular carcinoma was measured.

**METHODS:** The anti-adhesion effect of β2 on the highly metastatic hepatocellular carcinoma cell line HCCLM6 cells and fibronectin (FN) was assayed by the MTT assay. The inhibition of invasion of HCCLM6 cells by β2 was observed using a Transwell (modified Boyden chamber) and matrigel. Using the hepatocellular carcinoma metastasis model and LCI-D20 nude mice, the influence of β2 on the metastasis and recurrence of hepatocellular carcinoma after early resection was investigated.

**RESULTS:** HCCLM6 cells co-incubated with 100 μmol/L, 50 μmol/L, 20 μmol/L or 10 μmol/L β2 for 3 h showed an obvious decrease in adhesion to FN. The adhesion inhibition ratios were 11.8%, 21.7%, 29.6% and 48.7%, respectively. Additionally, HCCLM6 cells cultured with 100 μmol/L β2 had a dramatic decrease in cell invasion. β2 was also observed to inhibit the incisal edge recurrence and the distant metastasis of nude mice hepatocellular carcinoma after early resection ($P < 0.05$).

**CONCLUSION:** The β2 peptide can specifically block the adhesion and invasion of HCCLM6 cells, and can inhibit HCC recurrence and metastasis of LCI-D20 model post-thepatectomy *in vivo*. Thus, β2 should be further studied as a new anti-tumor drug.



**Key words:** β peptide; Hepatocellular carcinoma; Anti-adhesion; Invasion; Metastasis; Recurrence

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## INTRODUCTION

Despite significant advances in the treatment of human hepatocellular carcinoma (HCC) and the prevention of postoperative metastasis, the 5-year postoperative recurrence rate of HCC is still very high[1,2]. Many efforts have been made to develop a more efficient treatment to inhibit and prevent tumor metastasis, as the recurrence and metastasis of HCC is still a large problem in clinical practice. It is well known that the metastatic process is very complex, including tumor cells dissociating from the primary locus, invading the surrounding tissue, entering and extravasating from the circulation, and growing in distant organs[3,4]. During this process, cell adhesion is one of the most important events[5]. Many studies have been focused on the synthesized anti-adhesion peptides[6-8]. However, the application of these short peptides is limited due to their short half-life and high dosage required. To prolong the peptide's half-life, the polymer and a derivative of synthesized peptides were designed[9-11]. The anti-tumor metastasis effect of the repeat sequence of synthesized peptides was stronger than that of non-repeat peptides[12,13].

Integrins are a family of adhesion molecules located on cells and in the extracellular matrix. The expression level of integrins is related closely to a cell's migration ability[14,15]. The anti-adhesion peptide β (DLYYLMDLSYSMK, β1) was designed by Liu *et al*[16], according to the conserved sequence of the integrin α and β unit. This peptide can block the

interaction between tumor cells and the extracellular matrix and can also inhibit intrahepatic and pulmonary metastases after carcinosectomy in a nude mouse model with human HCC of high metastatic potential (LCI-D20)[17-21]. On the basis of these studies, here we have designed and synthesized the dimeric peptide β (β2). The effects of β2 on the adhesion of human liver cancer cell line HCCLM6 cells to fibronectin (FN), the invasion of HCCLM6 cells to reconstituted basement membrane, as well as liver cancer recurrence and metastasis after hepatectomy in a nude mouse model were investigated.

## MATERIALS AND METHODS

### Cell culture

The highly metastatic hepatocellular carcinoma cell line HCCLM6, initially established and preserved by the Liver Cancer Institute, Fudan University, was cultured in Dulbecco's modified eagle's medium (DMEM, Gibco, UK), supplemented with 10% fetal bovine serum, 100 U/mL penicillin and grown at 37℃ under an atmosphere of 5% $CO_2$. The medium was replenished every three days to maintain cell growth.

### Coating the 96 well high bind microplate with FN

Ten μg/mL FN (Sigma, USA) solution (containing 10 μg/mL FN, 20 mmol/L Tris-Cl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 1 mmol/L $MnCl_2$) was added to a 96-well high bind microplate (Corning, USA) (100 μL per well), and allowed to incubate at 4℃ overnight. The plate was then incubated with blocking buffer (10 mmol/L Hepes, pH 7.4, 140 mmol/L NaCl, 5.4 mmol/L KCl, 5.56 mmol/L glucose, 3% BSA, 1 mmol/L $MgCl_2$, 2 mmol/L $CaCl_2$, 1 mmol/L $MnCl_2$) at 37℃ for 2 h and air dried for further use.

### Cell adhesion assay

β2 peptide was designed in our laboratory using the sequence DLYYLMDLSYSMKGGDLYYLMDLSYS MK. The peptide was synthesized by Shanghai Sangon Bioengineering Company. 100 μL of a HCCLM6 suspension ($2 \times 10^5$/mL) was plated in each well of an FN coated 96-well high bind microplate. 100 μL DMEM medium containing β2 at a concentration of 200 μmol/L, 100 μmol/L, 40 μmol/L or 20 μmol/L was added to the cells concomitantly. The same volume of cell culture medium in place of β2 was added to the control group. 200 μL of cell culture medium only was added in the plate for the blank group. The assay was conducted in quintuplicate for each sample. After incubation for 3 h at 37℃, under an atmosphere of 5% $CO_2$, the unattached cells were gently washed away with HANKS buffer. The attached cell number in each well was measured by MTT. The inhibition rate of β2 on cell adhesion to FN was calculated with the following equation: Cell adhesion inhibitory rate = (average OD of control well-average OD of β2-treated well)/(average OD of control well-average OD of blank well) × 100%

### MTT assay

The number of attached cells in each well was examined by the MTT assay, as previously described[22], and quantified by a micro-titer plate reader (Amersham, USA). Briefly, after incubation for 3 h at 37℃ in 5% $CO_2$, the unattached cells were removed by gentle washing with HANKS buffer. 100 μL DMEM and 20 μL MTT (5 mg/mL) (Sigma, USA) were added to each well. After incubation at 37℃ for 4 h, the medium was discarded. 200 μL of 0.04 mol/L hydrochloric acid in isopropanol was added to each well. The amount of MTT formazan product, which reflects the number of cells adhering to FN, was determined by measuring absorbance with a microplate reader at a test wavelength of 570 nm and a reference wavelength of 630 nm.

### Invasion assay

Invasion assays were performed as described previously[23]. Briefly, the upper portion of Transwell chambers (Corning, USA) were coated with 75 μL of Matrigel (BD, USA) diluted 1:10 in serum-free DMEM and incubated at 37℃ for 2 h. The supernatants of HCCLM6 cells containing DMEM with 10% FCS were harvested after the cells had grown to confluence, and after adding FN at a final concentration of 5 μg/mL, resulting in conditioned medium. The trypsinized cells were harvested and diluted to a $2 \times 10^6$/mL cell suspension with serum-free DMEM. 100 μL of the cell suspension and 100 μL of 200 μmol/L β2 peptides in serum-free DMEM or serum-free DMEM only as a control were added in the upper chambers. Concurrently, 600 μL of conditioned medium was added to the bottom chamber of the Transwell plate. After incubation at 37℃ for 48 h under a 5% $CO_2$ atmosphere, the non-invading cells and the gel were gently removed from the upper chamber with cotton-tipped swabs. Cells were rinsed with PBS, and the cells on the filters were fixed with Formaldehyde and stained in Giemsa staining solution for 30 min. The number of invaded cells on the filters was counted in 5 randomly selected high-powered (× 200) fields per filter under a microscope (Leica, Switzerland). Invasion inhibitory rate was expressed as the following equation: Invasion inhibitory rate = [1 - (invaded cell number in β2 chamber/invaded cell number in control chamber)] × 100%.

### Animal model and treatment

Twelve 5-wk-old male nude mice (BALB/cA) weighing 17-20 g were obtained from the Shanghai Institute of Materia Medica, Chinese Academy of Sciences. The nude mouse model of human hepatocellular carcinoma with high metastatic potential (LCI-D20), which was established in Zhongshan Hospital Liver Cancer Institute, Fudan University, was used in this study. A tumor block of LCI-D20 nude mice human liver cancer metastasis model was implanted into the left lobe of the nude mouse liver as described previously[24]. Briefly, a left upper abdominal transverse incision was made under anesthesia; the left lobe of the liver was exposed and a part of the liver surface was mechanically injured with scissors. Next, a tumor block of 0.2 cm × 0.2 cm × 0.2 cm was fixed within the liver tissue. After the operation, mice were kept in laminar-flow cabinets under specific-pathogen-free conditions and given free access to mouse chow. Liver cancer early resection

**Table 1 The inhibitory effects of β2 on the invasion ability of HCCLM6 cells ($n$ = 5)**

| Group | Mean of invaded cell (SD) | Invasion inhibitory rate (%) |
|---|---|---|
| Control group | 19.30 (9.3) | - |
| β2 group | 12.20 (6.2) | 36.80% |

**Table 2 Liver cancer recurrences in incisal margins in nude mouse models after early resection**

| Group | Number of mice tested | Mean weight of recurrent lesion (g) (SD) | Number of mice with recurrent lesion |
|---|---|---|---|
| Control group | 6 | 2.31 (0.64) | 6 |
| β2 group | 6 | 0.50 (0.41)[a] | 4 |

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

was performed 0.2 cm from the edge of the cancer at day 10 after implantation, prior to metastasis. At day 1 after resection, the animals were subcutaneously administrated 100 μL of 1 mg/mL of β2 or NS as a control every other day for 10 doses. Mice were harvested at day 55 postimplantation, and lungs were fixed in 10% formalin, embedded in paraffin, cut into 5 μm slides and metastatic nodes were observed and counted under a microscope. If recurrence of the incisal margin of cancer was found, the lesion would be resected and weighed.

All of the animal experiments were conducted in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

### Statistical analysis

All data were entered into Excel spreadsheets (Excel, Microsoft, Seattle, USA). We used the SAS program (SAS Institute Inc., Cary, NC, USA) for statistical analysis. Comparisons for dimensional outcomes employed the Student's *t*-test, or the Mann Whitney *U* test when the data were not normally distributed. Values of $P < 0.05$ in a two-tailed fashion were considered to be statistically significant.

## RESULTS

### The inhibitory effect of β2 on the adhesion of HCCLM6 cells to FN

The inhibitory effect of β2 on the adhesion of HCCLM6 cells to FN is shown in Figure 1. HCCLM6 cells co-incubated with 100 μmol/L, 50 μmol/L, 20 μmol/L and 10 μmol/L β2 for 3 h led to an obvious decrease in cellular adhesion. The adhesion inhibition ratios were 11.8%, 21.7%, 29.6% and 48.7%, respectively. This observation indicates that β2 is able to inhibit the adhesion of HCCLM6 cells to FN, and thus β2 might obstruct the invasion of HCC cells to paratumor liver parenchyma.

### The inhibitory effect of β2 on the invasion ability of HCCLM6 cells

After incubation with 100 μmol/L β2, the number of invaded HCCLM6 cells was decreased. The inhibitory rate

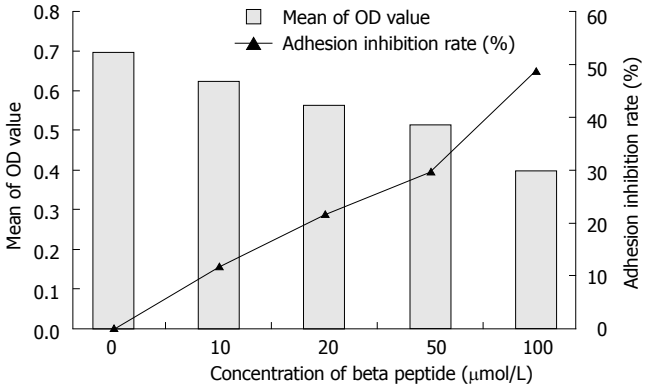


**Figure 1** The inhibitory effect of β2 on the adhesion of HCCLM6 cells to fibronectin ($n$ = 5).

was 36.8% (Table 1). Thus, β2 might block HCC cells from invading the surrounding tissue and entering and extravasating from the circulation *in vivo*.

### The influence of β2 on the intrahepatic recurrence of the LCI-D20 model after early resection

On the 10th d post-tumor-implantation, LCI-D20 tumors were resected, and β2 or the same volume of saline was subcutaneously injected. On day 55, mice were sacrificed to check for intrahepatic recurrence. The recurrent tumor was located around the incisal margins. Compared with the control group, the weight of the intrahepatic recurrent tumor of the β2 group was markedly decreased and statistically significant. There were 4 (4/6) mice with intrahepatic recurrent tumor in the β2 group, while there were 6 (6/6) mice with an intrahepatic recurrent tumor in the control group (Figure 1 and Table 2). These results indicate that β2 have inhibitory effects on tumor recurrence in the incisal margin.

### The inhibitory effects of β2 on metastasis of liver cancer in nude mouse models after early resection

On the 55th day after tumor implantation, the number of metastatic nodes was calculated under a microscope. The result showed that there were fewer metastatic nodes in the β2 treatment group compared to the control group, and there was a statistical difference between the β2 group and the control group. Furthermore, all of the 6 mice in the control group (6/6) had metastatic nodes, but only 4 (4/6) mice had metastatic nodes in the β2 group. These results indicate that β2 have a significant preventive and therapeutic effect on the metastasis of liver cancer (Table 3).

## DISCUSSION

The adhesion molecules on the surface of both tumor cells and endothelial cells are associated with tumor metastasis and recurrence. Blocking the interaction between tumor cell adhesion molecules and their ligands is a major target in the prevention of cancer metastasis[25,26]. Many studies have focused on the synthesized anti-adhesion peptides[27,28]. One such peptide is RGD[29,30], derived from the common conserved sequence of the main matrix

**Table 3 The lung metastasis in liver cancer nude mouse models after early resection**

| | Number (*n*) | The total number of metastatic nodes in lung | The number of mice with lung metastatic nodes |
|---|---|---|---|
| Control group | 6 | 30 | 4 |
| β2 group | 6 | 11[a] | 2 |

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

proteins such as fibronectin, collagen and fibrinogen. A second peptide is YIGSR[31], which originated from the basement membrane protein laminin. The third peptide is EILDV[32], which stemmed from the core sequence of fibronectin. The application of these short peptides was limited due to their short half-life, the ease with which they are degraded and the requirement for a high dosage. To prolong the peptides' half-life, the polymer and derivative of synthesized peptides were designed. The anti-tumor metastatic effect of repeat sequence of synthesized peptides was stronger compared to non-repeat peptides. The more times the sequence is repeated, the stronger the anti-metastasis effect is.

FN is an important cell adhesion molecule in the extracellular matrix. It mediates cell adhesion and migration, and plays a significant role in tumor invasion and metastasis. Assaying FN adhesion to tumor cells is a method commonly used for studying tumor cell metastasis. In this study, the extracellular matrix was simulated by coating cell culture plates with FN, after which the inhibitory effects of β2 peptide on FN adhesion to liver cancer cells were investigated. The results demonstrated that after co-culturing the peptides with HCCLM6 cells for 3 h, a distinct and specific inhibitory effect of β2 peptide on FN adhesion to tumor cells was observed.

Tumor cells must penetrate the basement membrane for at least three times during metastasis; i.e. dislodging from the original site, entering blood circulation, and migrating from blood flow into remote sites. Matrigel, used as a basement membrane matrix, is produced from mouse Engelbreth-Holm-Swarm sarcoma rich in extracellular matrix protein. The artificial basement membrane is plated on a Millipore filter in Transwell culture chambers, and forms a membrane structure similar to natural basement membrane. Invasive, metastatic tumor cells can penetrate the membrane under the induction of chemotactics, simulating tumor cells' invasion of the basement membrane *in vivo*. The results indicated that β2 exerted significant inhibitory effects on the invasion of HCCLM6 cells.

Metastasis and recurrence of liver cancer is a major determinant for the prognosis and long-term survival of liver cancer patients. Polypeptide therapy is a newly developed treatment for tumors[31], but its clinical application is restricted by the degradation of these peptides. β peptides can inhibit the metastasis and recurrence of human liver cancer in nude mouse models after early excision, and can also block the recurrence of cancer at the incisal margins.

The β peptide blocked tumor cell adhesion to FN through two possible mechanisms. First, the β peptide took up the integrin binding site competently through binding to the RGD sequence of the matrix protein. Next, the β peptide also interacted with integrin because the β peptide was designed according to the conserved sequence of the integrin α and β unit.

Taken together, these cell and animal studies demonstrated that the β2 peptide can prevent and treat liver cancer adhesion and metastasis and recurrence. Therefore, the β peptide is worthy of further investigation, as it is a potential drug for blocking tumor metastasis and recurrence.

## COMMENTS

### Background

Despite significant advances in the treatment of human hepatocellular carcinoma (HCC), metastasis and recurrence remain the main obstacles for HCC patients gaining a better outcome and long-term survival. It is well known that during the metastatic process, cell adhesion is one of the most important events. The adhesion molecules on the surface of both tumor cells and endothelial cells are associated with tumor metastasis and recurrence. So, blocking the interaction between tumor cell adhesion molecules and their ligands has become a major target in prevention cancer metastasis.

### Research frontiers

To prevent tumor metastasis and recurrence through inhibiting the adhesion of tumor cells, many studies have focused on the synthesized anti-adhesion peptides such as RGD, YIGSR and EILDV. These peptides are derived from the common conserved sequence of the main matrix proteins such as fibronectin, collagen, fibrinogen and laminin. Liu *et al* designed a new anti-adhesion peptide β (DLYYLMDLSYSMK, β1) according to the conserved sequence of the α and β unit of integrins. These peptides can inhibit the adhesion of tumor cells and cancer metastasis and recurrence. But their application is limited due to the short half-life and high dosage required.

### Innovations and breakthroughs

On the basis of Liu's study, to prolong the peptide's half-life, the dimmer of β peptide (DLYYLMDLSYSMKGGDLYYLMDLSYSMK, β2) was designed and synthesized and the anti-adhesion and anti-invasion effect of it on hepatocellular carcinoma cells, as well as it's influence to the metastasis and recurrence of mouse hepatocellular carcinoma were measured. The result showed that β2 can inhibit the adhesion of HCCLM6 cells to FN in dose-effect manner. And the number of invaded HCCLM6 cells was decreased when incubated together with 100 μmol/L β2. Compared with the control group, the weight of the intrahepatic recurrent tumor and the number of metastatic nodes in lung of the β2 group were markedly decreased.

### Applications

β2 might obstruct the invasion of HCC cells to paratumor liver parenchyma and block HCC cells from invading the surrounding tissue and entering and extravasating from the circulation *in vivo*. In addition, β2 have inhibitory effects on tumor recurrence in the incisal margin and a significant preventive and therapeutic effect on the metastasis of liver cancer. Taken together, these cell and animal studies demonstrated that the β2 peptide can prevent and treat liver cancer adhesion, metastasis and recurrence.

### Peer review

On the basis of previous work, the β2 peptide (DLYYLMDLSYSMKGGDLYYLMDLSYSMK, β2) was designed and synthesized. After co-culturing with HCCLM6 cells for 3 h, a distinct and specific inhibitory effect of β2 peptide on FN adhesion to tumor cells was observed. And also β2 showed significant inhibitory effects on the invasion of HCCLM6 cells. Furthermore, β2 peptides can inhibit the metastasis and recurrence of human liver cancer in nude mouse models after early excision, and can also block the recurrence of cancer at the incisal margins. These results indicate that β2 have a significant preventive and therapeutic effect on the metastasis of liver cancer.

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*RAPID COMMUNICATION*

# A case-control study of the relationship between hepatitis B virus DNA level and risk of hepatocellular carcinoma in Qidong, China

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## Abstract

**AIM:** To investigate the role of hepatitis B virus (HBV) replication in the development of hepatocellular carcinoma (HCC), a nested case-control study was performed to study the relationship between HBV DNA level and risk of HCC.

**METHODS:** One hundred and seventy cases of HCC and 276 control subjects free of HCC and cirrhosis were selected for this study. Serum HBV DNA level was measured using fluorescein quantitative polymerase chain reaction at study entry and the last visit.

**RESULTS:** In a binary unconditional logistic regression analysis adjusted for age, cigarette smoking, alcohol consumption and family history of chronic liver diseases, the adjusted odds ratios (95% confidence intervals) of HCC in patients with increasing HBV DNA level were 2.834 (1.237-6.492), 48.403 (14.392-162.789), 42.252 (14.784-120.750), and 14.819 (6.992-31.411) for HBV DNA levels $\geqslant 10^4$ to $< 10^5$; $\geqslant 10^5$ to $< 10^6$; $\geqslant 10^6$ to $< 10^7$; $\geqslant 10^7$ copies/mL, respectively. Forty-six HCC cases were selected to compare the serums viral loads of HBV DNA at study entry with those at the last visit. The HBV DNA levels measured at the two time points did not differ significantly.

**CONCLUSION:** The findings of this study provide strong longitudinal evidence of an increased risk of HCC associated with persistent elevation of serum HBV DNA level in the $10^4$-$10^7$ range.



**Key words:** Hepatitis B surface antigen; Viral replication; Asymptomatic carriers; Viral load

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## INTRODUCTION

Chronic hepatitis B virus (HBV) infection is still a worldwide health problem[1], with approximate 400 million patients persistently infected[2,3]. Although most of the HBV carriers are asymptomatic, about one-third (25%-40%) die from cirrhotic complications or hepatocellular carcinoma (HCC)[4]. The relative risk of HBV carriers for the development of HCC is up to 200:1, which is one of the highest relative risks known for a human malignancy[5]. Due to the high incidence of recurrence and secondary primary tumor, the survival rate of HCC after any treatment is still low[6]. Therefore, looking for the predictive factors for HCC in patients with chronic Hepatitis B will have a profound impact on the prevention and treatment of chronic HBV infection.

The precise mechanisms by which chronic Hepatitis B leads to HCC are not clearly understood. Viral, host (sex, age and genetic susceptibility) and environmental factors may play interactive roles in hepatocarcinogenesis[7-12]. Recent studies have indicated that serum level of HBV DNA may be a risk factor for HCC[13-16]. Tang *et al*[17] have previously reported that adult HBV carriers who maintain high-titer serum HBV DNA are at higher risk for development of HCC. In Taiwan, a 12-year follow-up study of 4841 men who were Hepatitis B surface antigen

(HBsAg) positive has demonstrated that the risk of HCC is 2.7-10.7-fold higher in patients with baseline HBV DNA levels of 4.0 $\log_{10}$ copies/mL to $\geqslant$ 6.0 $\log_{10}$ copies/mL[18]. However, it is important to study different endemic regions to verify the relationship between active HBV replication and development of HCC, because there is a geographic distribution of HBV genotypes. In particular, the data are largely lacking in mainland of China, where chronic HBV infection is highly endemic and accounts for half of the chronic hepatitis B in the world.

The township of Qidong, at the mouth of the Yangtze River, is one of the highest endemic regions for chronic HBV infection and HCC in China[19]. Between October 1996 and February 2006, we followed a total of 2387 HBsAg-positive adult residents in Qidong city. The aims of this study were to determine whether chronic HBV carriers who maintain high serum HBV DNA level are at higher risk for development of HCC in Chinese patients with chronic Hepatitis B.

## MATERIALS AND METHODS

### Study population

In October 1996, about 18 000 male residents between the ages of 20 and 65 yr living in 17 townships in Qidong county, China were invited to participate in a prospective study. All of those invited were tested for serum HBsAg, alanine aminotransferase (ALT) and $\alpha$-fetoprotein (AFP). A total of 2387 participants who were seropositive for HBsAg and confirmed to be free of HCC by AFP level and abdominal ultrasonography were followed up with abdominal ultrasonography and serological tests including ALT, AFP, HBV serological markers (HBsAg) and anti-Hepatitis C virus (HCV) antibody until February 2006. Each study participant provided informed written consent and a structured questionnaire on sociodemographic characteristics, habits of alcohol and tobacco consumption and family histories. A serum specimen was collected from each participant at every interview. All of the serum samples were stored at -30℃ before analysis. This study was approved by the research ethics committee at Zhongshan Hospital, Fudan University, Shanghai, China.

### Laboratory testing

Serum HBsAg and anti-HCV antibody were tested by commercially available enzyme immunoassay kits (Shanghai Kehua Bio-engineering Co. Ltd., China). Serum ALT level was determined by ultraviolet-lactate dehydrogenase (UV-LDH) method and serum AFP level was determined by ELISA (Shanghai Kehua Bio-engineering Co. Ltd).

### Fluorescein quantitative polymerase chain reaction (FQ-PCR)

The serum HBV DNA levels were determined using the FQ-PCR detection system (Taqmen; Roche USA), according to the manufacturer's instructions. HBV DNA was extracted using the commercial Kit (Shanghai Shenyou Biotech Company) from 50 μL serum. The PCR reaction was carried out as follows: 37℃ for 120 s, 94℃ for 180 s, followed by 40 cycles of 94℃ for 10 s, 55℃ for 30 s and 72℃ for 40 s. The lower limit of detection of this assay was 500 copies/mL with a linear range of up to $10^8$ copies/mL.

### Statistical analysis

The $\chi^2$ test was used to compare baseline characteristics between patients and controls subjects. Wilcoxon signed ranks test has been used to compare the constancy of the viral replication at two time points. For statistical comparisons, a value of 500 copies/mL was assigned, the detection limit of the assay, to samples that had undetectable levels of HBV DNA. Samples of the two groups were divided into six subgroups, according to the level of serum HBV DNA expressed as the logarithmic equivalent (LGE) per milliliter, subgroup 1 (< 500 copies/mL, undetectable), subgroup 2 (2.69 $\log_{10}$ to 3.99 $\log_{10}$ copies/mL), subgroup 3 (4.0 $\log_{10}$ to 4.99 $\log_{10}$ copies/mL), subgroup 4 (5.0 $\log_{10}$ to 5.99 $\log_{10}$ copies/mL), subgroup 5 (6.0 $\log_{10}$ to 6.99 $\log_{10}$ copies/mL), and subgroup 6 ($\geqslant$ 7.0 $\log_{10}$ copies/mL). Binary unconditional logistic regression analysis was used to evaluate relative risks. Potential confounders including age, cigarette smoking, alcohol consumption and family history of chronic liver diseases were adjusted. SPSS 13.0 for Windows was used for all statistical analyses. $P < 0.05$ was considered statistically significant.

## RESULTS

### Demographic characteristic of HCC and control patients

No participants had any clinical evidence of HCC at study entry. By December 31, 2004, 243 participants died of HCC. The data were obtained from medical records and searches of computer files of death certification and cancer registry systems. To ensure complete ascertainment, we also contacted relatives by mail to identify cases. HCC was diagnosed on the basis of either surgical biopsy or an elevated serum AFP level ($\geqslant$ 400 ng/mL), combined with at least one positive image on sonography, computed tomography and/or magnetic resonance imaging. Seventy-three patients diagnosed with HCC within the first two years of our study probably had subclinical HCC at study entry, and were therefore, excluded from the analysis, which left 170 cases of HCC. The paired serum samples were available only in 46 cases, both at study entry and at the time of HCC, for determining the change in serum HBV DNA level over time. Two hundred and seventy-six subjects with chronic Hepatitis B infection and normal ALT level at each follow-up, and free of evidence of cirrhosis or HCC, were selected as controls.

At baseline, there were no significant differences in age, cigarette smoking and alcohol consumption between HCC and control patients, while the family histories of HBV-associated chronic liver diseases were significantly different between the two groups. 85/170 (50%) of cases had a family history, while only 92/276 (33.3%) of control subjects had (Table 1).

### Baseline serum HBV DNA level in HCC patients and controls

186/276 (67.4%) samples of control subjects had undetectable levels of serum HBV DNA. Compared with those with undetectable levels of serum HBV DNA, the adjusted odds ratios of HCC for subjects with increasing

**Table 1 Demographic data in HCC and control patients *n* (%)**

| | HCC patients (*n* = 170) | Control patients (*n* = 276) | $\chi^2$ | *P* value |
|---|---|---|---|---|
| Age at recruitment (yr) | | | 8.347 | $P > 0.05$ |
| 20-29 | 6 (3.5) | 15 (5.4) | | |
| 30-39 | 52 (30.6) | 88 (31.9) | | |
| 40-49 | 72 (42.4) | 87 (31.5) | | |
| 50-59 | 35 (20.6) | 68 (24.6) | | |
| ⩾ 60 | 5 (2.9) | 18 (6.5) | | |
| Smoking | | | 0.131 | $P > 0.05$ |
| Yes | 88 (51.8) | 138 (50.0) | | |
| No | 82 (48.2) | 138 (50.0) | | |
| Alcohol use | | | 0.989 | $P > 0.05$ |
| Yes | 103 (60.6) | 154 (55.8) | | |
| No | 67 (39.4) | 122 (44.2) | | |
| Family history | | | 12.209 | $P < 0.01$ |
| Yes | 85 (50.0) | 92 (33.3) | | |
| No | 85 (50.0) | 184 (66.7) | | |

**Table 2 Association between HBV DNA level at study entry and subsequent risk of HCC *n* (%)**

| HBV DNA level ($log_{10}$ copies/mL) | HCC patients (*n* = 170) | Control patients (*n* = 276) | Adjusted odds ratio (95% CI) |
|---|---|---|---|
| 1 undetectable | 44 (25.9) | 186 (67.4) | 1.000 (reference) |
| 2 (2.69-3.99) | 5 (2.9) | 46 (16.7) | 0.465 (0.172-1.259) |
| 3 (4.00-4.99) | 12 (7.1) | 19 (6.9) | 2.834 (1.237-6.492) |
| 4 (5.00-5.99) | 30 (17.6) | 4 (1.4) | 48.403 (14.392-162.789) |
| 5 (6.00-6.99) | 38 (22.4) | 5 (1.8) | 42.252 (14.784-120.750) |
| 6 (⩾ 7.00) | 41 (24.1) | 16 (5.8) | 14.819 (6.992–31.411) |

Adjusted for age at enrollment (continuous variable), cigarette smoking, alcohol consumption and family history of chronic liver diseases.

HBV DNA level were 0.465 (95% CI 0.172-1.259), 2.834 (1.237-6.492), 48.403 (14.392-162.789), 42.252 (14.784-120.750), and 14.819 (6.992-31.411). The analysis has been adjusted for age, cigarette smoking, alcohol consumption and family history of chronic liver diseases. The risk of HCC was increased with increasing HBV viral load in 4.0 $log_{10}$ to 7.0 $log_{10}$ copies/mL (Table 2).

### *Change of serum HBV DNA level over time*

All the control subjects in our study were followed up for 10 years with persistently normal ALT level, and had no history of interferon-α or nucleoside analogue therapy. HBV DNA levels were compared between entry and last visit in asymptomatic HBV carriers (controls). There was a statistically significant difference in serum HBV DNA level at the two time points (Table 3). For the 46 patients for whom the serum samples were collected both at study entry and at or after the time of HCC diagnosis, the time interval between collection of the two samples ranged from 24 to 94 mo. The log HBV DNA levels measured at the two time points did not have a statistically significant difference.

## DISCUSSION

Family history of liver carcinoma is one of the main risk factors for HCC, especially in the Chinese population[20-22]. In our study, 85/170 (50%) of cases had a family history of HBV-associated chronic liver diseases. However, only 92/276 (33.3%) of control subjects did.

**Table 3 Comparison of serum levels of HBV DNA at study entry and at last visit in asymptomatic HBV carriers (controls) *n* (%)**

| HBV DNA level ($log_{10}$ copies/mL) | At study entry (*n* = 276) | At last visit (*n* = 276) | *Z* | *P* value |
|---|---|---|---|---|
| 1 undetectable | 186 (67.4) | 221 (80.1) | - 4.904 | $P < 0.01$ |
| 2 (2.69-3.99) | 46 (16.7) | 30 (10.9) | | |
| 3 (4.00-4.99) | 19 (6.9) | 9 (3.3) | | |
| 4 (5.00-5.99) | 4 (1.4) | 6 (2.2) | | |
| 5 (6.00-6.99) | 5 (1.8) | 3 (1.1) | | |
| 6 (⩾ 7.00) | 16 (5.8) | 7 (2.5) | | |

In China, HBV DNA levels > 5.0 $log_{10}$ copies/mL have been considered clinically significant, and are suggested by clinical practice guidelines for making a decision on antiviral therapy in chronic carriers of Hepatitis B. The guidelines are supported by the findings of a meta-analysis of 26 trials of statistical significance and consistent correlations between viral load and histological grading, and biochemical and serological response[23]. However, the relationship between different levels, especially lower levels, of HBV DNA and risk of HCC remains uncertain.

During the past 10 years, longitudinal studies have been used to evaluate HBV DNA level as risk factors of HCC in HBV carriers. A significant biological gradient of HCC risk by serum HBV DNA level from 4.0 $log_{10}$ to 7.0 $log_{10}$ copies/mL was observed in our cohort. Similar to previous results[24], the HCC risk started to increase significantly at a serum HBV DNA level of 4.0 $log_{10}$ copies/mL, which is much lower than the level of 5.0 $log_{10}$ copies/mL suggested by clinical practice guidelines for making decisions on antiviral therapy in carriers of chronic Hepatitis B. Viral loads < 4.0 $log_{10}$ copies/mL have been thought to be characteristic of an inactive carrier state and a much lower risk of HCC. Moreover, it is important to know that compared to viral loads between 5.0 $log_{10}$ and 7.0 $log_{10}$ copies/mL, patients with HBV DNA levels > 7.0 $log_{10}$ copies/mL were at lower risk of developing HCC. Chronic HBV carriers with mid-high viral loads (4.0 $log_{10}$ to 7.0 $log_{10}$ copies/mL) tended to be in the phase of immune clearance, while the majority of those with viral load levels of 7.0 $log_{10}$ copies/mL were immunotolerant and at lower risk of HCC. Our findings are partly consistent with studies in different areas. In Japan, Ohata *et al*[25] have investigated the risk factors for HCC in 73 patients with HBV-associated liver disease. A high viral load of HBV DNA, together with age and histological fibrosis, were found to be linked to the occurrence of HCC. Yang *et al*[26] have reported that HCC risk increased with the increasing HBV viral load above 7.5 $log_{10}$ copies/mL. They have also found that HCC risk is associated with Hepatitis B e antigen (HBeAg) positivity among HBsAg-positive men in Taiwan. Based on these results, the serum level of HBV DNA may be used as a prominent risk predictor for HCC, independent of age, histological

fibrosis and HBeAg status.

To the best of our knowledge, there have been few studies on longitudinal stability of HBV DNA level in HBV carriers over time in mainland China. In the 276 control subjects in our study, all the HBV DNA levels in samples at the last visit were compared with those collected at study entry. 186/276 (67.4%) samples of control subjects had undetectable levels of serum HBV DNA at study entry, while 221/276 (80.1%) samples had undetectable levels of serum HBV DNA at the last visit. During a follow-up period of 10 years, the HBV DNA levels of those asymptomatic carriers tended to decrease. Forty-six case patients were selected whose serum samples were collected both at study entry and after the time of HCC diagnosis. Compared with serum HBV DNA levels at study entry, viral load after HCC onset remained at high levels. This implied that for chronic HBV carriers free of antiviral therapy, HCC was preceded by persistently high replication activity of HBV and viral levels did not decline with progression of HCC.

It is generally agreed that antiviral treatment is suitable in patients with active HBV replication ($\geqslant$ 5.0 $\log_{10}$ copies/mL) and elevated ALT level (at least twice the upper limit of the normal range)[27] or advanced fibrosis present upon liver biopsy. In clinical trials, among patients with chronic Hepatitis B and advanced stage fibrosis, longer term lamivudine therapy reduces the risk of HCC[28,29]. Although individuals with low viral load (< 4.0 $\log_{10}$ copies/mL) are at decreased risk for HCC, continued monitoring is essential because of the fluctuating nature of chronic HBV infection. Treatment choices for patients with serum HBV DNA levels < 5.0 $\log_{10}$ copies/mL are still controversial. In our study, HBV carriers with HBV DNA levels > 4.0 $\log_{10}$ copies/mL have 2.834 times excess risk of HCC compared with HBV carriers with lower HBV DNA levels. Therefore, among patients with HBV DNA levels > 4.0 $\log_{10}$ copies/mL, liver tests should be carefully monitored at 3-4-mo intervals, irrespective of age and ALT levels. Antiviral treatment should be advised when hepatitis flares and/or advanced fibrosis is present upon liver biopsy.

In conclusion, serum HBV DNA levels were found to be associated with increased risk of HCC. For chronic HBV carriers without antiviral therapy, HBV DNA levels changed little with the progression of HCC. Based on these findings, it is conceivable that patients with a high viral load have a high potential for hepatocarcinogenesis, and should be subjected to closer clinical monitoring[30] and even antiviral treatment.

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## COMMENTS

### Background

Chronic Hepatitis B virus (HBV) infection is still a worldwide health problem. The precise mechanisms by which chronic Hepatitis B leads to hepatocellular carcinoma (HCC) are not clearly understood. Viral, host (sex, age and genetic susceptibility) and environmental factors may play interactive roles in hepatocarcinogenesis. Recent studies have indicated that serum level of HBV DNA may be a risk factor for HCC. However, the data are largely lacking in mainland China, where chronic HBV infection is highly endemic and accounts for half of the chronic Hepatitis B in the world. It is important to study different endemic regions to verify the relationship between active HBV replication and development of HCC, because there is geographic distribution of HBV genotypes.

### Research frontiers

Study on the prognostic factors in patients with chronic Hepatitis B, the relationship between Hepatitis B virus genotype and HBV DNA level, and HCC and treatment of chronic Hepatitis B patients who are resistant to antiviral therapy.

### Innovations and breakthroughs

The township of Qidong, at the mouth of the Yangtze River, is one of the highest endemic regions for chronic HBV infection and HCC in China. However, this is believed to be the first study of the relationship between HBV replication and development of HCC in that region.

### Applications

Base on our current findings, it is conceivable that patients with a high viral load have a high potential for hepatocarcinogenesis, and should be subjected to closer clinical monitoring and even antiviral treatment. The results provide a data-supported approach to patients with Hepatitis B.

### Peer review

This case-control study examined the relationship of HBV DNA quantitative levels and the risk of HCC in Qidong, China. They confirm other studies from Taiwan and elsewhere that demonstrate the risk of HCC occurs across a gradient of HBV DNA levels. This study is important.

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*RAPID COMMUNICATION*

# Predictive value of MTT assay as an *in vitro* chemosensitivity testing for gastric cancer: One institution's experience

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## Abstract

**AIM:** To investigate the predictive clinical value of *in vitro* 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay for directing chemosensitivity in patients with gastric cancer.

**METHODS:** Results of a total of 353 consecutive patients with gastric cancer treated with MTT-directed chemotherapy or physician's empirical chemotherapy from July 1997 to April 2003 were reviewed and analyzed retrospectively.

**RESULTS:** The overall 5-year survival rate of MTT-sensitive group (MSG) and control group (CG) was 47.5% and 45.1%, respectively. The results of subgroup analysis with Cox proportional-hazards model were favorable for the MSG-sensitive group. However, no statistically significant difference in survival rate was observed between the two groups.

**CONCLUSION:** Individualized chemotherapy based on *in vitro* MTT assay is beneficial, but needs to be confirmed by further randomized controlled trials.



**Key words:** Gastric cancer; Chemosensitivity testing; Chemotherapy; MTT assay; Survival rate

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## INTRODUCTION

Gastric cancer ranks second of all cancers and is the leading cause of cancer-related deaths worldwide[1,2]. The incidence of gastric cancer worldwide is reported to be especially high in Asia, South America, and Eastern Europe[2-5]. Gastric cancer patients are treated in clinical practice with various therapies, such as chemotherapy and radiation, though further improvement and progress would be required. With the development of new anti-cancer drugs, such as taxanes, CPT-11, oxaliplatin, gefitinib and S-1, significant improvements in the efficacy of chemotherapies against gastric cancer have been achieved[4,6-8]. However, some patients still fail to respond to chemotherapy and finally die of the critical toxicity of intensive chemotherapy[9]. Thus, new therapies and technologies are desperately needed for the treatment of gastric cancer. Advances in this area would have a major impact on the outcome of a large number of patients with this disease. Hence, chemosensitivity assay has been developed to individualize chemotherapy for gastric cancer patients[10]. 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay is a rapid and quantitative colorimetric method for determination of cell viability by measuring the anticancer drug effectiveness on human tumor cells. Several studies on advanced gastric cancer using this approach revealed that *in vitro* sensitivities are associated with *in vivo* tumor responses[11-15]. However, most of these studies were small-scale trials (< 100 patients).

In this study, MTT assay was used to predict the efficacy of individualized assay-directed chemotherapy for Chinese gastric cancer patients, and to prove whether *in vitro* chemosensitivities are associated with *in vivo* tumor responses by survival analysis.

## MATERIALS AND METHODS

### Patients

This was a retrospective study. The medical records of

patients registered for adjuvant chemotherapy from July 1997 to April 2003 were reviewed. The criteria for case inclusion were as follows: (1) a diagnosis of histologically or cytologically proven gastric cancer, (2) without prior chemotherapy or radiotherapy, (3) adequate blood counts (hemoglobin ⩾ 10 g/L, WBC count of 3000/μL, and platelets of 100 000/μL), normal renal function (creatinine clearance ⩾ 60 mL/min), and normal liver function (serum transaminase level less than double the normal upper limit). Patients with esophageal cancer, small cell carcinoma, lymphoma, and squamous cell carcinoma were excluded from the study. A total of 353 eligible records of patients were collected and analyzed. The patients were divided into MTT-sensitive group (MSG) and control group (CG). One hundred and fifty-seven patients in the MSG-sensitive group were treated by chemotherapy containing at least one sensitive drug based on the MTT assay results, and 196 patients received physician's empirical chemotherapy. The chemotherapeutic drugs used were cisplatin (CDDP), 5-fluorouracil (5-Fu), mitomycin (MMC), doxorubicin (DOX), paclitaxel (PAC) and docetaxel (DOC). The protocols of chemotherapy have been described elsewhere[6,16,17].

### MTT assay

Fresh tumor tissue obtained from the surgically resected specimens was tested within 6 h. The tumor tissue was cut into pieces (smaller than 1 $mm^3$) and passed through No. 100 and No. 200 stainless steel meshes respectively into a complete medium containing RPMI 1640 solution, 100 μg/mL penicillin, and 100 μg/mL streptomycin, and washed twice gently with the same solution. The viable cells were assessed using trypan blue exclusion method. Cell viability was measured by MTT assay to assess the chemosensitivity of tumor cells. Cell suspension was collected into sterile 96-well flat-bottomed microtiter plates ($1 \times 10^5$ cells/per well) with or without chemotherapeutic agents. The drug and testing drug concentrations used were 25 μg/mL cisplatin (CDDP), 100 μg/mL -fluorouracil (5-Fu), 10 μg/mL mitomycin (MMC), 4 μg/mL doxorubicin (DOX), 100 μg/mL paclitaxel (PAC) and 30 μg/mL docetaxel (DOC). Each drug was tested in triplicate. The plates were then incubated at 37℃ in a humidified atmosphere containing 50 mL/L $CO_2$ for 72 h. Microtiter wells containing tumor cells but no anticancer agents were used to control cell viability, in which the total number of tumor cells was equivalent to that in the test wells, and wells containing only a complete medium were used as blank controls for nonspecific dye reduction. After incubation, MTT solution was added to each well at a final concentration of 1 mg/mL per well and the plates were incubated at 37℃ for another 4 h. Then the mixture containing the medium, the drug, and the unconverted MTT was removed. DMSO was added to each well to dissolve the formazan and absorbance was read at 550 nm using a spectrophotometric microplate reader (Labsystems, Finland). The inhibition rate of tumor cells for each drug with different concentrations was calculated following the formula: inhibition rate (%) = $(1 - OD_{drug\ exposure}/OD_{control}) \times 100$. The effective anticancer activity was regarded as sensitive when the tumor inhibitory rate was greater than or equal to 70%.

**Table 1 Baseline clinical and pathological characteristics of the patients *n* (%)**

| Characteristic | MSG (*n* = 157) | CG (*n* = 196) |
|---|---|---|
| Gender | | |
| Male | 104 (66.2) | 137 (69.9) |
| Female | 53 (33.8) | 59 (30.1) |
| Age | | |
| < 60 yr | 87 (55.4) | 110 (56.1) |
| 60-69 yr | 54 (34.4) | 67 (34.2) |
| 70-80 yr | 16 (10.2) | 19 (9.7) |
| Median year | 58 | 62 |
| Range year | 29-80 | 31-78 |
| Cancer stage, TNM classification | | |
| Ⅰ B | 3 (1.9) | 4 (2.0) |
| Ⅱ | 85 (54.1) | 103 (52.6) |
| Ⅲ | 54 (34.4) | 71 (36.2) |
| Ⅳ | 15 (9.6) | 18 (9.2) |
| Histologic type | | |
| Differentiated | 73 (46.5) | 103 (52.6) |
| Undifferentiated | 84 (53.5) | 93 (47.4) |

### Toxicity

All patients who started treatment were considered assessable for toxicity. Toxicity was analyzed following the National Cancer Institute Common Toxicity Criteria (version 2.0).

### Statistical analysis

All statistical analyses were done using the SAS 6.12 statistical software (SAS Institute, Cary, NC). The clinical and pathological characteristics, including gender, age, cancer stage (TNM), and histological type (differentiated *versus* undifferentiated type), were evaluated by Mann-Whitney's *U*-test and the Kruskal-Wallis test. The overall probability was calculated using the Kaplan-Meier method for censored failure time data, and the statistical significance was analyzed by the log-rank test for comparison of survival rate between the two groups. The Cox proportional-hazards model was used to calculate the hazard ratios. $P < 0.05$ was considered statistically significant. All *P* values were two-tailed and unadjusted for potential multiple comparisons.

## RESULTS

### Patient characteristics

The clinical and pathological characteristics of the patients are outlined in Table 1. Between the MSG and CG arms, there was no significant difference in baseline clinical characteristics and pathological findings which were considered to be related to the prognosis of gastric cancer patients.

### Overall survival analysis

The overall 5-year survival rate of the patients was 47.5% and 45.1% in the MSG-sensitive group and CG group, respectively, with no statistical difference (Figure 1). The

**Table 2 Severe adverse effects and toxicities (NCI-CTC version 2.0)**

| Toxicities | MSG ($n$ = 157) | | | CG ($n$ = 196) | | |
|---|---|---|---|---|---|---|
| | Grade Ⅲ (No.) | Grade Ⅳ (No.) | Grade Ⅲ/Ⅳ (%) | Grade Ⅲ (No.) | Grade Ⅳ (No.) | Grade Ⅲ/Ⅳ (%) |
| Hematologic toxicity | | | | | | |
| Leukopenia | 2 | 2 | 2.5 | 4 | 1 | 2.6 |
| Anemia | 2 | 0 | 1.3 | 2 | 2 | 2.0 |
| Thrombocytopenia | 0 | 0 | 0.0 | 1 | 0 | 0.5 |
| Non-hematologic toxicity | | | | | | |
| Diarrhea | 2 | 0 | 1.3 | 3 | 0 | 1.5 |
| Stomatitis | 1 | 0 | 0.6 | 0 | 0 | 0.0 |
| Nausea | 2 | 1 | 2.5 | 2 | 0 | 1.0 |
| Vomiting | 2 | 0 | 1.3 | 3 | 0 | 1.5 |
| Anorexia | 8 | 2 | 6.4 | 6 | 3 | 4.6 |
| Fever | 0 | 0 | 0.0 | 2 | 0 | 1.0 |
| Rash | 1 | 0 | 0.6 | 1 | 0 | 0.5 |
| Elevated aminotransferase | 2 | 0 | 1.3 | 3 | 1 | 2.0 |
| Hyperbilirubinemia | 1 | 0 | 0.6 | 2 | 0 | 1.0 |
| Elevated creatinine | 0 | 0 | 0.0 | 1 | 0 | 0.5 |

hazard ratio for deaths in the MSG-sensitive group, as compared with the CG group, was 0.92 [95% confidence interval (CI) = 0.69 to 1.23, $P$ = 0.57].

### Subgroup analysis

The overall survival rate of the patients was analyzed according to sex, age, cancer stage (TNM classification), and histologic type. The hazard ratio of deaths was favorable for the MSG-sensitive group (Figure 2). There were no significant interactions between the two groups and any of the variables studied.

### Adverse events and treatment compliance

Data on the 157 patients in the MSG-sensitive group and 196 patients in the CG group were analyzed for adverse events. The main emergent adverse toxicities (grades 3 and 4) related to treatment are listed in Table 2. The severe adverse events (defined according to NCI-CTC version 2.0), including hematologic and nonhematologic toxic effects, did not occur more frequently in the MSG-sensitive group than in the CG group.


**Figure 1** Kaplan-Meier curves of overall survival probabilities for gastric cancer patients, there was no significant difference between the two groups.

## DISCUSSION

Conventional chemo-/radio-therapy for gastric cancer is limited to improve the treatment outcomes and quality of survival/life of human patients[18,19]. Physicians' empirical choice of chemotherapeutic regimen for gastric cancer is based on the data obtained from clinical trials[20]. However, even the same gastric cancer behaves so differently that the response rate of cancers to the chemotherapeutic agents varies. These variations are partly contributed to the failure of treatment of gastric cancer patients. The effectiveness of current chemotherapies for cancer is limited mainly due to its heterogeneity[21]. To overcome this problem, selecting a sensitive chemotherapeutic regimen *in vitro* for individual gastric cancer patients appears to be an attractive way. Chemosensitivity testing is an *ex vivo* means of determining the cytotoxic and/or cytostatic, or apoptosis-inducing effect of anticancer drugs. The most common *in vitro* assays include MTT and ATP-TCA assays, *etc*[22]. These assays have been successfully used in the assay-guided chemotherapy for certain cancers, including breast, ovarian, melanoma and colorectal cancers[23-25]. MTT assay has been most widely used in different cancers, and is sensitive, accurate, and efficient in the *in vitro* evaluation of anticancer or immunological agents prior to preclinical and clinical testing. Some research groups have used MTT assay to guide individual adjuvant chemotherapy for gastric cancer[10], showing that the therapy based on the chemosensitivity testing can improve the clinical outcomes of cancer patients. In the present study, based on the criteria for chemosensitivity *in vitro*, we predicted and evaluated the efficacy of chemotherapy for 353 gastric cancer patients according to the result of MTT assay. The overall survival rate of the patients, treated with chemotherapy regimen containing at least one sensitive agent, was higher in the MSG-sensitive group than in the CG group treated with the physicians' empirical therapy. The hazard ratio of most subgroups was favorable for the MSG-sensitive group as demonstrated in Cox proportional-hazards mode. However, no significant difference between the two groups was observed. These results indicate that MTT

| Baseline characteristic | MSG No. of deaths/total | CG No. of patients |
|---|---|---|
| Gender | | |
| Male | 58/104 | 69/137 |
| Female | 25/53 | 38/59 |
| Age | | |
| < 60 yr | 46/91 | 54/111 |
| 60-69 yr | 31/55 | 44/71 |
| > 70 yr | 6/11 | 9/14 |
| Cancer stage, (TNM classification) | | |
| Ⅱ | 43/85 | 44/103 |
| Ⅲ | 27/54 | 49/71 |
| Ⅳ | 11/15 | 14/18 |
| Histologic type | | |
| Differentiated | 37/73 | 48/103 |
| Undifferentiated | 46/84 | 59/93 |
| Total | 83/157 | 107/196 |

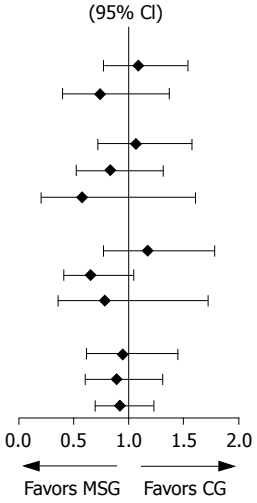


**Figure 2** Hazard ratios of deaths for the baseline characteristics among gastric cancer patients. NS: No significant difference.

assay can lead to additional clinical outcomes as compared with physicians' empirical therapy. The real benefit of MTT assay for chemosensitivity testing is to predict which agent is useful or not useful. Although the results of this retrospective study are not all concordant with previous studies[11,12,22,26], and do not definitively support the clinical values of MTT assay in detecting chemosensitivity to the adjuvant chemotherapy for patients with gastric cancer, they support chemosensitivity testing in patients with gastric cancer. Since the frequency of toxicity in patients is not reported in previous studies[11,12,26], we compared the frequency of severe toxicity of grades 3 and 4 between the two groups, showing that chemotherapy regimen based on MTT assay could not reduce its the adverse effect and toxicity.

At present, although some studies have shown a potential clinical benefit of chemotherapy for patients with drug-sensitive cancer[22,27], chemosensitivity testing has not been widely accepted by physicians. Meanwhile, prediction of chemosensitivity in clinical practice is a challenge because *in vitro* chemosensitivity testing systems have not considered the pharmacokinetic and pharmacodynamic variables affecting drug action *in vivo*. Because of varied pharmacogenetic make-ups of cancer patients, leading to interpatient variations in drug half-life, volume of distribution, types of metabolites formed, and route of elimination, dependence on *in vitro* and *in vivo* results is often not a straightforward process[28]. Ultimately, individualized chemotherapy based on cellular and genetic characteristics of cancer patients may be on the horizon[29-31]. The potential clinical benefits of individualized chemotherapy based on chemosensitivity testing need to be confirmed by further randomized controlled trials in comparison with standard chemotherapy.

## COMMENTS

### Background

Since cancer patients with histologically similar tumors respond differently to standard drug treatment, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) chemosensitivity assay is performed to provide predictive information to help physicians choose sensitive chemotherapeutic agents for eliminating potentially ineffective agents used in chemotherapeutic regimens for each cancer patients.

### Research frontiers

At present, several new chemosensitivity assays, such as histoculture drug response assay (HDRA), collagen gel-droplet-embedded culture drug sensitivity test (CD-DST) and fluorometric microculture cytotoxicity assay (FMCA), are used in selection of an appropriate chemotherapeutic drug, showing the predictive value of chemotherapy for cancer patients.

### Innovations and breakthroughs

There is no evidence for the clinical benefits of MTT chemosensitivity assay. The present study evaluated the clinical usefulness of MTT chemosensitivity assay in gastric cancer patients, and showed no significant differences in clinical outcomes between the MTT-sensitive group (MSG) and the control group (CG), indicating that the potential value of MTT assay for patients with gastric cancer is limited.

### Applications

Although some studies have shown a potential clinical benefit for patients with drug-sensitive cancer, MTT assay of chemosensitivity is not widely accepted by physicians because there is no sufficient evidence obtained in the clinical setting. The potential clinical benefits of individualized chemotherapy based on chemosensitivity assay for gastric cancer patients need to be confirmed by further randomized controlled trials in comparison with standard chemotherapy.

### Terminology

MTT assay is a laboratory test and a standard colorimetric assay for measuring cellular proliferation. Yellow MTT is reduced to purple formazan in the mitochondria of living cells. A solution (usually dimethyl sulfoxide) is added to dissolve the insoluble purple formazan products into a colored solution. The absorbance of this colored solution can be quantified at a certain wavelength with a spectrophotometer.

### Peer review

This is an interesting report on the predictive value of MTT assay as an *in vitro* chemosensitivity testing for gastric cancer patients. Individualized chemotherapy based on *in vitro* MTT assay has clinical benefit, but needs to be confirmed by further randomized controlled trials.

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*RAPID COMMUNICATION*

# Significance of Bcl-xL in human colon carcinoma

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## Abstract

**AIM:** To investigate the clinical significance of Bcl-xL gene in the pathogenesis of human colon carcinoma.

**METHODS:** Fifty-six pair tissue samples from patients with colon cancer were collected, and protein level of the Bcl-xL gene was measured by immunohistochemistry method. The correlation of Bcl-xL expression with clinical index was evaluated. After human colon cancer cell line HT29 was transfected with Bcl-xL small interfering RNA (siRNA), the anchorage-independent growth of cancer cells was detected by colony formation in soft agar and invasion ability of cancer cells was determined by a transwell model.

**RESULTS:** The Bcl-xL expression was higher in cancerous tissue samples than in normal tissue samples (38.78 ± 11.36 *vs* 0.89 ± 0.35, $P < 0.001$), and was associated with the pathological grade, lymphnode metastasis and Duke's stage of colorectal carcinoma. Transfection with Bcl-xL siRNA inhibited the colony formation and invasion ability of human colon cancer cell line HT29 *in vitro*.

**CONCLUSION:** Bcl-xL gene plays an important role in carcinogenesis of human colorectal carcinoma and is associated with malignant biological behaviors of human colorectal carcinoma.



**Key words:** Colorectal carcinoma; Bcl-xL; Clinical significance

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Zhang YL, Pang LQ, Wu Y, Wang XY, Wang CQ, Fan Y. Significance of Bcl-xL in human colon carcinoma. *World J Gastroenterol* 2008; 14(19): 3069-3073 Available from: URL: http://www.wjgnet.com/1007-9327/14/3069.asp DOI: http://dx.doi.org/10.3748/wjg.14.3069

## INTRODUCTION

Colorectal cancer is the third most common malignant neoplasm worldwide[1] and the second leading cause of cancer-related death[2]. Despite recent advances in diagnostic and therapeutic measures, the prognosis of colorectal cancer patients with distant metastasis still remains poor. Enhanced understanding of the signaling mechanisms that regulate metastasis of colon cancer may provide important insights into more effective therapeutic strategies.

Cells harboring multiple genetic alterations are normally eliminated by apoptosis. Diminished apoptosis plays a critical role in tumor initiation, invasion, metastasis, progression, and drug resistance. Results of numerous scientific and clinical studies link altered expression of apoptosis-regulatory proteins to the development of a lot of cancer cells. Among them, the Bcl-2 family of genes, which share sequence homology domains, plays a key role in the regulation of apoptotic cell death induced by a wide variety of therapeutic stimuli[3]. These genes can form homodimers and/or heterodimers that modulate one another's function, whereby their relative concentrations function as a rheostat for the apoptotic program[4]. Of them, Bcl-xL gene has been well characterized as a potential gene involved in the apoptotic signal pathway.

Bcl-xL, a mitochondrial membrane protein, promotes cell survival by regulating the electrical and osmotic homeostasis of mitochondria in response to a variety of stimuli[5,6]. Over-expression of Bcl-xL is reported to confer a multidrug resistance phenotype[7,8]. Moreover, inhibition of Bcl-xL expression by some ways results in an altered ratio of BAX to Bcl-xL and subsequent mitochondria-mediated cell death[9]. Thus, Bcl-xL might serve as an ideal molecular target of anticancer therapy

However, previous studies about Bcl-xL gene have mainly focused on the regulation of apoptosis and drug resistance. There is little information about the linkage of Bcl-xL with invasion in cancer cells. Increasing data show

that Bcl-xL over-expression might be related to invasion and metastasis of some solid tumors, such as breast cancer[10,11], hepatocellular carcinoma[12], ovarian cancer[13], glioma[14], and lung carcinoma[15]. We have found in previous works that human colon cancer cells transfected with signal transducer and activator of transcription 3 (STAT3) small interfering RNA (siRNA) can inhibit the invasion ability of cancer cells. Meanwhile, expression of Bcl-xL protein is also markedly down-regulated in transfected cancer cells[16]. However, the possible role of Bcl-xL in invasion of human colon cancer is not clear.

In the present study, we investigated the linkage of Bcl-xL with the invasion of human colon cancer *in vivo* and *in vitro*.

## MATERIALS AND METHODS

### Tissue samples

A total of 56 paired colon cancer tissue and distant normal colon tissue samples were obtained from 56 patients undergone surgical operation. Tumor histotype and grade of differentiation were defined according to the WHO criteria. The clinical and pathological stages were defined according to Duke's staging. These patients did not receive any chemotherapy or radiotherapy before operation. This study was approved by the Medical Ethical Committee of Affiliated Hospital of Jiangsu University, and all patients provided their written informed consent to participate in the study. All the specimens were fixed in 10% neutral-buffered formalin, dehydrated in ascending series of ethanol and routinely embedded in paraplast. Sections were cut at 4 μm, stained with hematoxylin and eosin for histopathological and immunohistochemical evaluation. The clinicopathological parameters are summarized in Table 1.

**Table 1 Relationship between Bcl-xL expression and clinical parameter in 56 cases of colorectal carcinoma (mean ± SD)**

| Characteristic | *n* | Bcl-xL PU | *P* |
|---|---|---|---|
| Sex | | | > 0.05 |
| Male | 30 | 39.22 ± 11.35 | |
| Female | 26 | 37.36 ± 12.18 | |
| Age (yr) | | | > 0.05 |
| ≤ 55 | 25 | 39.89 ± 15.78 | |
| > 55 | 31 | 38.66 ± 12.56 | |
| Tumor differentiation | | | < 0.05 |
| Well | 12 | 31.58 ± 12.69 | |
| Moderate | 19 | 39.77 ± 16.55 | |
| Poor | 25 | 53.95 ± 17.89 | |
| Lymph node metastasis | | | < 0.05 |
| Negative | 27 | 32.19 ± 13.35 | |
| Positive | 29 | 56.36 ± 11.95 | |
| Duke's staging | | | < 0.05 |
| A + B | 28 | 31.55 ± 12.39 | |
| C + D | 28 | 58.78 ± 11.68 | |

### Immunohistochemical analysis and quantitative evaluation

All the tissue sections were deparaffinized, rehydrated and incubated in a citrate buffer (0.01 mol/L, pH 6.0) for 1 min at 121℃. The endogenous peroxidase activity was blocked by covering the sections with 3% $H_2O_2$/methanol for 15 min. The sections were then incubated in a 1:100 dilution of goat antihuman Bcl-xL IgG at 4℃ overnight. After washed with PBS containing 0.05% Tween, the tissue sections were incubated in a 1:50 dilution of biotinylated donkey anti-goat IgG (Santa Cruz) for 30 min. The SABC reagents were used to amplify the immunoreactivity that was detected using 3'-diaminobenzidine according to the manufacturer's instructions. The sections were counterstained with hematoxylin. The positive uniT (PU) represents the relative concentration of positive staining according to previous data[17]. Each section was observed randomly at five areas and the mean PU was assembled and calculated.

### Sequence of Bcl-xL siRNAs

The anti-sense sequence of siRNA (5'-CTCTGAT ATGCTGTCCCTG-3') corresponding to Bcl-xL mRNA with dTdT on 3'-overhangs was designed and chemically synthesized according to the recommendation of the manufacturer (Dharmacon Research, USA). The scrambled siRNA served as a control, and its sequences are 5'-UUCU CCGAACGUGUCACGUTdTd-3' and 5'-ACGUGACAC GUUCGGAGAATdTdT-3'.

### Cell culture and Bcl-xL siRNA transfection

Human colon cancer cell line HT29 (Institute of Cell Biology, Shanghai, China) was cultured in RPMI 1640 (Invitrogen, Inc.) supplemented with 10% fetal bovine serum (FBS) in an atmosphere containing 50 mL/L $CO_2$ at 37℃. siRNA was transfected with a commercial reagent, oligofectamine (Invitrogen, USA) in 6-well plates following its manufacturer's instructions. Briefly, On the day before transfection, confluent layers of cells were trypsinized, counted and re-suspended. Cells ($1 \times 10^5$) were plated into each well of the 6-well plates, so that they could become about 70% confluence next day at the time of transfection. Oligofectamine was diluted in serum-free RPMI 1640 and mixed with siRNA at a 1:2 ratio (4 μL of 20 μmol/L of siRNA formulated with 8 μL of oligofectamine). The cells were then incubated for other 48 h. The number of cells was determined using a hemocytometer before subsequent assays.

### RNA isolation and complementary DNA synthesis

Total cellular RNA was isolated from cancer cell lines using Trizol. Final RNA pellets were dissolved in 20 μL of diethyl pyrocarbonate-treated water. RNA yield was determined by spectroscopy. For complementary DNA (cDNA) synthesis, 2 μg of total RNA was transcribed with cDNA transcription reagents using 0.2 μg of the oligo(dT)18 primer for subsequent quantitative, real-time polymerase chain reaction (RT-PCR).

### Real time transcription polymerase chain reaction (RT-PCR)

Real-time RT-PCR analyses were performed on an ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, CA). For Bcl-xL amplification, primers with the sequences 5'-TCCTTGTCTACGCTTT CCACG-3' and 5'-GGTCGCATTGTGGCCTTT-3' were

used in combination with a sequence 5'-ACAGTGCCC CGCCGAAGGAGA-3'. Primers, Taqman and TaqMan probes were designed by the Primer Express TM 1.0 (Applied Biosystems) software and the probes were labeled at 5' end with the reporter dye molecule FAM (6-carboxy-fluorescein) and at 3' end with the quencher dye molecule TAMARA (6-carboxytetramethyl-rhodamine). Real-time PCR was conducted in a total volume of 50 μL with 1 × TaqMan Master Mix (Applied Biosystems) and primers. Thermal cycle parameters included one cycle at 95℃ for 3 min, and 45 cycles involving denaturation at 95℃ for 30 s, annealing at 52℃ for 45 s, extension at 72℃ for 45 s, followed by a final extension at 72℃ for 10 min. The relative amount of each cDNA in each sample was calculated by dividing the CT value with the corresponding value of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All reactions were performed in triplicate.

### Western blotting analysis for Bcl-xL protein

HT29 cells were harvested and lysed in a buffer containing 10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA (pH 8.0), 2 mmol/L phenylmethylsulfonyl fluoride, 2 mg/L aprotinin, 2 mg/L leupeptin, and 1 mmol/L $Na_3VO_4$. For Western blotting analysis, 30 μg of total extracted proteins was applied per lane before SDS-PAGE. Following transfer to nitrocellulose membranes, protein expression levels were detected using anti-Bcl-xL (Alpha Diagnostics International, TX). The expression of β-actin (Sigma-Aldrich, MO) was used as a normalization control for protein loading.

### Anchorage-independent growth assay

For the anchorage-independent growth experiments, HT29 cells ($8 \times 10^3$ cells/well) were seeded in 0.3% Difco Bactoagar (Difco, MI) supplemented with a complete culture medium. This suspension was layered over 0.5 mL of 0.8% agar-medium base layer in 24 multiwell cluster dishes (Becton Dickinson, Italy). After 15 d, the colonies were stained with nitroblue tetrazolium, and colonies larger than 50 μm were acquired with a micro-Scopeman camera system (Moritex Europe Ltd, Italy) and analyzed with Image-Pro Plus (Media Cybernetics, MD) computer program.

### Cell invasion assay

Transwell invasion assays were performed using HT29 cells cultured in 12-well plates containing either 8 μm pore matrigel-coated inserts according to the manufacturer's instructions (Becton Dickinson, Bedford, MA). The membranes were rehydrated with warm serum-free (SF) Dulbecco's modified Eagle's medium (DMEM) (1.0 mL/chamber) for 2 h. The upper chamber was filled with $1 \times 10^5$ cells in L-15 medium containing 5% FBS. The lower chamber was filled with L-15 medium containing 25% FBS as a chemo-attractant. After the chambers were incubated for 24 h at 37℃ in an atmosphere containing 50 mL/L $CO_2$, non-invading cells were removed from the upper surface of the membrane by scrubbing, and invading cells on the lower surface of the membrane were fixed and stained with HE. The number of cells penetrating the filter was counted by a technician blinded to the experimental settings in four microscopic fields of each filter, under × 20 magnification. The percentage of invasion was expressed as the ratio of the mean cell number from the invasion chamber to the mean cell number from the control chamber according to the manufacturer's recommendation.

### Statistical analysis

All analyses were performed with *t* test and ANOVA using SPSS 11.5 software (Statistical Package for Social Science). $P < 0.05$ was considered statistically significant.

## RESULTS

### Expression of Bcl-xL PU in human colon cancerous and normal tissue samples

Bcl-xL expression was rarely expressed in normal large intestinal mucosa. However, Bcl-xL was mainly expressed in cytoplasm of the para-cancerous or cancer cells. The nuclei were stained brownish yellow, located sporadically or in the form of sheets. Quantitative immunohistochemistry analysis is summarized in Table 1. Bcl-xL PU was significantly higher in cancerous tissue samples than in normal tissue samples ($38.78 \pm 11.36$ *vs* $0.89 \pm 0.35$, $P < 0.001$).

### Relationship between Bcl-xL PU and clinicopathological parameters

Correlation of Bcl-xL expression with clinicopathological parameters was evaluated. Bcl-xL PU, positive lymph nodes and Duke's C/D stage were higher in cancerous tissue samples with low differentiation than in cancerous tissue samples with high differentiation ($P < 0.05$, Table 1). However, Bcl-xL expression was not correlated with sex, age of the patients.

### Suppression o f Bcl-xL by siRNA

To further clarity the role of Bcl-xL gene, siRNA was used to knockdown the Bcl-xL expression in human colon cancer cells. Bcl-xL siRNA was transfected into the colon cancer cell line HT29. The ability of siRNA to down-regulate Bcl-xL expression was quantified by real time RT-PCR analysis and Western blot assay, respectively. siRNA significantly reduced the Bcl-xL mRNA and protein level in a dose- and time- dependent manner (Figure 1). However, the control scrambled siRNA treatment had no effect on Bcl-xL expression, thus supporting the specificity of Bcl-xL siRNA.

### Bcl-xL siRNA inhibited anchorage-independent growth of human colon cancer cells

Next we evaluated the biological effects of Bcl-xL suppression on human colon cancer HT29 cells using several different types of assays. Colony formation in soft agar is a property closely associated with malignancy. Treatment with Bcl-xL siRNA significantly inhibited the anchorage-independent growth of human colon cancer cells in a dose-dependent manner (Figure 2A).

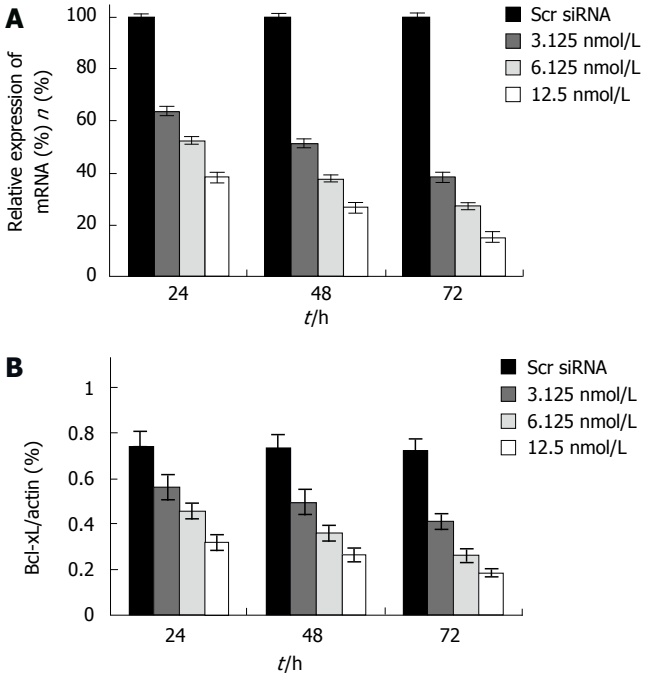


**Figure 1** Effects of siRNA on Bcl-xL expression in human colon cancer HT29 cells. **A**: Bcl-xL mRNA level; **B**: Bcl-xL protein level.

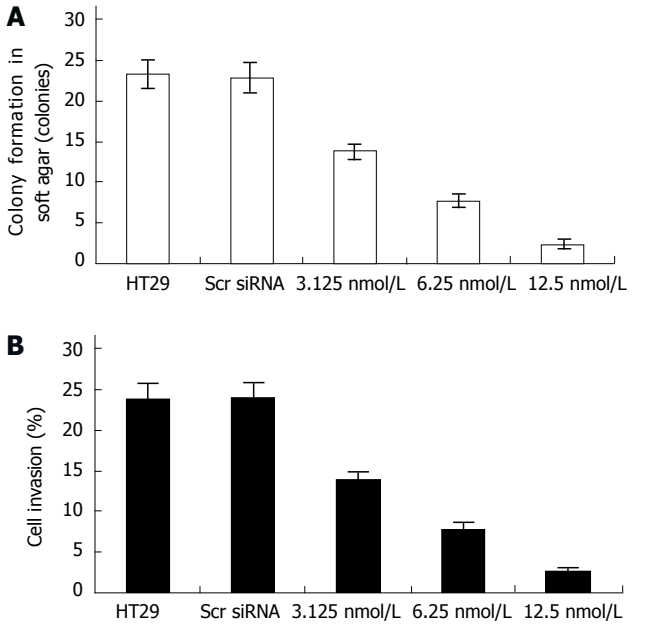


**Figure 2** Effects of Bcl-xL siRNA on anchorage-independent growth (**A**) and invasion ability (**B**) of human colon cancer HT29 cell.

### *Down-regulation of Bcl-xL decreased the ability of human colon cancer cells to grow in vitro*

Given the known role of Bcl-xL siRNA in down-regulation of anchorage-independent HT29 cell growth, we attempted further to evaluate whether the Bcl-xL gene contributes to cell invasion of colon cancer cells. Cell invasion studies were performed using the matrigel matrix assays. The results showed that Bcl-xL siRNA treatment resulted in a dramatic low level of invasion potential of HT29 cells (Figure 2B), but not scrambled siRNA treatment.

## DISCUSSION

The Bcl-2 family is characterized by the presence of Bcl-2 homology domains and falls into two main groups: anti-apoptotic proteins, such as Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1, and proapoptotic proteins, such as Bax, Bak, Bad, Bid, and Bcl-xS[6]. The Bcl-x gene encodes two proteins, a long form (Bcl-xL) and a short form (Bcl-xS), through an alternative splicing mechanism. Bcl-xL, displaying remarkable amino acids and an overall structural homology to Bcl-2, can effectively block apoptosis, whereas Bcl-xS, lacking 63 amino acids in Bcl-xL, is a dominant inhibitor of Bcl-2 activity and thereby acts as a proapoptotic factor[9].

Although there is evidence of cell apoptosis and Bcl-xL gene, the relationship between Bcl-xL and invasion of malignant tumors remains unclear. It was reported that Bcl-xL is related to the invasion and metastasis of some solid tumors. Zhang *et al*[18] and Takada *et al*[19] have reported the inhibition of invasion of cancer cells after treated with the Bcl-xL gene. Our previous study showed that STAT3 siRNA tranfection inhibits the invasion ability of human colon cancer cell line HT29 and that the Bcl-xL protein is significantly inhibited in transfected cancer cells[16], suggesting that Bcl-xL contributes to the invasion of human colon cancer cells. To verify it, we studied the relationship between Bcl-xL and invasion of human colon cancer *in vivo* and *in vitro*.

The expression of Bcl-xL protein in human colon cancer was determined by immunohistochemistry assay, showing that Bcl-xL protein was over-expressed in colon cancer tissue samples compared to normal tissue samples ($P < 0.001$). Meanwhile, Bcl-xL expression had no significant correlation with sex and age of the patients, but was greatly correlated with differentiation stage, lymph node metastasis, and Duke's stage of colorectal carcinoma ($P < 0.05$), indicating that Bcl-xL over-expression is related to the development and invasion of human colon cancer.

In order to further investigate the relationship between Bcl-xL and invasion of human colon cancer cells, we studied the effects of Bcl-xL down-regulated by siRNA on the invasion ability of human colon cancer cells. SiRNA is a short oligonucleotide consisting of 21-23 nucleotides that can be used *in vitro* to induce sequence specific gene silencing of mammalian cells[20]. To elucidate the role of Bcl-xL gene in human colon cancer, siRNA was used to knockdown the Bcl-xL expression in human colon cancer cell line HT29. Real time RT-PCR and Western blot analysis showed that the expression of Bcl-xL in HT29 cancer cells transfected with siRNA was significantly reduced in a dose- and time-dependent manner. In addition, tranfection of human colon cancer HT29 cells with Bcl-xL siRNA decreased the invasion ability and anchorage-independent growth of human colon cancer cells. The data *in vitro* suggest that the Bcl-xL gene plays an important role in regulating the invasion of human colon cancer cell.

In conclusion, the Bcl-xL gene is relevant to the invasion and progression of human colon cancer, and can be used in evaluating the carcinogenesis of human colon cancer. However, the precise mechanism of Bcl-xL underlying the

carcinogenesis of human colon cancer is still unclear, and further study is needed.

## COMMENTS

### Background

Despite recent advances in diagnostic and therapeutic measures, the prognosis of colorectal cancer patients with distant metastasis still remains poor. Enhanced understanding of the signaling mechanism underlying metastasis of colon cancer may provide important insights into more effective therapeutic strategies.

### Research frontiers

The results of this study indicate that the Bcl-xL gene plays an important role in the carcinogenesis of human colorectal carcinoma and is associated with the malignant biological behaviors of colorectal carcinoma.

### Innovations and breakthroughs

The results of the present study suggest that the Bcl-xL gene is relevant to the invasion and progression of human colon cancer and can be used in evaluating the carcinogenesis of human colon cancer.

### Applications

The paper helps to clarify the mechanism underlying the invasion and metastasis of colon cancer and contributes to the choice of therapeutic strategies.

### Peer review

This interesting article indicates that the Bcl-xL gene is relevant to the invasion and progression of human colon cancer, and might be used in evaluating the carcinogenesis of human colon cancer.

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*RAPID COMMUNICATION*

# Detection of *RASSF1A* promoter hypermethylation in serum from gastric and colorectal adenocarcinoma patients

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## Abstract

**AIM:** To evaluate the diagnostic role of serum *RASSF1A* promoter hypermethylation in gastric and colorectal adenocarcinoma.

**METHODS:** Methylation-specific polymerase chain reaction (MSPCR) was used to examine the promoter methylation status of the serum *RASSF1A* gene in 47 gastric adenocarcinoma patients, 45 colorectal adenocarcinoma patients, 60 patients with benign gastrointestinal disease (30 with benign gastric disease and 30 with benign colorectal disease), and 30 healthy donor controls. A paired study of *RASSF1A* promoter methylation status in primary tumor, adjacent normal tissue, and postoperative serum were conducted in 25 gastric and colorectal adenocarcinoma patients who later were underwent surgical therapy.

**RESULTS:** The frequencies of detection of serum *RASSF1A* promoter hypermethylation in gastric (34.0%) and colorectal (28.9%) adenocarcinoma patients were significantly higher than those in patients with benign gastric (3.3%) or colorectal (6.7%) disease or in healthy donors (0%) ($P < 0.01$). The methylation status of *RASSF1A* promoter in serum samples was consistent with that in paired primary tumors, and the MSPCR results for *RASSF1A* promoter methylation status in paired preoperative samples were consistent with those in postoperative serum samples. The serum *RASSF1A* promoter hypermethylation did not correlate with patient sex, age, tumor differentiation grade, surgical therapy, or serum carcinoembryonic antigen level. Although the serum *RASSF1A* promoter hypermethylation frequency tended to be higher in patients with distant metastases, there was no correlation between methylation status and metastasis.

**CONCLUSION:** Aberrant CpG island methylation within the promoter region of *RASSF1A* is a promising biomarker for gastric and colorectal cancer.



**Key words:** Gastric cancer; Colorectal cancer; Gene methylation; *RASSF1A*

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## INTRODUCTION

Gastric and colorectal cancers are two of the most common causes of cancer-related death worldwide. Development of efficient diagnostic methods to enable their early detection plays an essential role in increasing the survival rate of patients with these diseases. Although endoscopy is considered the most sensitive screening tool for gastric and colorectal cancers, its use is limited due to its considerable cost and risk, and patients' lack of acceptance of the invasive procedure. Therefore, reliable noninvasive test, preferably blood test, for screening and diagnostic purposes are obviously needed.

Conventional tumor markers in serum, such as carcinoembryonic antigen (CEA), are generally insensitive for screening purposes[1]. Consequently, novel serum biomarkers are clearly needed for the early detection of gastric and colorectal cancers.

Aberrant DNA methylation, a feature of many human cancers, frequently occurs as an early event in tumorigenesis and is characterized by general hypomethylation and regional hypermethylation[2]. The hypermethylation of CpG islands within the promoter and/or upstream exon regions is an important epigenetic mechanism underlying the inactivation of tumor-suppressor genes (TSGs)[3]. It was reported that quite a few TSGs, including the *Ras association domain family 1A* (*RASSF1A*) gene, are epigenetically silenced by aberrant promoter hypermethylation in gastric and colorectal cancer[4-10]. *RASSF1A* is a newly identified candidate TSG located in the 3p21.3 region[11], and promoter hypermethylation of *RASSF1A*, which is its most common inactivation mechanism, has been observed in many human solid tumors, including gastric and colorectal cancers[11-17].

It has been long known that the serum level of free DNA is increased in cancer patients, which is believed to be released from cancer cells[18,19]. It was reported that genetic and epigenetic alterations in serum DNA (such as point mutation, gene amplification, loss of heterozygosity, microsatellite instability, and aberrant methylation) are identical to those found in primary human cancers[20-24]. Because the promoter methylation status of TSGs in primary tumors and matched serum samples was consistent with each other[4,25,26], promoter hypermethylation of TSGs in serum DNA may become a promising biomarker for gastric and colorectal cancers.

In the present study, we attempted to identify the *RASSF1A* promoter methylation status both in serum DNA and in available paired tumor genomic DNA from patients with gastric and colorectal adenocarcinomas by using methylation-specific polymerase chain reaction (MSPCR). We also analyzed the correlation between serum *RASSF1A* gene promoter hypermethylation and patients' clinicopathologic parameters to further evaluate the clinical significance of this molecular change.

## MATERIALS AND METHODS

### Study population

This study included 47 gastric adenocarcinoma patients and 45 colorectal adenocarcinoma patients diagnosed at Departments of General Surgery, Gastroenterology, and Medical Oncology of Jinling Hospital (Nanjing, China) between August 1, 2006 and November 30, 2007. All diagnoses were based on pathologic evidence, and only patients with adenocarcinoma, the most common histologic type of gastric and colorectal cancer, were included. The clinicopathologic characteristics of these patients are summarized in Table 1.

**Table 1 Clinicopathologic characteristics of patients with gastric and colorectal adenocarcinoma**

| Characteristics | | Patients (*n*) | |
|---|---|---|---|
| | | Gastric cancer | Colorectal cancer |
| Sex | Male | 29 | 24 |
| | Female | 18 | 21 |
| Age (yr) | ≤ 60 | 21 | 31 |
| | > 60 | 26 | 14 |
| Differentiation grade | G1/Broders' Ⅰ | 2 | 4 |
| | G2/Broders' Ⅱ | 23 | 34 |
| | G3/Broders' Ⅲ & Ⅳ | 22 | 7 |
| Stage | TNM Ⅰ/Duke's A | 4 | 5 |
| | TNM Ⅱ/Duke's B | 15 | 16 |
| | TNM Ⅲ/Duke's C | 16 | 14 |
| | TNM Ⅳ/Duke's D | 12 | 10 |

The control population consisted of 60 patients with benign gastrointestinal diseases (30 with benign gastric disease and 30 with benign colorectal disease, such as chronic gastritis, gastric ulcer, benign polyp, nonmalignant adenoma, and ulcerative colitis; data not shown) and 30 healthy donors.

Gastric adenocarcinoma was staged according to the sixth edition of the TNM staging system[27], and colorectal adenocarcinoma was staged according to the Duke's staging system. Gastric and colorectal adenocarcinoma differentiation was graded according to the World Health Organization grading system and the Broders' grading system, respectively.

Our study was approved by the ethical committee of the hospital and informed consent was obtained from all patients.

### Sample collection

Five mL of peripheral venous blood was collected from each patient 1 day after the patients were admitted to our hospital. At this time, the patients did not start their treatment. Any previous treatment (surgery and/or chemotherapy), if given, was discontinued at least 4 wk earlier. Fresh tumor tissue and paired adjacent normal tissue were obtained from 16 gastric and 9 colorectal adenocarcinoma patients who later were underwent to surgical therapy in Jinling Hospital. An additional 5 mL peripheral venous blood was collected from these 25 patients 4 wk after surgery for a comparative study. All blood samples were kept in tubes containing clot activator at 4℃ for 2 h, and samples were centrifuged at 3000 r/min for 10 min to isolate sera. Thirty serum samples from healthy donors were obtained from the Blood Center of Jinling Hospital as normal controls. All serum and tissue samples were stored at -80℃ until use.

**Table 2 Sequences of the primers used in MSP**

| Primer | Sequence (5'-3') | Amplicon location[1] | Annealing temperature | Product size (bp) |
|---|---|---|---|---|
| MF | GGGTTTTGCGAGAGCGCG | 17882-18050 | 64℃ | 169 |
| MR | GCTAACAAACGCGAACCG | | | |
| UF | GGTTTTGTGAGAGTGTGTTTAG | 17883-18051 | 59℃ | 169 |
| UR | CACTAACAAACACAAACCAAAC | | | |

[1]GenBank accession number of *RASSF1A* is AC002481. F: Forward; R: Reverse; M: Methylated; U: Unmethylated.

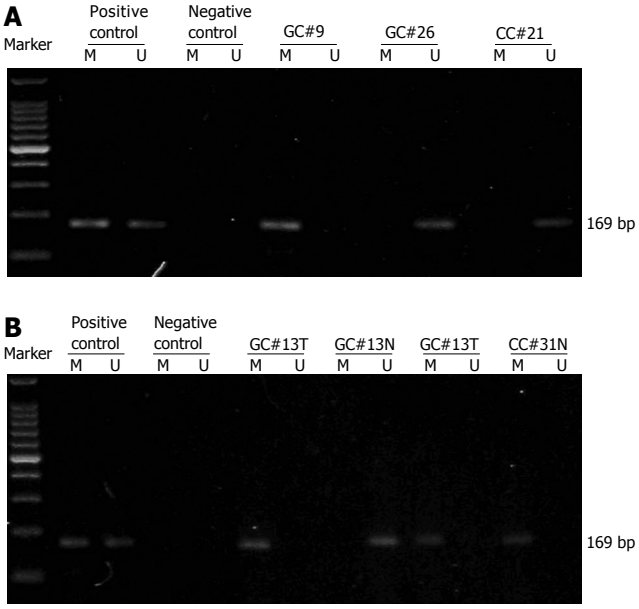


**Figure 1** Representative results showing *RASSF1A* promoter methylation status identified by MSPCR in gastric and adenocarcinoma patients. Identification of *RASSF1A* promoter methylation status in serum samples from gastric and colorectal adenocarcinoma patients (**A**) and in paired tumor and adjacent normal tissue from gastric and colorectal adenocarcinoma patients (**B**). A 100-bp DNA ladder marker (TaKaRa, Shiga, Japan) was used. Lanes M and U indicate the amplified products with primers recognizing specific methylated and unmethylated sequences, respectively. GC: Gastric adenocarcinoma; CC: Colorectal adenocarcinoma; T: Tumor tissue; N: Paired adjacent normal tissue.

### DNA extraction and bisulfite treatment

Serum DNA, extracted with the QIAamp blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, was stored at -80℃ until use. Genomic DNA isolated from tissue samples was prepared using standard phenol/chloroform extraction protocols.

The extracted DNA was modified according to Herman *et al*[28] with minor modifications, to convert all unmethylated cytosines to uracils. Briefly, 1 μg of genomic DNA, or serum DNA extracted from 5 mL blood plus 1 μg of salmon sperm carrier DNA (Sigma, St. Louis, MO, USA), in a total volume of 50 μL, were denatured by NaOH (0.3 mol/L final concentration) at 40℃ for 15 min. After 30 μL of freshly prepared 10 mmol/L hydroquinone (Sigma) and 520 μL of freshly prepared 3 mol/L sodium bisulfite (Sigma) at pH 5.0 were added, the samples were incubated under mineral oil at 55℃ in darkness for 14 h. The modified DNA was purified using the Wizard DNA clean-up system (Promega, Madison, WI, USA), following its manufacturer's protocol. Modification was completed by NaOH (0.3 mol/L final concentration) treatment for 10 min at room temperature, followed by ethanol precipitation. The modified DNA was resuspended in sterile deionized water (100 μL for genomic DNA and 25 μL for serum DNA) and used immediately or stored at -80℃.

### MSPCR

Two sets of primers, described elsewhere[29], were used to discriminate between methylated and unmethylated alleles (Table 2). The PCR system has been described previously[30]. Briefly, the PCR mixture containing 2.5 μL of 10 × reaction buffer (100 mmol/L Tris-HCl (pH 8.3), 500 mmol/L KCl, 15 mmol/L $MgCl_2$), 10 μL of modified DNA, 15 pmol of each primer (Shenery Biocolor, Shanghai, China), 2 μL of deoxynucleotide triphosphates (200 μmol/L each, final concentration), and 1 U TaKaRa Taq™ polymerase (Hot Start Version, TaKaRa, Shiga, Japan) was adjusted by $H_2O$ to a final volume of 25 μL. The cycling conditions consisted of an incubation period at 95℃ for 15 min, 40 cycles of denaturation at 94℃ for 30 s, annealing at 64℃ or at 59℃ for 50 s (Table 2), extension at 72℃ for 30 s, and a final extension at 72℃ for 10 min. PCR products were separated in 2% agarose gel and visualized under UV illumination.

Lymphocyte DNA, original or methylated *in vitro* by excessive CpG (Sss I) methylase (New England Biolabs, Beverly, MA, USA), was used as an unmethylated and methylated DNA positive control, respectively (Figure 1A). Water blank was used as a negative control.

### Statistical analysis

We analyzed the correlation between methylation status of serum *RASSF1A* promoter and clinicopathologic parameters. Chi-square test or Fisher's exact test was conducted to examine the association of two categorical variables using SAS software (SAS Institute, Cary, NC, USA). All statistical tests were two-sided, and $P < 0.05$ was considered statistically significant.

## RESULTS

### Serum RASSF1A promoter hypermethylation profile in gastric and colorectal adenocarcinoma patients

First we analyzed the methylation status of CpG islands within the *RASSF1A* promoter in serum DNA from 47 gastric adenocarcinoma patients, 45 colorectal adenocarcinoma patients, 60 benign gastrointestinal disease patients (30 with benign gastric disease and 30 with benign colorectal disease), and 30 healthy donors. Hypermethylation of the *RASSF1A* promoter was detected in 16 gastric adeno-

**Table 3 Correlation between serum *RASSF1A* gene promoter methylation status and clinicopathologic parameters in gastric and colorectal adenocarcinoma patients**

| Clinicopathologic parameters | | Gastric cancer | | | Colorectal cancer | | |
|---|---|---|---|---|---|---|---|
| | | *RASSF1A* promoter status | | *P* value | *RASSF1A* promoter status | | *P* value |
| | | M | U | | M | U | |
| Sex | Male | 9 | 20 | 0.5807[1] | 7 | 17 | 0.9649[1] |
| | Female | 7 | 11 | | 6 | 15 | |
| Age (yr) | ≤ 60 | 8 | 13 | 0.5982[1] | 8 | 23 | 0.5024[2] |
| | > 60 | 8 | 18 | | 5 | 9 | |
| Differentiation grade | G1/Broders' Ⅰ | 0 | 2 | 0.2280[1] | 1 | 3 | 0.9830[1] |
| | G2/Broders' Ⅱ | 6 | 17 | | 10 | 24 | |
| | G3/Broders' Ⅲ & Ⅳ | 10 | 12 | | 2 | 5 | |
| Surgical resection | Yes | 7 | 20 | 0.2203[1] | 5 | 10 | 0.7325[2] |
| | No | 9 | 11 | | 8 | 22 | |
| Distant metastasis | Yes | 7 | 5 | 0.0746[2] | 5 | 5 | 0.1237[2] |
| | No | 9 | 26 | | 8 | 27 | |
| Serum CEA | Elevated | 7 | 7 | 0.2365[2] | 6 | 7 | 0.1232[2] |
| | Normal | 3 | 10 | | 3 | 14 | |

[1]Chi-square test; [2]Fisher's exact test. CEA: Carcinoembryonic antigen; M: Methylated; U: Unmethylated.

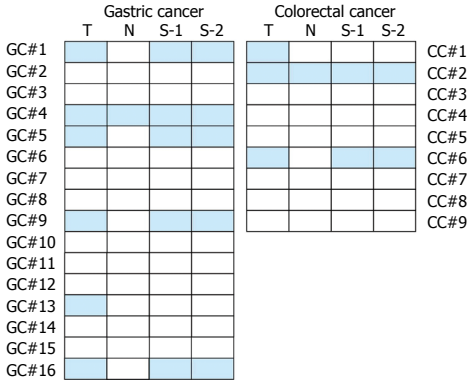


**Figure 2** Comparison of *RASSF1A* promoter methylation status in tissue and serum samples. For each patient, the *RASSF1A* promoter methylation status was analyzed in tumor tissue (T), adjacent normal tissue (N), preoperative serum (S-1), and postoperative serum collected 4 wk after surgery (S-2). Solid boxes indicate methylation, blank ones indicate unmethylation of *RASSF1A* promoter. GC: Gastric adenocarcinoma; CC: Colorectal adenocarcinoma.

carcinoma patients, 13 colorectal adenocarcinoma patients, 1 benign gastric disease patient (chronic fundal gastritis), and 2 benign colorectal disease patients (both colon adenomas). The representative agarose gel electrophoresis results are shown in Figure 1A. The frequencies of detection of serum *RASSF1A* promoter hypermethylation in gastric (34.0%) and colorectal (28.9%) adenocarcinoma patients were significantly higher than those in benign gastric disease patients (3.3%), benign colorectal disease patients (6.7%) and healthy donors (0%), respectively ($P < 0.01$).

### *RASSF1A promoter hypermethylation profile in paired tissue and serum samples from gastric and colorectal adenocarcinoma patients*

Next we compared the *RASSF1A* promoter methylation status in paired tissue and serum samples from 16 gastric and 9 colorectal adenocarcinoma patients who later were underwent to surgical resection in Jinling Hospital. For each patient, the *RASSF1A* promoter methylation status was analyzed in tumor tissue, adjacent normal tissue, preoperative serum, and postoperative serum collected 4 wk after surgery. The representative agarose gel electrophoresis results and the paired MSPCR results are shown in Figure 1B and Figure 2, respectively. In seven patients, the *RASSF1A* promoter hypermethylation was detected both in cancer tissue samples and in serum samples. In two patients, the hypermethylated *RASSF1A* promoter was present in tumor tissue samples but not in paired serum samples. The *RASSF1A* promoter hypermethylation was never detected in serum samples if it was not present in tumor tissue. In addition, the preoperative and postoperative serum *RASSF1A* promoter methylation status remained unchanged in all patients.

### *Correlation between serum RASSF1A promoter hypermethylation and clinicopathologic parameters in patients with gastric and colorectal adenocarcinoma*

We further analyzed the relationship between serum *RASSF1A* promoter methylation status and clinicopathologic features in gastric and colorectal adenocarcinoma patients. The results are listed in Table 3. As indicated in the table, there was no correlation between *RASSF1A* promoter methylation status and patients' sex, age, tumor differentiation grade, or serum CEA levels. No difference in serum *RASSF1A* promoter hypermethylation frequencies was detected between postoperative patients and those whose tumor was not resected. Although the serum *RASSF1A* promoter hypermethylation frequency tended to be higher in patients with distant metastases, no correlation between methylation status and metastasis was found.

## DISCUSSION

RASSF1A protein is actively involved in microtube regulation, genomic stability maintenance, cell-cycle regulation, apoptosis modulation, cell motility and invasion control[31-39]. The frequent inactivation of TSG *RASSF1A* due to aberrant promoter methylation has been reported in various tumor types[13], suggesting that it plays a pivotal

role in human cancer development. It was reported that *RASSF1A* is inactivated by promoter hypermethylation in gastric and colorectal cancer, but the frequencies of aberrant *RASSF1A* methylation vary widely[8,9,14-16,40,41]. In addition, serum promoter methylation of *RASSF1A* in gastric and colorectal cancer has not been extensively studied, and few comparative studies using both primary tumor and serum samples are available. To our knowledge, there is only one related study with a limited sample size[10]. In the present study, we identified the *RASSF1A* promoter methylation status both in serum DNA and in available paired tumor genomic DNA from patients with gastric and colorectal adenocarcinoma, showing that serum *RASSF1A* promoter hypermethylation is a potential biomarker for gastric and colorectal cancer diagnosis.

In the present study, serum *RASSF1A* promoter hypermethylation was detected in 34.0% of patients with gastric adenocarcinoma and in 28.9% of those with colorectal adenocarcinoma. The frequencies were slightly higher than those reported by Tan *et al*[10] (25% in gastric cancer and 24% in colorectal cancer, respectively). The serum *RASSF1A* promoter hypermethylation frequencies in gastric and colorectal adenocarcinoma patients were significantly higher than those in patients with benign gastric or colorectal disease or in healthy donors ($P < 0.01$). The sensitivity of serum *RASSF1A* promoter hypermethylation in detecting gastric and colorectal cancer is relatively low. Perhaps a simultaneous analysis of the methylation status of a panel of TSGs would be more sensitive in detecting gastric and colorectal cancer. On the other hand, the specificity of serum *RASSF1A* promoter hypermethylation was very high (approximate 98.3%). Since clinical tests with a high specificity are usually useful in confirming the diagnosis, serum *RASSF1A* promoter methylation status is a potential marker for the diagnosis of gastric and colorectal cancer.

We also compared the *RASSF1A* promoter methylation status in paired tissue and serum samples from 25 gastric and colorectal adenocarcinoma patients. For the seven patients with hypermethylated *RASSF1A* promoter detected in their serum samples, *RASSF1A* promoter hypermethylation was also present in the primary tumor, which supports the presumption that circulating DNA in peripheral blood of cancer patients reflects the epigenetic change in the primary tumor. In two patients, however, hypermethylated *RASSF1A* promoter could be detected in the primary tumor samples but not in the paired serum samples, suggesting that not all cancer patients have detectable tumor-originating DNA in their peripheral blood.

*RASSF1A* promoter hypermethylation was detected in adjacent normal tissue from 2 patients, which can be explained by the invisible invasion of the primary tumor to the adjacent tissue. Another possible reason is the presence of aberrant promoter methylation of TSGs in precancerous lesions adjacent to the primary tumor. Lee *et al*[9] reported that *RASSF1A* promoter hypermethylation occurs in 2.1% of colorectal adenomas, and Derks *et al*[42] found that aberrant *RASSF1A* promoter methylation is present in 19.1% of non-progressed adenomas and in 24.4% of progressive adenomas. In our study, we also detected methylated *RASSF1A* promoter in the serum from one patient with chronic fundal gastritis and two patients with colon adenoma, believed to be precancerous lesions in gastric and colon cancer, respectively. These findings suggest that aberrant promoter hypermethylation of *RASSF1A* might be an early event in the development of gastric and colorectal cancer. Therefore, identification of serum *RASSF1A* promoter methylation status may contribute to the early diagnosis of gastric and colorectal cancer.

In the present study, no association was observed between *RASSF1A* promoter methylation status and patients' sex, age, tumor differentiation grade, distal metastasis, or surgical therapy. We also compared the methylation status of *RASSF1A* promoter in preoperative and postoperative serum samples from patients who were underwent to surgical therapy in our hospital, and the status remained unchanged in all patients. Theoretically, when the primary tumor is resected, tumor-specific methylated DNA would decrease considerably in peripheral blood. However, this does not seem to be the case. Fiegl *et al*[43] monitored the serum *RASSF1A* promoter methylation status in 148 breast cancer patients for up to 1 year after surgery, and only 21 patients showed positive to negative transition in MSPCR analysis of serum *RASSF1A* promoter. A possible source of persistently present methylated copy after surgery is the micrometastases that may present before surgery.

We investigated whether serum *RASSF1A* promoter hypermethylation is correlated with elevated serum CEA levels and found that there is no correlation between them. Koike *et al*[44] reported that the detection rate of TSG (*p16*, *E-cadherin*, and *RARβ*) hypermethylation is higher than that of conventional tumor marker (CEA and CA19-9) abnormalities in the serum from gastric cancer patients, and that there is no correlation between them. Since serum CEA and TSG hypermethylation are not correlated, a combinational analysis of serum *RASSF1A* promoter methylation status and serum CEA level may be useful in the diagnosis of gastric and colorectal cancer.

In conclusion, serum *RASSF1A* promoter hypermethylation is common in gastric and colorectal adenocarcinoma and aberrant CpG island methylation within the promoter region of *RASSF1A* is a promising biomarker for such cancers.

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## COMMENTS

### Background

*RASSF1A* inactivation by promoter hypermethylation in gastric and colorectal cancer has been reported. However, serum promoter methylation of *RASSF1A* in gastric and colorectal cancer has not been extensively studied. Particularly, comparative studies using both primary tumor and serum samples are indicated can evaluate the diagnostic role of serum *RASSF1A* promoter hypermethylation in gastric and colorectal cancer.

### Research frontiers

Circulating nucleotide acid is a hotspot in the early diagnosis of cancer. Characterization of molecular changes in serum DNA reflecting the genetic and

epigenetic alterations in primary tumor would provide an alternative approach to the early detection of cancer.

### Innovations and breakthroughs

This is the first comprehensive study on *RASSF1A* promoter hypermethylation status both in tumor and normal tissue samples and in pre- and post serum samples from gastric and colorectal cancer patients. Our results indicate that aberrant hypermethylation of *RASSF1A* promoter is a promising serum biomarker for gastric and colorectal cancer diagnosis.

### Applications

A combined study on promoter hypermethylation of a panel of relevant tumor suppressor genes in serum samples may have a bright future in the early diagnosis of gastric and colorectal cancer.

### Terminology

In DNA, methylation is the addition of a methyl group to a cytosine residue to convert it to 5-methylcytosine. DNA methylation is the main epigenetic modification in humans, and changes in methylation patterns play an important role in tumorigenesis. In particular, hypermethylation of normally unmethylated CpG islands in the promoter region of tumor suppressor genes correlates with their loss of expression and may confer growth advantages to those cells that favor cancer development.

### Peer review

This paper is very interesting. The study is well designed. The authors evaluated the role of serum *RASSF1A* promoter hypermethylation in diagnosing gastric and colorectal adenocarcinoma, showing that aberrant CpG island methylation within the promoter region of *RASSF1A* is a promising biomarker for gastric and colorectal cancer.

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*RAPID COMMUNICATION*

# Reoperation of biliary tract by laparoscopy: Experiences with 39 cases

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## Abstract

**AIM:** To evaluate the safety and feasibility of biliary tract reoperation by laparoscopy for the patients with retained or recurrent stones who failed in endoscopic sphincterotomy.

**METHODS:** A retrospective analysis of data obtained from attempted laparoscopic reoperation for 39 patients in a single institution was performed, examining open conversion rates, operative times, complications, and hospital stay.

**RESULTS:** Out of the 39 cases, 38 (97%) completed laparoscopy, 1 required conversion to open operation because of difficulty in exposing the common bile duct. The mean operative time was 135 min. The mean post-operative hospital stay was 4 d. Procedures included laparoscopic residual gallbladder resection in 3 cases, laparoscopic common bile duct exploration and primary duct closure at choledochotomy in 13 cases, and laparoscopic common bile duct exploration and choledochotomy with T tube drainage in 22 cases. Duodenal perforation occurred in 1 case during dissection and was repaired laparoscopically. Retained stones were found in 2 cases. Postoperative asymptomatic hyperamylasemia occurred in 3 cases. There were no complications due to port placement, postoperative bleeding, bile or bowel leakage and mortality. No recurrence or formation of duct stricture was observed during a mean follow-up period of 18 mo.

**CONCLUSION:** Laparoscopic biliary tract reoperation is safe and feasible if it is performed by experienced laparoscopic surgeons, and is an alternative choice for patients with choledocholithiasis who fail in endoscopic sphincterectomy.



**Key words:** Minimally invasive surgery; Reoperation; Choledocholithiasis; Laparoscopic common bile duct exploration

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## INTRODUCTION

In the past, laparoscopic surgery was contraindicated for patients undergone any prior abdominal surgery. With the advances in laparoscopic instrumentation and skills, increasingly complex procedures can be performed for patients with or without prior operations[1-5]. Prior open biliary surgery in particular is associated with difficulty in placing the initial trocar and obtaining adequate exposure of the biliary tract. Two major concerns that have prevented surgeons from using a laparoscopic approach when performing a repeated biliary tract surgery include the risk of injury to organs adherent to the abdominal wall when Veress needle or trocar is inserted, and the complications associated with adhesiolysis. With the increased experience in our institution, we have attempted laparoscopic surgery for patients with retained or recurrent stones who failed in endoscopic sphincterotomy. We reviewed the data collected from our cases to study the effect of prior biliary surgery on biliary tract reoperation using laparoscopy.

## MATERIALS AND METHODS

### Patients

Laparoscopic cholecystectomy was introduced in our institution in 1993. Based on the experiences with 16 605 laparoscopic cholecystectomies, 658 laparoscopic common bile duct explorations, and 851 laparoscopic

**Table 1 Diagnosis and prior surgery of 39 patients**

| Diagnosis | Prior surgery | | | |
|---|---|---|---|---|
| | LC | OC | OC + CBDE | OC + CBDE + left lateral lobectomy |
| Stones in residual gallbladder | 1 | 2 | | |
| Stones in CBD | | 22 | 11 | 3 |

LC: Laparoscopic cholecystectomy; OC: Open cholecystectomy; CBDE: Common bile duct exploration; CBD: Common bile duct.

**Table 2 Results of laparoscopic biliary tract reoperation for 39 cases**

| | Laparoscopic biliary tract reoperation ($n$ = 39) |
|---|---|
| Mean operating time (min) | 135 (45-185) |
| Conversion rate | 1 (2.5%) |
| Postoperative hospital stay (d) | 4 (1-6) |
| Intra-operative complication rate | 2.5% (1/39) |
| Post-operative complication rate | 5.1% (2/39) |

cholecystectomies for patients with prior upper or lower abdominal surgery, we attempted laparoscopic biliary tract reoperation for patients with retained or recurrent stones who failed in endoscopic sphincterotomy.

A total of 39 patients including 26 females and 13 males, with a mean age of 46.4 years (ranging 13-76 years) were underwent to laparoscopic biliary tract reoperations by two surgical teams between January 2001 and June 2007. Retained or recurrent stones were found at a prior biliary surgery for biliary stones. None of them had any other previous abdominal surgery. A prior surgery was performed at other hospitals for 36 of them. The time between prior surgery and reoperation ranged from 7 d to 28 years, with a mean time of 2 years. Right subcostal scars were present in 18 cases, while midline or right para-midline scars were present in 21 cases. The diagnosis and prior surgery history of the 39 cases are listed in Table 1.

Diagnosis of retained stones or recurrent stones was made by pre-operative ultrasonography, CT, and MRCP. Endoscopic sphincterotomy failed or was contraindicated in the 39 cases. As the study was begun at a time when our experience with endoscopic sphincterotomy was limited, endoscopic sphincterotomy was either contraindicated or failed due to stones greater than 1.5 cm in diameter in 16 cases, the presence of more than four stones in 12 cases, tortuous ducts in 4 cases, and periampullary duodenal diverticula in 7 cases, respectively. There were no contraindications for general anesthesia. The diameter of the common bile duct ranged from 1 cm to 2.2 cm in 36 cases of choledocholithiasis. Biliary stricture or neoplasms were ruled out by radiological examination and serological tumor markers.

### *Operative procedure*

General endotracheal anesthesia was used. The abdominal cavity was accessed near the umbilicus. If the previous scar was more than 3 cm from the umbilicus, the blind technique was used to insert the Veress needle. If the scar was less than 3 cm from the umbilicus, the open (Hasson) technique was used. Adhesions under the umbilical incision were dissected using blunt finger dissection.

After pneumoperitoneum was established, intraperitoneal adhesions were evaluated by a 30-degree angled laparoscopy. A 5 mm port was placed under direct vision into the right or left lower abdomen, 5 cm from the adhesions, allowing dissection of the prior surgical adhesions located below the scar using scissors, a harmonic scalpel. One 10 mm operative port and two 5 mm accessory ports were placed as a standard four-trocar technique of laparoscopic cholecystectomy.

To approach the hepatic-duodenal ligament, we freed the lateral parietes and then began dissection on the right side along the lateral inferior border of the liver, dissecting the adhesions on the right side of hepatic round ligament down to the hepatic-duodenal ligament. The common bile duct was identified by touching the stones, needle aspiration of bile from the duct, or by laparoscopic ultrasound.

After identification of the common bile duct, choledochotomy was performed. Stones in the common bile duct were retrieved by spontaneous evacuation at the incision of the duct, instrumental exploration with forceps, flushing of the common bile duct with saline, or Fogarty balloon catheter. Next, a fifth port (10 mm) was placed at the right subcostal margin, just above the gallbladder, through which a 5.0 mm fiberoptic choledochoscope (Olympus) was inserted to check the biliary duct and remove the stones.

As long as choledochoscopy certified a patent common bile duct and absence of stones, the incision was closed using absorbable 4/0 sutures with a running suture and intracorporeal knotting, otherwise a T-tube was placed for drainage, and intraoperative cholangiography was performed through the T tube. A No. 10 Jackson-Pratt drain tube was placed in the subhepatic space for all patients.

## RESULTS

Of the 39 cases, 38 were underwent to laparoscopic operation and 1 was converted to an open operation because of difficulty in exposing the common bile duct. The mean operative time was 135 min (range, 45-185 min) and the mean postoperative hospital stay was 4 d (ranging 1-6 d, Table 2). Procedures included laparoscopic residual gallbladder resection in 3 cases, laparoscopic common bile duct exploration and primary duct closure at choledochotomy in 13 cases and laparoscopic common bile duct exploration and choledochotomy with T tube drainage in 22 cases. The mean number of removed stones was 3 (ranging 1-15) and the mean diameter of removed stones was 1 cm (ranging 1-2.6 cm). The mean time of T tube drainage was 38 d (ranging 28-47 d).

There were no complications due to port placement. In one patient with a history of open cholecystectomy and common bile duct exploration, the duodenum perforation occurred during dissection was repaired laparoscopically. There were no mortality, postoperative bleeding, bile

or bowel leakage in any of the 38 cases. Asymptomatic hyperamylasemia present in 3 cases postoperatively was treated with conservative therapy. Retained stones found in 2 cases were removed by choledochoscopy through the sinus tract of the T tube. No recurrent stones or duct stricture formation was found during a mean follow-up period of 18 mo.

## DISCUSSION

Most patients with common bile duct stones are cured by minimally invasive endoscopic sphincterotomy[6-10]. In the absence of a remaining T-tube from a prior operation, endoscopic sphincterotomy is considered the procedure of choice for patients with retained or recurrent stones, and should be attempted before pursuing biliary tract reoperation. However, endoscopic sphincterotomy cannot be performed, and is itself associated with a significant morbidity[11-15]. Contraindications for endoscopic sphincterotomy, as mentioned above, include size of stones, number of stones, presence of tortuous ducts or presence of periampullary duodenal diverticula, *etc* and vary depending on institutional and individual techniques and experiences. With the advances in laparoscopic skills and instrumentation, laparoscopic common bile duct exploration[16-20] and other laparoscopic procedures have become an increasingly popular option for patients undergone any prior abdominal surgery[21-25], making laparoscopic reoperation of the biliary tract a reasonable choice for patients with a history of prior biliary surgery who have failed in endoscopic sphincterotomy. The results of our study indicate that laparoscopic surgery was not only minimally invasive, but also safe and feasible in cases of biliary tract reoperation, suggesting that it is the best method for patients who have failed in endoscopic sphincterotomy.

A primary concern when considering laparoscopic reoperation is the formation of adhesions after abdominal surgery, particularly after open biliary surgery. Adhesions from prior surgery are associated with difficulty in establishing pneumoperitoneum, placing the initial trocar, and obtaining adequate exposure of the biliary tract. To avoid the potential risk of injury to organs adherent to either the abdominal wall or the previous operative field, certain techniques and principles should be followed during Veress needle and trocar insertion as well as adhesiolysis.

Safe establishment of pneumoperitoneum and placement of an initial trocar are the prerequisite to any laparoscopic biliary tract reoperation and related with half of the complications of laparoscopic surgery[26-29]. In our study, blind Veress needle and initial trocar insertion more than 3 cm from the previous scar were safe for patients with previous biliary surgery. The open Hasson procedure performed in a previously unoperated field can avoid potential underlying adhesions or injury. In our study, no complications were related to the entrance into the peritoneum, indicating that previous biliary surgery is not a contraindication for minimally invasive procedures.

After access has been achieved, sufficient adhesiolysis should be performed to allow the insertion of a second port to aid in visualization, retraction and dissection, and to allow for additional ports as needed. The laparoscope can be moved to different port sites without the need to perform total adhesiolysis of all visible adhesions. Only the adhesions interfering with adequate access to the operative field or the performance of the procedure need to be lysed. Adhesions close to the abdominal wall should be dissected to avoid injury to the intestine. By using a harmonic scalpel to dissect adhesions, the operative time can be reduced, thus decreasing blood loss[30].

Once the gallbladder has been removed or the common bile duct has been explored, dense adhesions are usually found during reoperation in the healed fossa and near the common duct. In many instances, the upper edge of the duodenum is tented sharply cephalad into the gallbladder fossa. At times, because it is difficult to recognize the anatomy or identify the common bile duct, one should approach to the hepatic hilum by freeing the lateral parietes, and then begin dissection on the right side along the lateral inferior border of the liver. This gives a better mobility of structures so the hepatic flexure of the colon and the lateral edge of the second part of the duodenum can be identified before beginning dissection in the area of dense adhesions. The adhesions on the right side of the hepatic round ligament should be dissected from Glisson's capsule down to the hepatic-duodenal ligament. When adhesions are dissected from Glisson's capsule, attempts at blunt dissection with heavy retraction can easily avulse the capsule and expose the bleeding liver parenchyma. Consequently, careful sharp dissection is a more expedient technique. To prevent thermal injury of the gastrointestinal tract, electrical cautery should be avoided. After exposure of the hepatic-duodenal ligament, the common bile duct can be identified by touching the stones and needle aspiration of bile or by laparoscopic ultrasound.

In summary, laparoscopic biliary tract reoperation has a reasonable operating time, low conversion rate, low intra-operative and postoperative complication rate, and short postoperative hospital stay. Given these results, a laparoscopic approach to biliary tract reoperation appears to be a minimally invasive, safe, feasible, and effective procedure when done by expert laparoscopic surgeons, and is a first choice of treatment for patients who have failed in endoscopic sphincterotomy.

## COMMENTS

### *Background*

In the past, a history of prior biliary tract surgery was considered a contraindication for performing a repeat biliary operation. In the absence of a remaining T-tube from a prior operation, endoscopic sphincterotomy is considered the procedure of choice for patients with retained or recurrent stones, and should be attempted before pursuing biliary tract reoperation. However, endoscopic sphincterotomy cannot be performed on everyone, and is itself associated with a significant morbidity. With the advances in laparoscopic skills and instrumentation, increasingly complex procedures have been performed in patients with or without prior operations.

### *Research frontiers*

It has previously been reported that laparoscopic common bile duct (CBD)

exploration is a common method for the management of choledocholithiae, and laparoscopic procedures are safe for patients undergone prior abdominal surgery. Few studies are available on the safety and feasibility of reoperation of biliary tract by laparoscopy for the patients with retained or recurrent stones who have failed in whom endoscopic sphincterotomy.

### Innovations and breakthroughs

This study showed laparoscopic biliary tract reoperation appears to be a minimally invasive, safe, feasible, and effective method when done by expert laparoscopic surgeons.

### Applications

Laparoscopic biliary tract reoperation is an alternative method for patients with choledocholithiasis who have failed in endoscopic sphincterectomy.

### Peer review

The authors describe, in this paper, their experience in laparoscopic biliary tract reoperation, which is of a certain clinical value.

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*CASE REPORT*

# Is infliximab safe to use while breastfeeding?

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## Abstract

Inflammatory bowel disease (IBD) often affects women around the age of conception and pregnancy. Most drugs used to treat IBD are safe in pregnancy, but physicians must consider the clinical implications of certain treatment regimens in young, fertile females. We report an informative case of a pregnant patient with IBD who underwent treatment with infliximab during her pregnancy and while nursing her infant. Serum and breast milk infliximab levels were monitored throughout this time period. This case report suggests that targeted monoclonal antibodies and other biologic agents can be used with caution in pregnant and breastfeeding patients.



**Key words:** Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Pregnancy; Breast-feeding; Monoclonal antibodies

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## INTRODUCTION

In recent years, targeted monoclonal antibodies and other biologic agents have been at the forefront of the numerous therapeutic options available to treat many immune-mediated disorders. A large number of young and fertile patients are afflicted with disorders like inflammatory bowel disease (IBD), rheumatologic diseases, asthma, and multiple sclerosis. These circumstances force patients and physicians to consider the safety of biologic agents during the peripartum time period.

## CASE REPORT

A 22-year-old female ($G_1P_1$) was referred to the Gastroenterology Clinic for treatment of fistulizing ileocolonic Crohn's disease (CD). The patient was initially treated with high dose corticosteroids, 6-mercaptopurine, metronidazole, and mesalamine with only mild improvement in her symptoms. The patient was eventually treated with infliximab and had a positive clinical response allowing her to be weaned off corticosteroids. Unfortunately, her 6-mercaptopurine was discontinued because of high thiopurine methyltransferase (TMPT) activity resulting in excessive production of the hepatoxic 6-methylmercaptopurine metabolite. The patient's CD continued to respond modestly to 3.6 mg/d mesalamine (1200 mg tid) and 5 mg/kg infliximab (500 mg) Ⅳ infusions every 8 wk. The patient responded well to the medications but continued to have progressive symptoms requiring a stepwise increase in the maintenance dose of infliximab to 10 mg/kg (1000 mg) Ⅳ infusions every 4 wk.

Three years after her diagnosis with CD, the patient was discovered to be pregnant with her second child. The patient was successfully treated with mesalamine and infliximab when she was discovered to be 12 wk pregnant. The patient was informed that her disease could potentially worsen, nutritional deficiencies could develop, and that her medications could be potentially harmful to the fetus. The patient understood the risks and decided to proceed with the pregnancy after multiple discussions regarding the side effects and potential teratogencity of her medications. She continued to take daily mesalamine and received a total of six doses of infliximab during her pregnancy with the last infusion occurring approximately 2 wk before delivery.

A healthy male infant weighing 7 pounds 6 ounces was born at thirty-nine weeks' gestation by an uncomplicated caesarian birth. The patient desired to breastfeed the infant while continuing to receive her mesalamine and infliximab. Again, the potential dangers of her medications were discussed with particular emphasis on their impact on breastfeeding. After taking the discussion under advisement, the patient decided to attempt to begin breastfeeding and to continue treatment with infliximab.

In an effort to determine if the infliximab was actually excreted into the breast milk, the patient's breast milk was collected and sent to the laboratory for analysis

**Table 1 IBD medications during pregnancy**

| Low risk | Limited data | Not recommended | Contraindicated |
|---|---|---|---|
| Oral mesalamine | Olsalazine | Tetracycline | Methotrexate |
| Topical mesalamine | Azathioprine | Sulfonamides | |
| Sulfasalazine | 6-Mercaptopurine | | |
| Ampicillin | Metronidazole | | |
| Cephalosporins | Ciprofloxacin | | |
| Corticosteroids | Infliximab | | |
| Cyclosporine | Adalimumab | | |
| Loperamide | | | |

**Table 2 IBD medications during nursing**

| Low risk | Limited data | Not recommended | Contraindicated |
|---|---|---|---|
| Oral mesalamine | Olsalazine | Tetracycline | Methotrexate |
| Topical mesalamine | Infliximab | Sulfonamides | Cyclosporine |
| Sulfasalazine | Adalimumab | Azathioprine | |
| Corticosteroids | | 6-Mercaptopurine | |
| | | Loperamide | |
| | | Metronidazole | |
| | | Ciprofloxacin | |

(Prometheus Laboratories, San Diego, CA) with an enzyme-linked immunosorbent assay. A spike and recovery study was performed to investigate whether any non-specific binding by breast milk components was interfering with the assay. A sample of breast milk was spiked with 40 ng/mL solution of infliximab, a concentration comparable to the mother's serum concentration. A dilutional analysis (1:2, 1:4, and 1:8) was also performed and the infliximab was detected by the laboratory in all the spiked breast milk samples, but was not identified in her regular breast milk. The patient then received her regularly scheduled infliximab infusion (10 mg/kg) and her breast milk was collected daily for 30 d. No infliximab was identified in any of the breast milk samples, even with dilutional analysis. At 27 mo, no developmental abnormalities were noted in the child.

## DISCUSSION

New medications and aggressive treatment approaches to medical management have put more women with IBD in the position of being healthy enough to consider pregnancy. In women with IBD, the key to a healthy pregnancy is adequate control of disease activity throughout pregnancy[1]. Biologic agents are increasingly becoming a mainstay in the treatment regimens of both CD and ulcerative colitis (UC). Unfortunately, little information is available about the short-term and the long-term consequences of treatment with target monoclonal antibodies on the maturing fetus[2,3]. The safety of IBD medications during pregnancy and nursing are summarized in Tables 1 and 2.

Infliximab (Remicade; Centocor Inc, Malvern, PA) is a chimeric monoclonal antibody to tumor necrosis factor-alpha (TNF-α)[4]. It is indicated for inducing and maintaining clinical remission in moderately to severely active CD and UC patients that have had an inadequate response to conventional therapy and maintenance of remission[5]. Infliximab is increasingly used to treat pregnant women and data on its safety during pregnancy are scarce. Infliximab is listed as a pregnancy category B medication and the product label states that "It is not known whether infliximab can cause fetal harm when administered to a pregnant woman[4]". Most clinicians believe that the chimeric structure of the infliximab molecule containing a human IgG1 constant region, limits placental transfer during the first trimester[6]. However, the safety of infliximab beyond the first trimester is unknown because IgG subclasses are readily passed into the fetus during the second and third trimesters[7]. Until recently, the medical literature contained no evidence that engineered therapeutic antibodies could cross the placenta when administered to expectant mothers. A recent case report documents clinically significant fetal exposure to infliximab *via* placental transfer and a prolonged half-life of the medication in newborns[2]. The presumed mechanism of fetal exposure to infliximab is transplacental maternal IgG antibody transfer beginning in the second trimester and peaking at term. No fetal abnormalities were apparent in this case, but the long-term implications of infliximab exposure during early childhood development are unknown. These findings suggest that pregnant patients should avoid therapeutic antibody treatments after thirty weeks' gestation and if necessary, the expectant mother can be bridged with steroids to control the disease activity until delivery[2,8].

Limited clinical data are available on the safety of infliximab in pregnancy, because no controlled study is available in pregnant women. The manufacturer's safety database contains information on the outcomes of 131 pregnant women who received infliximab for rheumatoid arthritis or IBD[9]. An analysis performed on this safety database suggests no significant difference in pregnancy outcomes in women with infliximab exposure[7]. A published retrospective review of 10 pregnancies in CD patients in which infliximab was continued throughout the course of the pregnancy reported favorable fetal and maternal outcomes[7]. The limited clinical results available suggest that the benefits of infliximab in attaining response and maintaining remission in pregnant IBD patients might outweigh the risks of drug exposure to the fetus[10].

The primary concern of the case we report is the safety of infliximab while breastfeeding, because many drugs and immunoglobulins are excreted in human milk. The infliximab product label states that "It is not known whether infliximab is excreted in human milk or absorbed systemically after ingestion[4]". A commercially available infliximab assay was used to measure drug levels in breast milk taken daily from our patient over a 30 d time period. No infliximab was detected in our patient's breast milk. Other published reports only tested breast-feeding mothers for one or two days but the results were consistent with our data[2]. We believe the daily testing performed on our patient's breast milk before and immediately after receiving an infliximab infusion clearly demonstrates that infliximab is not excreted in breast milk in any clinically significant amount.

Several case reports have recently emerged describing

the off-label usage of other biologics during pregnancy. A pregnant woman with treatment-refractory CD who failed treatment with infliximab was successfully treated with adalimumab (Humira; Abbott Laboratories, Chicago, IL), a recombinant human IgG1 monoclonal anti-TNF antibody[11,12]. The pregnancy was uncomplicated and at 6 mo, the infant showed normal growth and development[13]. Another case reported the use of etanercept (Enbrel; Amgen, Thousand Oaks, CA), a soluble TNF receptor fusion protein that binds to and inactivates TNF, in an uneventful pregnancy of a patient with refractory rheumatoid arthritis[14]. Etanercept has been shown to be excreted in breast milk, but it is not known whether the drug can be absorbed orally because it is such a large protein[15].

In conclusion, therapeutic monoclonal antibodies and other biologic agents are used to a greater extent to treat immune-mediated disorders in pregnant patients. The limited clinical data currently available show no significant difference in pregnancy outcomes of patients exposed to infliximab during pregnancy compared to a healthy population. Physicians should be aware that the fetus may be exposed to therapeutic monoclonal antibodies when administered to pregnant patients and the long term implications on the child's developing immune system are unknown at this time. While physicians must remain cautious about maternofetal exposure to medications like therapeutic monoclonal antibodies, additions to the literature from reports like this one will hopefully assuage some of the fears faced by gastroenterologists, obstetricians, and patients, alike.

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*CASE REPORT*

# Abscesses of the spleen: Report of three cases

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## Abstract

Abscess of the spleen is a rare discovery, with about 600 cases in the international literature so far. Although it may have various causes, it is most usually associated with trauma and infections of the spleen. The latter are more common in the presence of a different primary site of infection, especially endocarditis or in cases of ischemic infarcts that are secondarily infected. Moreover, immunosuppression is a major risk factor. Clinical examination usually reveals a combination of fever, left-upper-quadrant abdominal pain and vomiting. Laboratory findings are not constant. Imaging is a necessary tool for establishing the diagnosis, with a choice between ultrasound and computed tomography. Treatment includes conservative measures, and surgical intervention. In children and in cases of solitary abscesses with a thick wall, percutaneous catheter drainage may be attempted. Otherwise, splenectomy is the preferred approach in most centers. Here, we present three cases of splenic abscess. In all three, splenectomy was performed, followed by rapid clinical improvement. These cases emphasize that current understanding of spleen abscess etiology is still limited, and a study for additional risk factors may be necessary.



**Key words:** Spleen; Abscess; Splenectomy; Infections; Trauma

**Peer reviewer:** Ronan A Cahill, Dr, Department of General Surgery, Waterford Regional Hospital, Waterford, Cork, Ireland

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## INTRODUCTION

Abscess of the spleen is a rather rare clinical entity. About 600 cases have been described so far in the international literature[1]. Most of these refer to patients with recognized risk factors. These include the synchronous presence of conditions that compromise the immune system, such as endocarditis, diabetes mellitus, congenital or acquired immunodeficiency and the administration of immunosuppressive medication (e.g. post-transplantation or as part of the treatment of connective tissue disorders)[2-5]. Trauma is an additional predisposing factor for splenic abscesses[6]. Instances of splenic abscesses are relatively increased among intravenous drug addicts. On the other hand, splenic abscesses are most uncommon in the general population. From an epidemiological point of view, they are more frequently detected in middle-aged and older individuals, with no obvious preference for either sex[1-3].

The clinical manifestations of splenic abscesses usually include abdominal pain, exclusively located or, at least, more intensely described in the upper-left-quadrant area. Fever, nausea, vomiting and anorexia may be also present in various combinations[7-9]. Laboratory findings are consistent with the acute phase of infection, but their exact nature is determined by the pathogen isolated from the abscess[10,11]. The most common pathogens detected include *Staphylococcus* and *Streptococcus*[2,12]. Imaging by common abdominal X-ray or ultrasound may be suggestive, but the lesion is usually revealed *via* computed tomography (CT). Due to the seriousness of the potential implications, including a threat to life itself, the most usual treatment currently applied is splenectomy, which is followed by rapid clinical improvement[13-15].

## CASE REPORTS

### *Case 1*

A 45-year-old man presented to our hospital's outpatient clinic with persistent pain in the upper-left-quadrant area of the abdomen. He was working as a clerk, having previously spent 10 years as a member of embassy personnel in Africa. The referred pain had initiated about 1 mo previously, with periods of temporary improvement and relapse. The pain was not altered after food intake or sleep. The patient recognized no other major symptoms, such as vomiting, nausea or fever. Moreover, the patient was not treated for any other disease at the time (including recent infection or operation), nor had he ever been admitted to the hospital in the past. Clinical examination reproduced localized sensitivity in the area of the spleen, with no other significant findings. Laboratory testing (standard hematological and

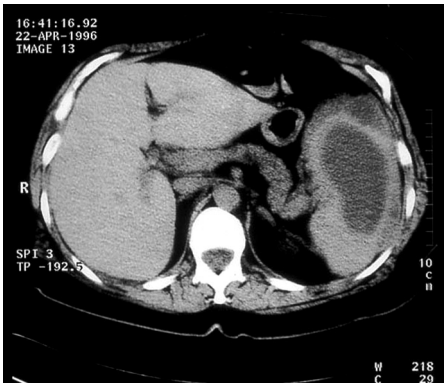


**Figure 1** Abdominal CT scan of a 45-year-old man. The spleen contained a large single abscess of 8 cm × 4 cm.

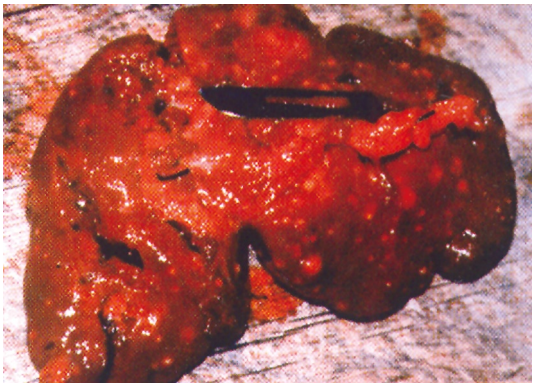

**Figure 2** Macroscopic image of the dissected spleen. Notice the extent of the pathological tissue.

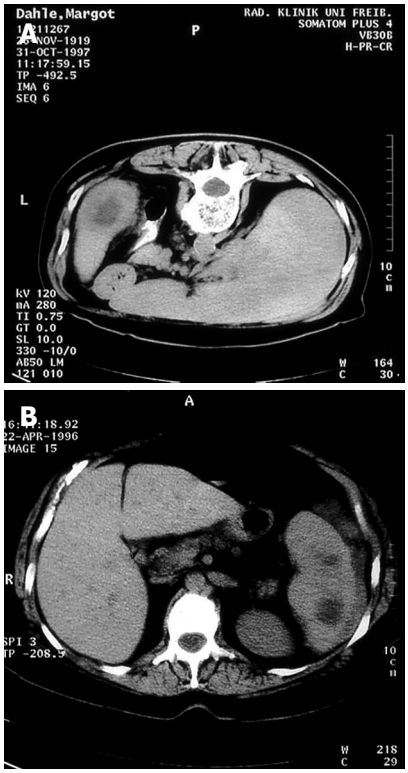


**Figure 3** **A**: CT scan of a 50-year-old woman. The spleen contained a single abscess of 3 cm × 3 cm; **B**: CT scan of a 40-year-old male sailor, which featured multiple splenic abscesses.

biochemical controls) revealed a mild increase in the number of leukocytes, which was otherwise within the normal range. Blood and urine cultures failed to reveal any pathogens. Imaging included chest and abdominal X-ray, followed by a CT scan of the upper abdomen. The latter detected a large abscess of the spleen, of an average size of 8 cm × 4 cm (Figure 1). Aspiration of the abscess was performed under CT guidance and the material obtained was cultured, which led to the development of several colonies identified as *Streptococcus* spp. No other pathogen of any kind was detected in the cultures. Owing to the abscess being symptomatic and of considerable size, the decision to perform splenectomy was made. The operation was completed successfully (Figure 2). Follow-up 1 year later has revealed a completely asymptomatic postoperative period.

### *Case 2*

A 50-year-old woman visited the outpatient clinic of our hospital with referred acute abdominal pain, which was not related to dietary habits and/or sleep. She also reported mild fever for about 1 wk (up to 38.5℃). Prior to surgery, she had been evaluated by a physician at the internal medicine clinic, who reported no clinical findings other than localized pain in the left-upper-quadrant area of the abdomen. Our clinical examination verified this finding. The patient had no history of recent infection or surgery, nor had she received medication of any kind. Moreover, she did not suffer from diabetes, human immunodeficiency virus (HIV) infection, or any other condition that would justify a degree of immunosuppression/immunocompromise. Laboratory testing was performed, which showed signs of on-going bacterial infection (leukocytosis, increased C-reactive protein and fibrinogen, and a mild decrease in blood albumin). Blood and urine cultures failed to reveal microbial infection. Imaging was also performed, including thoracic and abdominal X-ray, followed by CT of the upper and lower abdomen. The latter detected a single, small abscess of the spleen of 3 cm × 3 cm (Figure 3A). Subsequently, percutaneous aspiration of the lesion was performed and the material was sent for culture. The microbiological report referred to the presence of *Staphylococci*, a rather common finding in splenic abscesses. Due to the symptomatic nature of the lesion, the patient was advised to undergo surgical treatment, which she accepted. She was operated a week later and total splenectomy was performed under general anesthesia. A follow-up session took place 1 year after the operation, which revealed complete resolution of all symptoms. The patient has remained asymptomatic ever since.

### *Case 3*

A 40-year-old male sailor visited our hospital as part of a yearly check-up, as instructed by his employers. During a brief review of his medical history, he mentioned a persistent chronic pain in the upper-left quadrant of the abdomen. The pain was not directly related to food intake or sleep, although it was occasionally combined with mild episodes of nausea and anorexia. Moreover, the patient referred to moderate fever (up to 38.8℃) that had occurred 3-4 times in the past 3 mo, each time lasting for about 3 d, and retreated after the use of non-steroidal anti-inflammatory drugs, administered every 6 h. The patient also had a long history of infections of the respiratory

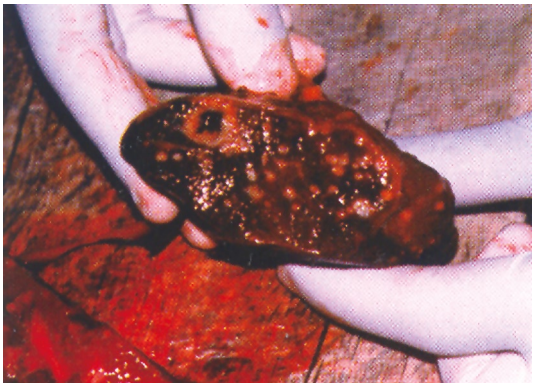

**Figure 4** Macroscopic postoperative image of the dissected spleen. Abscess areas are easily recognized.

and gastrointestinal tracts, although he reported that all of these had been treated successfully, leaving no remnant disease. Clinical examination revealed nothing significant, other than sensitivity in the abdomen, which was more intense in the upper-left quadrant. Laboratory findings were consistent with acute-phase reaction (leukocytosis, and increased acute-phase proteins). Imaging included thoracic and abdominal X-ray, abdominal ultrasound, and finally, CT. The latter revealed multiple abscesses in the spleen (Figure 3B). No abscesses were detected via imaging in any other organ examined, including the liver. Cultures from blood and urine samples were negative, but the sample obtained from the abscesses themselves developed numerous colonies of bacteria identified as *Streptococcus* spp. Evaluation of the clinical condition, laboratory and imaging data, as well as professional danger (minimal access to immediate medical referral in case of an emergency), led to the decision to perform splenectomy (Figure 4). The surgery was completed without any complications and the patient has been found healthy and free from all referred symptoms to this date.

## DISCUSSION

Abscesses of the spleen are rather rare, especially in developed countries in which the frequency of parasitic infections is low[2,13-17]. From this point of view, the random discovery of three such cases in a single Greek hospital in the course of two years may at first appear to signify an increased incidence. However, this may be misleading, since our hospital is a tertiary medical referral center for a densely populated area of about 4 million citizens. Moreover, it is closely located to the central port of the country, Pireaus, which makes access for foreign visitors, immigrants and Greeks returning from high-prevalence countries particularly easy[18]. Indeed, in case 3, the subject was a sailor and in case 1, there was a history of residence in Africa.

The age of the individuals in our small series is 40-50 years, which is consistent with the peak age group for initial diagnosis of splenic abscesses described in the literature[3,4,9,19,20]. However, it should be noted that in all three cases, medical care was only infrequently demanded prior to consultation by our department and therefore, it might be suggested that the disease evolved over a considerable time prior to our diagnosis. The personal history of long-term presence abroad strengthens this assumption.

The clinical manifestations, and laboratory and imaging findings in all three individuals were similar, although some variety was definitely observed (e.g. presence, height and duration of fever, abdominal pain characteristics and blood leukocyte count). In fact, all the symptoms described by the patients are included in the most frequent clinical findings lists provided by other reported studies, which proves that the current general understanding of the disease's pathophysiology is reasonably accurate[1,10,21].

Perhaps the most interesting parameter in our three cases of splenic abscess, however, is the lack of any obvious risk factors in any of the individuals[22,23]. Indeed, a detailed medical history and clinical examination were performed initially and post-diagnosis, in an attempt to reveal any of the factors known to be associated with the development of abscesses in the spleen and other organs. However, no such findings occurred, with the only exception of potential occupational risk in cases 1 and 3. This discovery, along with the detection of common pathogens in the abscess itself (*Streptococcus* and *Staphylococcus* spp., respectively), may imply that further factors, apart from those already described, must contribute to the etiology of the disease[6,10,24]. Their exact nature and involvement in immunity modification and regulation of the reaction to infectious agents remains to be determined in future[20].

As far as treatment is concerned, our department proceeded to classic total splenectomy in all three cases discussed. This led to rapid and complete relief from all disease-associated symptoms, without any major complications. This policy is still considered the standard of care for splenic abscesses[1,6]. However, more recent studies have also referred to alternative options, including laparoscopic splenectomy and spleen-preserving protocols, such as percutaneous imaging-guided drainage[1,15,25,26]. These methods are minimally invasive and are expected to result in smaller operative risk and overall treatment period, although of course this may differ according to the exact cause of the abscess[27-31]. Although initial results from the application of these novel methods are most promising and the potential advantages most welcome, current experience is still rather limited to justify their place in splenic abscess treatment. Therefore, the current policy is to limit their use in centers with adequately trained surgeons and only for a selected subgroup of patients.

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*CASE REPORT*

# Hepatic cyst misdiagnosed as a gastric submucosal tumor: A case report

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## Abstract

We describe here a case of 51-year-old woman with a symptomatic hepatic cyst that was misdiagnosed as a gastric submucosal tumor (SMT) with endoscopic ultrasound (EUS) and CT scan. The patient presented with an epigastric pain for two months. On endoscopy, a submucosal tumor was found on the cardia of the stomach. Based on EUS and abdominal CT scan, the lesion was diagnosed as a gastric duplication cyst or a gastrointestinal stromal tumor (GIST). The operative plan was laparoscopic wedge resection for the GIST of the gastric cardia. A cystic mass arising from the left lateral segment of the liver was found at the laparoscopic examination. There was no abnormal finding at the gastric cardia. She was treated by laparoscopic hepatic wedge resection including the hepatic cyst using an endoscopic linear stapler.



**Key words:** Hepatic cyst; Submucosal tumor; Stomach

**Peer reviewer:** Filip Braet, Associate Professor, Australian Key Centre for Microscopy and Microanalysis, Madsen Building (F09), The University of Sydney, Sydney NSW 2006, Australia

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## INTRODUCTION

Although submucosal tumor (SMT) is benign and asymptomatic, it should be evaluated by follow-up examinations. Certain gastric SMTs that are considered to be gastrointestinal stromal tumor (GIST) or symptomatic SMT require operative intervention because it is very difficult to confirm its malignant potential with endoscopic biopsy[1].

SMT is usually asymptomatic and most often discovered accidentally at surgery and autopsy or at performing diagnostic procedures. Unspecific symptoms, such as abdominal pain, obstruction, hemorrhage and intussusception, may occur. Two advanced tools have been generally accepted for the diagnosis and treatment of gastric SMTs. Endoscopic ultrasound (EUS) is one useful accurate diagnostic method, and the other is a laparoscopic procedure that allows minimally invasive treatment for SMTs.

Extragastric compression may mimic the symptoms and endoscopic findings of gastric SMTs. EUS and CT scan can accurately differentiate extragastric compression from true SMTs. However, cases may arise that cannot be differentiated even after various methods are used. We report here a case of hepatic cyst which was misdiagnosed as a gastric submucosal tumor in a patient undergone various diagnostic modalities, including endoscopy, EUS and abdominal CT scan.

## CASE REPORT

A 51-year-old woman presented with epigastric pain for two months. Initial examination showed that she had tenderness in the epigastrium. The patient was taking no medications. Her past medical history and familial history were unremarkable. Routine laboratory data on admission did not show any abnormal findings.

Gastrointestinal endoscopy revealed a submucosal tumor in the cardia of the stomach (Figure 1). On EUS, the lesion was a hypoechoic mass (3.6 cm in diameter) suggestive of a gastric duplication cyst or a GIST (Figure 2). Abdominal CT scan showed a cystic lesion at the submucosal layer of the gastric cardia, and the impression of the radiologist was a gastric duplication cyst or a GIST with necrosis (Figure 3). The operative plan was laparoscopic wedge resection for the GIST of the gastric cardia. Laparoscopic exploration was performed for the patient under general anesthesia. On the laparoscopic examination, a cystic mass arising from the left lateral segment of the liver was found (Figure 4A). There was no abnormal finding at the gastric cardia. After the

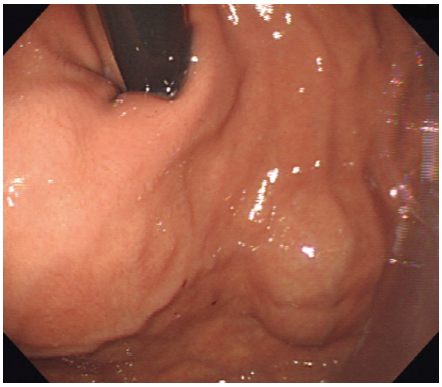

**Figure 1** Endoscopic photograph demonstrating a protruding mass on the cardia of stomach.

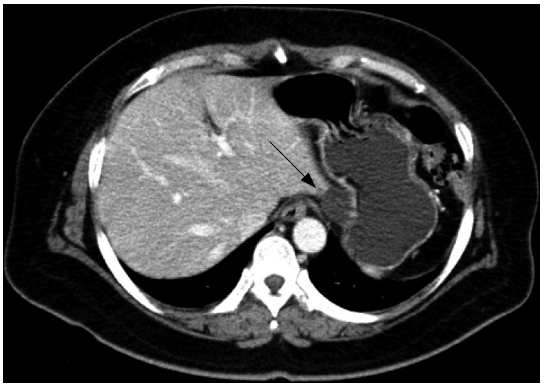

**Figure 3** Abdominal CT scan showing a low density lesion in the submucosal layer of the gastric cardia (arrow).

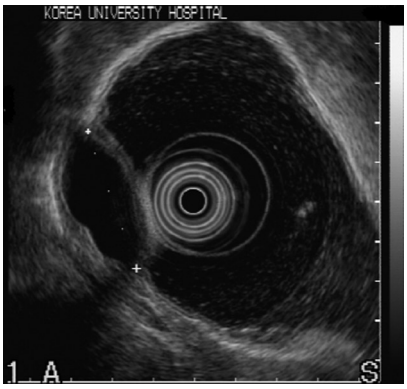


**Figure 2** EUS showing a hypoechoic mass (3.6 cm in diameter) which was suspicious of a gastric duplication cyst or a GIST.

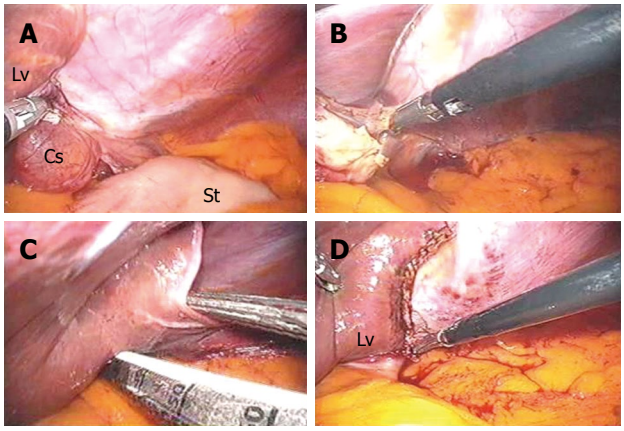


**Figure 4** Operative procedures for the hepatic cyst in the left lateral segment of liver (**A**), after dissection of the triangular ligament (**B**), liver wedge resection using an endoscopic linear stapler (**C**, **D**). Lv: Liver, Cs: Hepatic cyst, St: Stomach.

triangular ligament was divided with electrocautery (Figure 4B), wedge resection of the left lateral segment of the liver, including the hepatic cyst, was performed using an endoscopic linear stapler (Figure 4C and D). There was no specific complication during the procedure. An oral diet was permitted on the 1st postoperative day. She was discharged from the hospital on the 3rd postoperative day. The mass was diagnosed as a simple hepatic cyst.

## DISCUSSION

Hepatic cysts are usually asymptomatic and not associated with defective hepatic function. They are incidentally found at laparotomy or laparoscopy, and even at routine ultrasound or CT scan. However, they may become symptomatic if they grow. The symptoms depend on the size and location of the cyst. The patients may have a vague upper abdominal pain, a right upper quadrant abdominal mass, postprandial fullness, dyspnea and vomiting[2]. In our case, the hepatic cyst was located at the edge of the left lateral segment of the liver and caused epigastric pain by compressing the gastric cardia.

A left hepatic cyst may rarely mimic a SMT arising from the gastric cardia or fundus[3]. Various conditions can mimic gastric SMT due to extragastric compression. The most common source of extraluminal compression in the stomach is from the spleen and splenic vessels[4,5]. Other sources of extraluminal compression include normal abdominal organ structures such as liver and gallbladder, and pathologic lesions such as tumors, abscesses, pancreatic pseudocysts and enlarged lymph nodes.

Whether the lesion is due to intramural or extrinsic compression can be distinguished by changing the patient's position to see if the location and appearance of the mass change. Also, a change in appearance of the mass with either air insufflation or deflation is helpful in determining if the lesion is due to extrinsic compression, yet it can be difficult to differentiate. It was reported that the sensitivity and specificity of endoscopy are 87% and 29%, respectively for distinguishing intramural lesion from extramural compression[6]. On the other hand, EUS is 100% accurate for differentiating extragastric compression from submucosal tumor and for identifying the compressing organ[7].

In our case, however, the hepatic cyst was misdiagnosed as a GIST although various diagnostic methods such as EUS and CT scan were used.

In the best of our knowledge, this is the first report of a patient with asymptomatic left hepatic cyst that was misdiagnosed as a GIST and treated by laparoscopic resection of the hepatic cyst.

Surgical treatment for hepatic cyst is indicated when the cyst causes complaints and the diameter is at least 5 cm

or rapid growth is observed. Possible surgical treatments of the cyst include unroofing, extirpation or resection of the cyst. Conservative treatment (aspiration, sclerotherapy and percutaneous drainage) is not often recommended because of frequent relapse of the disease[2,8].

With the advances in minimally invasive surgery, laparoscopic unroofing is generally recommended for the treatment of hepatic cyst[8]. In our case, since the cyst was located at the left edge of the liver and relatively small, laparoscopic wedge resection of the hepatic cyst was easily performed by using an endoscopic linear stapler.

In conclusion, a left hepatic cyst may mimic a SMT arising from the gastric cardia and cause nonspecific abdominal symptoms. For such a case, laparoscopic procedure is a useful option for making the accurate diagnosis, and laparoscopic resection of the hepatic cyst is a minimally invasive treatment.

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*CASE REPORT*

# Aortoduodenal fistula and aortic aneurysm secondary to biliary stent-induced retroperitoneal perforation

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## Abstract

Duodenal perforations caused by biliary prostheses are not uncommon, and they are potentially life threatening and require immediate treatment. We describe an unusual case of aortic aneurysm and rupture which occurred after retroperitoneal aortoduodenal fistula formation as a rare complication caused by biliary metallic stent-related duodenal perforation. To our knowledge, this is the first report describing a lethal complication of a bleeding, aortoduodenal fistula and caused by biliary metallic stent-induced perforation.



**Key words:** Stents; Retroperitoneal perforation; Aortic aneurysm; Fistula

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Lee TH, Park DH, Park JY, Lee SH, Chung IK, Kim HS, Park SH, Kim SJ. Aortoduodenal fistula and aortic aneurysm secondary to biliary stent-induced retroperitoneal perforation. *World J Gastroenterol* 2008; 14(19): 3095-3097 Available from: URL: http://www.wjgnet.com/1007-9327/14/3095.asp DOI: http://dx.doi.org/10.3748/wjg.14.3095

## INTRODUCTION

Endoscopic or percutaneous biliary stenting is the preferred method of palliative treatment for malignant biliary strictures[1-4]. As the stents are used frequently and for long periods of time, biliary stent-related duodenal perforation is not an uncommon complication, which is potentially life threatening[5-9]. Biliary metallic stent-induced retroperitoneal perforation resulting in aortoduodenal fistula has not been reported as yet. To our knowledge, this is the first report describing the lethal complication of a bleeding aortoduodenal fistula following biliary metallic stent-related duodenal perforation.

## CASE REPORT

A 69-year-old woman presented herself with symptoms of abdominal pain and melena that started to worsen 2 d or 3 d before. An uncovered biliary wall stent (Boston Scientific, Marlboro, MA), 5 cm in length, was inserted two years before when the patient was diagnosed with a locally advanced pancreatic cancer. One year later, endoscopic removal of the uncovered wall stent was attempted because of stent clogging and tumor ingrowth. However, the attempt was unsuccessful, and resulted in a partial deformity of the distal end of the stent. A covered biliary wall stent, 6 cm in length, was reinserted into the stent. The patient received gemcitabine chemotherapy for 2 mo and recently took analgesics. She also frequently received folk remedies, such as massage of the epigastrium with downward palm-pressure.

Upon presentation, clinical examination revealed mild epigastric tenderness and abdominal distension without rebound tenderness. Laboratory tests showed 21.88 × $10^9$/L (4.0-10.8 × $10^9$/L) white blood cells, 8.5 g/dL (13-18 g/dL) hemoglobin, 36 IU/L (60-160 IU/L) amylase, 10 IU/L (0-60 IU/L) lipase, and 152 IU/L (39-117 IU/L) alkaline phosphatase. Comparisons of two simple abdominal X-rays, one taken recently and the other 2 mo before, found that the stents were slightly migrated distally and the outer stent's distal tip was compressed, shooting out radially in all directions (Figure 1). Abdominal computer tomography (CT) scan demonstrated a biliary stent with lesions arising from the head of the pancreas, compressing the second part of the duodenum. Air bubble densities were traced from the pancreatic head to the lower paraaortic lesions (Figure 2).

An endoscopy demonstrated that the bare metal

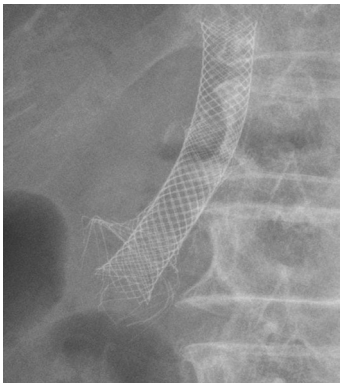

**Figure 1** Simple abdomen examination demonstrating compression of deformed distal tip of the outer biliary metallic stent shooting out radially in all directions.

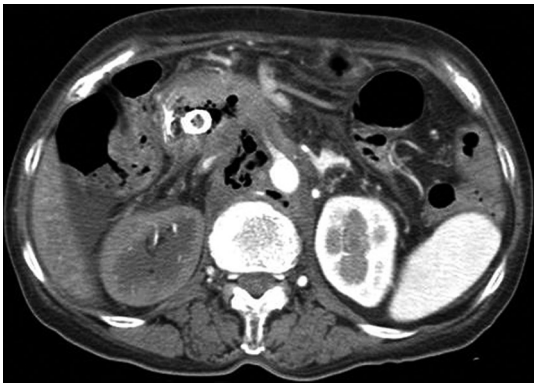

**Figure 2** Abdominal CT scan showing biliary metallic stents with a lesion arising from the pancreatic head and the trajectory of air bubble densities traced from the pancreatic head to the lower paraaortic lesions.

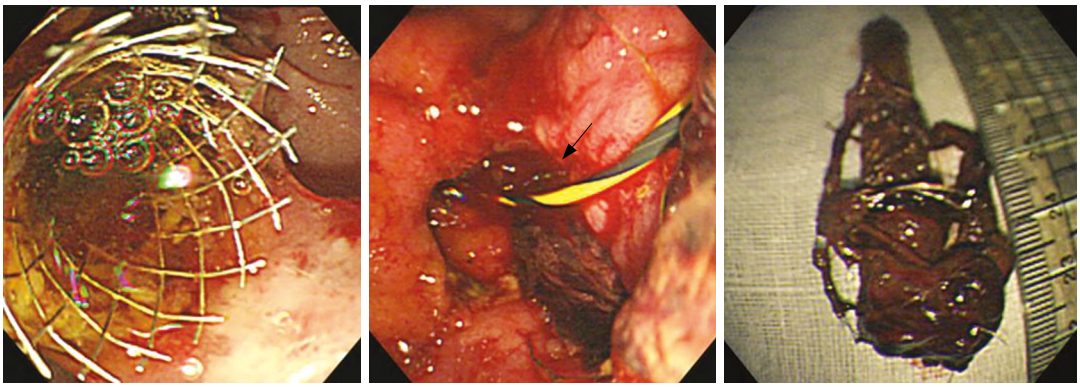

**Figure 3** EGD. **A**: On previous admission (one year ago), placement of a stent into a stent due to clogging; **B**: On the present admission, a circular hole with bleeding (arrow) caused by stent-induced perforation following removal of stents; **C**: Retrieved biliary metallic stents showing deformed barbs of the uncovered wall stent tip on the distal portion of the covered wall stent.

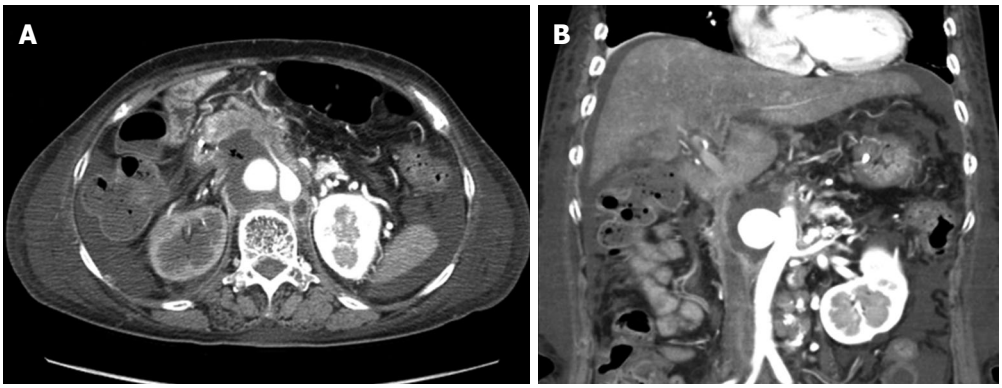


**Figure 4** Abdominal CT scan showing decreased air densities in the pancreatic head to the paraaortic area (**A**) and a circular contrast collecting aneurysm of aorta (**B**).

barbs deformed in the stent were seen to have impacted and penetrated the neighboring wall of the duodenum. Following removal of the stents with a rat-tooth forceps without any additional injury, a circular hole with bleeding corresponding to the perforation was evident (Figure 3). We then planned to place a covered self-expandable metal stent into the duodenum, instead of placing a nasobiliary drainage for a short time due to desaturation and instability of the patient. After removal of the stent and treatment with parenteral nutrition and intravenous antibiotics, the patient felt well with no abdominal pain. However, 3 d later, she complained again of abdominal pain and melena. So a follow-up abdominal CT was performed, which showed decreased air densities in the same area. However, a newly developed circular contrast collecting aortic aneurysm was found in the adjacent para-aortic lesion (Figure 4). On the following day, unexpected and massive hematemesis and hematochezia occurred, and the patient died due to bleeding.

## DISCUSSION

Early occurrence of iatrogenic duodenal perforations is generally apparent during papillotomy and stent placement.

Late presentations of duodenal perforations caused by biliary prosthesis are much rarer, but they are potentially life threatening and require immediate management[10-12].

Although the uncovered wall stent is easily embedded in the bile duct epithelium[13,14], the prolonged *in situ* uncovered wall stent may lose its framework, causing the stent to become weak because of its woven structures. In the present case, the stent's distal end was partially destroyed by repeated tries to remove it and it might have migrated distally during the follow-up. Under these circumstances, with repeated external abdominal pressure, the inner covered stent might have acted on the distal tip of the outer uncovered wall stent as a vehicle with a straining and compressing force. These factors are believed to have increased the intensity of trauma to the adjacent duodenal wall. Consequently, the deformed wire barbs of the outer wall stent's distal tip caused stent-induced retroperitoneal perforation and fistula.

The mainstays of treatment for early perforations without systemic upset are nasogastric suction, antibiotics, bowel rest and parenteral nutrition[15]. Our patient received conservative treatment and improved after stent removal. However, the patient suddenly bled to death due to the onset of an aortic aneurysm, the aortic wall might have been eroded as a result of retroperitoneal para-aortic irritation or inflammation through the fistula.

In summary, we can learn some lessons from this case. One is that stent-induced late perforation and related lethal complications may develop as a result of a distally migrated uncovered stent with sharp distal barbs and deformity caused by repeated stent removal trials and external abdominal pressure. The other is that early diagnosis and management are essential to prevent significant complications.

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*CASE REPORT*

# Tuberculous lymphadenitis as a cause of obstructive jaundice: A case report and literature review

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## Abstract

Obstructive jaundice secondary to tuberculosis (TB) is extremely rare. It can be caused by TB enlargement of the head of the pancreas, TB lymphadenitis, TB stricture of the biliary tree, or a TB mass of the retroperitoneum. A 29-year-old man with no previous history of TB presented with abdominal pain, obstructive jaundice, malaise and weight loss. Ultrasonography (US), computer tomography (CT) scan and endoscopic retrograde cholangiopancreatography (ERCP) were suggestive of a stenosis of the distal common bile duct (CBD) caused by a mass in the posterior head of the pancreas. Tumor markers, CEA and CA19-9 were within normal limits. At operation, an enlarged, centrally caseous lymph node of the posterior head of the pancreas was found, causing inflammatory stenosis and a fistula with the distal CBD. The lymph node was removed and the bile duct resected and anastomosed with the Roux-en Y jejunal limb. Histology and PCR based-assay confirmed tuberculous lymphadenitis. After an uneventful postoperative recovery, the patient was treated with anti-tuberculous medication and remained well 2.5 years later. Though obstructive jaundice secondary to tuberculous lymphadenitis is rare, abdominal TB should be considered as a differential diagnosis in immunocompromised patients and in TB endemic areas. Any stenosis or fistulation into the CBD should also be taken into consideration, and biliary bypass surgery be performed to both relieve jaundice and prevent further stricture.



## INTRODUCTION

Abdominal tuberculosis (ATB) is rare and obstructive jaundice caused by tuberculosis (TB) is extremely rare. ATB can mimic more common noninfectious abdominal syndromes and is often overlooked because of its low incidence. The mechanisms by which ATB causes bile duct obstruction are varied. We describe a patient with biliary obstruction caused by enlarged tuberculous lymph nodes.

## CASE REPORT

A 29-year-old man presented to our unit with epigastric pain and tenderness on examination, and jaundice, steatorrhea, malaise and weight loss of 7 kg over the preceding 6 mo. Total bilirubin was 163 μmol/L and direct bilirubin 88 μmol/L; SGOT, SGPT, gamma GT and alkaline phosphatase were moderately elevated. Other laboratory tests including the tumor markers CEA and CA19-9 were all within normal limits. HBsAg and HCV were negative. Abdominal ultrasonography (US) revealed a semi-solid hypoechogenic lesion 39 mm × 40 mm in size around the head of the pancreas, with two enlarged lymph nodes lying above this, and a common bile duct measuring 10 mm in diameter. Computer tomography (CT) scan showed a low density mass on the posterior aspect of the head of the pancreas with a contrast enhancing solid-rim (Figure 1). Pancreatography was normal, however severe narrowing of the distal common bile duct (CBD) was seen on endoscopic retrograde cholangiopancreatography (ERCP) (Figure 2A and B).

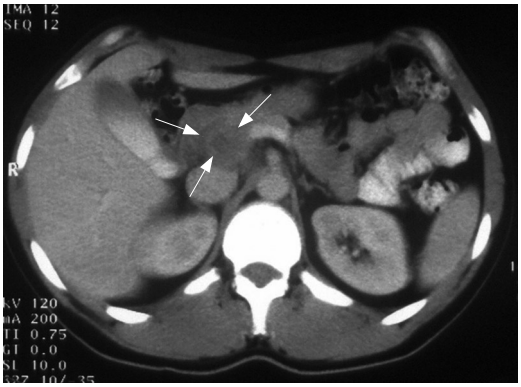

**Figure 1** Abdominal CT-scan showing a low density mass on the posterior aspect of the head of the pancreas with contrast enhancing solid rim (arrow).

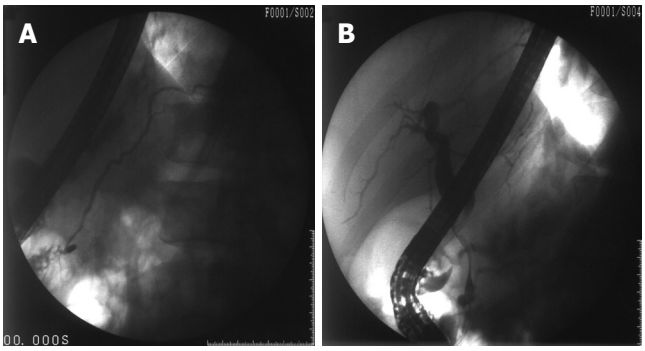


**Figure 2** **A**: ERCP with a normal pancreatogram; **B**: A smooth long severe narrowing of the distal common bile duct.

The patient underwent open surgery, and at operation the liver was found to be slightly firm, gallbladder moderately dilated, Lund's lymph node enlarged (about 1.5 cm), common bile duct moderately dilated and two lymph nodes close to the common hepatic artery also enlarged (2 cm and 3 cm). After mobilizing the duodenum and the head of the pancreas, an enlarged (4 cm) soft lymph node adherent to the distal CBD, was removed. The lymph node had a solid surface with a soft and caseous centre, and had a fistulous connection with the posterior aspect of the CBD. Frozen section histology of the lymph node revealed chronic granulomatous inflammation. The gallbladder, Lund's lymph node, the two other enlarged lymph nodes lying close to the common hepatic artery, and a specimen of liver was removed and sent for histology. The narrowed distal CBD was resected, the distal end over sewn, and the proximal end anastomosed with a Roux-en-Y jejunal limb. The resected specimen included the fistulous opening on the posterior wall of the CBD (Figure 3).

The patient had an uneventful postoperative recovery, and bilirubin levels normalised within two weeks. Histology of the liver and gallbladder was normal. The resected CBD showed epithelial ulceration and inflammation with a number of necrotizing granulomata. The lymph nodes had a chronic granulomatous appearance with large merged necrotic areas, and smaller epitheloid-type granulomata with occasional multinuclear giant cells (Figure 4A and B) suggestive of tuberculous lymphadenitis. The diagnosis was confirmed with a polymerase chain

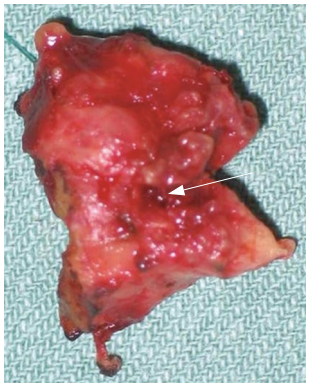

**Figure 3** The resected part of the common bile duct with fistula on the posterior wall (arrow).

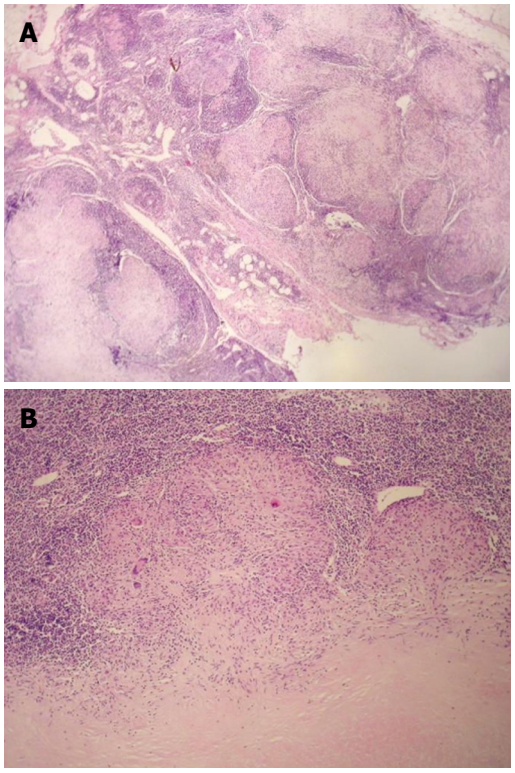


**Figure 4** **A**: Extensive chronic granulomatous lymphadenitis (HE, × 13); **B**: Focal tuberculoid granuloma formation (HE, × 64).

reaction (PCR) using automated analyzer Cobas/Roche/, with the Amplicor Mycobacterium tuberculosis assay. No previous specific risk for TB was found in the patient. He was treated with anti-tuberculous quadruple therapy and achieved gradual clinical improvement, with resolution of pain and malaise, and a weight gain of 10 kg over the next 6 mo. He remained well at 2.5 years postoperatively.

## DISCUSSION

Obstructive jaundice secondary to abdominal TB is extremely rare. Four mechanisms have been described: TB of the pancreas itself may cause pseudoneoplastic obstructive jaundice[1-10]; it may be secondary to TB lymphadenitis causing compression and inflammation of the lymph nodes and the CBD[6,11-19], as in our case, with caseation of the lymph node causing fistulation into the CBD; biliary TB itself may lead to single or multiple

strictures, mimicking cholangiocarcinoma[20-25]; and TB can create a retroperitoneal mass leading to biliary tree obstruction[26].

The diagnosis of abdominal TB should be considered in the context of a mass in the head of the pancreas in the immunocompromised patients and in countries with endemic TB[7], after the exclusion of malignancy and other biliary inflammation. TB lymphadenitis can be suspected when a contrast-enhanced CT scan demonstrates low density masses surrounded by an enhancing solid rim[14], or when ERCP demonstrates a normal pancreatogram with a smooth narrowing of the CBD[18], as were seen in our patient. FDG-PET scanning has not been shown to be useful in distinguishing TB from pancreatic malignancy, as both conditions have an increased uptake of the FDG metabolite[18]. US or CT-guided percutaneous fine needle aspiration (FNA) of the enlarged lymph nodes may be useful[7], but is often not definitive[21]. Cytology of CBD aspirate, however, obtained by ERCP, may be confirmatory in the presence of the acid-fast bacillus (*Mycobacterium tuberculosis*); alternatively PCR of the aspirate may be diagnostic[19]. However, in the case of a periportal lymphadenopathy causing obstructive jaundice, as in our patient, these FNA tests are only positive if a fistula exists between the TB lymph node and the CBD, allowing bacilli to pass into the CBD[19]. Other potential diagnostic methods include obtaining tissue specimens by laparoscopy[17] or endoscopic ultrasound with FNA[27]. Though in practice, the diagnosis is often established at operation[6,7,18,26] or even after surgery by histology[4] or PCR-based assay[2,4,6,8,10], as was the case in our patient.

The great benefit of a preoperative diagnosis of TB causing the obstruction is that a more conservative path could be followed, involving removal of the obstructing lymph node alone, followed by anti-TB medications[18]. In our case more elaborate CBD resective surgery was undertaken for presumed malignancy.

However, even though TB lymphadenitis was suspected in our patient after intraoperative frozen section, resection of the involved part of the CBD was necessary as the bile duct was already strictured, and eventual closure of the fistula would probably result in additional stenosis or even complete obstruction of the CBD. Thus inexplicable stenosis of the CBD should be taken into consideration in the context of pancreatic or TB lymphadenitis associated with obstructive jaundice and be treated by biliary bypass surgery[7] in addition to anti-TB medication.

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*CASE REPORT*

# Primary lymphoblastic B-cell lymphoma of the stomach: A case report

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## Abstract

Primary stomach lymphoblastic B-cell lymphoma (B-LBL) is a rare tumor. We describe a primary stomach B-LBL in a 38 years old female who presented with nonspecific complaints of fatigue and vomiting for 2 mo. Gastrofiberscopy revealed a large gastric ulcer, which was successfully resected. Pathology showed a lymphoblastic cell lymphoma arising from the stomach, and there was no evidence of disease at any extrastomach site. Immunohistochemical staining and gene rearrangement studies supported that the stomach tumor was a clonal B-cell lymphoma. Therefore, the diagnosis of B-LBL was made based on the stomach specimen.



**Key words:** Primary stomach lymphoma; Lymphoblastic lymphoma; B-cell

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## INTRODUCTION

Precursor B-cell lymphoblastic lymphoma (B-LBL) is a neoplasm of lymphoblasts committed to the B-cell lineage, which is an uncommon type of lymphoma and accounts for less than 10% of the total cases of lymphoblastic lymphoma. It usually affects people at a younger age. Most cases reported in a literature review are less than 18 years of age, some patients are under 35 years of age and the median age is 20 years[1]. Unlike precursor T-cell lymphoblastic lymphoma, precursor B-cell lymphoblastic lymphoma commonly involves lymph nodes or extranodal sites, mediastinal masses are infrequent. The most frequent sites of B-LBL lesions are the skin, bone, soft tissue, and lymph nodes[1-3].

Primary stomach lymphoma is defined as an extranodal non-Hodgkin's lymphoma of any cell type, with no evidence of extrastomach dissemination. The majority of stomach cases reported in the English literature are extranodal marginal zone B cell lymphoma of mucosa-associated lymphoid tissue (MALToma), diffuse large B cell lymphoma (DLBCL), nasal type NK/T cell lymphoma, *etc*[3-5]. Primary stomach B-LBL is rare. Here we present a case of primary B-LBL involving the stomach.

## CASE REPORT

The patient was a 38 years old female who had an unremarkable past medical history. She presented with nonspecific complaints of fatigue and vomiting for 2 mo. Gastrofiberscopy revealed a large gastric ulcer. During surgery, a large neoplastic ulcer was found in the stomach and gastric wall was diffusely thickened. The tumor was successfully resected with adjacent portions of the stomach. Good macroscopic margins were obtained. No other masses or enlarged lymph nodes were present. Examination of the bone marrow showed 13% immature lymphoid cells, the leukocyte count of peripheral blood was $12 \times 10^9$/L, and the percentage of peripheral blood lymphocytes was 35%.

Grossly, the greater and lesser curvatures of the resected stomach measured 22 cm and 11 cm, respectively. There was a well circumscribed neoplastic ulcer measuring 10 cm × 8 cm × 2.5 cm in antro-pyloric region of the stomach (Figure 1). A small necrosis was found on surface of the neoplastic ulcer. The cut surface of the tumor was grey and firm. Tumor tissue was found in the gastric serosa. Portions of tumor tissue were fixed in formalin and embedded in paraffin and cut into sections which were stained with HE for routine histomorphology. Additional sections of paraffin-embedded tissue were used

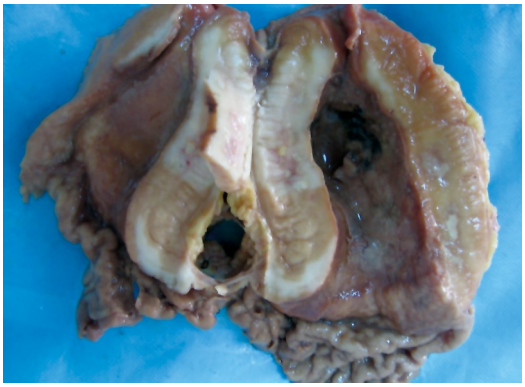

**Figure 1** A large neoplastic ulcer in the stomach involving the whole gastric wall.

for immunohistochemical staining and gene rearrangement analysis.

Microscopically, the tumor cells were uniform, medium-sized immature lymphoid cells, their nuclei contained evenly dispersed nuclear chromatin with a high nuclear/cytoplasmic ratio. The nuclei were round or oval or convoluted in shape. A large number of mitotic figures were appreciated. The tumor cells were diffusely distributed in the gastric glands, and found in all layers of the gastric wall. There were tumor emboli within the gastric wall lymphatic vessels. Lymphoepithlial lesions were not found (Figure 2).

Immunohistochemistry analysis revealed immature lymphoid cells positive for cytoplasmic CD10 and CD79a, nuclear TdT and CD99 antigens, and negative for CD20 antigen. About 50%-70% of the tumor cells were reactive for Ki-67 (Figure 3). Gene rearrangement analysis showed monoclonal immunoglubin high chain gene rearrangement (Figure 4).

The morphology, immunophenotype, and gene rearrangement of the neoplastic cells supported the diagnosis of stomach precursor B-LBL. There was no evidence that supported the diagnosis of precursor B lymphoblastic leukemia in the bone marrow and peripheral blood.

## DISCUSSION

Primary stomach lymphomas are in the minority, most of which are mucosa- associated lymphoid tissue B cell lymphoma, diffuse large B cell lymphoma, extranodal nasal type NK/T cell lymphoma, *etc*. Primary stomach B-LBL is rare[1-5].

Ninety percent of lymphoblastic lymphomas are of precursor T cell lineage and only 10% are of precursor B cell lineage[1,2]. Precursor B lymphoblastic lymphoma (B-LBL)/leukemia (B-ALL) are originated from B cell lineage. ALL and LBL represent different clinical presentations of the same neoplasm and are grouped in the category of precursor B-cell lymphoblastic leukemia/lymphoma by the revised World Health Organization classification of lymphoid neoplasms[1]. Because of the biologic unity of B-ALL and B-LBL, the criteria for distinguishing B-LBL from B-ALL are also arbitrary and applied inconsistently. In some studies[2,6,7], patients with B-LBL and acute leukemia are included, but in other studies[3,4,6], patients with leukemic involvement are excluded. According to the new WHO classification of lymphoid neoplasms, when the process is confined to a mass lesion without any or minimal evidence of blood and marrow involvement, the diagnosis is lymphoma; when extensive marrow and blood are involved, lymphoblastic

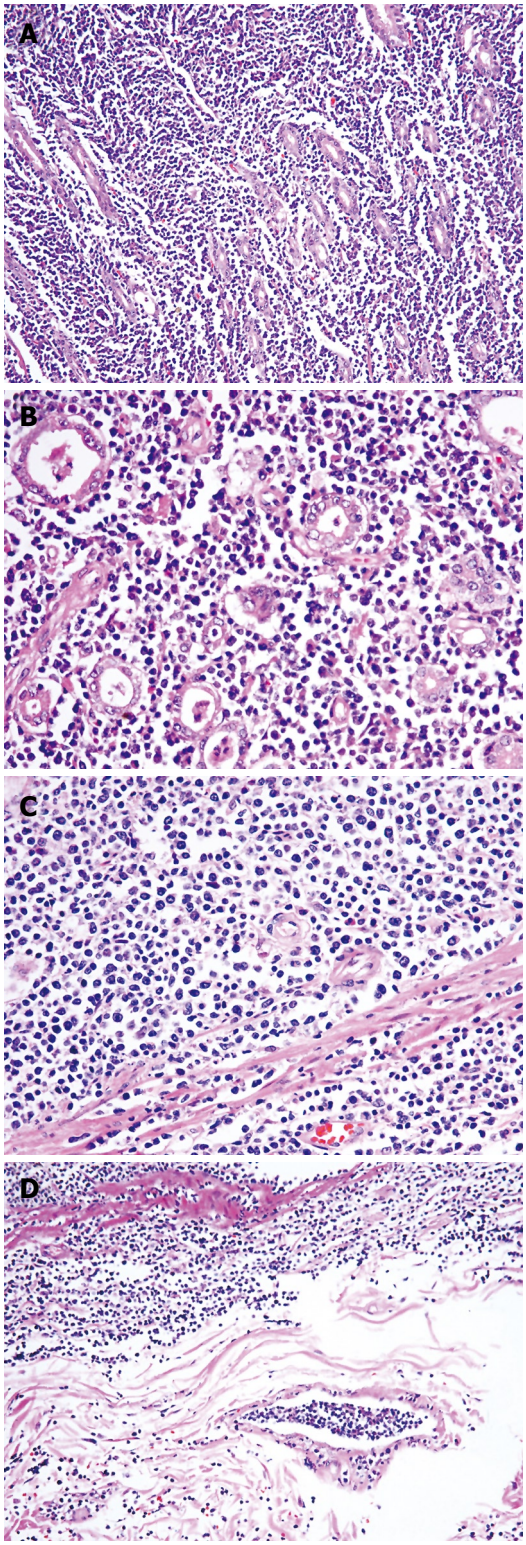


**Figure 2** Diffuse proliferation of lymphoblastic cells between gastric glands of stomach B-LBL. **A**: HE, × 100; **B**: HE, × 200; **C**: HE, × 200; **D**: Tumor emboli within lymphatic vessels of the gastric wall (HE, × 100).

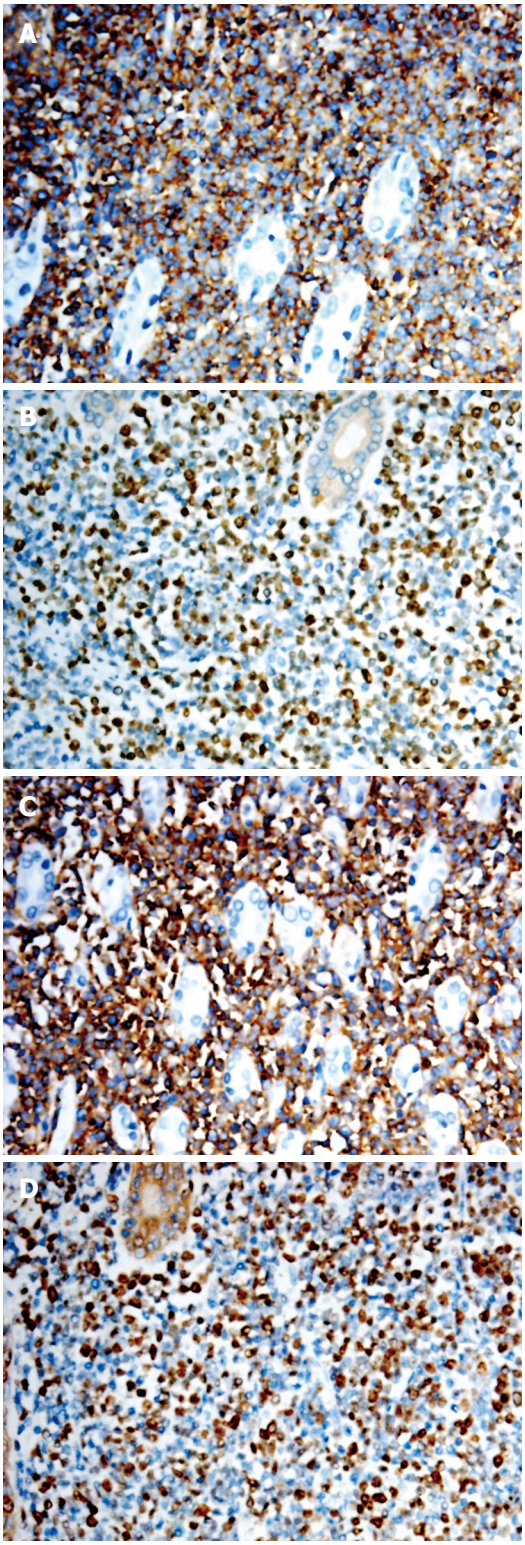


**Figure 3** Stomach B-LBL (EnVision, × 200). **A**: Lym-phoblasts diffusely stained with anti-CD79a; **B**: A large number of lymphoblasts positive for nuclear antigen TdT; **C**: Lymphoblasts diffusely stained with anti-CD10; **D**: A large number of lymphoblasts positive for Ki-67.

leukemia is the appropriate term; if a patient presents with a mass lesion and the number of lymphoblasts is less than 25% in the marrow, the diagnosis of lymphoma is preferred[1,3-5]. Precursor B lymphoblastic B-LBL most commonly involves the skin, bone, soft tissue, and lymph nodes, whereas stomach B-LBL is uncommon[6,8].

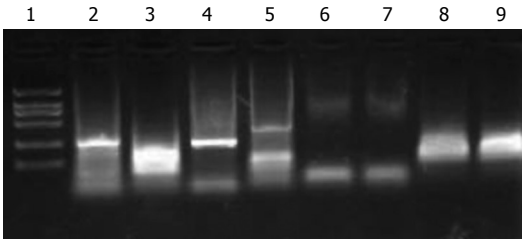


**Figure 4** Polymerase chain reaction showing rearrangement of immunoglobulin heavy-chain genes. Lane 1: Marker; lane 2: FR2, sample collected from stomach B cell lymphoblastic lymphoma showing monoclonal pattern; lane 3: FR3A, sample collected from stomach B cell lymphoblastic lymphoma showing a smear; lane 4: FR2, B cell lymphoma cell line Raja used as positive control; lane 5, FR3A, B cell lymphoma cell line Raja used as positive control. Lane 6, JⅥ, sample collected from stomach B cell lymphoblastic lymphoma showing a negative pattern; lane 7: JⅦ, sample collected from stomach B cell lymphoblastic lymphoma showing a negative pattern; lane 8: JⅥ, T cell lymphoma cell line Jurkat used as a positive control; lane 9: JⅦ, T cell lymphoma cell line Jurkat used as a positive control.

Leukemic presentation with involvement of peripheral blood and bone marrow is most common. Extramedullary involvement is frequent with particular pre-direction for the central nervous system, lymph nodes, spleen, liver, and gonads. The leukocyte count may be decreased, normal or markedly elevated. The etiology of B-LBL is unknown. However, there is evidence that suggests a genetic factor in some cases[9,10]. The neoplastic cells of B-LBL are morphologically indistinguishable from those of B-ALL and typically small to medium size blast cells, with scant cytoplasm, moderately condensed to dispersed fine chromatin, inconspicuous nucleoli, and a high mitotic rate. Immunophenotypically, the neoplastic cells express terminal deoxynucleotidyl transferase (TdT) and B-cell antigens, such as CD79a, CD10, CD19, and CD22[1,2,9].

Our review of the literature revealed few case reports of stomach B-LBL lymphoma. In this report, we describe an exceptional primary stomach precursor B-LBL with no evidence of disease at any extrastomach site. The number of lymphoblasts was less than 25% in the marrow and there was no evidence in the peripheral blood. Since immunohistochemical study showed the neoplastic lymphoid cells of precursor B-cell type, the diagnosis of B-LBL was made in this stomach case[1,9]. Precursor B-LBL, a potentially curable disease, an aggressive therapy is very important[7,11-12]. In this report, after surgical resection and final pathologic diagnosis of B-LBL, the patient was treated with chemotherapy. Now, she is followed-up at the outpatient clinic and in complete remission half a year after the initial diagnosis.

The differential diagnosis of precursor B-LBL includes myeloid lymphoma/leukemia, Burkitt's lymphoma, and precursor T-LBL, *etc*. The tumor morphology and immunophenotype help differential diagnosis. Meanwhile, the precursor B-ALL should be excluded[1,13].

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*LETTERS TO THE EDITOR*

# Perivascular epithelioid cell tumour of the liver

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## Abstract

Perivascular epithelioid cell tumour is not uncommon in the liver but seldom malignant.



**Key words:** Perivascular epithelioid cell tumour; Liver

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## TO THE EDITOR

I read with great interest the work by Fang, Zhou, Jin and Hu, dealing with perivascular epithelioid cell tumour (angiomyolipoma) of the liver[1]. It is right that, in mesenchymal tissues, the tumour is common in the uterus. The most frequent localization generally is, however, in the kidney[2,3]. In the liver, 110 cases had been described by 1999[3].

It is true that the malignant cases are much fewer.

Our interest in this entity was arisen, when we scrutinized a hepatic tumour, described from our department as an oncocytic adenoma[4]. However, the tumour later turned out to be positive for the melanocytic marker HMB-45 and is in fact a perivascular epithelioid cell tumour. The journal refused to publish the revised diagnosis!

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*LETTERS TO THE EDITOR*

# Role of silis in esophageal cancer

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## Abstract

Association of silica with diseases like cancers has been determined previously. This study was designed to determine the quantity of silis in flour produced in Golestan Province, and its relation to esophageal cancer (EC). We took flour samples from all flour millings in Golestan Province. Base-melting method in nickel cruise was used at 550℃. The extract was reduced with acids. Different silis concentrations in various regions were compared. $P < 0.05$ was considered statistically significant. The median silis concentration was 0.0030 g, the mean silis concentration was 0.008760 ± 0.004265 g in each 100 g flour. The difference of mean silis concentrations in various regions was not significant. No high level of silica was found in the flour of Golestan Province. We could not find any significant difference in various areas between silica contaminations. Studies on the consumed bread and rice in various regions of Golestan Province can be helpful.



**Key words:** Silis; Esophageal cancer; Flour; Miling; Iran

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## LETTER TO THE EDITOR

Silica ($SiO_2$) is an oxide of silicon. Its existence in food products is a presentation of contamination. Some studies revealed that silica exposure may play a role in diseases like cancer[1], although its definite effect has not been confirmed in some cancers like esophageal caner. The International Agency for Research on Cancer (IARC) has classified crystalline silica as a known human carcinogen in lung cancer[2]. The excess risk of esophageal cancer (EC) mortality among caisson workers with silicosis explains best by the very heavy exposure to free silica dust in their working environment[3-8] and their silicosis as an underlining disease.

A case-control study of the relationships among silica exposure, gastric cancer, and EC in Japan, suggested that gastric cancer and EC are related to silica exposure and silicosis in that area, although they did not reach a statistically significant level[9].

O'Neill *et al*[9] reported that the contamination with fibrous silica contaminant is high in the diet of north-east of Iran. Low quality of wheat and its contamination with weed and sand are considered important[10]. The northeastern part of Iran in Golestan Province (Turkmen Sahra) is known to have the highest incidence of EC in the country and to be one of the highest areas in the world[11]. Golestan Province is located on the hot spots that are along a presumptive belt starting from northern China, extending along the southern parts of the former Soviet Union and ending in the Caspian littoral in northern Iran.

In 1982, O'Neill *et al*[9] reported an association of silica fibers in the millet bran and esophageal tumors in another study. In 1986, Newman[1] found that certain plants contain structures consisting of biogenic silica, which has been supposed as a causative agent in the high cancer areas of Southern Africa, Northeast Iran and North of China. It is hypothesized that these plant mineral fibers are involved in the etiology of EC in Iran and in other high incidence areas. Phalaris minor is a known common weed in the Mediterranean area, but it is not considered a region with a high incidence and prevalence of EC.

Some findings suggest that silica particles might be involved in the etiology of EC. In fact, different results are available about the significant relationship between silis exposure and EC, some are in agreement and suppose that silis plays a role in the etiology of EC, and others are in disagreement.

In our study, silis but not its compound or its biologic derivatives was considered a carcinogen. Flour samples from all flour millings in Golestan Province were taken. Base-melting method in nickel cruise was used at 550℃ and the extract was reduced with acids. The complex was evaluated with a spectrophotometer (820 nanometer wavelength). Five control samples of wheat seeds and pedicles were examined, too. The different silis median concentrations in

wheat seeds, pedicles and flour were statistically significant. However, the total silis in the flour was in the normal range because a great deal of silis was omitted from the flour during the preparing process. The modern and new purification technologies may be effective in producing these results, so the previous contaminants can be supposed less important.

The mean silis concentration was 0.012, 0.01 and 0.003 in the central, western and eastern parts of Gorgan City, respectively. The differences were not statistically significant. The Golestan province was divided into 3 areas according to the incidence of EC and we matched the data with the location of flour millings on the map.

Our findings suggest that there are no significant differences in flours of various areas, revealing a less important role of silis in EC. However, from a medical point of view it is important. There is a great variation in the incidence of EC between countries and regions. The distribution of EC in Golestan Province is not concordant with the amount of silis reported in this study. Silis concentration is higher in the west part but EC is higher in the east.

Despite the high incidence of EC in Northeast Iran, no significant differences were seen between silis in wheat flour of this area and standard measures. It seems that silis could not play a major role in the etiology of EC or is considered a predisposing factor when we eat it. Perhaps, oral or inhalation absorption of silis has an effect on its carcinogenicity. This hypothesis becomes acceptable when we pay more attention to the main component of earth crust. Silis, an abundant mineral in rock, sand, and soil, is in contact with our skin, but it is not supposed as a carcinogen or a predisposing factor.

Previous studies have reported a considerable concentration of silica in the flour produced in this area and suggested a relationship between EC and silis. In this study, no high level of silica was found in the flour of Golestan Province. Thus, on the one hand, we could not confirm the hypothesis of high contamination of the flour in this area, which is considered a high risk of EC in Golestan Province. On the other hand, we could not rule out the probable role of this element in the etiology of EC. Studies on the consumed bread and rice in various regions of the province can be helpful.

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*SCIENTOMETRICS*

# Variations of author origins in *World Journal of Gastroenterology* during 2001-2007

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## Abstract

**AIM:** To discuss the variations and distributions of authors who published their papers in *World Journal of Gastroenterology* (*WJG*) during 2001-2007 and evaluate the development of *WJG* and gastroenterology core journals in recent years by comparing the contributions of the authors.

**METHODS:** *WJG* articles published in 2001-2007 were searched from *MEDLINE* database (by ISI Web of Knowledge). The variations (cooperation degree, cooperation rate) and distributions of the first authors were analyzed with bibliometric methods. SCIE was used to collect articles published in *Am J Gastroenterol*, *Gastroenterology*, *Scand J Gastroenterol* and *WJG* in 2007, and comparison of the data was made. Comparison indicators included the article number of annual journals, cooperation degree of authors, cooperation rate, mean number of articles published in each *WJG* issue, number of countries of the first *WJG* authors, geographical distribution and article contribution ratio of all *WJG* authors and domestic authors.

**RESULTS:** Of the 5851 articles covered in *MEDLINE*, 173, 236, 633, 826, 1496, 1382 and 1105 articles were cited from 2001 to 2007. The cooperation degree was 5.11, 5.56, 5.75, 5.76, 6.31, 5.90 and 5.64 respectively. The cooperation rates was 94.80%, 99.15%, 98.89%, 98.55%, 99.13%, 96.67% and 95.66%, respectively. The mean number of articles published in each *WJG* issue from 2001 to 2007 was 28, 39, 52, 34, 31, 28 and 23, respectively. The number of countries of the first *WJG* authors was 8, 8, 27, 32, 49, 61 and 56, respectively. The first authors of *WJG* came from 3 continents in 2001 and covered 6 continents in 2006-2007. The number of articles written by Asian authors was 136 (79.07%), 227 (96.19%), 575 (90.98%), 713 (87.81%), 1111 (75.32%), 712 (53.98%) and 555 (53.21%), respectively in 2001-2007. The number of articles written by European & American authors increased from 36 (20.93%) and 8 (3.39%) in 2001-2002 to 563 (42.68%) and 452(43.34%) in 2006-2007. The number of countries except for China contributing papers was increased. The number of articles written by first authors of Japan rose from 0 (0%) in 2001-2002 to 287 (12.15%) in 2006-2007. The number of articles written by American authors increased from 6 (1.47%) in 2001-2002 to 158 (6.69%) in 2006-2007. The number of articles written by Chinese authors was 136 (79.07%), 227 (96.19%), 548 (86.71%), 669 (82.39%), 884 (59.93%), 380 (28.81%) and 320 (30.68%), respectively, in 2001 to 2007. The number of articles published in *Am J Gastroenterol, Gastroenterology*, *Scand J Gastroenterol* and *WJG* was 565, 586, 238 and 1118, respectively in 2007. The cooperation degree was 4.77, 6.14, 5.95 and 5.64, respectively, in 2007. The cooperation rate was 95.40%, 84.18%, 96.63% and 95.66%, respectively, in 2007. The number of countries of authors contributing papers was 44, 35, 42 and 62, respectively, in 2007.

**CONCLUSION:** The geographical distribution of *WJG* authors is wide for the past 2 years. *WJG* has made a step onto international publishing, and drawn even more attentions from gastroenterology researchers. Its authors are distributed over 74 countries in 6 global continents, and the journal has become the main intermediary for international gastroenterology researchers to demonstrate their research accomplishments.



**Key words:** Bibliometrics; *World Journal of Gastroenterology*; Science citation index

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## INTRODUCTION

*World Journal of Gastroenterology* (*WJG*) was first published

in 1995. This English journal is edited and published by The WJG Press and can be retrieved with the following citation tools: Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, Index Medicus, *MEDLINE* and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, Nature Clinical Practice Gastroenterology and Hepatology, CAB Abstracts and Global Health, *etc*. In recent years, Ma *et al*[1] has analyzed the articles covered in SCIE during 1998-2004, claiming that the self-citation rate is decreased. However, the citation rate by others is increased and the journal citation status is improved. The variations of *WJG* authors' data in 2001 to 2007 were comparatively analyzed. The cooperation degree, cooperation rate, number of countries and author publishing ratios of domestic journal issues in *American Journal of Gastroenterology*, *Gastroenterology*, *Scandinavian Journal of Gastroenterology*, were also comparatively analyzed using the SCIE database.

A bibliometric analysis of the variations in distributions of authors was made to show the improvements and shortcomings of *WJG* and speed up its development.

## MATERIALS AND METHODS

*WJG* articles were searched from *MEDLINE* (by ISI Web of Knowledge)[2] in 2001-2007. Variations (cooperation degree, cooperation rate) and distributions of the first authors were analyzed with biliometric methods. Articles published in *Am J Gastroenterol*, *Gastroenterology*, *Scand J Gastroenterol and WJG* covered in SCIE[3] in 2007 were analyzed using Web of Science (meeting summaries were not covered). The authors, titles, addresses and other relevant data of the four journals in 2007 were processed through the SCIE's 'Refine Results' function, and countries of authors, *WJG* authors, research institutions and their distribution were closely consistent with the current status and authors of articles published in *WJG* experienced difficulties.

## RESULTS

### *WJG articles retrieval status with MEDLINE citation in 2001-2007*

*WJG* was published bimonthly in 2001-2002, monthly in 2003, semimonthly in 2004, and weekly from 2006. In 2001 -2007, 173, 236, 633, 826, 1496, 1382 and 1105 articles published in *WJG* were covered in *MEDLINE*. The number of articles published in each issue of *WJG* was 28, 39, 52, 34, 31, 28 and 23, respectively, in 2001-2007.

### *Co-author articles published in WJG*

A total of 5851 articles published in *WJG* during 2001-2007 were cited. The number of authors of these papers was 34415 and the cooperation degree was 5.11, 5.56, 5.75, 5.76, 6.31, 5.90 and 5.64, respectively, with a mean cooperation degree of 5.88. The number of articles written by a single author was 137, accounting for 2.34% of all articles. The number of co-author articles published in 2001-2007 was 5714 and the cooperation rate was 94.80%, 99.15%, 98.89%, 98.55%, 99.13%, 96.67% and 95.66% respectively, with a mean cooperation rate of 97.66%. The cooperation degree was slightly increased from 2001 to 2005 (Table 1).

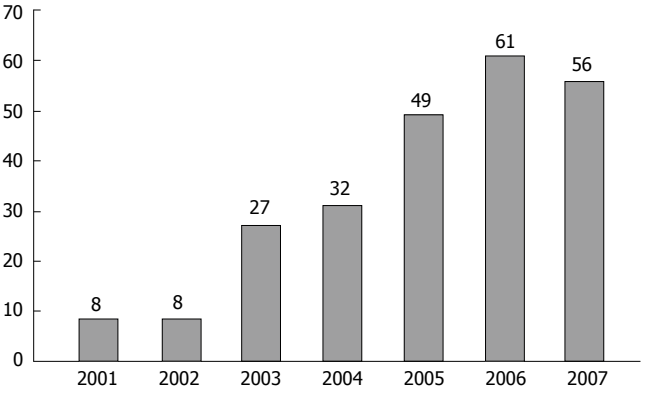


**Figure 1** Distribution of first authors and countries in 2001-2007.

### *Distribution of first authors in WJG*

Only addresses of the first authors were marked in *MEDLINE*, and 5851 articles published in *WJG* were retrieved in 2001-2007, in which only 5689 articles had available addresses. The number of countries with their articles covered in *MEDLINE* was 8, 8, 27, 32, 49, 61 and 56, respectively (Figure 1). The geographical distribution of *WJG* authors was increasingly broadened, especially in 2006 and 2007 during which the number of countries increased multiple folds.

The geographical distribution of the authors with addresses in 5689 articles was categorized into 6 continents (Table 2). During 2001-2005, the majority authors were from Asia, accounting for136 (79.07%), 227 (96.19%), 575 (90.98%), 713 (87.81%) and 1111 (75.32%), respectively. During 2006-2007, the number of authors from Asia was 712 (53.98%), 555 (53.21%) respectively, showing that the number of Asian authors is declining. During 2006-2007, the geographical distribution of *WJG* authors covered all the 6 continents and the number of European and North America authors increased from 36 (20.93%) and 8 (3.39%) in 2001-2002 to 563 (42.68%) and 452 (43.34%) in 2006-2007 respectively.

### *Geographical distribution of the first authors*

In order to reflect the geographical distributions of authors, a comparison of the distribution of *WJG* authors was performed. The number of articles contributed to *WJG* by the top 15 countries (Table 3) was 5167 (90.82%), the number of articles contributed to *WJG* by Chinese authors was 136 (79.07%), 227 (96.19%), 548 (86.71%), 669 (82.39%), 884 (59.93%), 380 (28.81%) and 320 (30.68%), respectively, in 2001-2007. The number of articles contributed by Japanese authors increased from 0 (0%) in 2001-2002 to 287 (12.15%) in 2006-2007, the number of articles contributed by the American authors was also increased from 6 (1.47%) in 2001-2002 to 158 (6.69%) in 2006-2007. All countries, except for China showed an increased number of contributed articles.

**Table 1 Co-author articles published in *WJG* during 2001-2007**

| Yr | Distribution of co-author articles | | | | | | | | | | | Total (articles) | Authors | Cooperation degree | Cooperation rate (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | > 11 | | | | |
| 2001 | 9 | 18 | 20 | 33 | 26 | 20 | 20 | 14 | 5 | 2 | 6 | 173 | 884 | 5.11 | 94.80 |
| 2002 | 2 | 20 | 23 | 44 | 35 | 39 | 30 | 20 | 10 | 2 | 11 | 236 | 1313 | 5.56 | 99.15 |
| 2003 | 7 | 30 | 65 | 88 | 131 | 110 | 79 | 55 | 25 | 22 | 21 | 633 | 3637 | 5.75 | 98.89 |
| 2004 | 12 | 47 | 82 | 112 | 146 | 154 | 105 | 71 | 43 | 25 | 29 | 826 | 4755 | 5.76 | 98.55 |
| 2005 | 13 | 59 | 113 | 188 | 243 | 273 | 202 | 154 | 86 | 56 | 109 | 1496 | 9434 | 6.31 | 99.13 |
| 2006 | 46 | 111 | 149 | 169 | 210 | 191 | 150 | 119 | 74 | 57 | 106 | 1382 | 8160 | 5.90 | 96.67 |
| 2007 | 48 | 107 | 129 | 148 | 158 | 139 | 120 | 83 | 48 | 59 | 66 | 1105 | 6232 | 5.64 | 95.66 |
| Total | 137 | 392 | 581 | 782 | 949 | 926 | 706 | 516 | 291 | 223 | 348 | 5851 | 34415 | 5.88 | 97.66 |

**Table 2 Geographical distribution of the authors in *WJG***

| Yr | Distribution of the authors in 6 continents | | | | | |
|---|---|---|---|---|---|---|
| | Africa | Asia | Europe | North America | Oceania | South America |
| 2001 | 0 | 136 | 32 | 4 | 0 | 0 |
| 2002 | 0 | 227 | 6 | 2 | 1 | 0 |
| 2003 | 1 | 575 | 45 | 6 | 2 | 3 |
| 2004 | 3 | 713 | 85 | 6 | 0 | 5 |
| 2005 | 7 | 1111 | 314 | 28 | 4 | 11 |
| 2006 | 11 | 712 | 453 | 110 | 10 | 23 |
| 2007 | 9 | 555 | 359 | 93 | 13 | 14 |

Among the top 15 countries, 7 are in Asia, 7 in Europe, and 1 in North America.

### Data comparisons of gastroenterology-related journals

The articles of *Am J Gastroenterol*, *Gastroenterology* and *Scand J Gastroenterol* were selected to compare with those of *WJG*. *Am J Gastroenterol* is an official publication of the American College of Gastroenterology, and its IF was 5.608 in 2006, ranking 5th in Journal Citation Report (JCR). *Gastroenterology* is the official journal of the American Gastroenterology Association (AGA) and its IF was 12.457 in 2006, ranking 1st in JCR. *Scand J Gastroenterol* published by Taylor & Francis Group is the membership journal of the Gastroenterologic Societies of Denmark, Finland, Iceland, Norway and Sweden, and its IF was 1.869 in 2006. These four journals are most typical of all journals related to the field of gastroenterology. The number of articles published in *Am J Gastroenterol, Gastroenterology*, *Scand J Gastroenterol* and *WJG* covered in SCIE was 565, 586, 238 and 1118, respectively, in 2007. The cooperation degree of authors was 4.77, 6.14, 5.95 and 5.64, respectively; the cooperation rate was 95.40%, 84.18%, 96.63% and 95.66% respectively, in 2007. The geographical distribution of authors' was 44, 35, 42 and 62, respectively (Table 4). In 2007, The number of American authors contributing to *Am J Gastroenterol* and *Gastroenterology* accounted for 47.43% and 50.85% respectively, the number of Swedish authors contributing to *Scand J Gastroenterol* accounted for 18.07%, the number of Northern Europe authors contributing to *Scand J Gastroenterol* accounted for 45.38%, the number of Chinese authors contributing to *WJG* accounted for 30.4%.

## DISCUSSION

In 2001-2007, the number of articles covered in *MEDLINE* was 173, 236, 633, 826, 1496, 1382 and 1105 respectively, the mean number of articles published in each issue was 28, 39, 52, 34, 31, 28 and 23 respectively. The number of articles published increased by 932 (638.73%) in 2007 compared to 2001.

**Table 3 Distribution of the top 15 countries in *WJG* during 2001-2007**

| Country name | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 | Total |
|---|---|---|---|---|---|---|---|---|
| China | 136 | 227 | 548 | 669 | 884 | 380 | 320 | 3164 |
| Japan | | | 6 | 19 | 133 | 170 | 117 | 445 |
| Germany | 10 | 2 | 4 | 10 | 68 | 92 | 66 | 252 |
| Italy | | | 9 | 15 | 56 | 79 | 56 | 215 |
| United States | 4 | 2 | 5 | 6 | 19 | 77 | 81 | 194 |
| Turkey | | | 17 | 24 | 31 | 43 | 62 | 177 |
| South Korea | | | 5 | 9 | 37 | 53 | 38 | 142 |
| Greece | | | 2 | 6 | 34 | 45 | 31 | 118 |
| United Kingdom | 18 | | 3 | 1 | 16 | 28 | 22 | 88 |
| India | | | 5 | 2 | 7 | 35 | 30 | 79 |
| Spain | | | 1 | 2 | 13 | 34 | 21 | 71 |
| Poland | | 1 | 2 | 6 | 31 | 17 | 4 | 61 |
| Hungary | | | 2 | 12 | 20 | 20 | 5 | 59 |
| Iran | | | 2 | 2 | 7 | 25 | 18 | 54 |
| Thailand | | | 1 | 4 | 14 | 16 | 13 | 48 |

In 2001-2007, the cooperation degree was 5.11, 5.56, 5.75, 5.76, 6.31, 5.90 and 5.64 respectively (mean 5.88), the cooperation rate was 94.80%, 99.15%, 98.89%, 98.55%, 99.13%, 96.67% and 95.66% respectively (mean 97.66%). The mean number of co-authors and single authors showed a tendency to increase from 2001 to 2005, while slightly decreased in 2006 to 2007.

The geographical distributions of authors in *WJG* were expanded from 4 continents in 2001 to the 6 continents in 2006-2007, the number of countries increased in multiple folds. The number of authors from Europe and North America increased while that from Asia decreased. The number of countries increased from 8, 8, 27, 32 and 49 in 2001-2005 to 61 and 56 in 2006-2007.

The number of Chinese authors accounted for 79.07%, 96.19%, 86.71%, 82.39%, 59.93%, 28.81% and 30.68% respectively, in 2001-2007, showing a maximum decrease of 67.38%. The number of Japanese, American, German and Italian authors increased greatly, showing an increasing trend of international authors contributing to *WJG*.

When compared with *Am J Gastroenterol, Gastroenterology, Scand J Gastroenterol*, the geographical distribution of

**Table 4 Data comparisons of the 4 representative gastroenterology journals in 2007**

| Journal name | Articles published in 2007 | Cooperation degree in 2007 | Cooperation rate in 2007 | Geographical distribution of authors | Ratio of articles contributed by domestic authors (%) |
|---|---|---|---|---|---|
| *Am J Gastroenterol* | 565 | 4.77 | 95.40 | 44 | United States 47.43 |
| *Gastroenterology* | 586 | 6.14 | 84.18 | 35 | United States 50.85 |
| *Scand J Gastroenterol* | 238 | 5.95 | 96.63 | 42 | Sweden 18.07 |
| *WJG* | 1118 | 5.64 | 95.66 | 62 | China 30.4 |

authors in *WJG* was greatly expanded in the order of China, Asia and 6 continents. The mean number of published articles in each issue showed a prominent decrease, which may improve the quality of articles published in *WJG*. The cooperation degree and rate were reasonable, and the number of Chinese authors was slightly increased in 2007.

In conclusion, the geographical distribution of *WJG* authors is worldwide. *WJG* has made a step onto international level, thus drawing more attentions from gastroenterology researchers. The journal has become the main intermediary for international researchers in gastroenterology to demonstrate their research accomplishments.

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# Meetings

## Events Calendar 2008-2009

FALK SYMPOSIA 2008
January 24-25, Frankfurt, Germany
Falk Workshop: Perspectives in Liver Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai - Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation - Management of Adeno- carcinomas
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April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

*No author given*
6 21st century heart solution may have a sting in the tail. *BMJ*

2002; **325**: 184 [PMID: 12142303]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

**Books**

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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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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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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