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# WJG

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<sup>[1]</sup>Passed away on October 20, 2007

<sup>[2]</sup>Passed away on June 14, 2008



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## Pernicious anemia: New insights from a gastroenterological point of view

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### Abstract

Pernicious anemia (PA) is a macrocytic anemia that is caused by vitamin B<sub>12</sub> deficiency, as a result of intrinsic factor deficiency. PA is associated with atrophic body gastritis (ABG), whose diagnosis is based on histological confirmation of gastric body atrophy. Serological markers that suggest oxyntic mucosa damage are increased fasting gastrin and decreased pepsinogen I. Without performing Schilling's test, intrinsic factor deficiency may not be proven, and intrinsic factor and parietal cell antibodies are useful surrogate markers of PA, with 73% sensitivity and 100% specificity. PA is mainly considered a disease of the elderly, but younger patients represent about 15% of patients. PA patients may seek medical advice due to symptoms related to anemia, such as weakness and asthenia. Less commonly, the disease is suspected to be caused by dyspepsia. PA is frequently associated with autoimmune thyroid disease (40%) and other autoimmune disorders, such as diabetes mellitus (10%), as part of the autoimmune polyendocrine syndrome. PA is the end-stage of ABG. Long-standing *Helicobacter pylori* infection probably plays a role in many patients with PA, in whom the active infectious process has been gradually replaced by an autoimmune disease that terminates in a burned-out infection and the irreversible destruction of the gastric

body mucosa. Human leucocyte antigen-DR genotypes suggest a role for genetic susceptibility in PA. PA patients should be managed by cobalamin replacement treatment and monitoring for onset of iron deficiency. Moreover, they should be advised about possible gastrointestinal long-term consequences, such as gastric cancer and carcinoids.

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**Key words:** Pernicious anemia; Autoimmune diseases; Atrophic gastritis; Intrinsic factor; Autoantibodies; Parietal cells; Vitamin B<sub>12</sub> deficiency; *Helicobacter pylori*

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### INTRODUCTION

Pernicious anemia (PA) (also known as Biermer's disease<sup>[1]</sup> and Addisonian anemia<sup>[2]</sup>) is a macrocytic anemia due to vitamin B<sub>12</sub> (cobalamin) deficiency, which, in turn, is the result of deficiency of intrinsic factor, a protein that binds avidly to dietary vitamin B<sub>12</sub> and promotes its transport to the terminal ileum for absorption<sup>[3]</sup>. The deficiency of intrinsic factor is a consequence of the presence of atrophic body gastritis (ABG), which results in the destruction of the oxyntic mucosa, and thus, the loss of parietal cells, which normally produce chlorhydric acid as well as intrinsic factor<sup>[4]</sup>. The term PA is sometimes used as synonym for cobalamin deficiency or for macrocytic anemia, but to avoid ambiguity, PA should be reserved for conditions that result from impaired secretion of intrinsic factor and atrophy of oxyntic mucosa<sup>[5]</sup>. However, differential diagnosis may sometimes be challenging due to the limit of available diagnostic tools.

PA is considered an autoimmune disorder due to the frequent presence of gastric autoantibodies directed against intrinsic factor, as well as against parietal cells. PA is often considered a synonym of autoimmune

gastritis, because PA is thought to be the end stage of an autoimmune process that results in severe damage of the oxyntic gastric mucosa<sup>[6]</sup>. Recent experimental and clinical data strongly suggest an involvement of long-standing *Helicobacter pylori* (*H pylori*) infection in the pathogenesis of ABG and PA, but it is still under debate whether PA may be included among the long-term consequences of *H pylori* gastritis<sup>[7]</sup>.

The present review focuses on novel aspects regarding the pathogenesis, clinical presentation, and diagnosis of PA, as well as the management of PA patients from a gastroenterological point of view.

## PA: AN AUTOIMMUNE DISORDER OR AN INFECTIOUS DISEASE?

PA is the end-stage of ABG and is generally considered an autoimmune disease. The autoimmune origin of PA is based on the presence of parietal cell and/or intrinsic factor autoantibodies, and the frequent association with other autoimmune disorders, such as autoimmune thyroid disease (ATD), type 1 diabetes, and vitiligo<sup>[6,8]</sup>.

ABG associated with PA is often called autoimmune gastritis or type A gastritis, which is defined as a type of chronic atrophic gastritis restricted to the body mucosa, characterized by a severe, diffuse atrophy of the oxyntic glands and hypochlorhydria, and a normal antral mucosa<sup>[4]</sup>. Another classical histological feature of ABG is the absence of *H pylori* on gastric mucosal biopsies<sup>[4]</sup>. It is now accepted that long-standing *H pylori* infection is able to induce atrophy of the gastric mucosa, and *H pylori* is considered the main causative agent of multifocal atrophic gastritis, in which the antrum is almost invariably involved<sup>[9]</sup>. Thus, ABG is generally considered a separate entity from *H pylori*-related atrophic gastritis, mainly because the prevalence of *H pylori* infection in patients with severe ABG and PA has been found to be low<sup>[10,11]</sup>. However, in the past few years, the question has been raised whether *H pylori* may be implicated in the pathogenesis of ABG, and, as a basic mechanism for the induction of gastric autoimmunity by *H pylori* infection, molecular mimicry has been proposed<sup>[7,12,13]</sup>. Molecular mimicry is defined as the possibility that sequence similarities between foreign and self-peptides are sufficient to result in the cross-activation of autoreactive T or B cells by pathogen-derived peptides. It is a phenomenon associated with some pathogens in which the antigens that evoke an immune response have enough similarity to the body's own proteins to cause an autoimmune reaction, such as in rheumatoid arthritis, mediated by cross-reactive T cells and/or circulating antibodies. In fact, gastric H<sup>+</sup>/K<sup>+</sup>-ATPase has been recognized as the major autoantigen in experimental and human ABG<sup>[14-16]</sup>, and autoreactive gastric CD4<sup>+</sup> T cells that recognize H<sup>+</sup>/K<sup>+</sup>-ATPase and *H pylori* antigens have been recently described in ABG<sup>[17,18]</sup>. Thus, PA and ABG seem to be an example of pathogen-induced, organ-specific autoimmunity, in which genetic susceptibility plays an important role in relation to the loss of immu-

nological tolerance<sup>[18]</sup>. In fact, the immunological basis of molecular mimicry lies in the recognition by T-cell antigen receptors of antigenic peptides bound to human leucocyte antigen (HLA) molecules on the surface of antigen-presenting cells, and inappropriate activation of T cells may occur as a result of the upregulation of HLA molecules in genetically susceptible individuals<sup>[19]</sup>. A specific HLA-DR pattern was suggested in PA patients several years ago<sup>[20]</sup>, and more recently, blocking experiments with anti-DR and anti-DQ antibodies have shown that DR antigen probably represents the HLA restriction element in ABG<sup>[17]</sup>. By using a DNA-based, sequence-specific oligonucleotide technology, we observed in our series of PA patients that the genotypes HLA-DRB1\*03 and DRB1\*04, which are known to be associated with other autoimmune disease (such as type 1 diabetes and ATD)<sup>[21]</sup>, were significantly associated with PA, compared to a control group (unpublished data), which supports the idea that genetic susceptibility for autoimmunity may play a role in PA.

Table 1 shows the literature regarding *H pylori* infection and related gastric histological features in some PA patients<sup>[10,22-24]</sup>. The presence of *H pylori* infection was diagnosed by histology in up to 30% (median 11%), but by serology (IgG) in up to 51% (median 20.5%) of PA patients. It is well known that the diagnosis of *H pylori* infection may be difficult in patients with ABG. *H pylori* may disappear over time due to the hostile gastric microenvironment, and past infection may be demonstrated by serological positivity to *H pylori* in a large majority of patients with ABG or PA<sup>[10,25-27]</sup>. A recent study has reported that seropositivity against *H pylori* antigens may be demonstrated in a very high percentage of patients with ABG by using *ad hoc* immunoblotting<sup>[28]</sup>: in this study 47.8% of ABG patients had PA and all but two of them presented with seropositivity against *H pylori* antigens, including CagA and VacA. As far as regards histological features of the gastric body, in the vast majority of PA patients (> 70%) this disorder is associated with severe body atrophy and the presence of intestinal metaplasia. From data reported in Table 1, another interesting observation emerges: irrespective of the presence of *H pylori* infection, in about half of PA patients, the gastric antrum is involved, and about one-third have antral atrophic gastritis, whose presence is strongly related to *H pylori* infection<sup>[9]</sup>. This observation challenges the widely accepted notion that PA occurs exclusively in association with the classical histological feature of corpus-restricted atrophic gastritis. All these data taken together support the idea that long-standing *H pylori* infection probably plays an important role in many genetically susceptible PA patients. In these patients, the active infectious process has been gradually replaced by an autoimmune process directed by autoreactive gastric CD4<sup>+</sup> T cells that recognize H<sup>+</sup>/K<sup>+</sup>-ATPase and *H pylori* antigens, which ends in a burned-out infection and the irreversible destruction of the gastric body mucosa. The failure to demonstrate *H pylori* infection in some of these individuals does not necessarily argue against the role of the bacterium in

Table 1 *H pylori* infection and related gastric histological features in a series of PA patients *n* (%)

First author/ publication year <sup>[Ref.]</sup>	No. of patients	Mean age (yr)	F:M ratio	Geographical origin	Severe body atrophy	Body intestinal metaplasia	Antral inflammation	Antral atrophic gastritis	Positive <i>H pylori</i> histology	Positive <i>H pylori</i> serology
Fong TL/1991 <sup>[22]</sup>	28	59	1.2:0.8	USA <sup>1</sup>	ND	18 (64)	14 (50)	2 (7)	3 (11)	2 (7)
Haruma K/1995 <sup>[23]</sup>	24	65	0.9:1.1	Japan	24 (100)	18 (75)	22 (92)	17 (71)	0 (0)	0 (0)
Sari R/2000 <sup>[24]</sup>	30	60	0.9:1.1	Turkey	15 (50)	13 (43)	14 (47)	8 (27)	12 (30)	ND
Annibale B/2000 <sup>[10]</sup>	81	62	0.9:1.1	Italy	56 (69)	70 (86)	43 (53)	27 (30)	8 (10)	41 (51)
Annibale B/ 2009 <sup>[unpublished data]</sup>	177	60	1:1	Italy	124 (70)	161 (91)	81 (46)	40 (23)	19 (11)	61 (34)

<sup>1</sup>Hispanic, *n* = 20; white, *n* = 3; black, *n* = 5. ND: Not done.

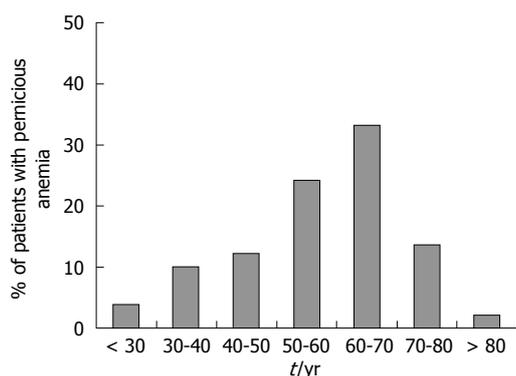


Figure 1 Age cohorts of a series of patients with PA (*n* = 177) consecutively diagnosed between 1992 and 2005 at an academic gastroenterology unit.

these patients, but more likely indicates that a point of no return may be reached beyond which the autoimmune process may no longer require the continued presence of the inducing pathogen<sup>[29]</sup>.

PA is frequently described as a disease of adults > 60 years of age<sup>[8,30,51]</sup>. Among our unpublished series of 177 PA patients, about one half were < 60 years of age; in particular, 4% of patients were < 30 years and 10% were 30-40 years of age (Figure 1). Table 1 shows that the mean age of PA patients in published studies ranges from 59 to 62 years. These data challenge the common notion that PA is an exclusive disease of the elderly, and suggest that, in clinical practice, PA is probably under-diagnosed in elderly and younger patients<sup>[32]</sup>. Stratification by age cohorts (< 20 years to > 60 years) of ABG patients identified by hypergastrinemia and positive parietal cell antibodies has shown a regular and progressive increase in mean corpuscular volume and levels of ferritin and gastrin, and a decrease in vitamin B<sub>12</sub> levels. However, the prevalence of *H pylori* infection has decreased from > 80% at age < 20 years to 12.5% at > 60 years<sup>[32]</sup>. This reminds us that: (1) iron deficiency is a complication of achlorhydria and may precede the development of PA<sup>[4]</sup>; (2) ABG patients frequently present with iron deficiency anemia<sup>[33-35]</sup>; and (3) iron deficiency may be present concomitantly with PA<sup>[36]</sup>. These findings further support the idea that PA seems to be a long-duration disease that is related to *H pylori*, gastric achlorhydria and atrophy, which begins many years before the establishment of clinical vitamin B<sub>12</sub> deficiency.

## CLINICAL PRESENTATION OF PA

The clinical presentation of PA is often insidious for various reasons. The onset and progression of PA are very slow. As a consequence, patients often are not aware of their symptoms related to anemia, because over time they have become used to them. In many such cases, the underlying disease may not be suspected until a complete red blood count has been performed. However, patients with PA may seek medical advice due to non-specific symptoms related to the presence of anemia *per se*, such as weakness, asthenia, decreased mental concentration, headache, and especially, in elderly patients, cardiological symptoms such as palpitations and chest pain<sup>[3,6]</sup>. Less frequently, patients with PA may present only with neurological symptoms, such as paresthesia, unsteady gait, clumsiness, and in some cases, spasticity. Indeed, vitamin B<sub>12</sub> deficiency may cause peripheral neuropathy and lesions in the posterior and lateral columns of the spinal cord (subacute combined degeneration) and in the cerebrum, and these lesions progress from demyelination to axonal degeneration and eventual neuronal death. It is particularly important to recognize these symptoms early, because the neurological lesions may not be reversed after replacement therapy with vitamin B<sub>12</sub><sup>[3,5]</sup>. Finally, the onset of PA may be observed in patients undergoing medical treatment for other autoimmune conditions frequently associated with PA, such as ATD, type 1 diabetes, and vitiligo, as part of the autoimmune polyendocrine syndromes<sup>[37]</sup>.

Although the primary cause of PA is ABG, rarely the disease may result from gastrointestinal tract symptoms. The reason for the apparent paradox may lie in the fact that ABG is associated with hypochlorhydria, and symptoms of the upper gastrointestinal tract are often related to the presence of chlorhydric acid. However, hypochlorhydria itself may cause impaired gastric emptying, which eventually leads to dyspeptic symptoms such as epigastric discomfort, postprandial bloating and fullness, and early satiety<sup>[38]</sup>. In our experience, awareness and concern about upper gastrointestinal or neurological symptoms often are not sufficient to seek medical advice, since patients over time become used to these slowly and insidiously presenting complaints. Only 3% of PA patients presented directly to our gastroenterology unit for long-standing dyspepsia, and only 3% were referred from a neurologist. At the time of diagnosis of

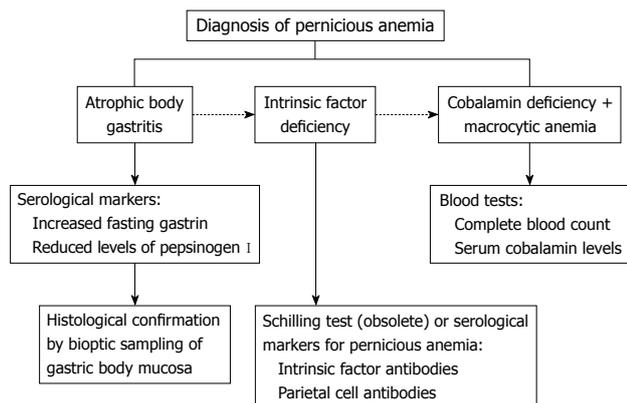


Figure 2 Diagnostic flow-chart for PA.

PA, dyspeptic symptoms were complained of by 28% of patients, and neurological symptoms were present in 19% (unpublished data).

An increased association of PA with other autoimmune diseases, such as type 1 diabetes (3%-4%)<sup>[39]</sup>, vitiligo (2%-8%)<sup>[3]</sup>, and in particular, ATD (3%-32%)<sup>[40]</sup> has been reported. Among our unpublished series of 177 PA patients, 41% had associated ATD and 10% presented with vitiligo or alopecia, which indicates that a subgroup of PA patients can be considered as having a type II autoimmune polyendocrine syndrome. In a recent study, we have observed that ABG and ATD occur in a closely linked fashion, with ATD being present in about 40% of ABG patients<sup>[41]</sup>. These data suggest that, in patients with autoimmune disorders, in particular ATD, a possible association with PA should be suspected and excluded. The diagnosis of concomitant autoimmune thyroiditis and PA may have an important clinical implication, in particular, in those patients who require replacement therapy with thyroxine. Recently, it has been reported that patients with impaired acid secretion may present with thyroxine malabsorption that requires an increased dose of the drug<sup>[42]</sup>, and in patients with PA, associated hypochlorhydria is always present, due to the loss of oxyntic mucosa<sup>[4]</sup>.

Useful information about which patients may have PA can be derived also from epidemiological data. According to the older literature, PA is thought to be particularly common among individuals of Scandinavian, English or Irish ancestry, whereas it appears to be much less common in Caucasians of Italian or Greek origin<sup>[43]</sup>. However, more recently, the disease has been reported in black and Latin American subjects<sup>[30,44]</sup>, and as shown in Table 1, series of PA patients have been diagnosed in the United States, Turkey and Italy, and even in Japan. The reason for the different distribution of PA among different ethnical groups is not known yet, but probably lies in their different genetic background, and in different awareness and diagnostic accuracy for this often overlooked disorder. In the so-called high-risk groups, about nine new cases are detected per 100 000 population per year, and about 0.13% of the population is affected<sup>[31]</sup>. A more recent population survey has reported that 1.9% of persons aged > 60 years have undiagnosed PA<sup>[30]</sup>.

A female preponderance ranging from 1.7 to 2.0:1 has been reported in white subjects<sup>[3]</sup>. This sex distribution has been confirmed in the more recent population survey of persons > 60 years old that was conducted in California, in which the prevalence of PA was 2.7% in women and 1.4% in men<sup>[30]</sup>. However, data reported in Table 1 concerning United States, Japanese, Turkish and Italian PA patients seem not to confirm the female preponderance described in older studies.

## DIAGNOSIS OF PA

PA is defined as the presence of a hemoglobin concentration < 13 g/dL for men and < 12 g/dL for women<sup>[45]</sup>, mean corpuscular volume  $\geq$  100 fL<sup>[5]</sup>, low levels of cobalamin (vitamin B<sub>12</sub>)<sup>[5]</sup>, together with the concomitant presence of ABG and intrinsic factor deficiency (Figure 2). By definition, PA is associated with ABG, and strict diagnostic criteria for ABG are based on the histological confirmation of gastric body mucosal atrophy and enterochromaffin-like (ECL) cell hyperplasia, associated with hypochlorhydria to pentagastrin stimulation<sup>[4]</sup>. Increased levels of fasting gastrin and decreased levels of pepsinogen I are well accepted serological markers<sup>[46,47]</sup>, which suggest the presence of oxyntic mucosa damage, which should be confirmed, however, by appropriate histological sampling of gastric body mucosa to diagnose ABG definitively.

As far as regards gastric mucosa histology, classical findings associated with PA are the presence of corpus-restricted atrophy with a spared antrum, as well as the presence of hyperplasia of ECL cells<sup>[4,6]</sup>. As shown in Table 1, in about 50% of PA patients, antral mucosa is not spared, and in about 27% of PA patients, a concomitant antral atrophic gastritis may be observed. These data strongly suggest that an extension of gastritis to the gastric antrum does not necessarily exclude the diagnosis of PA and the presence of gastric autoimmunity. The determination of ECL cell hyperplasia is helpful in the histological diagnosis of ABG, because the presence of this histological change may be considered an indirect confirmation of the presence of hypochlorhydria. This leads to hypergastrinemia, which in turn, is a trophic factor for ECL cells that leads to their hyperplasia, and eventually, to the development of gastric carcinoids<sup>[48]</sup>.

Intrinsic factor deficiency can be proven by the now obsolete Schilling test. To confirm that the cobalamin deficiency is the result of intestinal malabsorption due to intrinsic factor deficiency, urinary excretion of orally administered vitamin B<sub>12</sub> is low, and is increased by administration of vitamin B<sub>12</sub> and intrinsic factor. Unfortunately, the availability of this test is vanishing due to problems related to its radioactive reagents. Therefore, in clinical practice, the presence of intrinsic factor deficiency may not be proven, and increasing reliance is placed on the detection of intrinsic factor antibodies for the diagnosis of PA, which are viewed as useful markers of this disease<sup>[49]</sup>. Earlier studies have reported positivity for intrinsic factor antibodies in 40%-60% of patients with PA<sup>[50,51]</sup>, which rises to 60%-80% with increasing duration of disease<sup>[52]</sup>.

Table 2 Differential diagnosis of PA: other causes of macrocytic anemia and cobalamin deficiency

Other causes of macrocytic anemia	Other causes of cobalamin deficiency
Folate deficiency due to decreased intake, impaired absorption or increased requirements	Gastric causes of impaired absorption/mal-digestion:
Drugs (e.g. methotrexate, azathioprine, 6-mercaptopurine, acyclovir, 5-fluorouracil, phenobarbital)	Gastrectomy
Accelerated erythropoiesis: hemolytic anemia, response to hemorrhage	Corpus-predominant <i>H pylori</i> gastritis
Liver disease (alcoholic, advanced cirrhosis, poor dietary intake)	Long-term proton pump inhibitor therapy
Hypoplastic anemia, myelodysplastic syndrome	Intestinal causes of impaired absorption:
Chronic obstructive pulmonary disease	Ileal disease or resection
	Blind loop syndrome
	Fish tapeworm
	Severe pancreatic insufficiency
	Decreased intake due to vegetarianism

Recently, we reassessed the diagnostic performance of intrinsic factor and parietal cell antibodies in PA patients by using a novel ELISA<sup>[48]</sup>, which yielded for intrinsic factor antibodies, a sensitivity and specificity of 37% and 100%, respectively, and for parietal cell antibodies, a sensitivity and specificity of 81.5% and 90.3%, respectively. The combined assessment of both autoantibodies significantly increased their diagnostic performance, which yielded 73% sensitivity for PA, while maintaining 100% specificity. Thus, our data show that, by combining the assessment of intrinsic factor and parietal cell autoantibodies, the diagnostic performance of these surrogate markers for PA may be notably improved. Beyond being a specific hallmark of PA, the positivity for intrinsic factor and parietal cell antibodies may be interpreted as an expression of oxyntic mucosal damage, because a positive correlation between the increasing histological score of body mucosa atrophy and the titer of both antibodies can be observed<sup>[27,35]</sup>.

Accurate differential diagnosis of other causes of cobalamin deficiency is mandatory. As shown in Table 2, cobalamin deficiency may result from other causes of impaired absorption in the stomach or intestine, or by decreased intake due to vegetarianism. Among cases of mal-digestion, there are very rare cases related to severe pancreatic insufficiency, but more interesting is the recent evidence of mal-digestion of dietary cobalamin in patients with corpus-predominant *H pylori* gastritis, which leads to impaired acid secretion and consequent increased intragastric pH<sup>[53,54]</sup>. In fact, dietary cobalamin is bound to salivary proteins, which needs to be cleaved in the presence of chlorhydric acid before it can be bound to intrinsic factor and be absorbed in the terminal ileum<sup>[4]</sup>. In these cases of mal-digestion of dietary cobalamin, the Schilling test would be normal, which indicates that cobalamin deficiency is not due to intrinsic factor deficiency. Without performing a Schilling test, it may be challenging to discriminate between the presence of PA and mal-digestion of dietary cobalamin. However, from a practical point of view, the clinical management of these two groups of patients is similar. As observed<sup>[54,55]</sup>, when atrophy of the gastric body mucosa is mild and active *H pylori* infection is present, in patients with mal-digestion of dietary cobalamin, a reversal of body mucosal atrophy, anemia and cobalamin deficiency following eradication treatment may be achieved. An accurate differential diagnosis should be carried out also for macrocytic anemia,

which may underlie other causes such as folate deficiency and myelodysplastic syndrome (Table 2).

In this context, it should be kept in mind that, in order to diagnose vitamin B<sub>12</sub> deficiency, total vitamin B<sub>12</sub> measurement is used cost-effectively as the parameter of choice, but it has limited sensitivity and specificity, especially in persons with vitamin B<sub>12</sub> concentrations in the lower reference range (< 400 pmol/L). As an alternative, modern biomarkers for early diagnosis of vitamin B<sub>12</sub> deficiency, such as holotranscobalamin, also known as active B<sub>12</sub>, and methyl malonic acid as a functional B<sub>12</sub> marker, have been proposed<sup>[56]</sup>. Figure 2 shows a proposed diagnostic work-up when the presence of PA is suspected.

## CLINICAL MANAGEMENT OF PATIENTS WITH PA

The clinical management of patients with PA has two different aspects: firstly, the treatment of cobalamin deficiency and the monitoring of onset of iron deficiency; and secondly, the surveillance of these patients, to detect early the long-term consequences of PA, such as gastric cancer and carcinoids.

### Treatment of cobalamin deficiency and monitoring of iron deficiency

Cobalamin replacement treatment is able to correct the anemia, whereas the neurological complications may be corrected only if the replacement treatment is given soon after their onset. The therapeutic recommendations for PA with regard to dosage and administration of vitamin B<sub>12</sub> substitution treatment are divergent<sup>[57]</sup>. In the United States, patients usually receive vitamin B<sub>12</sub> injections of 1 mg/d in their first week of treatment; in the following month, they receive weekly injections and then monthly injections<sup>[58]</sup>. In Denmark, patients receive injections of 1 mg/wk cyanocobalamin during the first month and every 3 mo subsequently, or 1 mg hydroxycobalamin every other month<sup>[59]</sup>. According to our protocol, a higher dosage of cobalamin is used to prevent early relapse of cobalamin deficiency: firstly patients receive intramuscular injection of 5 mg/d cyanocobalamin for 5 d, which replenishes the cobalamin body stores; then, vitamin B<sub>12</sub> stores are maintained by intramuscular injection of 5 mg cyanocobalamin every 3 mo.

Furthermore, according to our protocol, PA patients are monitored at least yearly by complete blood count, and serum cobalamin and ferritin levels, to monitor the replacement treatment and to detect early the eventual onset of iron deficiency. Also patients with ABG with iron deficiency anemia or without hematological alterations are monitored in the same way, to detect early the eventual onset of cobalamin deficiency. Finally, PA patients are monitored by at least a yearly clinical interview, to verify the onset of new symptoms that are suspicious of long-term consequences of PA, such as dysphagia, epigastric pain, dyspeptic symptoms, loss of body weight, and/or iron-deficiency, which require immediate gastroscopic investigation.

### Long-term consequences of PA

Although PA is substantially a benign disorder for a large number of patients, it is epidemiologically and biologically linked to the development of intestinal-type gastric adenocarcinoma and gastric carcinoid type I<sup>[60,61]</sup>. Hypergastrinemia, secondary to hypochlorhydria in PA patients, is a well-known risk factor for ECL cell hyperplasia and gastric carcinoids<sup>[62,63]</sup>, and it has been reported that one in 25 patients with PA develops gastric carcinoids<sup>[64]</sup>. Moreover, the crucial role of hypochlorhydria, as a consequence of atrophy of the oxyntic mucosa, in the development of gastric cancer, has been highlighted<sup>[65]</sup>. Hypochlorhydria leads to overgrowth of nitrosamine-producing bacteria with potential carcinogen activity<sup>[66]</sup>. Also ascorbic acid, the main redox agent in the gastric juice with protective action against reactive oxygen species, is reduced in the presence of atrophy of the oxyntic mucosa. It has been described previously that the level of ascorbic acid in the gastric juice is reduced in patients with ABG, with an indirect correlation between ascorbic acid level and intragastric pH<sup>[67]</sup>.

In the literature, the annual incidence of gastric cancer in PA patients ranges from 0.1% to 0.5%<sup>[62,64,68]</sup>. A recent follow-up study of patients with ABG has reported an annual incidence risk of 0.14% for developing gastric cancer, during an observation period of 6.7 years<sup>[69]</sup>. To date, the need and cost-effectiveness of endoscopic/histological surveillance in patients with PA have not been established definitively<sup>[4]</sup>. One previous study<sup>[64]</sup> that has considered the relatively benign nature of gastric carcinoids in patients with PA has concluded that follow-up is indicated at 5-year intervals only in patients with ECL hyperplasia. As for gastric cancer, the same authors have concluded that the first gastroscopic follow-up after diagnosis of PA should be performed relatively soon, and that only PA patients with preneoplastic lesions and those with gastrointestinal symptoms should undergo endoscopic surveillance<sup>[64]</sup>. Another study has concluded that follow-up should be performed at 3-year intervals only in PA patients aged < 60 years<sup>[70]</sup>. A more recent study has compared the usefulness of 2- and 4-year follow-up in patients with ABG, and has shown that the first follow-up performed 4 years after the diagnosis seems to be safe and convenient for early detection of potentially neoplastic lesions<sup>[71]</sup>. As a result of the lack

of other prospective data, and considering the risk for developing neoplastic lesions over time in some PA patients, in our unit, PA patients are monitored regularly by gastroscopy with antral and corporal biopsies at 4-year intervals.

## CONCLUSION

PA is an often silent and under-diagnosed autoimmune disease, because its onset and progression are very slow and patients may become used to their complaints. Nevertheless, the clinical consequences of undiagnosed PA may be serious, including gastric neoplastic lesions. Thus, gastroenterologists should increase their awareness of this disorder, whose definite histological diagnosis may be preceded by reliable noninvasive serological screening.

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## Costimulatory molecule programmed death-1 in the cytotoxic response during chronic hepatitis C

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### Abstract

Hepatitis C virus (HCV)-specific CD8<sup>+</sup> T cells play an important role in the resolution of HCV infection. Nevertheless, during chronic hepatitis C these cells lack their effector functions and fail to control the virus. HCV has developed several mechanisms to escape immune control. One of these strategies is the up-regulation of negative co-stimulatory molecules such as programmed death-1 (PD-1). This molecule is up-regulated on intrahepatic and peripheral HCV-specific cytotoxic T cells during acute and chronic phases of the disease, whereas PD-1 expression is low in resolved infection. PD-1 expressing HCV-specific CD8<sup>+</sup> T cells are exhausted with impairment of several effector mechanisms, such as: type-1 cytokine production, expansion ability after antigen encounter and cytotoxic ability. However, PD-1 associated exhaustion can be restored by blocking the interaction between PD-1 and its ligand (PD-L1). After this blockade, HCV-specific

CD8<sup>+</sup> T cells reacquire their functionality. Nevertheless, functional restoration depends on PD-1 expression level. High PD-1-expressing intrahepatic HCV-specific CD8<sup>+</sup> T cells do not restore their effector abilities after PD-1/PD-L1 blockade. The mechanisms by which HCV is able to induce PD-1 up-regulation to escape immune control are unknown. Persistent TCR stimulation by a high level of HCV antigens could favour early PD-1 induction, but the interaction between HCV core protein and gC1q receptor could also participate in this process. The PD-1/PD-L1 pathway modulation could be a therapeutic strategy, in conjunction with the regulation of others co-stimulatory pathways, in order to restore immune response against HCV to succeed in clearing the infection.

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**Key words:** Chronic hepatitis; Exhaustion; Hepatitis C virus core; Hepatitis C virus; Programmed death-1; Programmed death-1 ligand

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### INTRODUCTION

Hepatitis C virus (HCV) is a hepatotropic non-cytopathic positive-strand RNA virus which belongs to the *Flaviviridae* family. HCV infection is a major public problem, affecting more than 200 million people worldwide<sup>[1]</sup>. Only around a quarter of acute HCV infections resolve within a few months, while in the majority of cases the virus establishes a persistent infection, and a significant proportion of cases progress to fibrosis, cirrhosis, liver failure or even hepatocellular carcinoma<sup>[2-4]</sup>. Nowadays standard

anti-HCV therapy resolves about 50% of chronic infections<sup>[5-7]</sup>, therefore new therapeutic strategies should be designed to control this disease. HCV-specific cytotoxic T lymphocytes (CTLs) play a major role in viral control during acute infection<sup>[8]</sup>. Nevertheless, during persistent infection HCV-specific CTL effector functions are significantly impaired, and this situation is a major cause of host inability to eliminate the persistent virus<sup>[9,10]</sup>. Appropriate activation of primed virus-specific CTLs in the infected site depends on the engagement between T cell receptor (TCR) and HLA-I/epitope complex plus interaction among positive co-stimulatory molecules and their ligands<sup>[11,12]</sup>. Virus-specific CTLs, after developing their effector function, express negative co-stimulatory molecules to switch-off their activity. The appropriate virus-specific CTL response development correlates with the adequate balance between positive and negative co-stimulatory signals (Table 1)<sup>[13,14]</sup>. Programmed death-1 (PD-1) is one of the negative co-stimulatory molecules. Engagement of PD-1 and its ligand (PD-L1) delivers a negative signal to the TCR activation pathway, avoiding proliferation, and interleukin (IL)-2 production, which leads to T cell anergy<sup>[15,16]</sup>. Evidence that PD-1 suppresses activation of the immune response comes from studies in which mice deficient in PD-1 developed autoimmune diseases, such as systemic lupus erythematosus, dilated cardiomyopathy, rheumatoid arthritis and type I diabetes mellitus, due to the uncontrolled persistent T cell activation against different epitopes<sup>[17,18]</sup>. The PD-1 induced exhaustion on virus-specific T cells was first described by Barber *et al.*<sup>[19]</sup>, in a murine model of lymphocytic choriomeningitis virus (LCMV) infection. The authors demonstrated that the majority of LCMV-specific CD8<sup>+</sup> T cells were anergic during the chronic phase of infection in association with PD-1 up-regulation. Mice treated with anti-PD-L1 monoclonal antibodies restored the LCMV-specific cytotoxic response and facilitated viral control. These experimental data suggested that the PD-1/PD-L1 pathway could play a major role in the development of persistent infections by non-cytopathic viruses, and different groups started to research the role of this pathway in different chronic viral infections in humans, such as hepatitis B virus (HBV), HCV and human immunodeficiency virus (HIV) infections. Bearing in mind these data, the PD-1/PD-L1 pathway could be an effective escape mechanism and its blockade could be a therapeutic target to reverse T-cell dysfunction. In this editorial, the current state of knowledge about the role of PD-1 expression on specific cytotoxic responses during HCV infection is reviewed.

## STRUCTURE AND EXPRESSION OF PD-1 AND PD-L1

PD-1 is a 55 kDa glycoprotein which belongs to the CD28 immunoglobulin superfamily of transmembrane proteins<sup>[20]</sup>. PD-1 shares a 23% homology with CTLA-4, which is another member of this family, although PD-1 has lost the MYPPPY motif for binding to B7 molecules<sup>[20]</sup>, and the cysteine residue necessary for

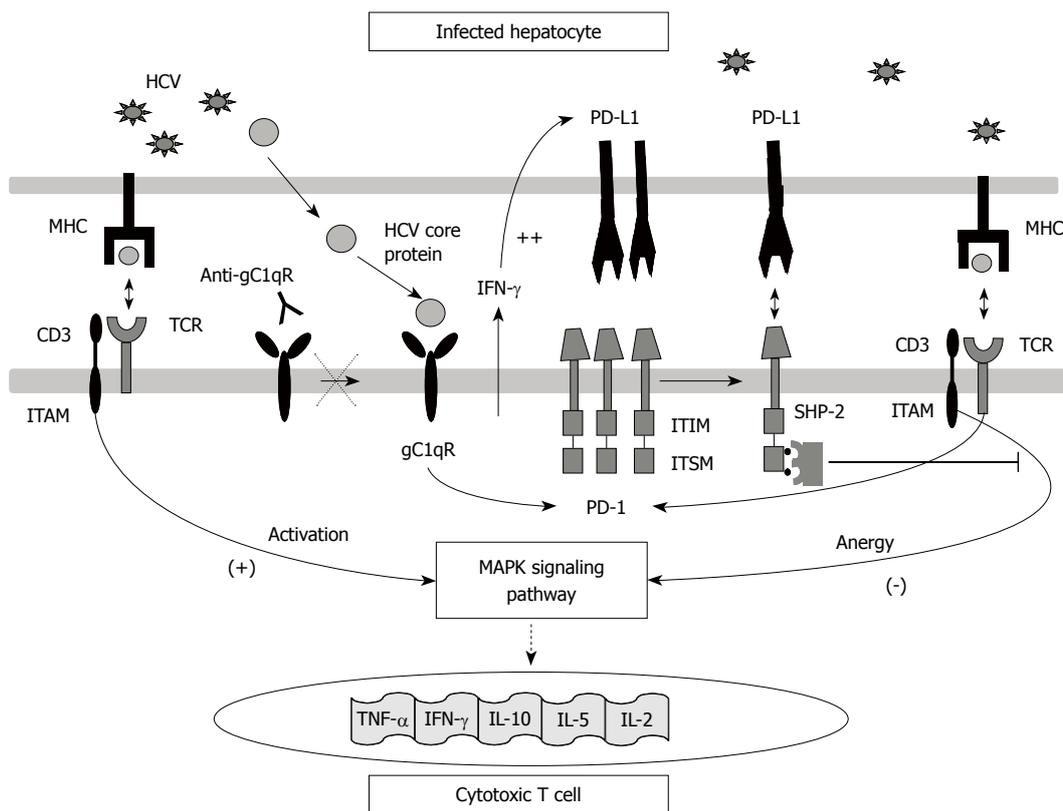
homodimerization<sup>[21]</sup>. PD-1 is expressed on activated T cells, B cells and myeloid cells (Table 1)<sup>[20]</sup>. The PD-1 structure consists of two regions; the extracellular region is formed by a single IgV-like domain and its cytoplasmic region contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM)<sup>[22,23]</sup>. Following antigen stimulation, PD-1 recruits the protein tyrosine phosphatase src homology 2 domain-containing tyrosine phosphatase 2 (SHP-2) to ITSM but not to the ITIM motif, and subsequently, SHP-2 dephosphorylates effector molecules downstream of the TCR-induced nuclear regulatory pathway<sup>[24,25]</sup>. The direct result of PD-1 mediated inhibition of T cell proliferation is cell cycle arrest in G0/G1 and the inhibition of IL-2 production<sup>[15,16]</sup> (Figure 1). The ligands for PD-1 (PD-L1 and PD-L2) are type I transmembrane proteins with IgV and IgC-like domains in the extracellular region<sup>[26,27]</sup>. PD-L1 is expressed on resting and activated B and T cells, and on non-lymphoid cells such as pancreas, placenta and heart, while PD-L2 is induced on dendritic cells (DC) and macrophages<sup>[26-30]</sup> (Table 1). Interestingly, PD-L1 can be up-regulated on hepatocytes by  $\alpha$ -interferon (IFN) and  $\gamma$ -IFN, and also by activated lymphocytes, and by direct viral infection (perhaps also through IFN pathways)<sup>[31-33]</sup>. PD-1 plays an important physiological role in regulating the cellular immune response, tuning-down the cellular effector functions after T cells have developed their tasks. This physiological function of PD-1 can be damaged by persistent viruses inducing a tolerogenic-like status on specific T cells to avoid immune viral control.

## PD-1 EXPRESSION IN THE LIVER

The liver is characterised by being an immunotolerant organ prepared to deal with intense contact with antigens from the gut, and PD-1/PD-L1 is expressed in resident and infiltrating liver cells to carry out this task<sup>[34]</sup>. The liver is also the primary site for HCV replication and disease pathogenesis<sup>[35]</sup>, and HCV can take advantage of the PD-1/PD-L1 pathway to impair the HCV-specific response reaching the infected liver in order to escape immune control. The liver is exposed to antigens and microbiologically-derived molecules which cause a unique microenvironment that requires liver immunological properties to induce tolerance rather than immunity<sup>[36-38]</sup>. Hepatic tolerance contributes to the common ineffectiveness of immune response against HCV which often results in chronic viral persistence<sup>[39]</sup>. When naive T cells reach the liver from the bloodstream they are activated by resident antigen presenting cells and are prone to become anergic, and this process could take part in the interaction between PD-1 and PD-L1 (Figure 2)<sup>[34,40]</sup>. On the other hand, primed effector HCV-specific T cells, reaching the infected liver, are also conducted through energy by several mechanisms. One of them is PD-1 up-regulation on T cells in the liver<sup>[41,42]</sup>, and the expression of its ligand on resident liver cells, such as hepatocytes, Kupffer cells and sinusoidal endothelial cells (Figure 2)<sup>[31]</sup>. Usually, PD-L1 is constitutively expressed in non-lymphoid tissues such as heart,

Table 1 Summary of CD28 family co-stimulatory and co-inhibitory pathways

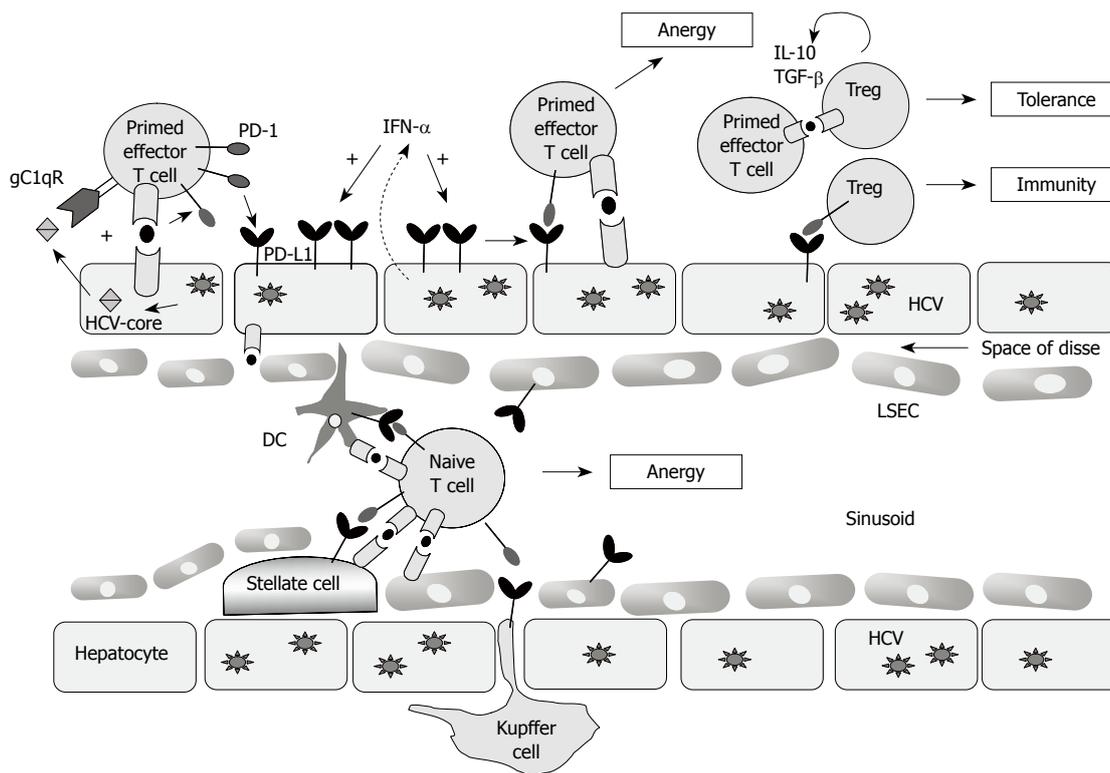
Receptor	Expression	Ligand	Ligand expression	T cell response regulation
CD28	T cells (naive and some memory)	CD80	B, T and DC, macrophages	Positive
PD-1	Activate T cells, B cells, macrophages	PD-L1	B, T and DC, macrophages, non-lymphoid cells	Negative
		PD-L2	(pancreas, placenta, heart)	
CTLA-4	Activate T cells and regulatory T cells	CD80	B, T and DC, macrophages	Negative
		CD86		
BTLA	B and T cells	PD-L2	Macrophages, DC	Negative
		B7-H3	T and B cells, NK	
		B7-H4		
ICOS	T cells (memory and effector)	ICOS-L	B, T cells, macrophages and DC	Positive



**Figure 1 Programmed death-1 (PD-1) structure and interactions.** Interaction between the PD-1 molecule expressed on T cells and its ligand PD-L1 expressed on antigen-presenting cells leads to immunoreceptor tyrosine-based switch motif (ITSM) motif phosphorylation in its cytoplasmic tyrosines which are recognized by src homology 2 domain-containing tyrosine phosphatase 2 (SHP-2). All of these interactions cause T cell anergy due to T cell receptor (TCR)-dependent MAP Kinase-pathway signalling inhibition which avoids interleukin (IL)-2 gene transduction. PD-1 expression is induced by TCR activation but could also be favoured by HCV-core protein through interaction with gC1qR. PD-L1 is up-regulated on antigen presenting cells by the effect of  $\gamma$ -interferon produced during HCV infection by activated lymphocytes.

lung, placenta, kidney, and liver<sup>[43-45]</sup>, but during chronic HCV infection, this molecule is up-regulated on parenchymal liver cells, as previously commented (Figure 3A). The regulation of HCV-specific effector CTLs is also controlled by intrahepatic CD4+CD25+FoxP3+ cells (regulatory T cells, Treg). These cells have an important role in maintaining the balance between tolerance and immunity in HCV infection<sup>[46-48]</sup>. The PD-1/PD-L1 pathway is also important in modulating the regulatory activity of these Treg cells. The PD-1/PD-L1 interaction on intrahepatic Treg suppresses their regulatory activity, favouring CTL response<sup>[49-51]</sup>. Nevertheless, when it is necessary to down-modulate HCV-specific CTL response in order to avoid liver damage, PD-1/PD-L1 engagement is not pro-

duced between Tregs and intrahepatic PD-L1 expressing cells, due to PD-L1 down-regulation on resident liver cells, allowing Tregs to down-modulate HCV-specific CTLs effector functions (Figure 2)<sup>[52]</sup>. The important role of PD-1 in liver pathogenesis during HCV chronic infection is evident, as it is shown by the high PD-1 expression on total intrahepatic T cells<sup>[53-56]</sup>, indicating that some non-specific HCV-dependent stimulus is acting in liver infiltrating T cells to favour PD-1 up-regulation. Previous reports suggest that this factor could be HCV-core protein and this will be discussed later. In addition to this non-specific stimulation on T cells, PD-1 expression is also induced by persistent specific TCR stimulation. PD-1 expression is higher on intrahepatic than in peripheral HCV-specific



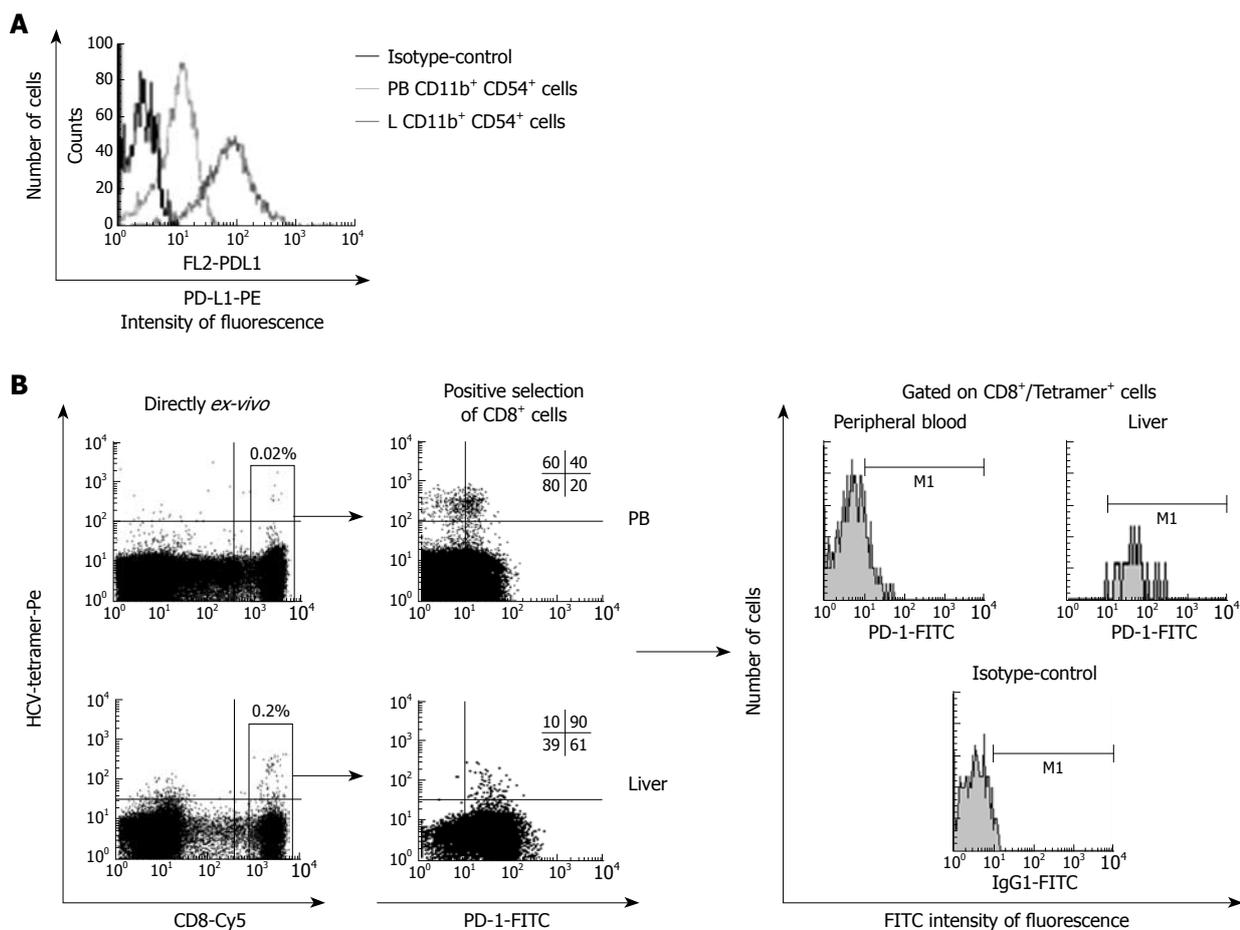
**Figure 2 Liver microenvironment.** Circulating HCV-specific CD8<sup>+</sup> T cells migrating through the hepatic sinusoid interact with resident liver cells [Kupffer cells, dendritic cells (DC), hepatocytes, liver sinusoidal endothelial cells (LSECs), stellate cells] that could act as antigen presenting cells. These cells up-regulate PD-L1 expression during persistent HCV infection and interact with the PD-1 molecule expressed on HCV-specific CD8<sup>+</sup> T cells. This interaction leads to T cell anergy. PD-1 up-regulation is produced by TCR stimulation in addition to the interaction between HCV-core protein and the complement receptor (gC1qR). In this micro-environment the regulatory T cells (Treg) also participate, whose activity is also regulated by the PD-1/PD-L1 pathway.

CD8<sup>+</sup> cells<sup>[41]</sup> (Figure 3B). These data suggest that the intense TCR activation produced in the liver in conjunction with the high level of HCV-core protein leads to the highest PD-1 up-regulation on HCV-specific CD8<sup>+</sup> cells. This high PD-1 up-regulation on intrahepatic specific CD8<sup>+</sup> cells is exquisitely HCV specific, so that PD-1 expression on other virus-specific CD8<sup>+</sup> T cells is not up-regulated during chronic HCV infection<sup>[41]</sup>. Therefore, liver environment conditions produce a huge PD-1 up-regulation on HCV-specific CTLs during persistent infection, and this could impair viral control by the cellular immune response through anergy induction<sup>[41]</sup>.

## DIFFERENTIAL PD-1 EXPRESSION IN ACUTE, CHRONIC AND RESOLVED HEPATITIS C VIRUS INFECTION

During the initial phase of acute infection, HCV-specific CD8<sup>+</sup> T cells are dysfunctional irrespective of the final outcome of the disease, and this impairment persists when infection becomes chronic<sup>[10]</sup>. In contrast, effector and memory CD8<sup>+</sup> T cells generated after acute onset are highly functional in cases of resolving infection<sup>[57,58]</sup>. One of the possible mechanisms responsible for impairment of virus-specific CTL response could be the exhaustion of these cells caused by PD-1 up-regulation. The exhaustion of virus-specific CD8<sup>+</sup> T cells has been observed in

different human infections such as HIV, HBV and HCV infections<sup>[56,59-65]</sup>. In HCV infection, during the early period of primo-infection irrespective of the final outcome, PD-1 is up-regulated on all HCV-specific CD8<sup>+</sup> T cells<sup>[53,66]</sup>. However, after the acute stage of the disease PD-1 expression is modulated depending on the progression. Therefore, during self-limited infection HCV-specific CD8<sup>+</sup> cells down-regulate PD-1 expression, and acquire a CD127<sup>+</sup> phenotype which correlates with appropriate effector functions (Figure 4)<sup>[67]</sup>. CD127 is the IL-7 receptor (IL-7R) which plays an essential role in mature lymphocyte survival through a pathway activated by the interaction with IL-7<sup>[67]</sup>. However, in persistent infection HCV-specific CD8<sup>+</sup> cells remain CD127 negative, and maintain high levels of PD-1 expression<sup>[66,68]</sup> (Figure 4). Therefore, PD-1<sup>+</sup> CD127<sup>-</sup> expressing HCV-specific CD8<sup>+</sup> cells during persistent infection are not only anergic, but also prone to apoptosis after antigen encounter due to the absence of CD127 expression. Furthermore, PD-1 up-regulation on peripheral and intrahepatic HCV-specific CD8<sup>+</sup> cells during the acute and chronic phases of infection is correlated with the apoptosis susceptibility of these cells<sup>[55]</sup>. As a result, the majority of high PD-1 expressing HCV-specific CD8<sup>+</sup> cells could follow an apoptotic process<sup>[69]</sup>, indicating that PD-1 is involved in anergy induction but could also be implicated in specific T cell deletion. Probably, both mechanisms are damaged by HCV infection to escape cellular immune response.

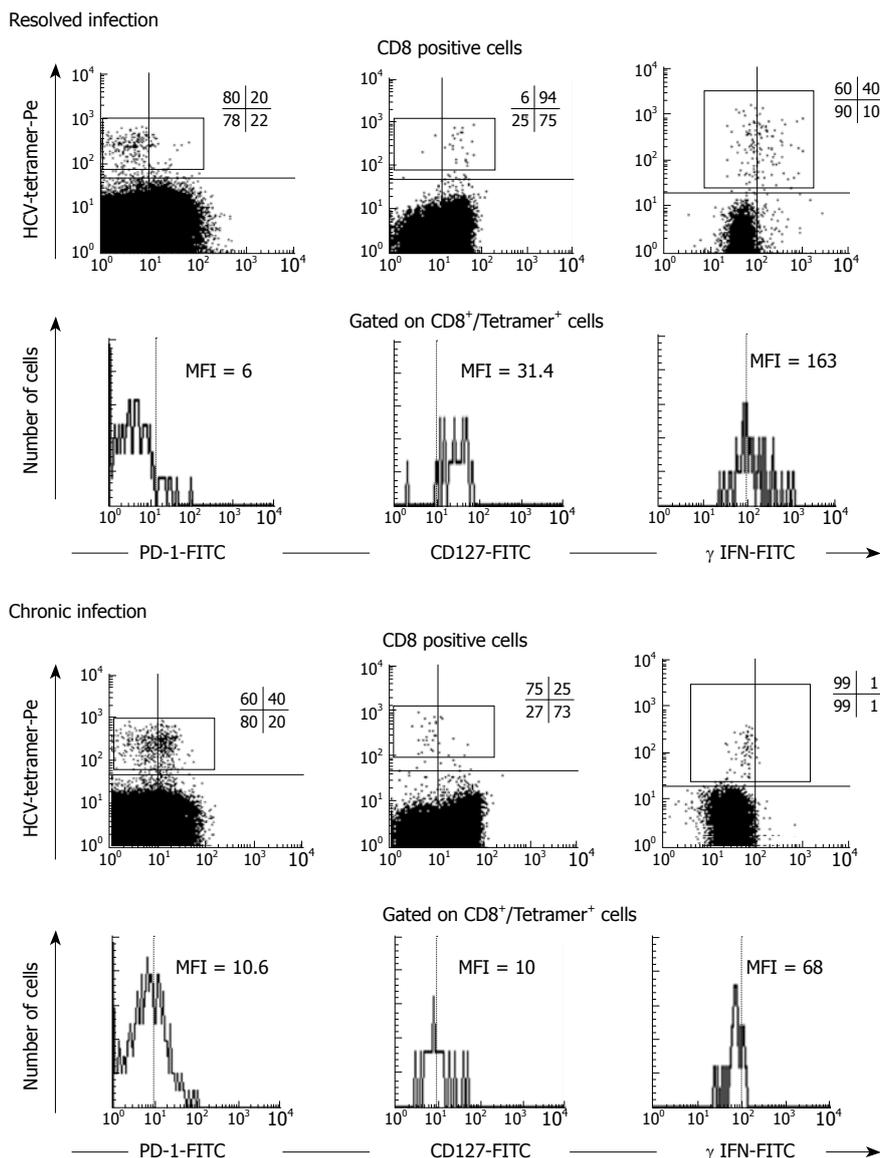


**Figure 3** PD-L1 expression on Kupffer cells and PD-1 expression on HCV-specific CD8<sup>+</sup> T cells. A: PD-L1-FITC FACS<sup>®</sup> histograms gated on CD11b<sup>+</sup> CD54<sup>+</sup> cells from liver (L) and peripheral blood (PB), showing a higher PD-L1 expression on intrahepatic Kupffer cells; B: FACS<sup>®</sup> dot-plots and histograms of peripheral blood and intrahepatic T cells stained with CD8-Cy mAb, HCV-tetramers-PE and PD-1-FITC mAb. Gated Tetramer<sup>+</sup>/CD8<sup>+</sup> cells are presented in the histograms for PD-1-FITC expression. PD-1 is up-regulated on intrahepatic HCV-specific CD8<sup>+</sup> cells.

### CORRELATION BETWEEN PD-1 EXPRESSION AND EFFECTOR FUNCTION IMPAIRMENT ON HCV-SPECIFIC CTLs

Once differential PD-1 expression on HCV-specific CD8<sup>+</sup> T cells between chronic and resolved patients has been described, the next point to address is to analyse whether this difference translates into different quality of HCV-specific CTLs effector functions. Cytotoxic T-cell exhaustion represents a spectrum of effector defects that are correlated with the level of PD-1 expression. Recent reports show that patients with HCV chronic infection, whose CTLs display high PD-1 expression, have impaired CTL capacity to synthesise type-1 cytokines, such as  $\gamma$ -IFN,  $\alpha$ -tumor necrosis factor (TNF) and IL-2, in addition to cytolytic molecules, such as perforin and granzyme B, after direct *ex-vivo* specific *in-vitro* challenge<sup>[41,54]</sup>. One of the variables determining viral control has been suggested to be the ability of virus-specific CD8<sup>+</sup> cells to clonally expand after antigen encounter<sup>[45]</sup>. HCV-specific CD8<sup>+</sup> T cells during persistent infection also displayed impaired proliferation ability after specific stimulation, which correlated with PD-1 expression level<sup>[70,71]</sup>. Because of the role of the

PD-1/PD-L1 pathway in proliferation impairment, subsequent works were aimed at trying to enhance HCV-specific CD8<sup>+</sup> T cell proliferation by modulating this pathway. Blocking the interaction between PD-1 and its ligand increased the proliferation ability of peripheral HCV-specific CD8<sup>+</sup> cells from some chronic HCV patients, characterised by high PD-1 expression, but did not occur in others, suggesting the presence of another anti-proliferative mechanism not yet described<sup>[54,63]</sup>. During HCV-specific CTL exhaustion, not all effector functions are altered at the same time; proliferative potential and IL-2 production are lost at an early phase, whereas cytokine production and cytolytic function are lost later<sup>[72]</sup>. This progressive impairment could be related to the level of PD-1 up-regulation. Interestingly, the exhaustion of CTLs during chronic HCV infection is highly antigen-specific and related to the level of antigenemia, not being present in either CTLs against other specificities or HCV-specific CTLs from patients with resolved infection<sup>[41,66]</sup>. In these two situations PD-1 is not up-regulated on specific CTLs. As commented before, intrahepatic HCV-specific CD8<sup>+</sup> T cells are highly PD-1 positive and they do not expand after antigen encounter and do not produce either  $\gamma$ -IFN or perforin, whereas intrahepatic specific CTLs against other



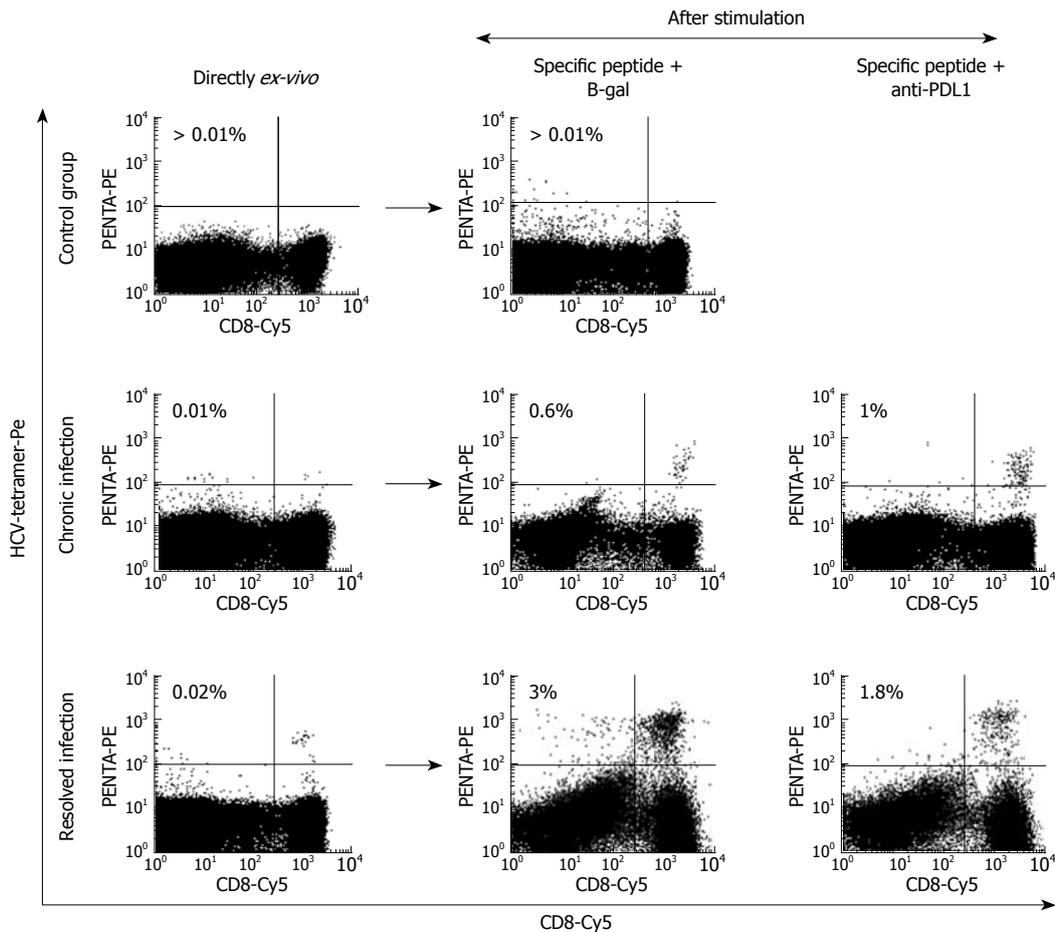
**Figure 4** Direct *ex-vivo* PD-1, CD127 expression and IFN- $\gamma$  production. FACS® dot-plots and histograms of peripheral blood T cells from two representative patients, one with persistent HCV infection and the other with resolved HCV infection. T cells are stained with PD-1-FITC, CD127-FITC,  $\gamma$ -IFN-FITC and CD8-Cy mAbs and HCV-tetramers. In persistent infection, HCV-specific CD8<sup>+</sup> cells maintain a PD-1<sup>+</sup>/CD127<sup>+</sup>/IFN- $\gamma$  phenotype while the resolved infection case displays an opposite phenotype PD-1<sup>-</sup>/CD127<sup>-</sup>/IFN- $\gamma$ <sup>+</sup>.

viruses, such as influenza virus-specific CD8<sup>+</sup> T cells, expand efficiently and present a high level of perforin expression, but interestingly they are PD-1 negative<sup>[41]</sup>. High PD-1 expressing intrahepatic HCV-specific CTLs do not respond to anti-PD-L1 treatment<sup>[41]</sup>. Therefore, when PD-1 expression is extremely up-regulated, treatment with anti-PD-L1 antibodies can not counteract the HCV-specific CTLs exhaustion, induced by the PD-1/PD-L1 pathway.

### HCV-SPECIFIC CTL FUNCTIONAL RESTORATION AFTER PD-1/PD-L1 INTERACTION BLOCKADE

Previous studies developed an LCMV infection animal model, and specific CTL function restoration during persistent infection after treatment with anti-PD-L1 monoclonal antibodies was shown<sup>[19,73]</sup>. This finding could have clinical implications in the treatment of persistent viral infections, as will be discussed later. In HCV infection, the *in-vitro* blockade of the PD-1/

PD-L1 pathway with anti-PDL-1 antibodies increases proliferation capacity after antigen encounter in peripheral PD-1 expressing HCV-specific CTLs from chronic patients (Figure 5). *In-vitro* treatment with anti-PD-L1 antibodies also restored  $\gamma$ -IFN, perforin, CD107a, IL-2 and IL-13 production after antigen specific stimulation<sup>[41,54,74]</sup>. However, this PD-1/PD-L1 pathway blockade is not efficient on intrahepatic HCV-specific CD8<sup>+</sup> T cells, which are characterised by a higher PD-1 expression, as previously discussed. These cells failed to proliferate and produce perforin,  $\gamma$ -IFN and CD107a after specific stimulation in the presence of anti-PD-L1 antibodies<sup>[41]</sup>. All these findings suggest that PD-1 expression level correlates inversely with HCV-specific CD8<sup>+</sup> T cells functional restoration by PD-1/PD-L1 blockade. PD-1/PD-L1 blockade may increase the functionality of peripheral HCV-specific CD8<sup>+</sup> T cells with intermediate PD-1 expression, whereas this blockade did not enhance the effector functions of intrahepatic PD-1<sup>high</sup> expressing HCV-specific CD8<sup>+</sup> T cells. High antigenic stimulation in the liver induces other negative co-stimulatory molecules, such as CTLA-4<sup>[41]</sup>,



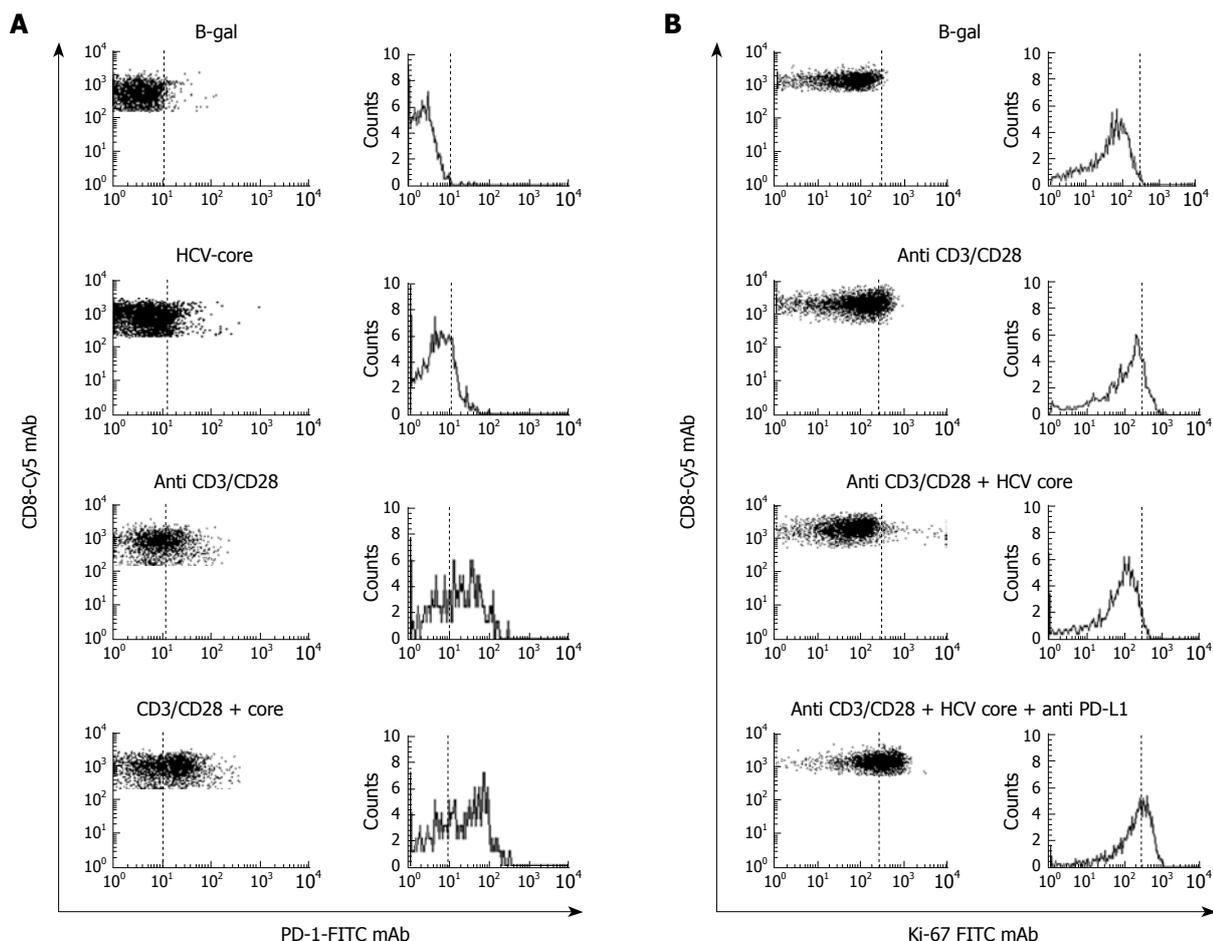
**Figure 5 Proliferation restoration after PD-1 blockade.** FACS® dot-plots of peripheral blood T cells from two representative patients, one with persistent HCV infection and the other with resolved HCV infection, and a control case, after specific stimulation in the presence or absence of anti-PD-L1 mAb. T cells were stimulated for 10 d with the HCV-specific peptide plus IL-2. After stimulation, T cells were stained with CD8-Cy mAbs and HCV-tetramers-PE. PD-1/PD-L1 pathway blockade by anti-PD-L1 antibodies increases the HCV-specific cell proliferation in the chronic patient that had a high level of PD-1 expression.

which could maintain the anergic status, despite blockage of the PD-1/PD-L1 pathway. Therefore, the functional restoration of intrahepatic HCV-specific CTLs could be obtained by the combined blocking of different negative co-stimulatory molecules. In fact, a previous report has shown that combined PD-1 and CTLA-4 blockade induces a restoration of intrahepatic HCV-specific CD8<sup>+</sup> T cell function in chronically HCV infected patients<sup>[75]</sup>. Obviously, the modulation of different co-stimulatory molecules on HCV-specific T cells, such as CD137<sup>[76]</sup>, OX40<sup>[77]</sup> and ICOS<sup>[78]</sup> should be tested in combination with PD-1/PD-L1 blockade in order to restore HCV-specific CTL effector functions<sup>[79,80]</sup>. Nevertheless, blocking the engagement between PD-1 and PD-L1 is not enough in many cases to restore peripheral HCV-specific CTL functionality in chronic patients, even in combination with the blockade of other negative co-stimulatory molecules. It is reasonable to assume that these cells, exposed to high persistent antigenic stimulus, are prone to apoptosis. Previous data on HBV chronic infection showed an up-regulation of the pro-apoptotic molecule Bim on HBV-specific CTLs<sup>[81]</sup>. In this chronic infection, only CD127<sup>+</sup> (IL-7R) cells maintained the ability to expand after antigen encounter. These CD127<sup>+</sup> cells could be protected from apoptosis due

to the antiapoptotic molecule Mcl-1, induced by IL-7. Otherwise, CD127<sup>+</sup> HBV-specific CTLs would die due to apoptosis after antigen encounter, mediated by the Bim pathway. Bearing in mind these data on HBV infection, it is possible that the benefit observed by blocking the PD-1/PD-L1 interaction may occur only in specific T cells protected against apoptosis by CD127 expression. This phenotype is quite rare in patients with long-standing HCV infection, and this could explain why not all PD-1 expressing HCV-specific CTLs respond to anti-PD-L1 treatment. This theoretical scenario should be tested in the near future.

## HCV CORE PROTEIN INDUCES PD-1 UP-REGULATION

The PD-1 up-regulation on intrahepatic total T cells<sup>[53,56]</sup> suggests that something other than TCR stimulation is involved in the PD-1 expression regulation during HCV infection. HCV-core protein binding to the complement receptor gC1q (gC1qR) is responsible for impairing T cell proliferation ability<sup>[82]</sup> through down-regulation of the high affinity IL-2 receptor<sup>[83]</sup>. A recent report suggests that this process could be mediated by PD-1 expression



**Figure 6 PD-1 up-regulation induced by HCV-core protein.** A: FACS® dot-plots and histograms of peripheral blood CD8<sup>+</sup> cells stained with PD-1-FITC and CD8-Cy mAbs from a healthy subject. CD8<sup>+</sup> cells were stimulated with B-galactosidase, HCV-core protein, CD3-CD28 mAb, and HCV-core protein plus CD3-CD28 mAbs. PD-1 expression was highly up-regulated on CD8<sup>+</sup> cells after non-specific stimulation by anti CD3/CD28 mAbs in the presence of HCV core protein; B: FACS® dot-plots and histograms of peripheral blood CD8<sup>+</sup> cells stained with Ki-67 FITC and CD8-Cy mAbs from the same healthy subject to test proliferation ability of CD8<sup>+</sup> cells after incubation with B-galactosidase, CD3-CD28 mAb, HCV-core protein plus CD3-CD28 mAb and HCV-core protein plus CD3-CD28 and anti-PD-L1 mAbs. HCV-core protein decreased the proliferation induced by CD3-CD28 mAb stimulation. This proliferation impairment induced by HCV-core protein was resolved by anti-PD-L1 mAb treatment.

induction<sup>[84]</sup>. In fact, in an intrahepatic HCV-core protein expressing mouse model, liver infiltration by PD-1 expressing cytotoxic T cells unable to clear the virus has been shown. However, the liver from HCV-core non-expressing mice was infiltrated by non-PD-1 expressing specific-CTLs which could control the viral infection<sup>[85]</sup>. These data suggest that HCV-core protein could play a role in early PD-1 induction on T cells, mainly in the liver environment where this protein is richly expressed<sup>[86,87]</sup>. At least *in-vitro*, PD-1 and PD-L1 expression are up-regulated on activated T cells in the presence of HCV-core protein<sup>[84]</sup>. PD-1 up-regulation induced by HCV-core protein translated into impairment of T cell proliferation ability<sup>[84]</sup>. However, this dysfunction could be partially restored by blocking the PD-1/PD-L1 pathway with anti-PD-L1 antibodies (Figures 1 and 6) and by blocking the interaction between HCV-core protein and gC1qR<sup>[84]</sup>. Probably the interaction between HCV-core protein and gC1qR co-operate with the continuous TCR stimulation to produce an early PD-1 up-regulation in order to induce a premature anergy on HCV-specific CTLs as an efficient HCV escape mechanism.

## PD-1/PD-L1 BLOCKADE AS A THERAPUTIC TOOL

As previously commented, a defective virus-specific cytotoxic T cell response is one of the most important causes of host inability to eliminate a persistent viral infection. Several studies have highlighted the role of the PD-1/PD-L1 pathway in the development of anergy on virus-specific CD8<sup>+</sup> T cells, and how PD-1/PD-L1 blockade could enhance virus-specific CD8<sup>+</sup> T cell functionality *in-vitro*<sup>[41,74,88-92]</sup>. Recently, several works have been carried out to analyse whether modulation of the PD-1/PD-L1 pathway could improve T cell response against persistent viral infections either directly, using anti PD-L1 antibodies alone, or in combination with a therapeutic vaccine. Therapeutic vaccine usually fails to induce a vigorous T cell response due to the tolerogenic-like status of HCV-specific T cells<sup>[93,94]</sup>. This scenario could be positive if negative co-stimulatory molecules, such as PD-1, were blocked when the therapeutic vaccine is administered in order to enhance the specific immune response against the supplied epitopes. In the chronic LCMV infection

animal model, the administration of a therapeutic vaccine in combination with PD-1/PD-L1 interaction blockade enhances expansion and improves the function of LC-MV-specific CD8<sup>+</sup> T cells. In addition, this combinatorial therapeutic vaccination accelerates viral control compared with either therapeutic vaccine or PD-1 blockade alone<sup>[95]</sup>. Moreover, the effect of anti-PD-L1 antibodies alone could also be effective in controlling persistent viral infection by restoring specific CTL response. The administration of anti-PD-L1 monoclonal antibodies during simian immunodeficiency virus (SIV) chronic infection in macaques resulted in a rapid expansion and restoration of SIV-specific CD8<sup>+</sup> T cells<sup>[96,97]</sup>. Although these results seem to be quite promising, the blockade of negative costimulatory pathways could lead to the development of autoimmune diseases<sup>[17,18]</sup>, which could prevent the use of this strategy as a therapeutic tool in humans. Therefore, more research is necessary in this field before blockade of the PD-1/PD-L1 pathway is suitable for the treatment of chronic HCV infection.

## CONCLUSION

In summary, the PD-1/PD-L1 pathway displays an important role in the induction of anergy on HCV-specific cytotoxic T cells, and could be important in the development of HCV persistent infection. Blocking the PD-1/PD-L1 interaction, probably in association with the modulation of other co-stimulatory molecules, could be an interesting strategy to restore HCV-specific CTL response in patients unresponsive to standard anti-HCV treatment.

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## Metabolic syndrome and risk of subsequent colorectal cancer

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hyperinsulinemia, elevated C-peptide, elevated body mass index, high levels of insulin growth factor-1, low levels of insulin growth factor binding protein-3, high leptin levels and low adiponectin levels are all involved in carcinogenesis. Understanding the pathological mechanism that links metabolic syndrome and its components to carcinogenesis has a major clinical significance and may have profound health benefits on a number of diseases including cancer, which represents a major cause of mortality and morbidity in our societies.

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### Abstract

The metabolic syndrome and visceral obesity have an increasing prevalence and incidence in the general population. The actual prevalence of the metabolic syndrome is 24% in US population and between 24.6% and 30.9% in Europe. As demonstrated by many clinical trials (NAHANES III, INTERHART) the metabolic syndrome is associated with an increased risk of both diabetes and cardiovascular disease. In addition to cardiovascular disease, individual components of the metabolic syndrome have been linked to the development of cancer, particularly to colorectal cancer. Colorectal cancer is an important public health problem; in the year 2000 there was an estimated total of 944 717 incident cases of colorectal cancer diagnosed world-wide. This association is sustained by many epidemiological studies. Recent reports suggest that individuals with metabolic syndrome have a higher risk of colon or rectal cancer. Moreover, the clusters of metabolic syndrome components increase the risk of associated cancer. The physiopathological mechanism that links metabolic syndrome and colorectal cancer is mostly related to abdominal obesity and insulin resistance. Population and experimental studies demonstrated that

### INTRODUCTION

The concept of metabolic syndrome has existed for at least 80 years and was first described by Kylin<sup>[1]</sup>, a Swedish physician, as a clustering of hypertension, hyperglycemia and gout, and later on by Vague<sup>[2]</sup> who added to the previous description the presence of abdominal obesity.

While the concept of metabolic syndrome has been accepted for a long time, there was no largely recognized international definition until 1998.

The first proposal came in 1998 from a consultation group for the definition of diabetes for World Health Organisation. This definition was then modified by the European Group for Study of Insulin Resistance in 1999, the National Cholesterol Education Program Adult Treatment Panel III (ATP III) in 2001 and revised in 2005, and the International Diabetes Foundation (IDF) in 2005. These definitions agree on the different components of metabolic syndrome but differ in details (Table 1)<sup>[3]</sup>.

More recently, the use of the term of “metabolic syndrome” has been questioned by the American

Diabetes Association and the European Association for the Study of Diabetes for several reasons: (1) Criteria are ambiguous or incomplete (for example it is unclear if the blood pressure definition is systolic blood pressure  $\geq 130$  mmHg and diastolic  $\geq 85$  mmHg or whether it is either  $\geq 130$  mmHg or  $> 85$  mmHg); (2) The value of including diabetes in the definition is questionable and the role of insulin resistance as unifying etiology is uncertain. Furthermore it is still unclear the extent to which an elevated cardiovascular (CVD) risk is due to insulin resistance itself *vs* isolated hyperinsulinemia; (3) There is no clear basis for including/excluding other CVD risk factors; the CVD risk associated with the syndrome appears to be no greater than the sum of its parts; (4) The treatment of the syndrome is not different from the treatment for each of its components.

A recent review of the ATP III definition broadened the etiological basis of the syndrome from insulin resistance alone to include "obesity and disorders of adipose tissue"<sup>[4]</sup>.

The actual prevalence of obesity is 30.5% and that of associated metabolic syndrome is 24% in the US population<sup>[3,5]</sup>.

In Europe, the age- and sex-adjusted prevalence of metabolic syndrome was 24.6% using the 2005 ATP III definition and 30.9% using the International Diabetes Federation definition, according to the MADRIC study (MADrid Rlsego Cardiovascular Study) performed on 1344 participants<sup>[6]</sup>. In this study, the authors found a good overall agreement between the ATP III and IDF definitions, much closer in women than in men ( $\kappa = 0.92 \pm 0.07$  *vs*  $\kappa = 0.66 \pm 0.06$ ). The prevalence of metabolic syndrome was greater according to the IDF definition than according to ATP III, because the former definition has a lower threshold of abdominal obesity.

A cross-sectional analysis of 10206 participants aged 20-89 years in the Nord-Trøndelag Health Study 1995-97 (HUNT 2) in Norway, found a prevalence of IDF-defined metabolic syndrome of 29.6%, compared to 25.9% using the 2005 ATP III criteria<sup>[7]</sup>.

In a meta-analysis, Cameron *et al*<sup>[8]</sup> found a variable prevalence of metabolic syndrome in urban populations from 8% (India) to 24% (USA) in men, and from 7% (France) to 43% (Iran) in women.

It is well known that the metabolic syndrome is associated with an increased risk of both diabetes and cardiovascular disease.

Many clinical studies outlined the interrelation between the metabolic syndrome and cardiovascular risk<sup>[9]</sup>.

Applying the ATP III criteria to 10537 NHANES III participants resulted in a significant association between the metabolic syndrome with prevalent myocardial infarction and stroke in a multivariate analysis: myocardial infarction [OR: 2.01, 95% confidence intervals (CI): 1.53-2.64], stroke (OR: 2.16, 95% CI: 1.48-3.16), and myocardial infarction/stroke (OR: 2.05, 95% CI: 1.64-2.57)<sup>[10]</sup>.

The INTERHART study performed on 15152 cases and 14820 controls in nearly 52 countries found a significant association between abnormal lipids, smoking,

hypertension, diabetes, abdominal obesity, psychosocial factors, consumption of fruits, vegetables, and alcohol, and regular physical activity and the risk of myocardial infarction. Collectively, these nine risk factors accounted for 90% of the population risks in men and 94% in women<sup>[11]</sup>.

In addition to CVD, individual components of the metabolic syndrome have been linked to the development of cancer<sup>[12]</sup>.

Colorectal cancer is an important health problem since one million new cases are diagnosed world-wide each year with half million related deaths<sup>[13]</sup>. The incidence rate of colon cancer according to Five Continents cancer registries varies from 3% in Africa (Algeria) up to 40% in North America. In Europe the incidence of colon cancer ranges from 12.1% in Belarus up to 30.5% in Italy<sup>[14]</sup>.

There is evidence that body composition and hormonal factors contribute to colorectal cancer etiology. In this paper we will highlight this association supported by epidemiological data and pathophysiological mechanisms arising from prospective human research studies.

## EPIDEMIOLOGY

In an analysis of nearly 58000 individuals who participated in the National Health Interview Survey (2002-2003), Garow *et al*<sup>[15]</sup> identified 1200 individuals with metabolic syndrome, 350 of them being diagnosed with colorectal cancer. After controlling for age, race, gender, obesity, smoking and alcohol use the individuals with metabolic syndrome had a 75% increased risk for colon or rectal cancer.

In a large prospective study of more than 900000 US adults (404576 men and 495477 women) conducted by Calle *et al*<sup>[16]</sup>, there were 57145 deaths from cancer during a follow up period of 16 years. The authors also studied the relationship between the relative risk (RR) of death and body mass index (BMI). For all cancer there was a trend in increasing death rate with BMI. For colorectal cancer the RR of death varied from 1.34 (95% CI: 0.94-1.34) for a BMI of 25-29.9, to 1.90 (95% CI: 1.46-2.47) and 4.52 (95% CI: 2.94-6.94) for an BMI between 30.0-34.9 and 35.0-39.9, respectively<sup>[16]</sup>.

Recent studies also provide information concerning the association between colorectal cancer incidence and the number of metabolic syndrome components, especially BMI, waist circumference (WC), lipid levels, plasma glucose and glycosylated hemoglobin (HbA1c). In an analysis of 14109 participants from the ARIC study (Atherosclerosis Risk in Communities), 194 incident colorectal cancers were identified. In this study baseline metabolic syndrome ( $> 3$  components *vs* 0 components) had a positive association with age-adjusted and gender-adjusted colorectal cancer incidence (RR: 1.49, 95% CI: 1.0-2.4). There was a dose-response association between colorectal cancer incidence and the number of metabolic syndrome components present at baseline ( $P$  for trend = 0.006) after multivariate adjustment<sup>[17]</sup>.

In another study, Trevisan *et al*<sup>[18]</sup>, used information

Table 1 Comparison of definitions of metabolic syndrome

WHO 1999	ATP III 2001	IDF 2005
Diabetes or impaired fasting glycemia or impaired glucose tolerance or insulin resistance Plus 2 or more of the following: Obesity: BMI > 30 or waist-to-hip ratio > 0.9 (male) or 0.85 (female) Dyslipidemia: triglycerides > 150 mg/dL or HDL cholesterol < 35 mg/dL (male) or < 39 mg/dL (female) Hypertension: blood pressure > 140/90 mmHg Microalbuminuria: albumin excretion > 20 µg/min	Three or more of the following: Central obesity: waist circumference > 102 cm (male), > 88 cm (female) Hypertriglyceridemia: triglycerides > 150 mg/dL Low HDL cholesterol: < 40 mg/dL (male), < 50 mg/dL (female) Hypertension: blood pressure > 130/85 mmHg Fasting plasma glucose > 100 mg/dL	Increased waist circumference > 94 cm in men and > 80 cm in women plus any 2 of the following: Hypertriglyceridemia: triglycerides > 150 mg/dL Low HDL cholesterol: < 40 mg/dL (male), < 50 mg/dL (female) Hypertension: blood pressure > 130/85 mmHg Fasting plasma glucose > 100 mg/dL

from the Risk Factors and Life Expectancy study, which pooled data from nine epidemiological studies conducted in Italy between 1978 and 1987, including 21 311 men and 15 991 women. In this study, low high density lipoprotein (HDL) and high triglyceride levels, hypertension and plasma glucose levels were also analyzed as individual components of the metabolic syndrome. For the presence of the cluster of metabolic abnormalities, the calculated hazard ratios and 95% CIs were 2.99 (1.27-7.01) when both sexes were combined. When analyzing the individual components, only glucose level was associated with an increased risk of death from colorectal cancer, and only in men and women combined (RR: 1.8, 95% CI: 1.05-3.09). The results of this study suggest that the effects of the individual components of metabolic syndrome are additive, because the RR of death from colorectal cancer was increased in cluster analysis compared with glucose alone.

The association between plasma glucose levels reflected by HbA1c and the incidence of colorectal cancer was outlined in a prospective analysis from the European Prospective Investigation into Cancer and Nutrition (EPIC) study<sup>[19]</sup>. Among 9605 participants in this study, aged between 45 and 79 years, there were 67 incident colorectal cancers. In this study population, the RR of colorectal cancer for men and women combined was 2.94 (95% CI: 0.80-10.85), age and sex adjusted for an HbA1c  $\geq 7\%$ , compared with RR, 1.13 (95% CI: 0.56-2.30), for HbA1c of 5.0%-5.9%. For the same HbA1c levels of > 7%, the age adjusted RR was higher in men than in women [RR: 4.94 (95% CI: 0.89-27.35) in men, and 1.58 (95% CI: 0.19-13.14) in women]. The association of higher HbA1c levels and increased colorectal cancer risk was also present in the CLUE II cohort<sup>[20]</sup>.

Conversely, to evaluate the association between metabolic syndrome and colorectal cancer, Stocks *et al*<sup>[21]</sup> evaluated the presence of metabolic syndrome components (C-peptide, HbA1c, leptin, adiponectin, BMI, hypertension and fasting glucose) in 306 individuals with known colorectal cancer. The presence of hypertension, obesity and hyperglycemia, correlated with a RR for three *vs* null factors of 2.57 (95% CI: 1.20-5.52, *P* trend = 0.00021).

The relationship between BMI and colon cancer was also studied in the recent EPIC study<sup>[22]</sup>, which was based on 984 cases of colon cancer. A 55% increased

risk of colon cancer was observed between the high and low quintiles of BMI in men, but no significant association was observed in women.

Some recent studies considered anthropometric measures of adipose distribution in addition to BMI in relation to the risk of colon cancer of adenoma. In most of these studies, the association between WC or waist-to-hip ratio and colon cancer risk was stronger than that between BMI and cancer risk. Moore *et al*<sup>[23]</sup>, in a retrospective analysis of 7566 subjects from the Framingham cohort, found 306 cases of incident colorectal cancer. The authors demonstrated a two-fold increased risk of colorectal cancer for a WC of > 99 cm in women and 101 cm in men; the risk increased linearly with increasing WC<sup>[23]</sup>. One Japanese study<sup>[24]</sup> of 51 consecutive patients aged  $\geq 40$  years, suggests that visceral adipose tissue rather than whole body adipose tissue correlates better with the risk of colorectal adenoma. Furthermore, in this study, low adiponectin level is a factor associated with the development of colorectal adenoma. It is known that adiponectin levels decrease in obesity, especially abdominal obesity in association with insulin resistance; thus, the results of this study offer an insight to understanding the relationship of colorectal carcinogenesis with abdominal obesity and insulin resistance which will be discussed later on this paper.

The fact that the metabolic syndrome is a risk factor for both CVD and colorectal cancer raised the question if there is any association between CVD and colorectal cancer. This correlation was found to be positive in several studies. In a pilot study of 63 patients with colorectal cancer, Hamoudi and Dumitrascu demonstrated a statistical association between CVD and colorectal cancer in men<sup>[25]</sup>.

The relationship between individual components of metabolic syndrome and the risk of colorectal cancer was also separately analyzed by several studies. Colangelo *et al*<sup>[26]</sup> found a 35% increased risk of colorectal cancer associated with high blood pressure. The results were confirmed by another study<sup>[17]</sup>. Both studies also underlined that the clustering of metabolic syndrome components significantly increased the risk of associated colorectal cancer.

High circulating triacylglycerols were associated in a large prospective study with a non-significant two-

fold elevation in risk of colorectal cancer in men, but no clear association was observed in women<sup>[17]</sup>. In another prospective study, there was a 40% increased risk of colorectal cancer for men and women in the top quartile of triacylglycerol levels, although this association was not significant<sup>[27]</sup>.

The association between C-peptide levels as a marker of hyperinsulinemia and colorectal cancer risk was also examined by several studies. In a case control study in the Physicians' Health Study, an increased concentration of plasma C-peptide was statistically significantly associated with an increased risk of colorectal cancer in men (RR for the highest *vs* lowest quintile of plasma C-peptide = 2.7, 95% CI: 1.2-6.2, *P* trend = 0.047), after adjusting for age, smoking status, fasting, BMI and alcohol consumption. The results of this study also suggest that elevated insulin production, as reflected by elevated concentrations of plasma C-peptide, may predict the risk of developing colorectal cancer, independently of BMI, factors related to insulin resistance, or levels of insulin growth factor (IGF)-1 and insulin growth factor binding protein (IGFBP)-3<sup>[28]</sup>. The interrelation between a high concentration of plasma C-peptide and colorectal adenoma was also demonstrated in women in a series of 380 patients with a multivariable relative risk (MVRR) top *vs* bottom quartile, 1.63, 95% CI: 1.01-2.66, *P* = 0.01, even after including BMI and physical activity in the statistical model<sup>[29]</sup>.

The findings of all these studies suggest that the clusters of the metabolic syndrome components may be predictors for developing colorectal cancer and for colorectal cancer mortality. The understanding of the underlying physiopathology that links the metabolic syndrome and cancer may play a key role in developing new strategies for prevention and treatment.

## PHYSIOPATHOLOGICAL LINKS BETWEEN METABOLIC SYNDROME AND COLORECTAL CANCER

### **Obesity, insulin resistance and insulin growth factors and binding proteins**

It has been hypothesized that insulin resistance is the most important underlying mechanism of the metabolic syndrome in close relationship to abdominal obesity. Insulin has been shown to affect growth of normal and neoplastic epithelial cells and to have mitogenic actions *in vitro* and in experimental models, either directly or indirectly through IGF-1<sup>[17]</sup>. At high concentrations, insulin can bind to IGF-1 receptors (IGF1Rs) or can act directly to promote IGF-1 biosynthesis, enhancing IGF-1 bioavailability and inhibiting the production of IGFBP-1, IGFBP-2 and IGFBP-3<sup>[30]</sup>.

IGF-1 is an important mitogen required for the progression through the cell cycle and has autocrine, paracrine and endocrine actions on cell proliferation and apoptosis<sup>[31]</sup>, increasing the risk of cellular transformation by enhancing cell turnover. In addition, IGF-1 increases the production of vascular endothelial growth

factor (VEGF), an angiogenic factor that can support cancer growth<sup>[32]</sup>.

It has been shown that normal colorectal epithelia and colon cancer cells have both insulin and IGF1Rs<sup>[17]</sup>. Tissue homeostasis in the normal colonic crypt relies on a balance between proliferation, differentiation and apoptosis, with apoptosis occurring at the top of the colonic crypt as the culmination of a differentiation pathway.

The link between IGF-1 and IGFBP-3 levels and the increased risk of colorectal adenoma and cancer came first to attention in acromegalic patients, characterized by chronically elevated growth hormone (GH) levels. GH excess leads to hepatic and peripheral insulin resistance and thus to hyperinsulinemia, a common feature of acromegaly and metabolic syndrome, that causes IGF-1 hypersecretion and low IGFBP-3 levels<sup>[33]</sup>.

The relationship between IGF-1 and IGFBP-3 levels and colorectal cancer was examined by Giovannucci *et al*<sup>[34]</sup> on 32826 women from Nurses' Health Study. Controlling for IGFBP-3 level, relative to women in the lowest tertile of IGF-1, those in the highest tertile were at elevated risk of intermediate/late-stage colorectal neoplasia adenoma (MVRR: 2.78, 95% CI: 0.76-9.76) and cancer (RR: 2.18, 95% CI: 0.94-5.08). Controlling for IGF-1 level, relative to women in the lowest tertile of IGFBP-3, women in the highest tertile of IGFBP-3 were at lower risk of intermediate/late-stage colorectal adenoma (RR: 0.28, 95% CI: 0.09-0.85) and cancer (RR: 0.28, 95% CI: 0.10-0.83). Neither IGF-1 nor IGFBP-3 had any appreciable relation with early-stage adenoma. These analyses indicate that high levels of circulating IGF-1 and particularly low levels of IGFBP-3 are associated independently with an elevated risk of large or tubulo-villous/villous colorectal adenoma and cancer. These results are concordant with those obtained previously in Physicians' Health Study<sup>[35]</sup>.

The role of IGFBP-3 in colorectal cancer was independently analyzed by Williams *et al*<sup>[36]</sup>. IGFBP-3 has been shown to enhance p53-dependent apoptosis after DNA damage. Therefore, loss of IGFBP-3 could contribute to the development of colonic adenomas that retain wild-type p53 function through suppression of p53-dependent apoptotic signals, allowing aberrant cell survival and tumor formation. Furthermore there is disruption in both adenoma and carcinoma tissue. This pattern is similar to that of TGF- $\beta$  distribution in normal, adenoma and carcinoma tissue<sup>[37]</sup>. Because it is known that TGF- $\beta$  is a potent growth inhibitor for colonic epithelium<sup>[36]</sup>, this similarity suggests that IGFBP-3 may have an important role in the regulation of differentiation and apoptosis in human colonic epithelium<sup>[37]</sup>.

The role of insulin resistance and hyperinsulinemia in colorectal cancer was directly assessed by Schoen *et al*<sup>[27]</sup> in a study performed on 5849 participants in the Cardiovascular Health Study cohort. The authors identified 102 cases of colorectal cancer. Fasting insulin was not related to an increased risk (RR = 1.2), whereas 2 h insulin was related to a significantly increased risk (RR = 2.0).

Giovannucci *et al*<sup>[38]</sup> found that BMI was not significantly

associated with an increased risk of distal colon adenoma irrespective of size, while WC and waist hip ratio were strong risk factors for large distal colon adenomas with diameter  $\geq 1$  cm but were unrelated to small adenomas with diameter  $< 1$  cm. The association of WC with an increased risk of cancer has been reported to be slightly stronger for distal colon cancer.

There are also several studies which determined the relationship between C-peptide (an indicator of insulin production) and the risk of colorectal cancer. As mentioned before, in the Physicians' Health Study, men with C-peptide in the top *vs* the bottom quintile had a 2.7-fold significantly higher risk of colorectal cancer after control for BMI and exercise; this RR increased to 3.4 after the analysis was controlled for indicators of the metabolic syndrome<sup>[28]</sup>. In a prospective study of 14275 women in New York State, a 3-fold higher risk of colorectal cancer was observed in those in the top quartile of C-peptide, and a 4-fold higher risk was observed for colon cancer alone<sup>[39]</sup>.

### **Adipokines and inflammatory cytokines**

Adipose tissue is a complex endocrine organ, responsible for the secretion and synthesis of hormones, cytokines and other signaling proteins, collectively termed as adipokines. Adipokines are a diverse group of signaling molecules that play roles in such processes as appetite and energy balance, inflammation, insulin resistance/sensitivity, angiogenesis, lipid metabolism, cell proliferation and atherosclerosis. Many of these functions are related to either the metabolic syndrome or cancer, and they may serve as a link between these two pathologies<sup>[40]</sup>.

### **Adiponectin**

Adiponectin, a 30-kDa complement C1q-related protein, is a key regulator of insulin sensitivity and inflammation and modulates several physiologic processes, such as metabolism of glucose and fatty acids. In contrast to other adipokines such as leptin, adiponectin circulating levels are decreased in obese individuals and in those with diabetes<sup>[41]</sup>. Decreased plasma adiponectin concentrations are associated with insulin resistance, type 2 diabetes and atherosclerosis. In addition, it was recently shown that adiponectin may play a role in the development and progression of various types of malignancies. Accumulating evidence suggests that adiponectin is an important regulator of cell proliferation. Adiponectin may act either directly on cancer cells or indirectly by regulating whole-body insulin sensitivity<sup>[42]</sup>.

### **Mechanisms that may link adiponectin with carcinogenesis**

In obesity, reduced adiponectin levels lead to the development of insulin resistance and compensatory, chronic hyperinsulinaemia. Increased insulin levels results in increased levels of bioavailable IGF-1. Insulin and IGF-1 signal through the insulin receptors and IGF1R, promote cellular proliferation and inhibit apoptosis in many tissue types up-regulating the secretion of VEGF, contributing thus to carcinogenesis<sup>[39]</sup>. Adiponectin has also

been shown to inhibit both the production of TNF- $\alpha$  in macrophages and its action in endothelial cells, thus promoting carcinogenesis through the altered effect of TNF- $\alpha$  on tumor cell proliferation and angiogenesis<sup>[43]</sup>.

Adiponectin can also protect from carcinogenesis through more direct effects.

Specifically, adiponectin has been found to be an important negative regulator of hematopoiesis and the immune system. Moreover, adiponectin may inhibit activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B), a transcription factor that upregulates VEGF<sup>[44]</sup>.

Several signalling molecules such as 50-AMP-activated protein kinase (AMPK), NF- $\kappa$ B, peroxisome proliferators activated receptor (PPAR)- $\alpha$  and p38 mitogen-activated protein kinase are known to mediate adiponectin-induced metabolic effects. AMPK might inhibit the growth and/or survival of cancer cells<sup>[45]</sup>. Finally, adiponectin may also regulate angiogenesis negatively (independently of AMPK) through induction of apoptosis in vascular endothelial cells by activating the caspase cascade, a group of apoptotic enzymes<sup>[46]</sup>.

The relationship between circulating adiponectin levels and colorectal cancer was demonstrated by several clinical and experimental studies.

Ferroni *et al*<sup>[47]</sup> demonstrated in a study involving 60 patients with non metastatic colorectal cancer that low adiponectin levels are inversely correlated with increases in tumor stage and were independent predictors of recurrent disease. Low adiponectin levels were found in 52% of relapsing patients, compared with 26% of non-relapsing patients<sup>[47]</sup>.

Similar results were obtained by Wei *et al*<sup>[48]</sup> in a prospective case-control study of 18225 men enrolled in the Health Professional Follow-up Study. Over the approximately 8 years of follow-up, the authors noted 25 cases of colorectal cancer in the 3645 men in the highest category of adiponectin compared with 54 cases of colorectal cancer in the 3645 men in the lowest quintile of adiponectin.

### **Leptin**

Leptin is a 16 kDa glycoprotein which is expressed almost exclusively ( $> 95\%$ ) by adipocytes. Initial interest in leptin focused on its role in obesity but recently leptin, has been associated with the inflammatory response, insulin signaling, and carcinogenesis.

Insulin and leptin interact at multiple levels within a complex network of adipose tissue signaling pathways, providing several mechanisms that could link leptin to colon cancer.

Of particular importance for cancer is the influence of leptin on suppressors of cytokine signaling 1 and 3 which in turn limits insulin signaling<sup>[49]</sup>.

Although data directly linking leptin to colon cancer are limited, some studies have shown increased risk of colon and colorectal cancer with high serum leptin levels.

Data from a cohort study in Norway detected an almost 3-fold increased risk of colon cancer among people with high leptin levels, independently of BMI<sup>[50]</sup>.

Another study found that men in the highest tertile of leptin concentrations had a 3.3-fold (95% CI: 1.2-8.7) increased adenoma risk compared with those in the lowest tertile<sup>[51]</sup>. The association between leptin concentration and colorectal cancer was also evaluated in women, in a case-control study conducted in Japan, suggesting that leptin increases substantially the risk of female colorectal cancer, independent of BMI<sup>[52]</sup>.

### **Inflammatory cytokines and colorectal cancer**

Accumulating evidence suggests that systemic inflammation might be a plausible mechanism for colon carcinogenesis. Studies have shown that genetic variations in inflammation-related genes, such as interleukin (IL)-6, IL-8, and IL-10, are associated with susceptibility to colorectal cancer and adenomas.

IL-6 appears to enhance tumorigenesis by a paracrine and autocrine mechanism, to stimulate cell growth and inhibit apoptosis. Also IL-6 concentrations reflected disease status and were commonly associated with metastatic disease<sup>[53]</sup>.

TNF- $\alpha$  activates NF- $\kappa$ B (by phosphorylation of its inhibitor I $\kappa$ B), which increases production of NO, a substrate for reactive oxygen species (ROS) formation, and stimulates other inflammatory cytokines<sup>[54]</sup>. With respect to cancer, ROS can damage DNA by several processes including DNA base modification, deletions, frame shifts, strand breaks, DNA-protein cross-links, and chromosomal rearrangements. DNA damage can occur in genes that are important in cell proliferation (such as ras), or cell survival (such as p53), which can then trigger cancer progression<sup>[55]</sup>.

There are several studies which demonstrated the correlation between high levels of IL-6, TNF- $\alpha$ , C-reactive proteins (CRP) and colorectal carcinogenesis. Moreover, a Greek study demonstrated that high levels of serum IL-6, TNF- $\alpha$  and CRP were correlated with larger tumor size. The relation to tumor size could be related to the fact, that larger tumors may trigger a more potent immunological response manifested by the circulation of proinflammatory cytokines such as TNF- $\alpha$ <sup>[56]</sup>.

### **PPAR- $\gamma$**

PPAR- $\gamma$ , a ligand-activated transcription factor, is a key regulator of adipogenic differentiation and glucose homeostasis. PPAR- $\gamma$  ligands have recently been demonstrated to affect proliferation and differentiation in cancer cell lines. A gradually increasing number of studies demonstrated the association between PPAR- $\gamma$  and colorectal cancer<sup>[57]</sup>.

A recent study demonstrated a positive PPAR- $\gamma$  immunostaining in 48 of 86 cases of colon cancer (56%). No association was found for PPAR- $\gamma$  positivity with different Dukes' stages, histological grade of differentiation, tumor location, presence of lymph node and liver metastasis, venous invasion, or tumor cell proliferating capacity assessed as Ki-67 overexpression. On the contrary, PPAR- $\gamma$  expression was statistically significant correlated with the expression of cell cycle-related molecules<sup>[58]</sup>.

Another recent study demonstrated that PPAR- $\gamma$  agonists have inhibitory effects on the proliferation of colon cancer cell lines associated with G1 cell cycle arrest and invasive activity. The latter effect is demonstrated in certain cell lines through the down-regulation of metalloproteinase-7 synthesis<sup>[59]</sup>.

## **CONCLUSION**

The association between metabolic syndrome and colorectal cancer is now supported by a large number of epidemiological studies<sup>[14,16,17,19,26]</sup>. The components of metabolic syndrome appear to have an additive effect on colon cancer development acting through different pathophysiological pathways. This evidence is based on studies of determinants of the metabolic syndrome (obesity, abdominal distribution of adiposity, physical inactivity), clinical consequences (type 2 diabetes, hypertension) of this syndrome, plasma or serum components of the definition of metabolic syndrome (hypertriglyceridemia, hyperglycemia, low HDL cholesterol), markers of hyperinsulinemia or insulin resistance (insulin, C-peptide), and serum inflammatory cytokines levels in relation to colon cancer or adenoma risk. High insulin and insulin resistance are common features of industrialized societies characterized by a large prevalence of overweight individuals and obesity, a diet rich in energy intake, and a lifestyle characterized by low calorie expenditure. Understanding the pathological mechanism that links metabolic syndrome and its components to carcinogenesis has a very important clinical significance. Controlling even one or two of the components of the metabolic syndrome may result in a longer, healthier and cancer-free life. Public health efforts aimed at reducing lifestyle patterns and dietary habits associated with this imbalance on insulin metabolism may have profound health benefits on a number of diseases including cancer, that represent major causes of mortality and morbidity in our societies.

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## EP4 agonist alleviates indomethacin-induced gastric lesions and promotes chronic gastric ulcer healing

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### Abstract

**AIM:** To investigate EP4-selective agonist effect on indomethacin-induced gastric lesions and on the spontaneous healing of chronic gastric ulcers.

**METHODS:** In a mouse model of gastric bleeding with high dose of indomethacin (20 mg/kg), an EP4-selective agonist was administered orally. Stomach lesions and gastric mucous regeneration were monitored. In a mouse model of chronic gastric ulcer induced by acetic acid, EP4 agonist effect on the healing of chronic gastric ulcer was evaluated in the presence or absence of low dose indomethacin (3 mg/kg). In cultured human gastric mucous cells, EP4 agonist effect on indomethacin-induced apoptosis was assessed by flow cytometry.

**RESULTS:** The EP4-selective agonist reduced high dose indomethacin-induced acute hemorrhagic damage and promoted mucous epithelial regeneration. Low-dose indomethacin aggravated ulcer bleeding and inflammation, and delayed the healing of the established chronic gastric ulcer. The EP4 agonist, when applied locally, not only offset indomethacin-induced gastric bleeding and inflammation, but also accelerated ulcer healing. In the absence of indomethacin, the EP4 agonist even accelerated chronic gastric ulcer healing and suppressed inflammatory cell infiltration in the granulation tissue. *In vitro*, the EP4 agonist protected human gastric mucous cells from indomethacin-induced apoptosis.

**CONCLUSION:** EP4-selective agonist may prevent indomethacin-induced gastric lesions and promote healing of existing and indomethacin-aggravated gastric ulcers, *via* promoting proliferation and survival of mucous epithelial cells.

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**Key words:** Prostaglandin E2; Non-steroidal anti-inflammatory drugs; Gastric bleeding; Gastric ulcer; EP4-subtype receptor

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### INTRODUCTION

Over 300 million patients use non-steroidal anti-inflammatory drugs (NSAIDs) in the world to treat pain, arthritis, fever and other diseases. Nearly 30% of the users suffer from gastric lesions and bleeding. Mechanisms for such actions of NSAIDs seem to be complex and multifactorial, including the inhibition of prostaglandin (PG) synthesis, induction of apoptosis and necrosis of gastric mucosal cells<sup>[1-3]</sup>, neutrophil penetration, dysfunction of microvessels, reduced secretion of bicarbonate and mucus, and increased gastric motility<sup>[4]</sup>.

Proton pump inhibitors (PPIs) have been the mainstay for the treatment of gastric ulcers, primarily due to its abilities to reduce acid secretion<sup>[5]</sup>. An alternative approach is to administer misoprostol, a non-selective prostaglandin E1 (PGE1) analogue. PGE2/E1 has been shown to protect isolated gastric glands from indomethacin, independently of neural, vascular and hormonal factors<sup>[6]</sup>. Misoprostol has successfully prevented NSAID-induced bleeding, perforation or gastric outlet obstruction in patients<sup>[5,7]</sup>, and reversed the negative effects of indomethacin on the maturation of granulation

tissue which PPIs were not able to do<sup>[8]</sup>. Misoprostol, however, produces severe side effects, such as diarrhea, gastrointestinal (GI) cramping pain, nausea, flatulence, dyspepsia, abortion, headache and poor tolerance<sup>[9]</sup>. Among the four PGE2 receptor subtypes (EP1-4), EP4 is constitutively expressed in gastric surface mucous cells, the first layer of lining cells of the stomach mucus<sup>[10-12]</sup>, and its agonists have been shown to inhibit the production of chemokines and cytotoxic cytokines from immune cells<sup>[13,14]</sup>, and to promote epithelial cell survival and growth by activating anti-apoptotic and proliferative cellular signaling pathways<sup>[15,16]</sup>. Previous studies, using a non-selective EP3/EP4 agonist or an EP4 antagonist, imply EP4's role in gastric ulcer healing<sup>[17]</sup>; however, direct evidence of EP4 agonists' role on gastric lesions is still missing. In the present study, we investigated therapeutic potentials of a highly-selective EP4 agonist for treatment of a mouse gastric ulcer model in the presence or absence of indomethacin at various levels.

## MATERIALS AND METHODS

### *The EP4-selective agonist*

Competition binding experiments were performed in a medium containing Hank's balanced salt solution, 20 mmol/L HEPES, pH 7.3, membranes (about 60 µg protein) or  $2 \times 10^5$  cells from HEK 293 cells stably expressing the human EP4 receptor, [<sup>3</sup>H] PGE<sub>2</sub> (10 nmol/L) and various concentrations of test compounds in a total volume of 300 µL, read with LS6500 multi-purpose scintillation counter (Beckman Coulter, CA). cAMP assay was carried out using AlphaScreen cAMP assay kits (PerkinElmer, Boston, MA) following manufacturer's instructions. Intracellular Ca<sup>2+</sup> was monitored using a FLIPR Tetra system and assay kits from Molecular Devices following manufacturer's instructions. All assays were carried out in HEK-293 cells heterologously and stably expressing each of the eight human recombinant prostanoid receptors. For Ca<sup>2+</sup> signals, hEP2, hEP4 and hDP were co-expressed with a chimeric G protein, Gqs, which converts the Gs signal to a Gq Ca<sup>2+</sup> signal, and hEP3 with a chimeric G protein, Gqi. Subtype-selective compounds used here were PGE<sub>2</sub> for EP1, EP2, EP3 and EP4; BW245C for DP; 17-phenyl PGF<sub>2</sub>α for FP, carbacyclin for IP and U-46619 for TP.

The PGE2 analog used in this study bound hEP4 with a Ki of  $6.7 \pm 0.7$  nmol/L, not other prostanoid receptors, and increased cAMP production with an EC<sub>50</sub> of  $0.25 \pm 0.03$  nmol/L. On the other hand, the drug at 10 µmol/L showed no detectable FLIPR signals in HEK 293 cells heterologously expressing hEP1, hFP, hIP and hTP, and also in hEP2 (Gqs), hEP4 (Gqs), hEP3 (Gqi), hDP (Gqs), respectively. This compound is unstable in liver microsomes and thus when locally applied, its systemic exposure was minimal.

### *Cell culture and apoptosis assay*

Human gastric mucosal cells (AGS) were purchased from the American Type Culture Collection (Manassas, VA), and maintained on Ham's F-12 medium (GIBCO-BRL)

with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin. The cells were seeded in 6-well plates at  $1 \times 10^5$  cells/well. After overnight (37°C, 5% CO<sub>2</sub>) culture, the cells were treated with indomethacin at 50, 100, 200 or 400 µmol/L for 24 h, under serum-free conditions, to induce apoptosis as reported elsewhere<sup>[18]</sup>. Cell apoptosis was quantitated with flow cytometry as below. To evaluate EP4 agonist effect on cell survival, 70% confluent cells were treated with the EP4 agonist at 0, 1, 3 or 10 nmol/L, respectively, followed by 400 µmol/L indomethacin 30 min later. Twenty-four hours later, cells were collected and washed in cold PBS, and fixed with cold 70% ethanol added drop by drop while vortex stirring. Following overnight fixation, the cells were stained with propidium iodide (10 µg/mL, Sigma) and RNase A (1 mg/mL, Sigma) for 30 min in the dark. The cells were sorted by flow cytometry using CellQuest software (Becton Dickson, San Diego, CA). The sub-2N population was quantified. The percentage of apoptotic cells was calculated by sub-2N population from each drug treatment minus vehicle treatment<sup>[19]</sup>.

### *Indomethacin-induced gastric damage model*

High dose indomethacin (20 mg/kg in 4% DMSO, corn oil) was orally gavaged to induce gastric damage in C57BL/6 mice (Charles River, Wilmington, MA) at 8 wk old<sup>[20,21]</sup>. Vehicle or the EP4 agonist at 0.1 mg/kg in 0.1 mL 4% DMSO-corn oil was orally gavaged, 24 h and 30 min prior to indomethacin. Gastric lesions were assessed 24 h after indomethacin administration. The stomachs were removed, inflated with 2% formalin, immersed in 2% formalin for 10 min and then opened along the great curvature. The area of hemorrhagic lesions was measured under a dissecting microscope (16 × magnification) with a square grid (× 10), summed per stomach, and used as a lesion score<sup>[20,21]</sup>. The stomachs were then fixed in 10% formalin and sectioned at 5 µm thickness. HE staining was performed as usual. To monitor cell proliferation, BrdU was injected intraperitoneally at 10 mg/mL in 0.1 mL of normal saline, 16 h prior to sacrifice. Paraffin-embedded sections were deparaffinized in xylene and rehydrated in ethanol. Antigen was retrieved with citrate buffer, pH 6.0, boiled for 5 min in a microwave and slowly cooled down at room temperature. Immunofluorescence staining of BrdU was then performed following manufacturer's instructions (Roche-Applied Science, Penzberg, Germany). Briefly, the sections were incubated with sufficient amount of anti-BrdU working solution at 4°C in a humid atmosphere for 24 h; after 3 × washing, anti-mouse-Ig-fluorescein was applied for 30 min at 37°C in a humid atmosphere. DAPI was used to co-stain the slides for 5 min. The slides were covered with antifade mount medium, and evaluated with a fluorescence microscope. Ten views from each section were randomly collected at original magnification, × 200. The percentages of BrdU-positive cells were counted in well-oriented glands of mucous layer.

### *Chronic gastric ulcer model and treatments*

C57BL/6 mice at 3 mo old were shaved in the epigastric region and anesthetized with isoflurane. Following

sterilization with betadine and 70% ethanol, a midline incision was made to expose the stomach. Five microliters of 40% acetic acid was added through a 3 mm curette onto the serosal surface of the anterior wall of the stomach (just proximal to the antral gland area). The curette was placed tightly on the stomach surface to limit the spread of acetic acid. Thirty seconds later, acetic acid was wiped off and the surface was cleaned with normal saline. The abdomen wall was closed by 6-0 silk sutures, and the skin was closed by staples<sup>[22]</sup>. We first performed a study to monitor the dynamic changes of ulcers and animals. The mice lost some weight initially and recovered in 2 d. Their ulcer sizes were peaked at day 3 and then spontaneously healed within 2 wk. Vehicle or the EP4 agonist (0.1 mg/kg per day) and/or indomethacin (3 mg/kg per day) were orally gavaged from day 3 to day 6 in 0.1 mL 4% DMSO-corn oil. Then the animals were assessed on day 7. To study EP4 agonist effect on chronic gastric ulcer healing (without indomethacin), the EP4 agonist (0.1 mg/kg per day) or vehicle were given from day 3 to day 10, and evaluated on days 7 and 11, respectively.

On the day of sacrifice, blood was withdrawn, and hematology analysis was conducted by personnel who did not know the treatments (ADVIA120 Hematology System, Bayer, Tarrytown, NY). The stomachs were inflated with 2% formalin for 10 min and opened along the greater curvature. The stomachs were flattened on 3M paper. The ulcers were photographed under dissection microscopy ( $\times 16$ ) with a hooked camera, and images stored in the computer and analyzed by SPOT software. The stomachs were then fixed in 10% formalin and processed for sectioning. A slice cutting through the biggest diameter of each ulcer was sectioned and stained by HE.

All animal use protocols were approved and performed according to the guidelines of Allergan's animal care and use committee. Data shown are mean  $\pm$  SE. Statistical analysis was conducted by student *t*-test.

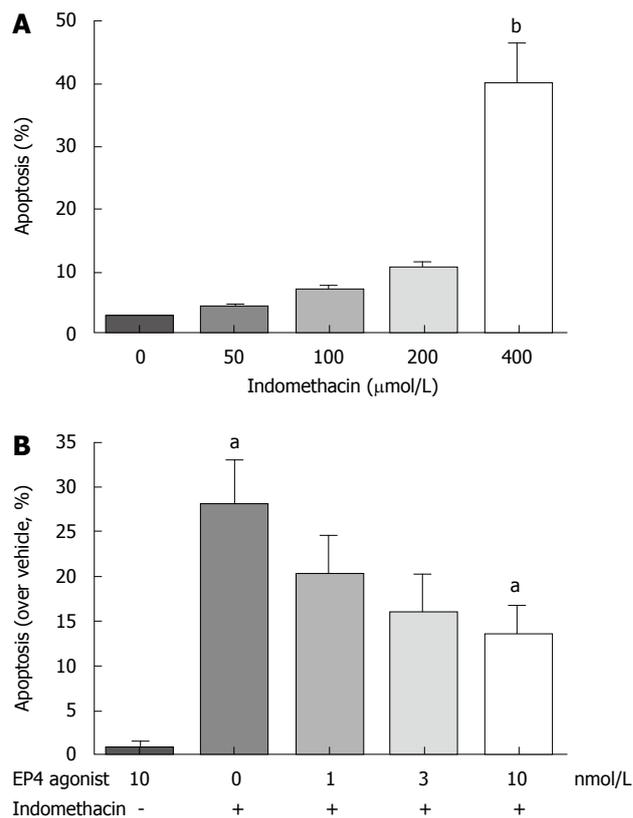
## RESULTS

### EP4 agonist decreased indomethacin-induced apoptosis

Exposure of human gastric mucous epithelial cells (AGS) to indomethacin (0, 50, 100, 200, 400  $\mu\text{mol/L}$ ) for 24 h concentration-dependently induced cell apoptosis as determined using flow cytometry analysis (Figure 1A). Particularly, indomethacin at 400  $\mu\text{mol/L}$  markedly increased apoptosis, nearly 10-fold greater than in the untreated cells, and its activity was significantly reduced upon treatment (30 min before) with a highly-selective and potent EP4 agonist (see materials and methods), in a dose-dependent manner (Figure 1B). The EP4 agonist at the highest dose, 10 nmol/L, significantly decreased indomethacin-induced apoptosis, by more than 50%.

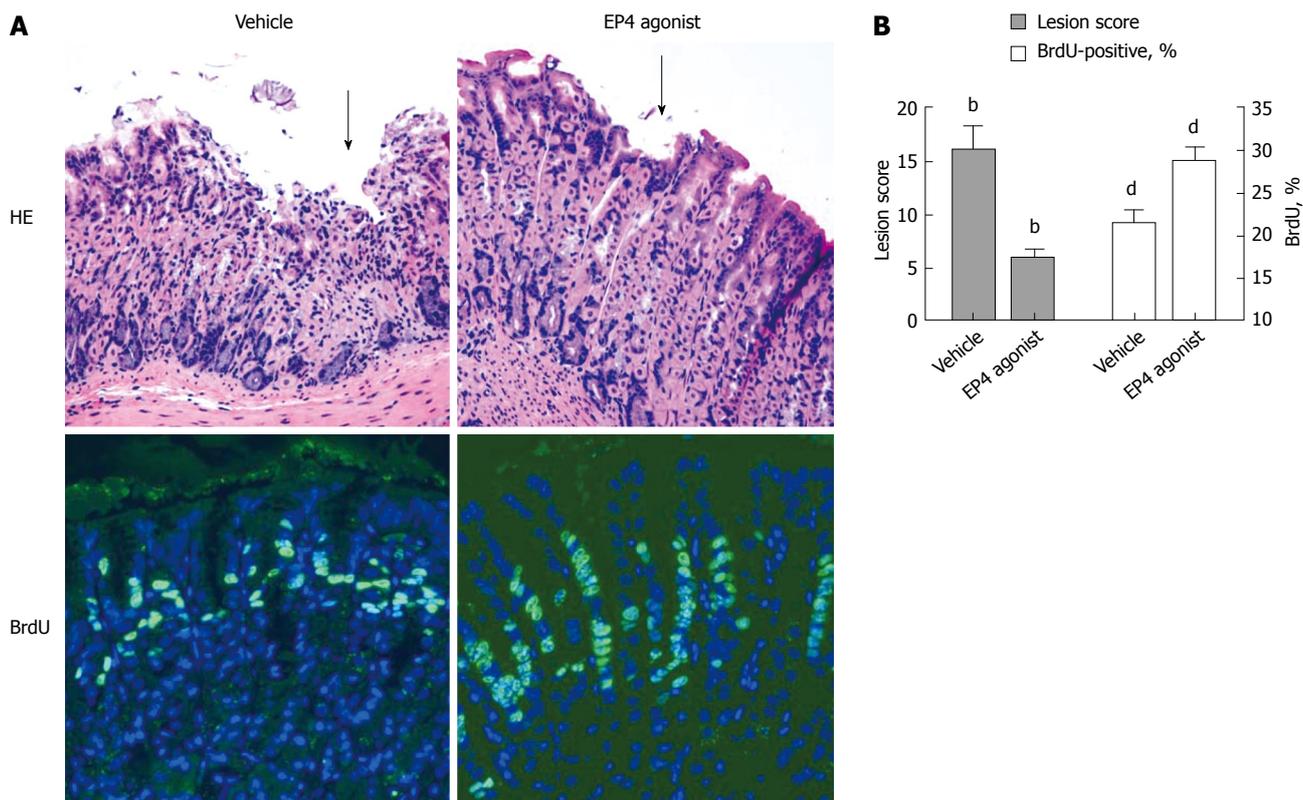
### EP4 agonist alleviated indomethacin-induced acute damage and promoted epithelial regeneration in mice

In rats, apoptosis of mucous epithelial cells contributes to indomethacin-induced lesions in stomachs<sup>[18]</sup>, and



**Figure 1** EP4 agonist effect on indomethacin-induced apoptosis in human gastric mucous epithelial cells (AGS). AGS cells were pre-treated with EP4 agonist for 30 min followed by addition of indomethacin for 24 h and sorted by flow cytometry after PI staining. Sub-2N population was quantified as apoptotic cells. A: indomethacin dose-dependently induced apoptosis, <sup>b</sup> $P < 0.0001$  vs the rest,  $n = 4$ ; B: shown are values minus vehicle controls, <sup>a</sup> $P < 0.005$ ,  $n = 5-9$ ; indomethacin dose was 400  $\mu\text{mol/L}$ .

here we examined whether the EP4 agonist protects the gastric mucus layer from indomethacin. Indomethacin at high dose (20 mg/kg) produced band-shaped hemorrhagic lesions in the mucous layer mostly at the glandular part of the stomachs, occurring 7 h post indomethacin application. Histologically, there was edema and disorganization of the mucous layer, patchy mucous epithelial cell exfoliation, shallow ulcer formation and bleeding with infiltration of inflammatory cells in vehicle treated mice. The mucous layer of EP4-treated mice remained largely intact except for some sparse, focal superficial defects in mucous cells (Figure 2A). Treatment with the EP4 agonist (concentration 0.002%), 24 h and 30 min before indomethacin, significantly reduced gastric lesion scores, from an average of 16 to less than 6 (Figure 2B). BrdU staining-positive cells were largely limited to the isthmus and neck region in the tubular glands of the stomach mucosa layer in vehicle-treated mice (Figure 2A). BrdU-positive cells migrated much higher and lower along the tubular glands in the EP4 agonist-treated mice than vehicle-treated mice (Figure 2A). BrdU-positive cells in mucous layer were on average 29% in EP4 treated mice, and 21% in vehicle treated mice (Figure 2B). Taken together, the EP4 agonist may stimulate proliferation and migration of gastric epithelial progenitors, so as to accelerate mucous repair.



**Figure 2** EP4 agonist effect on indomethacin-induced gastric lesion in mice. EP4 agonist was orally administered 24 h and 30 min prior to indomethacin dosing at 20 mg/kg. The stomachs were assessed for mucus lesions 24 h after indomethacin dosing. A: HE and BrdU immunohistochemistry staining of stomachs ( $\times 200$ ). Superficial mucosal cells had sloughed off gastric mucus with infiltration of inflammatory cells in vehicle-treated group (arrow points to one lesion site). The mucus of EP4 agonist-treated stomachs was almost normal, except for a sparse focal defect of superficial mucous cells without inflammatory cells (arrow points to one lesion site). BrdU labeling showed robust mucous epithelial regeneration and migration in EP4 agonist-treated rats compared with that of vehicle treatment; B: Quantification of gross lesion under dissection microscopy ( $\times 16$ ) and percentage of BrdU-positive cells among mucus cells. Shown are lesion scores,  $^bP < 0.0001$ ,  $n = 10$ ; and BrdU percentage,  $^dP < 0.01$ ,  $n = 10$ , respectively.

### Low dose indomethacin exacerbated chronic gastric ulcers in mice

A chronic gastric ulcer model was established by acetic acid application in mice. Low-dose indomethacin (3 mg/kg per day), which is sufficient to block *de novo* synthesis of PGE<sub>2</sub>, was applied 3 d post ulcer induction. On day 7, the indomethacin treatment increased gross ulcer areas by 76% as compared to vehicle-treated mice ( $P < 0.01$ , Figure 3A). Consistent with exacerbation of gastric ulcer sizes, hematology analysis revealed that indomethacin also worsened blood loss from the ulcers (Figure 3B), and higher lymphocyte surge as compared to untreated controls (Figure 3C). This supports the view that blocking of *de novo* synthesis of PGE<sub>2</sub> delayed spontaneous repair of established gastric ulcer, and exacerbated inflammation and bleeding, which is similar to human gastric ulcer's responses to NSAIDs.

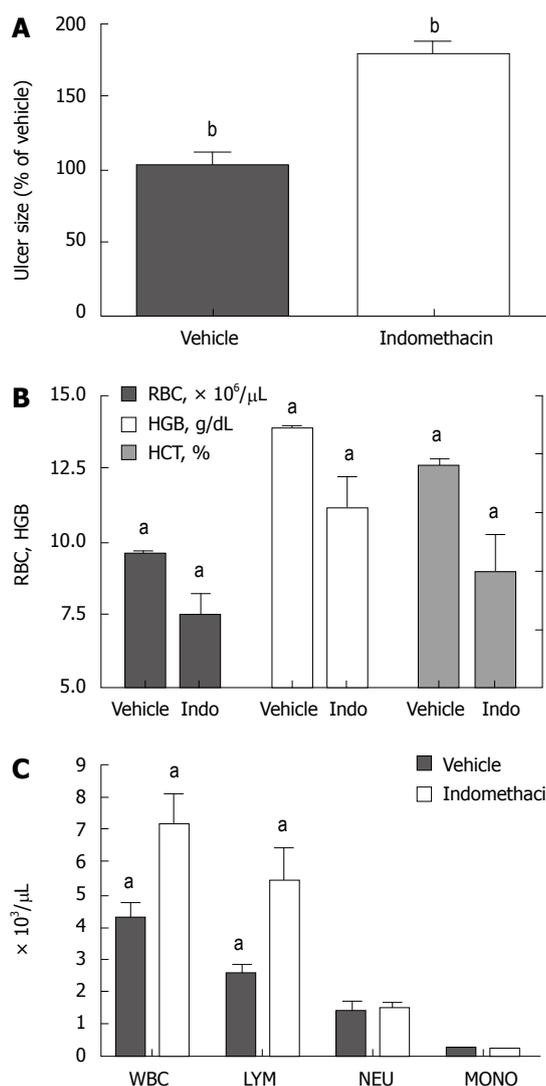
### EP4 agonist ameliorated indomethacin exacerbation on chronic gastric ulcer in mice

We next investigated whether exogenous EP4 agonist is capable of promoting ulcer healing in the presence of indomethacin treatment. Indomethacin (3 mg/kg) with EP4 agonist (0.002% in 0.1 mL) or with vehicle was orally administered to mice with established gastric ulcers from day 3 to day 7. Mice treated with EP4 agonist

had a smaller ulcer size than mice treated with vehicle,  $75.04\% \pm 7.06\%$  and  $100.02\% \pm 9.44\%$ , respectively, on day 7. Hematology analysis showed that EP4 agonist treatment significantly ameliorated loss of red blood cells, hemoglobin and hematocrit (Figure 4A). This may suggest that EP4 agonist-treated mice had either smaller ulcers or more mature granulation tissue than control mice. EP4 agonist leads to gastric mucous vasodilation, not vasoconstriction (mediated by EP3 receptor)<sup>[13]</sup> and mature granulation tissue is more resistant to noxious stimuli. The inflammation at ulcer sites was reflected by white blood cell counts in the peripheral circulation. EP4 agonist treatment decreased white blood cell counts from  $6900/\mu\text{L}$  to  $5600/\mu\text{L}$ , and lymphocyte counts from  $4690/\mu\text{L}$  to  $3330/\mu\text{L}$  ( $P < 0.05$ ) (Figure 4B).

### EP4 agonist accelerated the spontaneous healing of chronic gastric ulcer

We also examined the effects of EP4 agonist alone on gastric ulcer healing. By day 7, treatment with EP4 agonist reduced ulcer area by 40% as compared to that observed with vehicle treated mice (Figure 5A,  $P < 0.005$ ). By day 11, the drug further reduced ulcer size by 70% (Figure 5A). There was much less inflammatory cell infiltration and necrosis tissue in the ulcers of EP4 agonist-treated animals, compared with untreated mice.

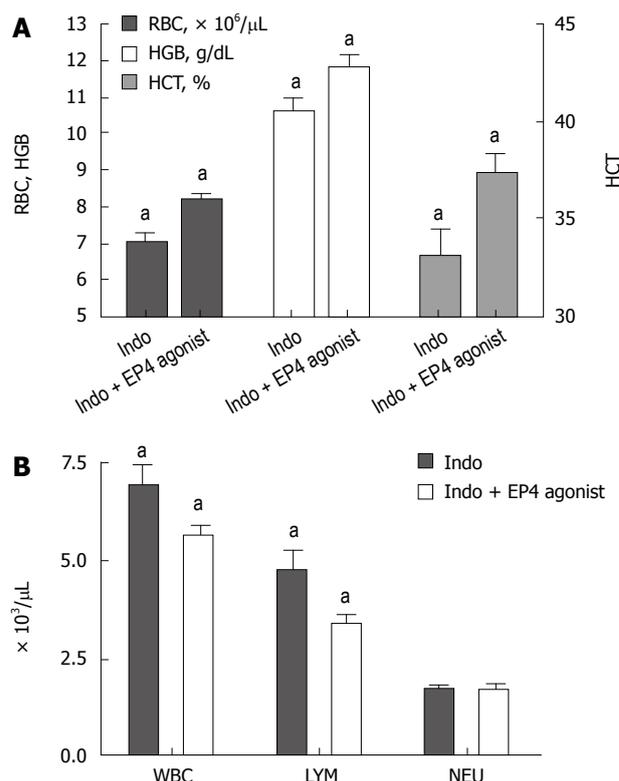


**Figure 3 Low dose indomethacin effect on chronic gastric ulcer of mice.** Chronic gastric ulcers were created by 40% acetic acid as detailed in "Methods". Then the animals were given indomethacin from day 3 to day 6 at 3 mg/kg per day and the ulcers were assessed after blood withdrawal on day 7 post-surgery. A: Ulcer sizes were measured under dissection microscopy with 16 × magnification; <sup>b</sup>*P* = 0.006, *n* = 18; B: Hematology analysis of ulcerated animals on day 7, <sup>a</sup>*P* < 0.05, *n* = 6; RBC: Red blood cell; HGB: Hemoglobin; HCT: Hematocrit; C: Hematology of ulcerated animals on day 7, <sup>a</sup>*P* < 0.05, *n* = 6; WBC: White blood cells; LYM: Lymphocytes; NEU: Neutrophils; MONO: Monocytes.

On sectioning slides, inflammatory cell scores were 1.8 ± 0.2 for the EP4-agonist-treated and 2.7 ± 0.2 for the vehicle-treated mice (*P* < 0.05) (Figure 5B).

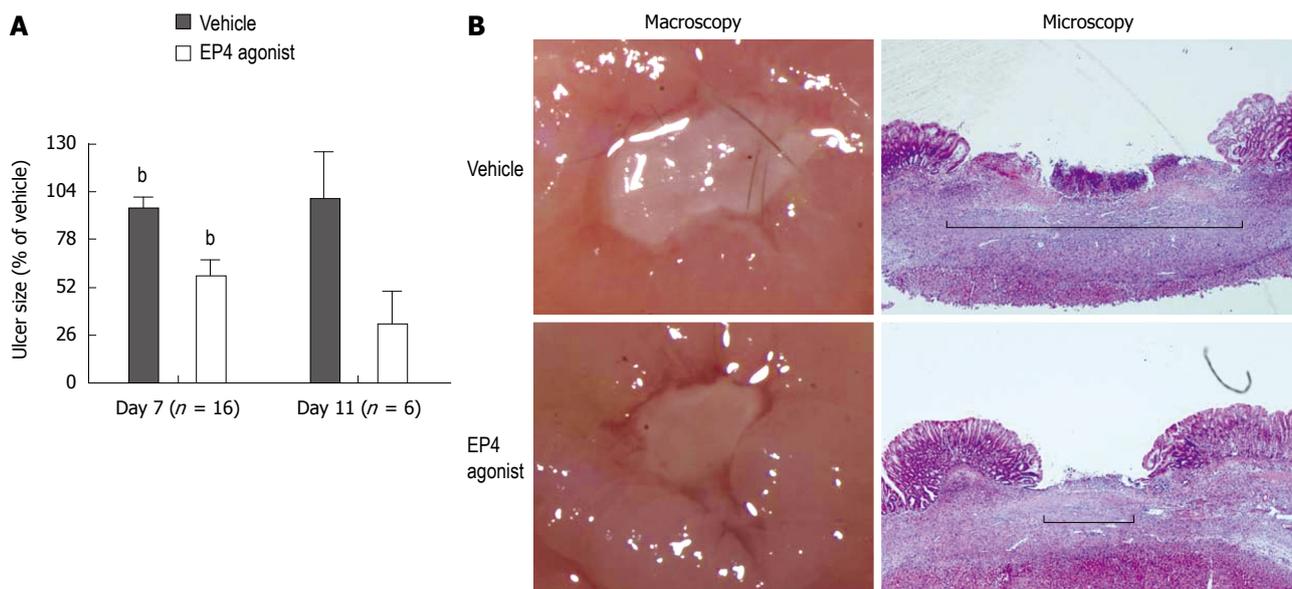
## DISCUSSION

In the present study, we have shown that indomethacin, a prototypic cyclo-oxygenase (COX) inhibitor, at high dose, induced gastric epithelial apoptosis and produced gastric hemorrhagic lesions, and that the EP4-selective agonist we used here reduced such indomethacin-induced gastric injuries. Also, the EP4 agonist ameliorated ulcer bleeding and inflammation exacerbated by indomethacin at a low dose on existing ulcers, and promoted the spontaneous healing of chronic gastric ulcers in the absence of indomethacin.



**Figure 4 EP4 agonist alleviated indomethacin effect on gastric ulcer in mice.** Mice with gastric ulcer were given low-dose indomethacin (3 mg/kg per day) with or without EP4 agonist from day 3 to day 6 post-surgery. The blood was withdrawn on day 7 for hematology analysis. A and B are results of hematology analysis, <sup>a</sup>*P* < 0.05, *n* = 20.

Such indomethacin-induced gastric injuries and PGE2 analogue-induced gastric protection appear to be somewhat similar to their actions observed at cellular level: NSAIDs are known to bring about mitochondrial damage, caspase cleavage, and eventually cell apoptosis in human gastric mucus as well as animal primary gastric epithelial cells<sup>[3,18,23,24]</sup>. PGE2 and its analogs, on the other hand, inhibit indomethacin-induced mitochondrial damage and apoptosis in gastric epithelial cells<sup>[23,25]</sup>, and a PGE1 analog, misoprostol, has been shown to reverse the inhibitory effect of NSAIDs on the regeneration of gastric mucous epithelial cells from human and animals<sup>[26]</sup>. 11-deoxy-PGE1 (EP3/EP4 agonist) reverses indomethacin-induced delay in the healing of chronic gastric ulcers<sup>[17]</sup>. One EP4-selective antagonist shows a deleterious effect on the spontaneous healing of chronic gastric ulcers with simultaneous suppression of vascular endothelial growth factor (VEGF) expression<sup>[17,27]</sup>. So gastric protective effects of PGE2 and its analogs may be partly caused by the activation of EP4, one of its 4 receptor subtypes, which is known to activate Gi- and Gs subtypes of G proteins, and to transduce PI3k/Akt/Erk1/2 and cAMP/PKA signaling<sup>[1,28,29]</sup>. Both pathways mediate pro-survival and proliferation signals in various epithelial cell lines, and are in line with our observation that the EP4 agonist inhibits indomethacin-induced apoptosis in human gastric mucosal cells (AGS), an established model for gastrointestinal effects of COX inhibitors<sup>[30]</sup>, and expressing high levels of EP4



**Figure 5** EP4 agonist promoted gastric ulcer healing in mice. Mice with gastric ulcers were treated with vehicle or EP4 agonist from day 3 to day 10 post-surgery. Gastric ulcers were assessed on day 7 or 11, respectively. A is quantification of ulcer sizes under dissection microscopy with 16 × magnification,  $^bP = 0.003$ ,  $n = 16$ ; B is representative images of gastric ulcers; the macroscopy images are at original magnification, × 16; the microscopy images are at original magnification, × 40 from slide sections.

transcripts (data not shown). Besides cell survival, the stimulation of intracellular cAMP from EP4 activation induces smooth muscle relaxation, and increases mucous blood supply and mucus secretion, which may additionally contribute to lessening indomethacin-induced injuries<sup>[25,31-33]</sup>.

In addition to acute damages produced by high-dose indomethacin, we also observed the chronic deleterious gastric effects of indomethacin at low-dose, which is more relevant to common clinical situations. Consistent with earlier reports<sup>[17,34,35]</sup>, low-dose indomethacin delayed healing of chronic gastric ulcers, exacerbated ulcer bleeding and inflammation, due to the inhibition of COX-2 expression and *de novo* synthesis of PGE<sub>2</sub>, and the inhibition of epithelial cell proliferation at the ulcer edge in both animals and humans. We have shown here that the EP4 agonist reversed such chronic effects induced by indomethacin at low dose.

Interestingly, we also observed here that the EP4 agonist accelerated the healing of chronic gastric ulcers under non-indomethacin challenged conditions. The major part of gastric ulcer healing is the restoration of gastric structure, which depends on the formation of the granulation tissue template made of gastric fibroblast cells and neovasculature. These fibroblasts express EP4 abundantly, and its activation is known to increase the synthesis of basic fibroblast growth factor, hepatocyte growth factor and VEGF<sup>[29,36-38]</sup>. All these factors may accelerate regeneration of fibroblasts and extracellular matrix, thus restoring ulcerated areas, and restituting epithelial cell layers<sup>[17,38]</sup>. Also the EP4 agonist showed anti-inflammatory activities as shown here and reported earlier<sup>[39]</sup>: fewer inflammatory cells in the blood and minimal infiltration in the ulcers from animals treated with the EP4 agonist. This should facilitate the healing process

since the control of inflammation is a pre-requisite to rapid healing<sup>[35]</sup>.

In summary, EP4 agonists may mimic the gastric protective effects of PGE<sub>2</sub> in the presence or absence of NSAIDs, and may show advantages over non-selective analogs such as misoprostol by minimizing adverse effects arising from activating all 4 subtype receptors of PGE<sub>2</sub>.

## ACKNOWLEDGMENTS

We are grateful to the excellent assistance from Wu-Xia Fu, Ke-Hui Wang and Margaret A Ramos. We also appreciate the discussion with Professor George Sachs at UCLA.

## COMMENTS

### Background

Non-steroidal anti-inflammatory drugs (NSAIDs) including both cyclooxygenase (COX)-1/2 and COX-2-selective inhibitors, such as indomethacin, ibuprofen and celecoxib, have been prescribed in the world for treatment of pain, arthritis, menstrual symptoms and cancer, to name a few. However, nearly 30% of patients suffer from gastric lesions and bleeding. To mitigate NSAIDs' adverse effects on the stomach, misoprostol, a non-selective PGE<sub>1</sub> analogue, has been prescribed as the first choice for prevention of NSAID-induced injuries in USA, but often induces severe adverse effects. There remain unmet medical needs for drugs with improved therapeutic profiles.

### Research frontiers

PGE<sub>2</sub>/E<sub>1</sub> interacts with 4 subtype receptors, EP<sub>1</sub>, 2, 3 and 4 in mammalian cells. Numerous studies have been performed to understand each subtype receptor's function and mechanism under various physiological and pathological conditions. High subtype receptor-selective ligands have been designed and tested to avoid adverse effects from non-selective drugs, such as misoprostol.

### Innovations and breakthroughs

This study employed a novel, highly selective EP4 agonist, reported direct evidence for its protective activities against NSAIDs in the stomach, and further disclosed that the EP4 agonist may in part function through promoting mucous

epithelial cell survival and regeneration. This is the first study to show that an EP4 agonist may facilitate chronic gastric ulcer healing, although similar activity of EP4 in the stomach has been implicated in several concurrent studies using non-selective EP4 agonists or antagonists.

### Applications

The concept from this paper would facilitate therapeutic developments of EP4-selective agonists for prevention of NSAIDs' adverse effects in the gastrointestinal (GI) tract as well as for monotherapy treatment of gastric ulcers. Further, EP4 agonists may provide gastric protection under conditions such as stress, radio/chemotherapy and other conditions compromising GI activities.

### Terminology

Prostaglandin E2 (PGE2) is synthesized *via* key enzymes COX-1/2, under normal conditions primarily by COX-1 and under pathological conditions by inducible COX-2. PGE2 is a paracrine or autocrine hormone, and is involved in inflammation and pain, and also plays an important role in functional stability of the GI tract.

### Peer review

In this manuscript, the authors investigated the effects of EP4-selective agonist on indomethacin-induced gastric lesions and spontaneous healing of chronic gastric ulcers in mice or cultured human gastric mucous cells. The study was well performed and very interesting.

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## Voxel-based analyses of magnetization transfer imaging of the brain in hepatic encephalopathy

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**CONCLUSION:** The distribution of MTR changes in HE points to an early involvement of basal ganglia and white matter in HE.

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**Key words:** Brain; Hepatic encephalopathy; Magnetic resonance imaging; Liver cirrhosis; Magnetization transfer imaging

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### Abstract

**AIM:** To evaluate the spatial distribution of cerebral abnormalities in cirrhotic subjects with and without hepatic encephalopathy (HE) found with magnetization transfer imaging (MTI).

**METHODS:** Nineteen cirrhotic patients graded from neurologically normal to HE grade 2 and 18 healthy control subjects underwent magnetic resonance imaging. They gave institutional-review-board-approved written consent. Magnetization transfer ratio (MTR) maps were generated from MTI. We tested for significant differences compared to the control group using statistical non-parametric mapping (SnPM) for a voxel-based evaluation.

**RESULTS:** The MTR of grey and white matter was lower in subjects with more severe HE. Changes were found in patients with cirrhosis without neurological deficits in the basal ganglia and bilateral white matter. The loss in magnetization transfer increased in severity and spatial extent in patients with overt HE. Patients with HE grade 2 showed an MTR decrease in white and grey matter: the maximum loss of magnetization transfer effect was located in the basal ganglia [SnPM (pseudo-) $t = 17.98$ ,  $P = 0.0001$ ].

### INTRODUCTION

Hepatic encephalopathy (HE) is a frequent complication of liver cirrhosis, which is characterized by sleeping disorders, asterixis, and deficits in motor skills and reaction time. Twenty to eighty percent of patients with cirrhosis suffer from minimal HE (mHE)<sup>[1]</sup>. Five years after the diagnosis of mHE, 26% of patients with liver cirrhosis have episodes of overt HE. This condition is associated with a poor prognosis<sup>[2]</sup>.

Studies using magnetization transfer contrast imaging (MTI) have demonstrated a decrease in the magnetization transfer ratio (MTR) in the brains of cirrhosis patients with mHE and overt HE<sup>[3-10]</sup>. MTR decrease in cirrhosis has been proposed to be an effect of astrocytic water retention<sup>[5-7,9,10]</sup>, demyelination<sup>[5]</sup> and axonal loss<sup>[5]</sup>, in addition to changes in blood flow and energy metabolism<sup>[9]</sup>.

MTI has been used successfully to monitor normalization of cerebral abnormalities in cirrhosis patients following liver transplantation<sup>[8]</sup>, and has been shown to detect increasing abnormalities following induced hyperammonemia<sup>[9]</sup>.

The cited studies evaluated MTR maps using region-of-interest (ROI) based analyses. The MTR of normal appearing white matter<sup>[5-10]</sup> and anatomically defined areas of deep grey matter<sup>[3-5,10]</sup> have been the subject of previous studies on HE. To the best of our knowledge, no systematic evaluation of the spatial distribution of MTR abnormalities in HE has been published so far. The purpose of the present study is to evaluate the spatial distribution of MTI changes caused by central nervous system (CNS) abnormalities in HE.

## MATERIALS AND METHODS

### Subjects

Approval was obtained from the institution's review board. All patients and volunteers gave written informed consent after the neuropsychological tests and magnetic resonance imaging (MRI) had been explained fully. In this prospective study (for details see Table 1), 19 patients (14 male and five female) with non-alcoholic cirrhosis and 18 age-matched controls (eight male and 10 female) were included. Cirrhosis was caused by hepatitis C ( $n = 9$ ), hemochromatosis ( $n = 2$ ), primary chronic cholangitis ( $n = 2$ ), hepatitis B ( $n = 1$ ), Wilson's disease ( $n = 1$ ) and cryptogenic cirrhosis ( $n = 4$ ).

Subjects with a history of drug abuse, including alcohol, and those suffering from neurological or psychiatric diseases were excluded. Also excluded were patients who were taking CNS-relevant medications such as benzodiazepines, benzodiazepine antagonists and antidepressants. Further exclusion criteria were severe diseases such as spontaneous bacterial peritonitis, severe renal failure, uncontrolled diabetes mellitus or coronary heart disease. Since asterixis and hyperreflexia, as well as other more severe neurological conditions such as stupor or somnolence may interfere with safe and artefact-free MRI, patients with higher degrees of HE (grade 3 or 4) who also had these conditions were not considered for investigation. Also, patients needed to cooperate for neuropsychological examination and the patients' willingness represented a limitation in testing subjects with HE grade 3 or 4.

The severity of liver disease was determined according to the Child-Pugh-score<sup>[11]</sup>: patients were graded Child-Pugh A in nine cases, B in five and C in five.

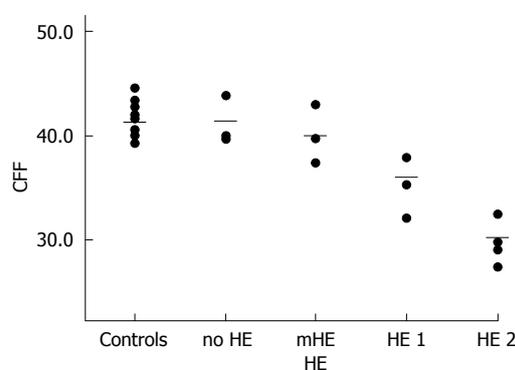
### Neuropsychological examination

Five computerized psychometric neurological tests were performed on each patient. The test battery included the visual pursuit test, motor performance series, Cognitron, Vienna reaction test and Tachistoscopic Traffic Test Mannheim for Screen (TAVTMB) as part of the Vienna Test System (Schuhfried GmbH, Mödling, Austria, 1999). mHE was diagnosed if a patient showed no clinical overt symptoms of HE and performed at  $< 1$  SD below the mean in at least two of the five computer psychometric tests of the test battery. Cirrhosis patients without clinical overt HE who performed at  $< -1$  SD in only one of the tests were graded as having no HE<sup>[12]</sup>. Overt encephalopathy was graded according to the

**Table 1** Nineteen patients with cirrhosis and 18 controls were enrolled (mean  $\pm$  SD)

	Controls	Cirrhosis
$n$ (male/female)	18 (8/10)	19 (14/5)
Age (yr)	55.7 $\pm$ 13.8	61.1 $\pm$ 12.4 NS
CFF (Hz)	41.3 $\pm$ 1.6	36.6 $\pm$ 4.9 <sup>b</sup>
Child-Pugh grading		
A		9
B		5
C		5
HE grading		
HE 0		5
mHE		3
HE 1		6
HE 2		5

<sup>b</sup> $P < 0.01$  vs controls (Mann-Whitney  $U$  test). NS: No significant difference.



**Figure 1** CFF of controls and patients. HE grading and CFF (Hz). Circles indicate individuals' CFF, bars represent means of groups (mean CFF of controls: 41.3 Hz; mean CFF of group with no HE: 41.4 Hz; mean CFF of group with mHE: 40.0 Hz; mean CFF of group with HE 1: 36.1 Hz; mean CFF of group with HE 2: 30.2 Hz).

West-Haven criteria<sup>[13]</sup>. Cirrhosis patients were graded as no HE in five cases, mHE in three, HE 1 in six and HE 2 in five cases (Table 1).

In order to assess the severity of HE, the critical flicker frequency (CFF) was introduced in 2002. CFF is used in the evaluation of neurological deficits in low-grade HE and has been shown to respond readily to an improvement or deterioration of neurological symptoms<sup>[12]</sup>.

CFF was determined in each patient using the Schuhfried Test System (Eberhard, G. Schuhfried GmbH, Mödling, Austria, 1994). A flickering light of 650 nm wavelength and 5.3 mcd was used for intrafoveal stimulation, while the flickering frequency decreased at 0.5-0.01 Hz/s. The CFF was defined as the frequency needed to perceive initially the stimulus as non-continuous. The means of eight repeat tests were determined (Figure 1).

### MRI protocol

All scans were acquired on a 1.5-T clinical scanner (Magnetom Vision Plus; Siemens Medical Solutions, Erlangen, Germany) using the standard head coil. MTI was performed with two 2D gradient-echo sequences (TR 700 ms, TE 12 ms, flip angle  $\alpha = 20^\circ$ , one acquisition, 20 slices of 5 mm thickness, 0.5 mm gap), using a matrix

of  $224 \times 256$  pixels with a field of view of  $240 \text{ mm} \times 240 \text{ mm}$ . The first set of images was obtained with no saturation pulse. The latter sequence used a saturation pulse 1.5 kHz below H<sub>2</sub>O frequency, with a bandwidth of 250 Hz, 7.68 ms length and a flip angle of  $500^\circ$ . Additionally, routine examinations including T2- and T1-weighted imaging were performed to exclude patients with asymptomatic infarction or chronic ischemic changes.

### Image processing

For each subject, the MTR was quantified as a percentage of signal loss between the two images according to the following equation:  $\text{MTR} = (S_0 - S_s)/S_0 \times 100\%$ .  $S_0$  is the mean signal intensity of a pixel obtained from the sequence using no saturation pulse, and  $S_s$  is the mean signal intensity with the applied saturation pulse. Gray values in these secondary images represent MTR data, which yielded an MTR map.

Pixels that contained skull and soft tissue in the MTR maps were removed using the MRIcro-Software<sup>[14]</sup>. Images without skull and soft tissue pixels were used, since this has been reported to improve the validity of voxel-based evaluation of brain imaging data<sup>[14]</sup>.

The MTR maps that contained only brain pixels were normalized into a standardized space using statistical parametric mapping (SPM5) software<sup>[15]</sup>. An MTR template was generated to ensure reliable normalization. For this, the images of all individuals were smoothed with a Gaussian kernel of 3 mm full width at half maximum, and normalized to the standard SPM EPI-template (Montreal Neurological Institute). The mean of the 37 normalized images was used as an MTR template for the normalization of each original MTR map. The default spatial normalization settings were applied. A Gaussian kernel of 3 mm full width at half maximum was used for the smoothing of the images.

It has been reported that brain atrophy is present in cirrhosis patients<sup>[16]</sup> and that comparison of atrophied and normal brains may lead to systematic errors<sup>[17]</sup>. To assess, whether atrophy interferes with voxel-based analysis in patients with liver cirrhosis, a test with binary images was performed. Binary images were generated from the normalized images of all patients and controls using MRIcro-Software. Pixels that contained brain were given the value 1. Pixels that contained cerebrospinal fluid (CSF) were set to 0.

### Statistical analysis

Statistical mapping analysis has been applied in functional brain imaging [fMRI or positron emission tomography (PET)]. Recently, the method's successful use has been demonstrated in diffusion-weighted imaging<sup>[18]</sup>, fluid-attenuated inversion-recovery imaging<sup>[19]</sup>, perfusion-weighted imaging<sup>[20]</sup> and MTI<sup>[21]</sup>.

We conducted a voxel-based analysis using the statistical non-parametric mapping (SnPM5b) software<sup>[22]</sup> based on SPM5<sup>[15]</sup>. A non-parametric approach was chosen because uniform variance across the volumes was not given and group size did not permit parametric tests. SnPM uses a permutation approach to account

for the multiple comparison problem in voxel-by-voxel evaluation. It does not make the assumptions derived from random field theory underlying the multiple comparison corrections used in SPM<sup>[22]</sup>.

For all statistical models employed, a threshold of  $P < 0.001$  was used to determine significance. Non-parametric testing was conducted with 10000 random permutations when possible permutations exceeded this number. The anatomical localization of the maximum statistics was determined by co-examination of the SnPM (pseudo-)t map and the customized MTR template. The results are displayed as SnPM (pseudo-)t-map images superimposed on MTR maps of representative subjects.

Based on the hypothesis that cirrhosis patients (no HE, mHE, HE 1 and HE 2) had lower MTR values than controls, two-sample, one-sided permutation tests were conducted. Subject age was included as a confounder in the group comparison tests. Correlation between the MTR maps and CFF was tested. The binary images were tested on the hypothesis of a decreased brain volume in cirrhosis patients compared with controls.

## RESULTS

### MTR group comparisons

Compared with controls, cirrhosis patients without neurological deficits (no HE) displayed significantly decreased MTR values in the basal ganglia and in the hemispheric white matter (Table 2). The maximum statistics were located in the right putamen [(pseudo-)  $t = 4.60$ ,  $P = 0.0004$ ] (Figure 2). Statistics in the left putamen were (pseudo-)  $t = 2.65$ ,  $P = 0.0006$ . In the group with mHE, a significant MTR decrease was found in hemispheric white matter, deep grey matter, brainstem and cerebellum. The cluster exhibiting the maximum statistics was in the right putamen (pseudo-)  $t = 8.57$ ,  $P = 0.0008$ . Contralateral statistics were [(pseudo-)  $t = 5.72$ ,  $P = 0.0004$ ].

The groups with overt HE showed an MTR decrease in the entire brain. In HE 1, the maximum statistics were detected in the left posterior white matter [(pseudo-)  $t = 8.82$ ,  $P = 0.0001$ ]. The statistics of the right posterior white matter were (pseudo-)  $t = 4.70$ ,  $P = 0.0001$ . In HE 2, the maximum statistics were found in the left globus pallidus [(pseudo-)  $t = 17.98$ ,  $P = 0.0001$ ]. The statistics of the right globus pallidus were (pseudo-)  $t = 14.06$ ,  $P = 0.0001$ .

### Binary data comparison

The SnPM results showed significant differences in external and internal CSF space between cirrhosis patients and controls (Figure 3). Spatial extent and maximum (pseudo-)  $t$  was higher in the patients with overt HE.

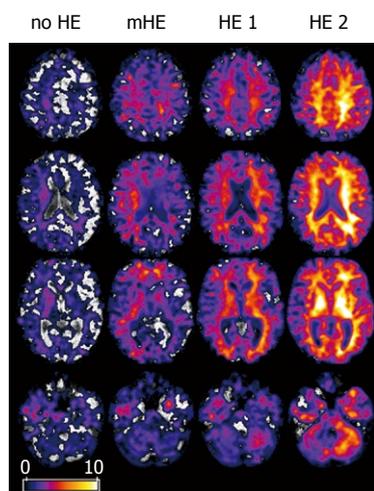
### Correlation of CFF and MTR

In the cirrhosis patients, a positive correlation between CFF and MTR was found in basal ganglia and in supra- and infratentorial white matter (Figures 4 and 5, Table 3). Largest statistics detected in a brain parenchyma cluster

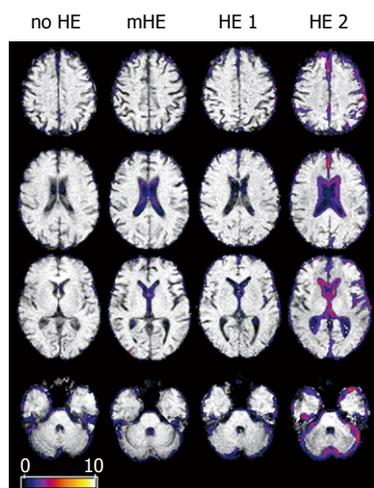
Table 2 Patients with no HE, mHE, HE 1 and HE 2 compared to controls (SnPM two-sample  $t$  test)

	Cluster level		Voxel level			SPM coordinates (mm)		
	k	$P$ (FWE corrected)	$P$ (FDR corrected)	(Pseudo-) $t$	$P$ (uncorrected)	x	y	z
Controls vs no HE	209966	0.4753	0.5034	4.60	0.0004	-53	18	38
		0.7301	0.5034	4.27	0.0019	32	-101	-14
		0.8160	0.5034	4.14	0.0002	-27	17	55
Controls vs mHE	2652240	0.0045	0.0140	8.57	0.0008	-8	54	1
		0.0090	0.0140	8.27	0.0008	37	18	32
		0.0135	0.0140	7.72	0.0008	-19	-40	40
Controls vs HE 1	288720	0.0003	0.0034	8.82	0.0001	-38	-74	1
		0.0010	0.0034	8.31	0.0001	-38	-59	-10
		0.0017	0.0034	7.91	0.0001	-30	-26	1
Controls vs HE 2	286240	0.0001	0.0006	17.98	0.0001	22	-6	-6
		0.0001	0.0005	14.33	0.0001	-19	-38	36
		0.0001	0.0005	14.31	0.0001	18	2	-4

k: Number of voxels in significant clusters; FWE: Family-wise error; FDR: False discovery rate corrected and uncorrected;  $P$  and SnPM (pseudo-) $t$  of most significant voxel clusters and their coordinates.



**Figure 2 (Pseudo-) $t$ -maps.** MTR of patients with no HE, mHE, HE 1 and HE 2 compared to controls. Axial views superimposed on MTR maps of representative subjects. Colored areas represent voxels with significant decrease in MTR. Grey and white matter are involved. Local statistical maxima were found in basal ganglia and posterior white matter.

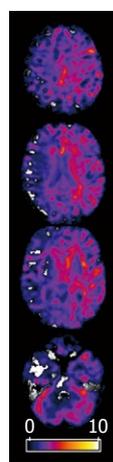


**Figure 3 (Pseudo-) $t$ -maps.** Binary data of patients with no HE, mHE, HE 1 and HE 2 compared to controls. Axial views superimposed on the binary images of representative subjects showing differences in brain size. Colored areas represent voxels with significant differences. CSF space was increased in lateral cerebral fissure; parietal, frontal and cerebellar gyration were prominent in overt HE.

were in the left frontal white matter: (pseudo-) $t = 7.06$ ,  $P = 0.0001$ . Contralateral frontal white matter statistics were (pseudo-) $t = 4.11$ ,  $P = 0.0004$ .

## DISCUSSION

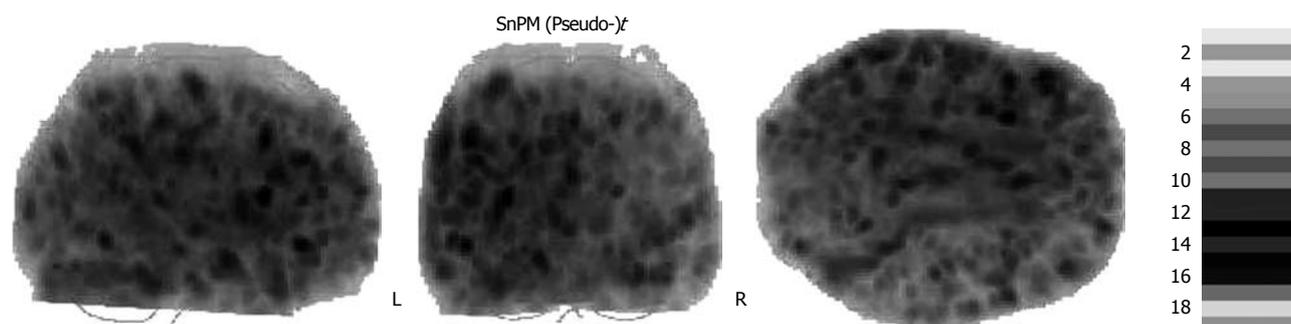
MTI is an application of MRI that is used to assess non-water components in tissue. As opposed to conventional



**Figure 4 (Pseudo-) $t$ -maps ( $P < 0.001$ ).** Areas with a correlation of subjects' CFF and MTR. Axial views superimposed on the MTR map of a representative subject (mHE). Colored areas represent voxels with significant positive correlation between CFF and MTR. The maximum statistics of a brain parenchyma cluster were found in left frontal white matter.

MRI, which is designed to represent different relaxation times in different tissues, MTI uses the exchange of magnetic properties between two brain tissue components: free water and macromolecules. Magnetization transfer takes place with and without physical exchange of protons between these two components. The extent of magnetization transfer is quantified using the ratio between two MR images. One image is acquired without and one with a high-frequency pulse that is designed to saturate the macromolecules. This results in a signal difference between the two images, as a result of the transfer of magnetization from free water protons towards macromolecules. The index derived from the two images can be calculated pixelwise and is rendered as an MTR map<sup>[23]</sup>. Changes in both components can lead to abnormal magnetization transfer. Depletion of macromolecules, a different macromolecule composition, as well as changes in brain water content may influence MTR.

Abnormal magnetization transfer is a known pathological feature in MRI of HE<sup>[24]</sup>. It has been reported to increase with the severity of HE. The present study provides a description of the distribution of these changes. In comparison to former studies that rely on simple ROI type analysis, a voxel-based approach was used to detect the localization of magnetization transfer changes in HE.



**Figure 5 (Pseudo-) $t$  results ( $P < 0.001$ ).** Voxel clusters with a correlation of subjects' CFF and MTR. SnPM glass brain representation of voxel clusters with a positive correlation between CFF and MTR (sagittal, coronal and axial view). A significant correlation was seen in all lobes.

**Table 3 Correlation of MTR and CFF (SnPM  $t$  test)**

	Cluster level	Voxel level				SPM coordinates (mm)		
	k	$P$ (FWE corrected)	$P$ (FDR corrected)	(Pseudo-) $t$	$P$ (uncorrected)	x	y	z
Controls vs no HE	280567	0.0031	0.0137	7.06	0.0001	-34	18	8
		0.0167	0.0137	6.26	0.0001	-53	18	38
		0.0205	0.0137	6.19	0.0001	-54	35	-17

### Spatial distribution of MTR abnormalities

MTR reduction in the brain of patients with cirrhosis involved white and grey matter, the brainstem and the cerebellum. The MTR decrease was more severe in patients with overt HE. The area of significantly reduced MTR was larger in patients with severe HE.

These results go beyond the findings of Iwasa *et al*<sup>[5]</sup>, who have reported decreased MTR values in anatomically defined areas of the brain (globus pallidus, putamen, thalamus, corona radiata and subcortical white matter) in 37 cirrhosis patients with mHE, compared to controls. They also support and exceed the reports of Córdoba *et al*<sup>[6]</sup>, Rovira *et al*<sup>[7]</sup> and Balata *et al*<sup>[9]</sup>, who have described a loss of magnetization transfer effect in frontal and parietal white matter. In patients without overt HE, the maximum statistics were in the right basal ganglia. Patients with HE 2 had a more severe loss of MTR, which was localized bilaterally and symmetrically.

The data suggest that encephalopathy in patients with liver cirrhosis is unlikely to be related to structural damage of a specific area of the brain. In the dysmetabolic situation that accompanies hepatic insufficiency, our data show a diffuse pattern of brain involvement.

Pathological MRI of basal grey matter in HE has been described before the advent of MTI. Hyperintensity in T1-weighted imaging in the globus pallidus of patients with cirrhosis is a frequent finding<sup>[25]</sup>. Basal ganglia hyperintensity has been demonstrated to be reversible after successful liver transplantation<sup>[26]</sup>. Manganese deposition is a suggested condition to shorten T1 in the basal ganglia of cirrhosis patients<sup>[3,27]</sup>, and to be an additive factor to the extrapyramidal symptoms in these patients<sup>[28]</sup>. In the mouse brain, significant shortening of T1 relaxation can be demonstrated after intravenous, intraperitoneal or subcutaneous administration of MnCl<sub>2</sub><sup>[29]</sup>. In a quantitative T1-mapping study of the brains of patients with liver cirrhosis and HE, Shah *et al*<sup>[30]</sup> have

reported T1-shortening for the globus pallidus, caudate nucleus, and posterior limb of the internal capsule. They have discussed manganese deposition as a possible reason for their findings. No significant correlation between HE severity and T1 relaxation time could be found in the putamen, frontal white matter, white matter of the corona radiata, white matter in the occipital lobe, the anterior limb of the internal capsule, visual cortex, thalamus, or frontal cortex<sup>[30]</sup>. Patients with stage 1-2 primary biliary cirrhosis have been shown to exhibit a decrease in pallidal MTR (normalized to putamen), which correlates with blood manganese concentration<sup>[31]</sup>.

In a study of diffusion-weighted imaging, Lodi *et al*<sup>[32]</sup> have reported increased diffusivity in white matter and basal ganglia, which suggests the presence of brain edema in chronic hepatic failure<sup>[32]</sup>. Increased diffusion in mHE and overt HE has been demonstrated to be reversible after successful treatment<sup>[33]</sup>. Both edema and manganese deposition may be explanations for the decrease in magnetization transfer effect.

White matter involvement in HE may be an effect of disturbed blood flow and energy metabolism<sup>[9]</sup>, demyelination<sup>[5]</sup>, axonal loss<sup>[5]</sup>, as well as an effect of astrocytic water retention<sup>[5-7,9,10]</sup>. The loss in magnetization transfer reflected in a decrease in MTR has been proposed to be associated with brain edema in neuropsychiatric systemic lupus erythematosus<sup>[34]</sup>, multiple sclerosis<sup>[35]</sup> and traumatic brain injury<sup>[36]</sup>. In non-cirrhotic patients with portal vein thrombosis, decreased MTR values of normal-appearing white matter have been found, which has been interpreted as an increase in free brain water<sup>[37]</sup>.

The presence of white matter edema in chronic liver dysfunction is supported by the above findings of Lodi *et al*<sup>[32]</sup> and Kale *et al*<sup>[33]</sup>, who reported increased diffusivity in white matter areas and in the basal ganglia.

MR spectroscopy has established the presence of a disturbed metabolic pattern in cirrhosis, which is considered

to represent a state of minimal brain swelling<sup>[26,38,39]</sup>. Reversibility after successful liver transplantation has been suggested to be an indicator of increased free cerebral water rather than structural damage<sup>[8]</sup>.

The maximum statistics in the groups without overt HE were in the right putamen. In patients with HE 1 and HE 2, the highest (pseudo-) *t* was located in the left hemisphere. In each of the four groups, the corresponding contralateral brain area exhibited strongly significant, yet slightly lower statistics. The limited group size and the lack of information about the patients' handedness hinder interpretation of this finding. Unilateral brain involvement is unlikely under dysmetabolic conditions, with clinical symptoms of lack of awareness, constructional apraxia, dyscalculia, personality change and asterixis. A non-focal alteration of the dominant hemisphere might be expected to yield a stronger correlation with neuropsychological test results, than the same alteration of the non-dominant hemisphere. Nevertheless, a structurally higher susceptibility of the dominant hemisphere to metabolic toxins might be worthy of discussion. Further studies will possibly be dedicated to this question.

### MTI and CFF

CFF assesses neurological deficits in cirrhosis as a continuous parameter, rather than offering the six-tier graduation used in the (revised) West-Haven criteria<sup>[40]</sup>. Since the test requires cooperation from the patients, its use in high-grade HE may be hindered. Patients suffering HE grade 3 and 4 were not enrolled in the present study. The CFF has been shown to be reliable in retest evaluation and responds rapidly to neurological deterioration or recovery<sup>[12]</sup>. Both parameters have been shown independently to decrease with increasing severity of HE<sup>[4,7,9,10,12]</sup>.

In an fMRI study, Zafiris *et al*<sup>[41]</sup> have found impaired activation of visual cortex and abnormal neuronal coupling in cirrhosis patients with decreased CFF. Alzheimer-II degeneration of glial Muller cells and mild visual impairment is another proposed mechanism of CFF decrease in HE<sup>[12]</sup>.

A positive correlation was found between CFF and cerebral MTR. Our findings indicate that the MTR of large areas of normal-appearing white matter and deep grey matter correlates with a decrease in CFF in patients with HE.

### Brain atrophy in HE

SnPM group comparison of the binary data sets showed prominent sulci, ventricles and lateral cerebral fissures as morphological correlations of brain atrophy.

In normal aging, a linear decrease in brain volume is known<sup>[42]</sup>. Although there was no significant difference between the mean age of patients ( $61.1 \pm 12.4$  years) and that of the control subjects ( $55.7 \pm 13.8$  years) in the present study, subject age was included in the analysis as a confounding variable. The findings from these images point towards an effect of cirrhosis on brain volume. Brain atrophy has been reported to be present in minimal and overt HE<sup>[27]</sup>, which correlates with poor psychometric performance<sup>[16,43]</sup>.

In voxel-based analysis following normalization, the comparison of normal and atrophied brains may lead to tests, in which voxels with mainly brain parenchyma in controls are tested against voxels that contain predominantly CSF in patients. This may result in non-valid differences. The topic of using the same spatial normalization algorithm for anatomically normal and atrophied brains has been addressed by Ishii *et al*<sup>[17]</sup>. Comparing SPM and NEUROSTAT normalization methods for the analysis of PET images in Alzheimer's disease, the authors have concluded that brains with atrophy tend to show artefacts caused by the anatomical standardization process.

In an MRI and PET study of schizophrenia, SPM results were deemed invalid because of incorrect spatial normalization of atrophied brains. This led to an underestimation of metabolic activity in the caudate nucleus in patients with schizophrenia<sup>[44]</sup>.

The present work shows a correlation between MTR and CFF in intraventricular and superficial voxels. This may be attributed to unreliable normalization of brains with atrophy in the patient group. Evaluating cortical or periventricular MTR values is limited by this effect.

In conclusion, in the brains of patients with non-alcoholic liver cirrhosis, MTR loss is seen. It involves basal ganglia and white matter, even in mild stages of HE. The changes are more severe and spatially more extended in patients with HE. Maximum statistics were found in the basal ganglia in overt HE, compared to healthy controls. The correlation of MTR and CFF is strongest in frontal white matter. Analyses of cerebral MTI of patients with liver cirrhosis might profit from inclusion of both basal ganglia and frontal white matter.

## COMMENTS

### Background

Hepatic encephalopathy (HE) is a frequent complication of liver cirrhosis and is associated with poor prognosis. Many studies have shown that magnetization transfer imaging (MTI) of the brain is sensitive to HE and subclinical forms of this condition [minimal HE (mHE)]. Treatment as well as induction of HE has been monitored successfully with MTI. Edema with water accumulation is a possible mechanism for MTI changes. Many brain areas have been investigated with MTI, but no systematic evaluation of the spatial pattern of MTI abnormalities in HE has been published.

### Research frontiers

In the area of brain imaging in liver cirrhosis, the research hotspot is whether magnetic resonance imaging (MRI) can be used to detect HE early and to monitor its treatment. MTI is a MRI technique that is used to assess the interaction of free water protons and macromolecules. MTI is a promising research tool to assess minimal brain edema in HE.

### Innovations and breakthroughs

In the previous application of MTI in HE, alterations have been reported for various brain areas such as the frontal and posterior white matter, basal ganglia and thalamus. More recent studies have demonstrated the sensitivity of MTI in mHE. These studies used simple region-of-interest (ROI) evaluations. The selection of ROIs was based on *a priori* assumptions from reports on white matter MRI anomalies and manganese deposits in the basal ganglia. To the best of the authors' knowledge, no systematic assessment of the distribution of brain MTI changes has been published on which to base the selection of ROIs in the imaging of HE. In the present study, they performed a voxel-based analysis (VBA) of MTI data of patients with HE and showed that abnormal signals were present throughout the brain. No one specific area was present where MTI changes in manifest HE were exclusive.

## Applications

The study results suggest that MTI changes in HE are present throughout the brain. Test results in frontal and posterior white matter, thalamus and basal ganglia were statistically significant in cases with mHE, which might be areas that could be used as ROIs in monitoring HE by means of MTI.

## Terminology

HE is a neurological condition with fatigue, sleeping disorders and motor deficits caused by liver cirrhosis. MTI is an MRI technique that is used to assess the interaction of free water protons and macromolecules, e.g. in mild brain edema. ROI is a means of evaluating MR images of the brain by measuring an image parameter in a defined area. VBA is a statistical evaluation of the entire brain MRI data of a study population.

## Peer review

The manuscript entitled "Voxel-based analyses of magnetization transfer imaging of the brain in hepatic encephalopathy" is an interesting study that aimed to evaluate the spatial distribution of cerebral abnormalities in cirrhosis patients with and without HE found in MTI.

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## Direct hepatic differentiation of mouse embryonic stem cells induced by valproic acid and cytokines

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### Abstract

**AIM:** To develop a protocol for direct hepatic lineage differentiation from early developmental progenitors to a population of mature hepatocytes.

**METHODS:** Hepatic progenitor cells and then mature hepatocytes from mouse embryonic stem (ES) cells were obtained in a sequential manner, induced by valproic acid (VPA) and cytokines (hepatocyte growth factor, epidermal growth factor and insulin). Morphological changes of the differentiated cells were examined by phase-contrast microscopy and electron microscopy. Reverse transcription polymerase chain reaction and immunocytochemical analyses were used to evaluate the gene expression profiles of the VPA-induced hepatic progenitors and the hepatic progenitor-derived hepatocytes. Glycogen storage, cytochrome P450 activity, transplantation assay, differentiation of bile duct-like structures and tumorigenic analyses were performed for the functional identification of the differentiated cells. Furthermore, FACS and electron microscopy were used

for the analyses of cell cycle profile and apoptosis in VPA-induced hepatic differentiated cells.

**RESULTS:** Based on the combination of VPA and cytokines, mouse ES cells differentiated into a uniform and homogeneous cell population of hepatic progenitor cells and then matured into functional hepatocytes. The progenitor population shared several characteristics with ES cells and hepatic stem/progenitor cells, and represented a novel progenitor cell between ES and hepatic oval cells in embryonic development. The differentiated hepatocytes from progenitor cells shared typical characteristics with mature hepatocytes, including the patterns of gene expression, immunological markers, *in vitro* hepatocyte functions and *in vivo* capacity to restore acute-damaged liver function. In addition, the differentiation of hepatic progenitor cells from ES cells was accompanied by significant cell cycle arrest and selective survival of differentiating cells towards hepatic lineages.

**CONCLUSION:** Hepatic cells of different developmental stages from early progenitors to matured hepatocytes can be acquired in the appropriate order based on sequential induction with VPA and cytokines.

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**Key words:** Hepatic differentiation; Embryonic stem cells; Histone deacetylase inhibitor; Progenitor cell; Cell cycle arrest

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### INTRODUCTION

Hepatocyte transplantation is an alternative therapy strategy for liver failure or end-stage liver diseases<sup>[1,2]</sup>.

However, the shortage of donor hepatocytes and the difficulties for large scale hepatocyte amplification and function maintenance limit the clinical application of this cell-based therapy<sup>[3]</sup>. Embryonic stem (ES) cells, known for their capacity to proliferate indefinitely and differentiate into almost all types of cells including hepatocytes, have raised the hope of cellular replacement therapy for liver failure. The essential prerequisite for this purpose is to develop well-defined protocols for directing cellular differentiation into hepatic lineage, followed by selective isolation and proliferation *in vitro*. There have been several protocols available up till now for hepatic fate specification from ES cells, for example, the protocols based on spontaneous differentiation, combination of growth factors, co-culture with non-parenchymal liver cells and gene modifications<sup>[4-8]</sup>. However, most of the protocols currently used were devised to induce mature hepatocytes by using embryoid bodies that may result in low yield or purity of functional hepatocytes. Little documentation exists about a strategy for acquiring different developmental hepatic cells, for example, guiding differentiation of ES cells to hepatic progenitors and then to an entire population of mature hepatocytes to meet the requirements of precise study regarding the mechanisms of hepatic differentiation and potential clinical applications. Furthermore, more concise and reproducible methods to acquire abundant hepatic cells still remain to be developed, among which the direct hepatic differentiation from an ES monolayer without using embryoid bodies is most promising<sup>[9]</sup>.

Valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, has been used as a new class of chemotherapeutic drug for cancer clinical purposes and as an inducer for stem cell differentiation<sup>[10-13]</sup>. It has exhibited profound therapeutic activity against hepatocellular carcinoma by inducing apoptosis and cell-cycle arrest<sup>[14-16]</sup>, and dramatic effects on cellular differentiation from stem cells, including cardiomyocyte differentiation from ES cells, neuronal differentiation from neural stem cells, and osteogenic and hepatic differentiation from bone marrow-derived mesenchymal stem cells (MSC)<sup>[17-20]</sup>. In a previous study, we established a method for hepatic differentiation from MSC using VPA, which provided preliminary evidence that VPA could facilitate hepatic differentiation<sup>[21]</sup>. However, little is known about whether VPA can induce the hepatic differentiation of ES cells. Here, we report such research and the development of a protocol for direct hepatic lineage differentiation, from early developmental progenitors to a population of mature hepatocytes, based on sequential induction with VPA and cytokines. Results show that VPA can direct the hepatic specification of ES cells and largely participates in the differentiation of ES cells into hepatic progenitors. Further differentiation of hepatic progenitors into mature hepatocytes requires supplementation with cytokines. The present study may not only be helpful for the clinical application of hepatocyte transplantation, but also provide an *in vitro* research model for the better investigation and understanding of the entire develop-

mental process of hepatocytes, from ES cells to hepatic progenitors, and then to mature hepatocytes. Furthermore, as VPA is an epigenetic modulator, so our results may also be of benefit to the research of mechanisms of epigenetic modifications during liver development.

## MATERIALS AND METHODS

### Reagents

VPA was purchased from Sigma (St Louis, MO); fetal bovine serum (FBS) was purchased from Hyclone (Rockville, MD); murine leukemia inhibitory factor (LIF) was purchased from Chemicon (Temecula, CA); mouse hepatocyte growth factor (mHGF), mouse epidermal growth factor (mEGF), oncostatin M (OSM), Insulin-Transferrin-Selenium (ITS), and collagen I were all from R&D systems (Minneapolis, MN); Matrigel was purchased from BD Biosciences (Palo Alto, CA); sheep anti-ALB antibodies were purchased from Biodesign (Saco, ME); rabbit anti-AFP, mouse anti-CK19 were from Dako (Copenhagen, Denmark); rat anti-OCT-4 was from R&D; mouse anti-SSEA-1 was from Developmental Hybridoma Bank of Iowa University; goat anti-mouse DLK was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rat anti-A6 was presented by Dr. Valentina Factor of NIH; FITC-conjugated bovine anti-sheep IgG, FITC-conjugated rabbit anti-goat IgG, FITC-conjugated goat anti-rabbit IgG, TRITC-conjugated goat anti-mouse IgG, TRITC-conjugated goat anti-rat IgG were all purchased from Dako; FITC-conjugated goat anti-mouse IgM was from Jackson Immunoresearch Laboratories Inc. All other reagents were from Sigma (St. Louis, MO).

### Culture of mouse ES cells

Mouse ES D3 cells, provided by the Cell Biology Institute of the Chinese Academy of Sciences, were cultured on mitomycin C inactivated MEF feeder layers in high-glucose DMEM supplemented with 15% FBS, 2 mmol/L L-Glu, 0.1 mmol/L N-ME, 1% NEAA and 10 ng/mL murine LIF as described previously<sup>[22]</sup>. Briefly, MEF feeder cells were isolated from ICR mice at embryo day 13.5 and cultured at 37°C and 5% CO<sub>2</sub>. At approximately 80% confluence, the feeder cells were incubated with 10 µg/mL mitomycin C for 4 h and washed three times with PBS. Then the cells were replated at  $8 \times 10^4$  cells/cm<sup>2</sup> on to tissue culture flasks. After allowed for attachment overnight, the ES cells were seeded.

### Differentiation of hepatic cells from mouse ES cells

A protocol was designed to obtain the hepatic progenitor cells and then mature hepatocytes from mouse ES cells in a sequential manner (Figure 1). For differentiation of hepatic progenitor cells, ES cells were cultured in DME medium as described above with the exception of the withdrawal of feeder layer and LIF, and treated with 1 mmol/L VPA for 4-6 d, then the VPA was removed and recombinant mouse HGF 10 ng/mL was added for

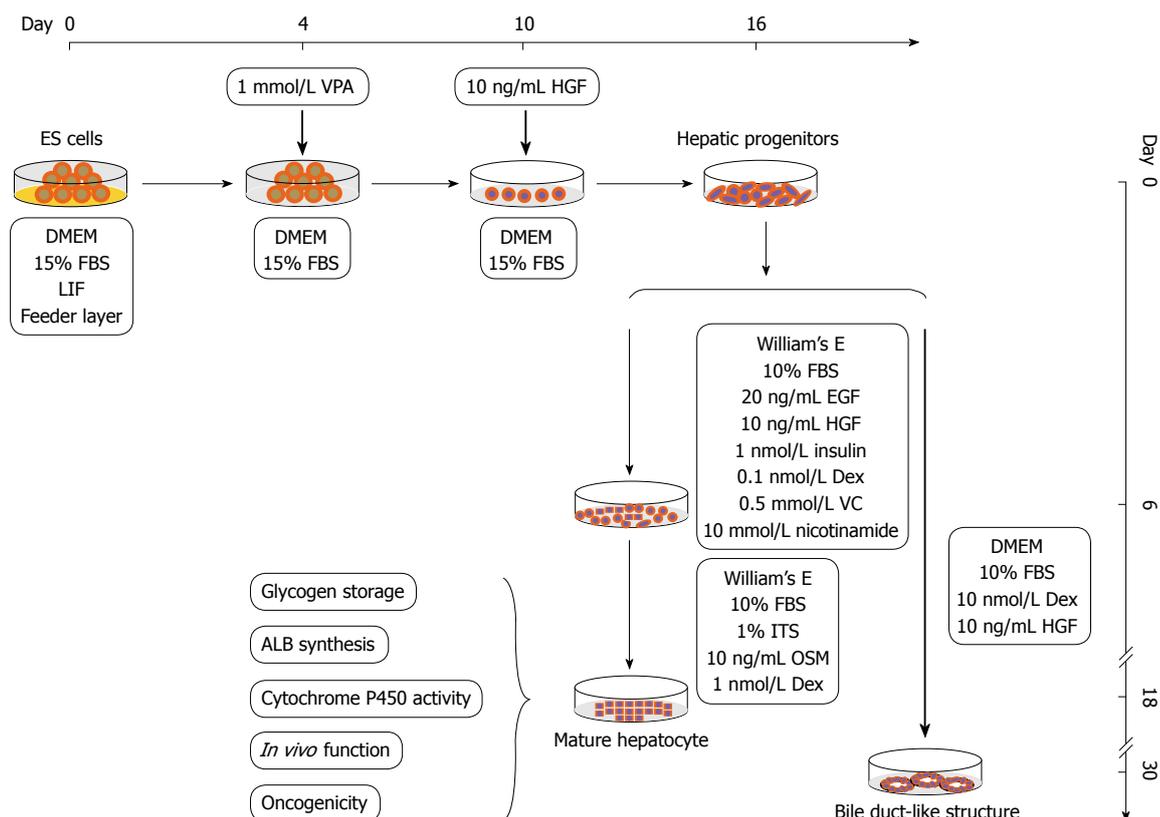


Figure 1 Schematic presentation of the protocol.

another 6-12 d until the hepatic progenitor cells became confluent. For differentiation of hepatocytes from hepatic progenitor cells, the progenitor cells were cultured in a William's E medium supplemented with 20 ng/mL mEGF, 10 ng/mL mHGF,  $10^{-6}$  mol/L insulin,  $10^{-7}$  mol/L Dex, 0.5 mmol/L ascorbic acid diphosphate, 10 mmol/L nicotinamide and 10% FBS (maturation medium I) for 6 d, and then replaced with another William's E medium containing 1% ITS, 10 ng/mL OSM and  $10^{-6}$  mol/L Dex (maturation medium II) for another 6-12 d.

#### Molecular and structural identification of the differentiated cells

**Gene expression analyses:** Total RNA was extracted from the ES cells, hepatic progenitor cells, mature hepatocytes and adult mouse liver using Nucleospin RNA Kits (BD Biosciences, Palo Alto, CA). RNA samples were digested with DNase I for 15 min at room temperature. cDNAs were synthesized from 1  $\mu$ g total RNA with a superscript III first-strand synthesis kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and amplified with the following program: initial denaturation at 95°C for 5 min followed by 30-40 cycles of 94°C for 30 s, 50-56°C for 30 s, 72°C for 30 s and final extension at 72°C for 10 min. The amplified products were analyzed by electrophoresis on 1.5% agarose gel and stained with ethidium bromide. All the primers used are listed in Table 1.

**Immunocytochemical analyses:** Cells were fixed using 4% paraformaldehyde (PFA) followed by ice-cooled

methanol, and incubated with primary antibodies overnight at 4°C after blocking with 5% normal goat serum. For the application of SSEA-1 antigen, the cells were fixed with PFA only. Following washing with PBS three times, the cells were incubated with fluorescence-conjugated secondary antibodies, and examined under a Carl Zeiss confocal laser-scanning microscope.

**Electron microscopy:** Cells were fixed in 2.5% glutaraldehyde in 0.1 mmol/L phosphate buffer saline (pH 7.4) at 4°C for 24 h, post-fixed in 1% OsO<sub>4</sub> for 1 h, and embedded in Epon 812. They were cut into 30-40 nm sections for ultrastructural evaluation using a Philips TECNAL-10 transmission electron microscope.

#### Functional identification of the differentiated cells

**Glycogen storage:** At the final stage of differentiation, the cells were assessed by periodic acid-Schiff reaction for glycogen storage as previously described<sup>[23]</sup>. Briefly, cells were fixed in Carnoy's solution, oxidized in 1% periodic acid, treated with Schiff's reagent and examined under a Nikon microscope.

**Cytochrome P450 activity:** Cytochrome P450 activity was examined by ethoxyresorufin O-dealkylase assay. Briefly, cells were maintained under the same conditions in the presence or absence of 5  $\mu$ mol/L phenobarbital for 24 h followed by treatment with 5  $\mu$ mol/L ethoxyresorufin for 2-3 h, and then observed under Carl Zeiss confocal laser-scanning microscope at 355 nm excitation and 581 nm emission.

Table 1 Primers and annealing temperatures used for RT-PCR

Gene	Sequence (5'-3')	Product size (bp)	Annealing temperature (°C)
<i>β-actin-F</i>	TTCCTTCTTGGGTATGGAAT	200	55
<i>β-actin-R</i>	GAGCAATGATCTTGATCTTC		
<i>AFP-F</i>	CACTGCTGCAACTCTTCGTA	300	52
<i>AFP-R</i>	CTTTGGACCCCTTCTGTGA		
<i>HNF3β-F</i>	GACCTCTCCCTTCTACCG	551	51
<i>HNF3β-R</i>	TTGAAGGCGTAATGGTGC		
<i>TTR-F</i>	TGCCTCGCTGGACTGGTAT	334	52
<i>TTR-R</i>	CAGAGTCGTGGCTGTGAA		
<i>DPPIV-F</i>	GATTCATACCCAAAGGC	587	55
<i>DPPIV-R</i>	GGTCACTAAGGCACCT		
<i>ALB-F</i>	TCTTCGTCTCCGGCTCTG	475	55
<i>ALB-R</i>	CTGGCAACTTCATGCAAA		
<i>OCT4-F</i>	GGCGTTCCTTTGGAAAGGTGTTT	313	57
<i>OCT4-R</i>	CTCGAACCACATCTTCTCT		
<i>AAT-F</i>	AGAACCATTATCAGGCAGAA	675	55
<i>AAT-R</i>	AATAAGGAACGGCTAGTAAGA		
<i>DLK-F</i>	GGGGTGAAGTCCGTTGC	510	52
<i>DLK-R</i>	GCTCCTCGCCGCTGTTAT		
<i>HNF4-F</i>	CTTCAAGAGCTGCAGATTG	517	55
<i>HNF4-R</i>	CTTGTAGGATTCAGATCCCG		
<i>G6P-F</i>	TCAATCTCCTCTGGGTGGC	602	52
<i>G6P-R</i>	GGCAAAGGGTGTAGTGCAAG		
<i>TAT-F</i>	CTTCAGTGTGGATGTTCCG	619	55
<i>TAT-R</i>	CAGGGATTGGACGGTGTGT		
<i>TDO-F</i>	TAAACAGAGCCAGCAAAG	868	56
<i>TDO-R</i>	ATGAGCGTGTCAATGTCC		
<i>BG-F</i>	CGTGAAGGATACGGGAGT	581	55
<i>BG-R</i>	CAGAGTTATTGACGAGGC		
<i>GGT-F</i>	TGTCCTGGTGAAATCCG	577	55
<i>GGT-R</i>	GGCATAGGCAAACCGAAA		

AFP:  $\alpha$ -fetoprotein; HNF: Hepatocyte nuclear factor; TTR: Transthyretin; DPPIV: Cytokeratin 18; ALB: Albumin; OCT4: Octamer-4; AAT:  $\alpha$ -1-antitrypsin; Dlk:  $\Delta$ -like (Pref-1, preadipocyte factor-1); G6p: Glucose-6-phosphatase; TAT: Tyrosine aminotransferase; TDO: Tryptophan 2,3-dioxygenase; BG: Biliary glycoprotein; GGT:  $\gamma$ -glutamyl transpeptidase.

**Transplantation assay:** To evaluate the *in vivo* function of differentiated hepatocytes, a transplantation assay was performed in CCl<sub>4</sub>-intoxicated mice as previously described<sup>[24]</sup>. Five ICR mice were injected intraperitoneally with 10% CCl<sub>4</sub> in olive oil (1 mL/kg body weight). After 6 h, mice underwent intrasplenic transplantation of differentiated hepatocytes at  $1 \times 10^6$  cells ( $0.1 \text{ mL}$  of  $1 \times 10^7$  cells/mL) per mouse. The injection site was ligated to prevent cell leakage and bleeding. Mice were then sacrificed 24 h after transplantation, and sera were collected separately. Liver function was assessed by measuring the total bilirubin (T-Bil), ALT, AST and urea levels. Ten CCl<sub>4</sub>-intoxicated and untreated mice were used as controls.

**Differentiation of bile duct-like structures:** To evaluate the differentiation potential of progenitors into cholangiocytes, a spontaneous and directed bile duct-like structural differentiation assay was designed. For spontaneous differentiation, the progenitors were inoculated into the collagen I coated 96-well plate, and cultured with William's E medium supplemented with  $10^{-6}$  mol/L Dex, 10 ng/mL mHGF for 20-40 d until the special bile duct-like structures appeared. For directed differentiation,

the progenitors were seeded on a layer of Matrigel basement membrane matrix and cultured in the medium supplemented with 100 ng/mL mHGF and 50 ng/mL mEGF until the bile duct-like structures formed. Media were changed every 3 d.

**Tumorigenic analyses:** Undifferentiated ES cells and the hepatic progenitor cells were collected and suspended in DMEM medium ( $1 \times 10^7$  cells/mL). A 0.2 mL aliquot was injected subcutaneously into the backs of Balb/C nude mice. Each experimental group contained three animals. The animals were kept under a controlled lighting schedule with a 12-h dark period. Food and water were available *ad libitum*. All animals received humane care in compliance with institutional guidelines. Mice were sacrificed 6-8 wk after transplantation, the tumors and tumor-like tissues were fixed with formalin. After additional fixation in 4% paraformaldehyde for 2 h at 4°C, tissues were embedded in paraffin. Sections were stained with hematoxylin and eosin, and then microscopically observed.

#### Cell cycle and apoptosis analyses

Flow cytometry was performed to measure cell cycle distribution and apoptosis of VPA-treated cells. The measurements were made with a Becton Dickinson FACS Calibur machine, adapted for excitation with a 488 nm argon laser, and 582/42 nm band-pass filter for detecting propidium iodide emission.

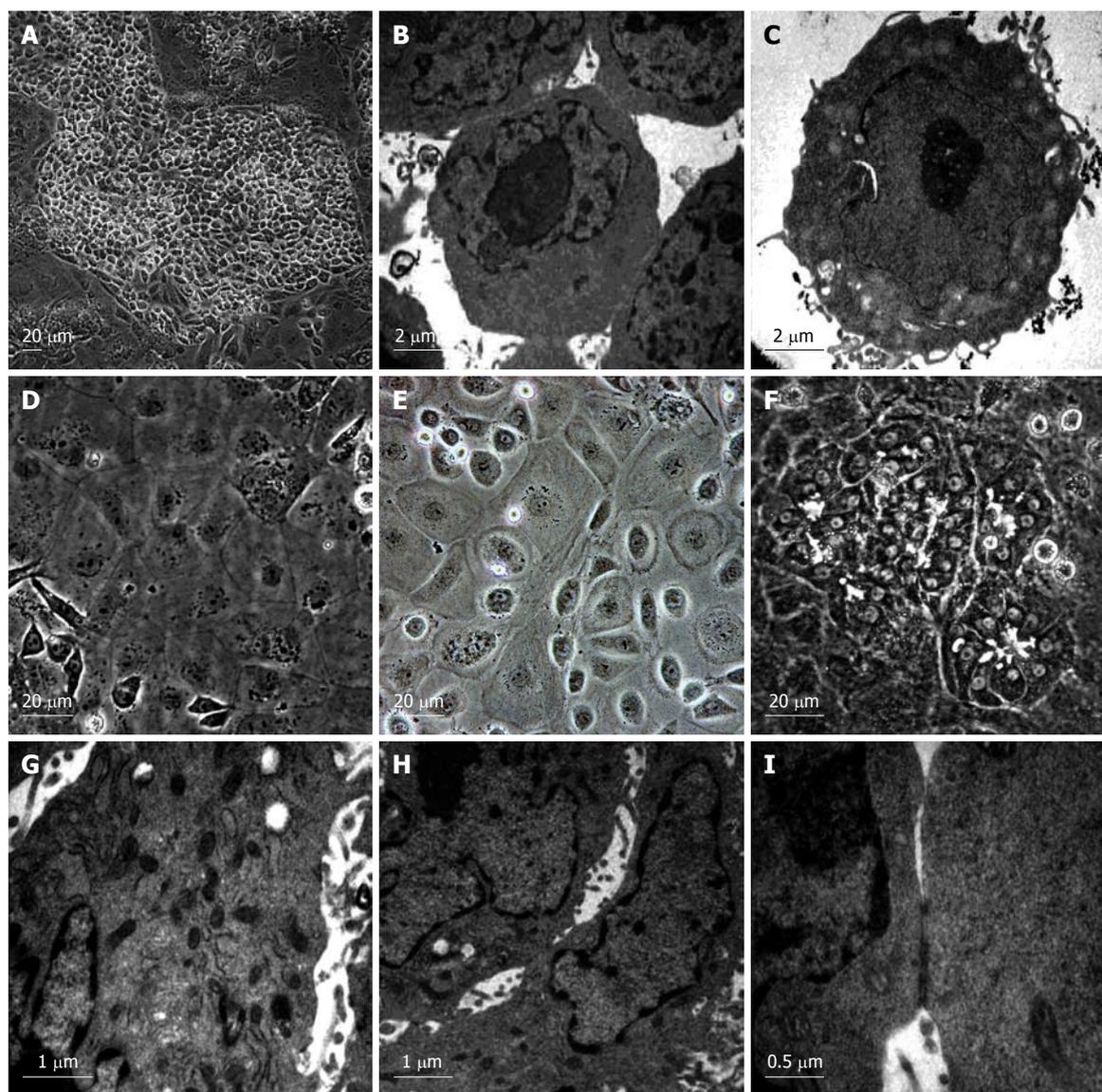
#### Statistical analysis

The data are presented as mean  $\pm$  SD. Statistical tests for the significance of differences between control and transplanted mice were performed by way of Student's *t* test.  $P < 0.05$  was considered as statistically significant.

## RESULTS

### Differentiation of hepatic progenitors from mouse ES cells induced by VPA

To examine whether VPA has an effect on hepatic fate differentiation from mouse ES cells, the ES cultures were subjected to VPA (1 mmol/L) after withdrawing feeder layers and LIF. After treatment for 4-6 d, a small round-shaped progenitor-like population appeared. These cells were then treated with recombinant mouse HGF (10 ng/mL) which allowed the promotion of cellular proliferation. After another 6-12 d, this progenitor-like population underwent rapid growth, and finally proliferated into confluence. The acquired progenitor-like cells exhibited 8-10  $\mu\text{m}$  in diameter, scant cytoplasm and a high nuclear to cytoplasmic ratio (Figure 2A), and resembled the blast-like oval cells proliferating during severe liver injury or the hepatoblasts found in fetal liver. Electron microscopic observation showed that the progenitor-like cells had abundant cell surface microvilli and shared scanty organelles except for a few mitochondria as in ES cells (Figure 2B and C). Noticeably, it was found that few mature functional hepatocytes



**Figure 2 Morphological observation of the differentiated cells.** A: Morphology of hepatic progenitor cells induced by VPA; B: Electron microscopic observation of the undifferentiated ES cells (control); C: Electron microscopic observation of the hepatic progenitor cells differentiated from ES cells; D, E: Morphological observation of typical hepatocytes differentiated from hepatic progenitor cells with flattened and cuboidal morphology, and acquired abundant granules in the cytoplasm; F: Morphological observation of another kind of hepatocyte-like cells differentiated from hepatic progenitor cells with rising and piled morphology, dark cytoplasm and light nuclei, and bile canaliculi-like structures found between these cells; G: Electron microscopic observation of the differentiated hepatocytes with abundant mitochondria in their cytoplasm; H: Electron microscopic observation of the bile canaliculi between adjacent cells as mentioned above (Figure 1F); I: The bile canaliculi between adjacent cells sealed with tight junctions.

could be acquired by treating the ES cells with VPA independently, and further differentiation into mature hepatocytes needed the combination of various cytokines, which indicated that VPA probably participates mainly in the regulation of early hepatic differentiation.

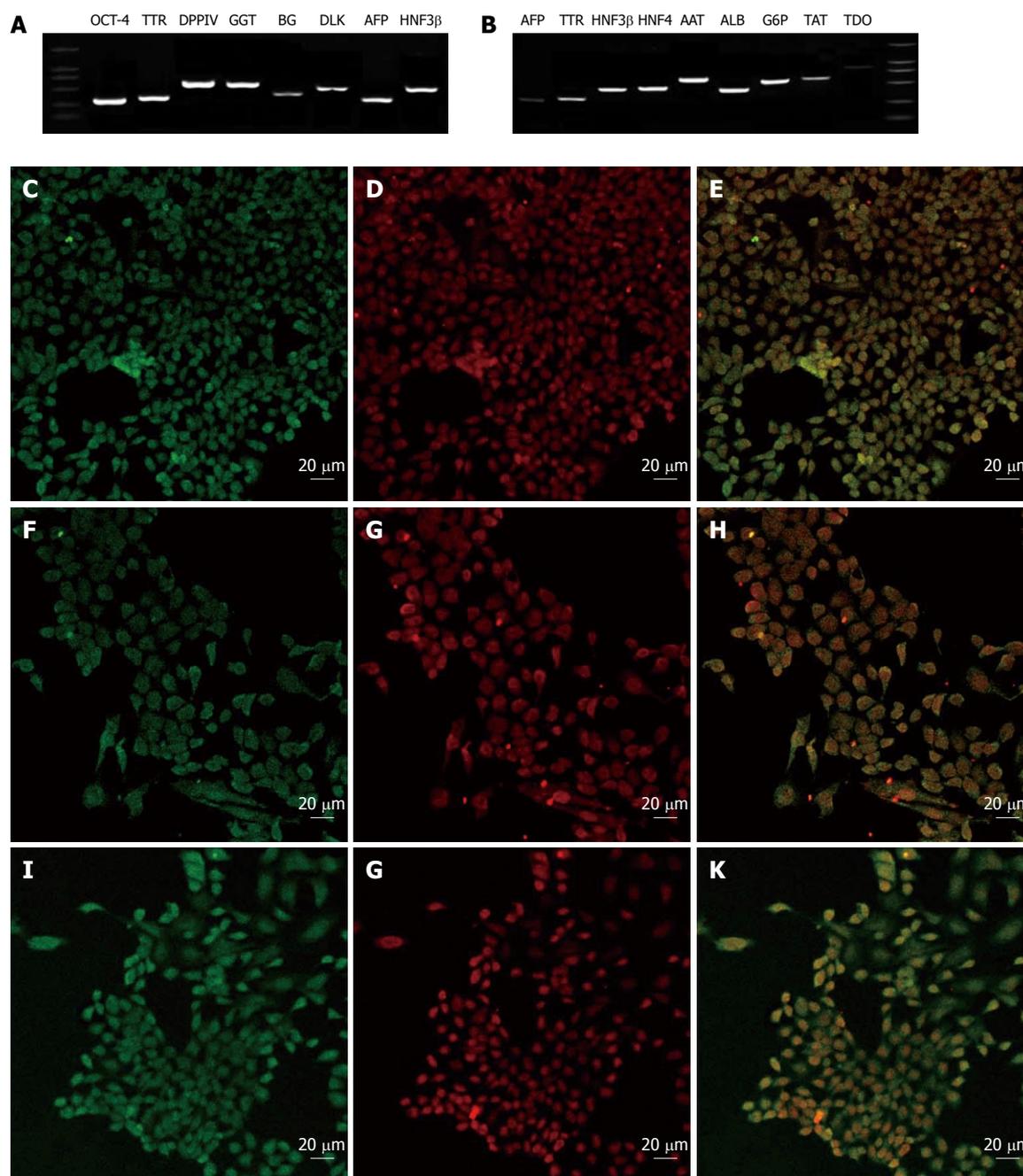
#### **Differentiation of hepatocytes from progenitors**

For maturation of functional hepatocytes from hepatic progenitors, the progenitor cells were further cultured in William's E medium (containing 10% FBS) supplemented with cytokines (EGF, HGF and insulin) and chemical inducers (Dex, ascorbic acid diphosphate and nicotinamide) for 6 d. Then, the cells were cultured in another medium containing ITS, OSM and Dex. After 6-12 d, two kinds of hepatocyte-like cells appeared. One exhibited typical hepatocyte features with a diameter of 20-40  $\mu\text{m}$ ,

flattened and cuboidal morphology, and had acquired abundant granules in the cytoplasm (Figure 2D and E). The other displayed a rising/piled morphology with dark cytoplasm and light nuclei, and bile canaliculi-like structures were often found between these cells (Figure 2F). Ultrastructural analyses revealed that both kinds of hepatocyte-like cells contained numerous mitochondria and endoplasmic reticulum (Figure 2G). Bile canaliculi were also observed between adjacent cells and sealed with tight junctions (Figure 2H and I). These results showed that functional hepatocytes could be acquired from VPA-induced progenitors under sequential induction combinations.

#### **Molecular identification of the hepatic lineage cells**

To evaluate the characterization of the VPA-induced hepatic progenitors and the hepatic progenitor-derived



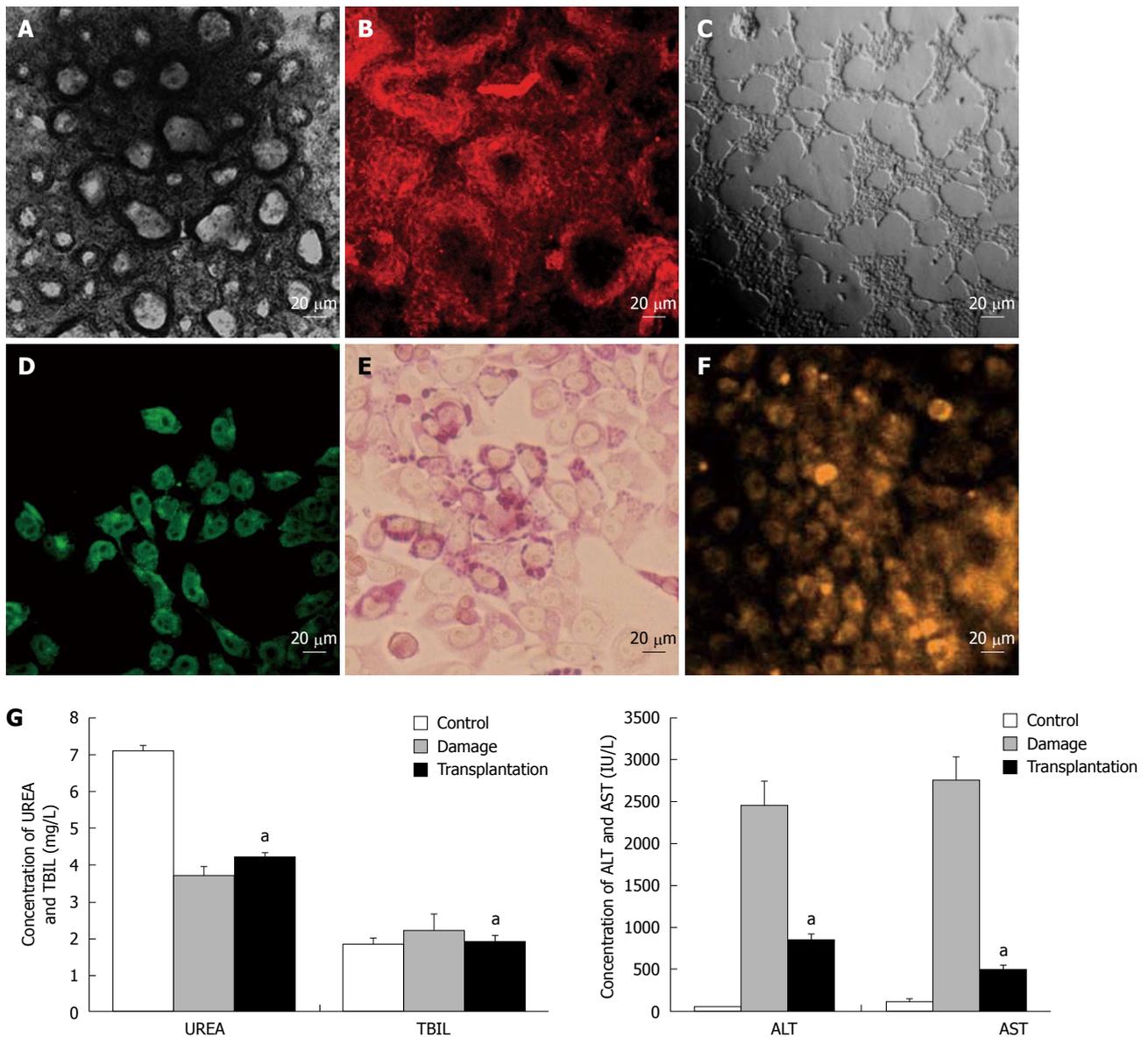
**Figure 3** Gene expression analysis of VPA-induced hepatic lineage cells by RT-PCR and immunofluorescence staining. A: RT-PCR showed hepatic progenitor cells after the treatment with valproic acid expressing most typical markers of hepatic/hepatic stem cells; B: The hepatic progenitor-derived hepatocytes expressing typical markers of mature liver cells; C-E: Immunofluorescent images of AFP (C) and OCT-4 (D) staining in VPA-induced hepatic progenitor cells (E is the merged image of C and D); F-H: Immunofluorescent images of AFP (F) and CK19 (G) staining in VPA-induced hepatic progenitor cells (H is the merged image of F and G); I-K: Immunofluorescent images of AFP (I) and DLK (J) staining in VPA-induced hepatic progenitor cells (K is the merged image of I and J).

hepatocytes, a number of gene expression profiles were examined at mRNA and/or protein levels. The results showed that the undifferentiated ES cells expressed SSEA-1 and OCT-4, but no hepatic markers (data not shown). However, the progenitor cells expressed most typical markers of hepatic/hepatic stem cells, such as AFP, TTR, DPPIV, GGT, BG, HNF3 $\beta$ , CK19 and Dlk (Figure 3), but did not express the ES marker SSEA-1 or mature hepatocyte marker ALB (data not shown). Interestingly, the progenitor cells expressed the ES cell marker OCT-4, which suggested that the VPA-induced

hepatic progenitor cells may share partial properties of ES cells (Figure 3A and D). Accordingly, the hepatic progenitor-derived hepatocytes expressed typical markers of mature liver cells, including ALB, AAT, HNF4, G6p, TAT, AFP, TTR, HNF3 $\beta$  and TDO (Figure 3B).

#### **Functional characterization of the hepatic lineage cells**

For functional evaluation of the progenitor cells, the *in vitro* bi-differentiation potential of the progenitors into bile duct-like structures and mature hepatocytes was determined. The results showed that bile duct-like structures

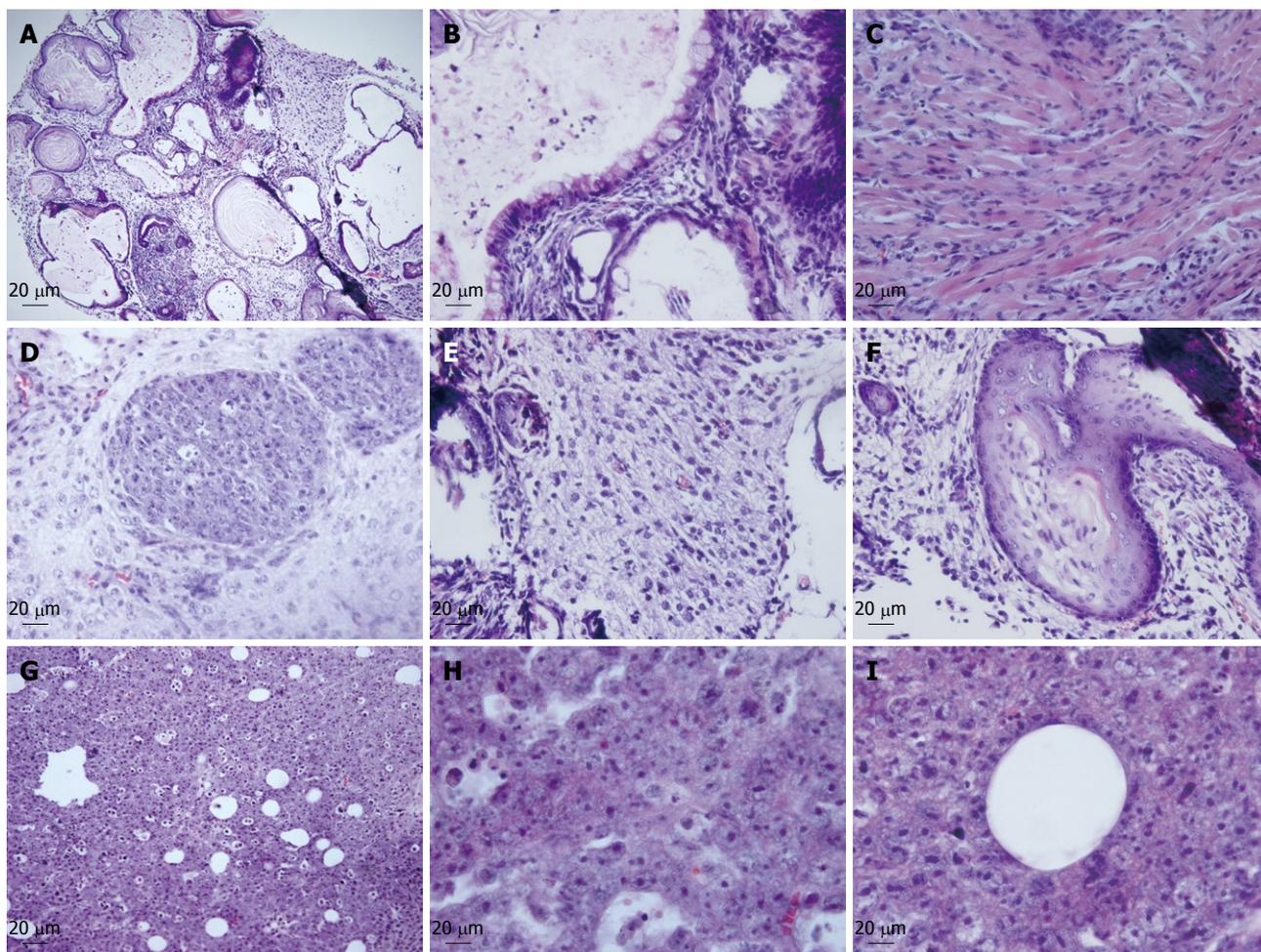


**Figure 4 Functional characterization of the hepatic lineage cells.** A: Bile duct-like structures formed from the VPA-induced hepatic progenitor cells cultured on collagen I coated dishes; B: Bile duct-like structures were stained CK19 positive; C: Bile duct-like structures formed from the VPA-induced hepatic progenitor cells cultured on Matrigel basement membrane matrix; D-F: Differentiated hepatocytes from hepatic progenitor cells could synthesize albumin (D), store glycogen (E) and possessed cytochrome P450 activity (F) after induction for 2-3 wk; G: When ES-HPCs derived hepatocytes were transplanted 6 h after liver intoxication, serum total bilirubin (TBIL), serum ammonia (UREA), AST and ALT levels were significantly improved towards normal at 1 d after the transplantation. <sup>a</sup> $P < 0.05$ .

could be formed from the progenitor cells in two ways. In the first way, the progenitor cells were cultured on collagen I coated dishes in the medium supplemented with  $10^{-6}$  mol/L Dex and 10 ng/mL mHGF for about 30 d until the foci of small dark cells appeared, and these foci became organized and developed as doughnut-like structures identical to bile duct units (Figure 4A). In the second way, the progenitor cells were seeded on a layer of Matrigel basement membrane matrix and cultured in the medium supplemented with 100 ng/mL mHGF and 50 ng/mL mEGF as previously described. Many well-defined duct-like structures comprised of neatly aligned cells were found throughout the dish 1-2 d after inoculation, which developed into spherical 3-dimensional structures consisting of tightly packed columnar epithelium along with a central lumen when further maintained

for another 15 d (Figure 4C). Immunofluorescence analysis demonstrated that the structures were CK19-positive (Figure 4B) and AFP-negative (data not shown), which are typical hallmarks seen in the bile duct.

Accordingly, typical hepatocytes could also be attained from the progenitors as mentioned above. Functional characterization analyses showed that the differentiated hepatocytes could synthesize albumin (Figure 4D), store glycogen (Figure 4E) and possessed cytochrome P450 activity (Figure 4F) after differentiation for 2-3 wk. Furthermore, an *in vivo* transplantation assay of hepatocytes in acute-injured liver was performed. The results showed that when the differentiated hepatocytes were transplanted 6 h after liver intoxication, serum T-Bil, serum ammonia, AST and ALT levels were significantly ( $P < 0.05$ ) improved towards normal levels compared to con-



**Figure 5** *In vivo* multi-differentiation potential and tumorigenic analyses. A-F: The teratomas derived from ES cells were composed of a variety of types of differentiated cells from all three primary germ layers including secretory glands (B), muscle (C), epithelium (D), neuroectodermal cells (E) and cartilage (F); G-I: The tumor-like bumps derived from ES-HPCs contained only epithelial (H) and mesenchymal-like cells, and duct-like structures (I) surrounded by several epithelial cells were also observed.

trol mice without hepatocyte transplantation (Figure 4G), suggesting that the hepatocytes functioned normally *in vivo* and their transplantation improved the liver function of CCl<sub>4</sub>-treated mice.

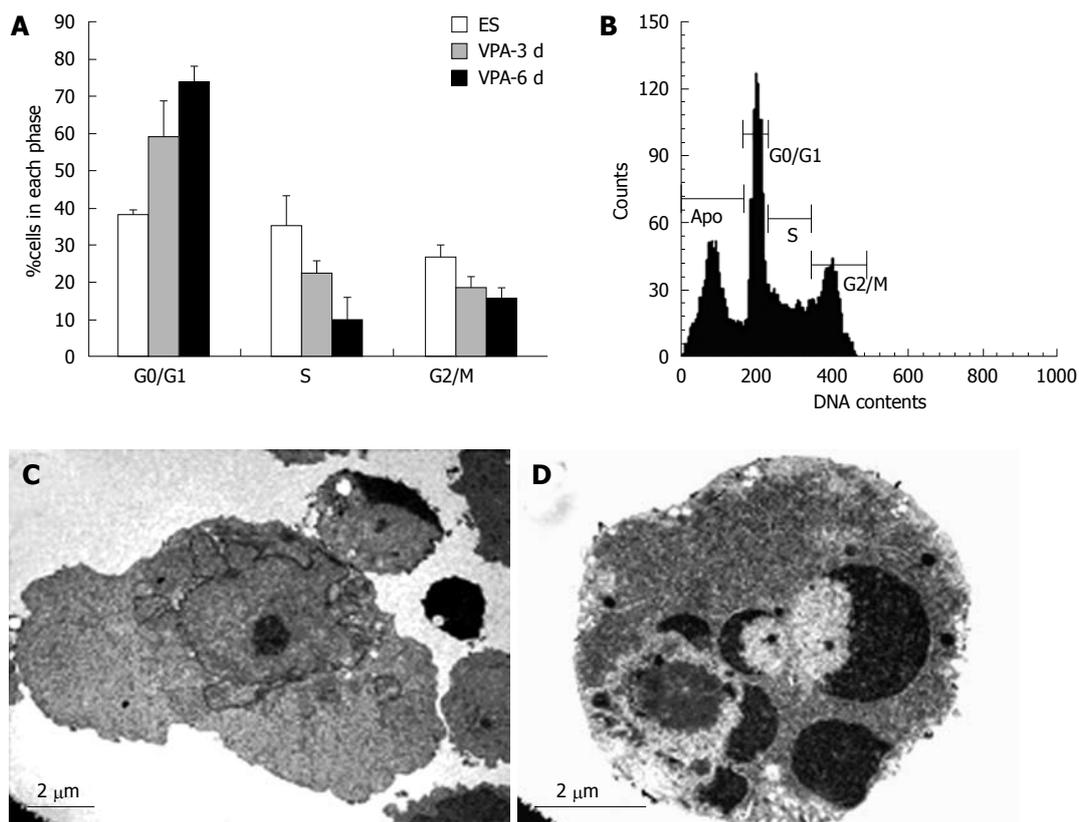
#### **Tumorigenic and *in vivo* differentiation potential analyses**

To evaluate the *in vivo* oncogenicity and differentiation potential of the progenitor cells, we introduced these cells and undifferentiated mES cells (as control) into Balb/c nude mice. Two weeks post-inoculation, the mice implanted with ES cells developed apparent teratomas at the injection site, while the mice with progenitor cells developed tumor-like bumps about 3-4 wk later. After the development of 6-8 wk, the teratomas and tumor-like bumps were fixed and examined with HE staining. The teratomas derived from ES cells were composed of a variety of types of differentiated cells from all three primary germ layers including neuroectodermal cells, adipocytes, muscle and epithelium (Figure 5A-F). However, the tumor-like bumps derived from the progenitor cells contained only epithelial and mesenchymal-like cells, and duct-like structures surrounded by epithelial cells were also observed (Figure 5G-I). The results further confirmed that

the progenitor cells have *in vivo* differentiation potential into hepatic lineages, and indicated the difference in *in vivo* potential between the undifferentiated ES cells and hepatic progenitor cells.

#### **Cell cycle and apoptosis analyses of VPA-induced hepatic differentiation**

To evaluate whether VPA had an effect on cell cycle profile, ES cells were exposed to 1 mmol/L VPA for 0, 3, 6 d and cell cycle analyses were performed with FACS. Results indicated that exposure to VPA decreased the proportion of cells in S phase and increased the G<sub>0</sub>/G<sub>1</sub> phase proportion. Approximately 75% of the cells were arrested in the G<sub>0</sub>/G<sub>1</sub> phase and only 10% of the cells were in the S phase after 6 d of treatment with VPA, whereas over 37% of control ES cells were in the S phase (Figure 6A). These data indicated that VPA could reduce the proliferation of ES cells and cause the inhibition of G<sub>1</sub>-S transition. In addition, DNA content analysis of the cells treated with VPA for 3 d showed that about 28% of the cells adopted apoptotic features (Figure 6B). Furthermore, the ultrastructural observations also showed that a considerable number of cells presented typical



**Figure 6** VPA-induced cell cycle arrest and apoptosis during hepatic differentiation. A: Cell cycle analysis revealed that exposure to VPA decreased the proportion of cells in S phase and increased the proportion of cells in the G0/G1 phase. Approximately 75% of the cells were arrested in the G0/G1 phase and only 10% of the cells in the S phase after 6 d of treatment with 1 mmol/L VPA, whereas greater than 37% of control ES cells were in the S phase; B: The analysis of apoptosis proportions during 3 d of treatment with VPA; C, D: Ultrastructural observations showed that some cells presented typical apoptotic morphology when treated with VPA for more than 5 d.

apoptotic morphology after being treated with VPA for more than 5 d (Figure 6C and D).

## DISCUSSION

To develop well-defined *in vitro* protocols for directing cellular differentiation into hepatic lineage has become critical for better investigating the mechanisms of hepatocyte differentiation, and for providing seed cells for hepatic tissue engineering as well as for clinical purposes. Most of the protocols currently developed to induce the hepatic differentiation from ES cells were devised by using embryoid bodies. However, several disadvantages may exist in this method. For example, it is time-consuming to prepare the embryoid bodies, and hepatic differentiation through embryoid bodies may result in low yield and purity of functional hepatocytes. Therefore, direct hepatic fate differentiation from an ES monolayer without using embryoid bodies is a most promising concept. A previous study has demonstrated that hepatocyte-like cells could be directly induced from human ES cells by using a combination of cytokines, providing preliminary evidence of the possibilities for this method<sup>[25]</sup>. However, the direct hepatic differentiation of ES cells still remains a challenge and needs to be further developed. In the present study, we report a novel strategy for the direct hepatic fate differentiation

of ES cells, which allows hepatic differentiation from progenitor cells to functional hepatocytes, based on a combination of VPA and cytokines. The results show this strategy has obvious advantages, such as being easy to operate, well reproducible and capable of acquirement of abundant and uniform progenitor cells and mature hepatocytes, the latter of which are suitable for the large-scale requirements of cell replacement therapy. Noticeably, following this strategy, we can harvest progenitor cells and mature hepatocytes in a sequential order. Thus, it may provide an *in vitro* research model which could meet the requirement of precise study regarding the mechanisms of hepatic differentiation at different developmental stages.

In addition, histone acetylation is considered to be one of the most important epigenetic regulation processes involved in gene expression, which is largely controlled by HDAC inhibitors including VPA. The observation that VPA could induce hepatic progenitor differentiation from ES cells suggested that epigenetic regulation mediated by histone acetylation might play an important role in early hepatic development. Therefore, the VPA-induced hepatic differentiation may also provide a model for the study of early hepatic developmental events, such as the relationship between the initiation of hepatic differentiation and epigenetic modification.

By FACS and ultrastructural analysis, it was found

that treatment of ES cells with VPA significantly reduced the proportion of the cells in the S phase and promoted the accumulation in the G0/G1 phase, which was accompanied by cellular apoptosis. This finding suggests that cell cycle arrest and apoptosis are involved in the VPA-induced hepatic specification. Further study is needed to elucidate the exact molecular and cellular mechanisms underlying the VPA-induced hepatic cell fate determination from ES cells.

Several lines demonstrated that the VPA-induced progenitor cells possessed some distinctive characteristics distinguished from ES cells or traditionally identified hepatoblasts or hepatic oval cells. For example, the progenitor cells exhibited typical epithelial morphology when cultured in a collagen coated dish, while ES cells formed multilayer compact colonies. There were many condensed-stained heterochromatin areas in the nucleus of ES cells, while the progenitor cells were almost all euchromatic. Also, the results of an *in vivo* differentiation assay revealed that ES cells formed typical teratomas containing the structures of three primary germ layers, while the progenitor cells formed tumor-like bumps containing only epithelial and mesenchymal cells. Moreover, the acquired progenitor cells expressed a number of typical markers of hepatoblasts or oval cells, such as AFP, TTR, Foxa2, DPPIV, GGT and BG. Importantly, Dlk, a marker of hepatic stem cells (hepatoblasts or hepatic oval cells) recently reported<sup>[26-28]</sup>, was also expressed by the progenitor cells. However, investigation of the hepatic oval cell marker, A6, was negative<sup>[29,30]</sup>. In addition, the progenitor cells expressed OCT-4, a marker of multi-lineage stem/progenitor cells<sup>[31-33]</sup>. These data indicated that the acquired progenitor cells may represent a novel subset at a developmental stage between ES cells and the known hepatic stem/progenitor cells, suggesting that a novel progenitor population was involved in the VPA-induced hepatic differentiation. This finding will be of benefit for understanding more about the early differentiation of hepatocytes.

Importantly, mouse is an often-used model species for human disease, so the investigation of hepatic differentiation of mouse ES cells will be of benefit for the better understanding of mechanisms underlying human hepatic differentiation and the development involved. Our results also support the establishment of new strategies to acquire human hepatic progenitor cells as well as hepatocytes, which will be helpful for the solution of obtaining cell sources for clinical cytotherapy.

## COMMENTS

### Background

Embryonic stem (ES) cells, known for their capacity to proliferate indefinitely and differentiate into almost all types of cells including hepatocytes, have raised the hope of cellular replacement therapy for liver failure. There have been several protocols available for hepatic fate specification from ES cells, however, most of the protocols currently used result in low yield or purity of functional hepatocytes. Valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, has been demonstrated to facilitate the hepatic differentiation of mesenchymal stem cells. However, little is known about whether VPA could induce the hepatic differentiation of ES cells.

### Research frontiers

The research hotspot is to develop well-defined protocols for directing cellular differentiation into hepatic lineage, followed by selective isolation and proliferation *in vitro*.

### Innovations and breakthroughs

In the present study, the authors report a novel strategy for the direct hepatic fate differentiation of ES cells, which allows the hepatic differentiation from progenitor cells to functional hepatocytes, based on using a combination of VPA and cytokines. The results show that this strategy has obvious advantages, such as being easy to operate, well reproducible and capable of acquirement of abundant and uniform progenitor cells and mature hepatocytes, the latter of which are suitable for the large-scale requirements of cell replacement therapy. Noticeably, following this strategy, they can harvest progenitor cells and mature hepatocytes in a sequential order. Thus, it may provide an *in vitro* research model which could meet the requirements of precise study regarding the mechanisms of hepatic differentiation at different developmental stages. In addition, the observation that VPA could induce hepatic progenitor differentiation from ES cells suggested that epigenetic regulation mediated by histone acetylation might play an important role in early hepatic development. Therefore, the VPA-induced hepatic differentiation may also provide a model for the study of early hepatic developmental events, such as the relationship between the initiation of hepatic differentiation and epigenetic modification.

### Applications

The present study may not only be helpful for the clinical application of hepatocyte transplantation, but also provide an *in vitro* research model for the better investigation and understanding of the entire developmental process of hepatocytes, from ES cells to hepatic progenitors, and then to a population of mature hepatocytes.

### Terminology

VPA, a HDAC inhibitor, has been used as a new class of chemotherapeutic drug for cancer clinical purposes and as an inducer for stem cell differentiation.

### Peer review

This is an important area for research to find new sources of hepatocytes for future clinical application. The authors have performed a good study.

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S- Editor Tian L L- Editor Logan S E- Editor Zheng XM

BRIEF ARTICLE

## Modification of sleep architecture in an animal model of experimental cirrhosis

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and rapid eye movement sleep (REM sleep) in most of the 11 wk. SWS I showed no significant variations. During the final weeks, a significant increase in REM sleep frequency was also observed. Histological analyses of the livers showed unequivocal signs of cirrhosis.

**CONCLUSION:** These data suggest that hepatic failure produced by CCl<sub>4</sub> administration is capable of modifying the sleep pattern even after only a few doses.

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**Key words:** Experimental cirrhosis; Sleep; Rapid eye movement sleep; CCl<sub>4</sub>; Wakefulness

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### Abstract

**AIM:** To analyze the polygraphic sleep patterns during cirrhosis progression in a rat model by repeated CCl<sub>4</sub> administration.

**METHODS:** Male Wistar rats received three weekly injections of CCl<sub>4</sub> for 11 wk, and were analyzed before and during the induction of cirrhosis. Rats were implanted with electrodes to record their sleep patterns. Polygraph recordings were made weekly over 11 wk for 8 h, during the light period. After a basal recording, rats received three weekly injections of CCl<sub>4</sub>. Histological confirmation of cirrhosis was performed after 11 wk.

**RESULTS:** The results showed a progressive decrease in total wake time that reached statistical significance from the second week of treatment. In addition, there was an increase in total time of slow wave sleep (SWS) II

### INTRODUCTION

The sleep-wakefulness cycle is an important circadian rhythm in mammals and is characterized by a reduction in the level of consciousness and by specific metabolic activities. Sleep is controlled by multiple areas of the brain and by several chemical factors, and can be readily modified by different activities, drugs, and pathological process, such as exercise, stress, alcoholism, depression, and metabolic diseases<sup>[1]</sup>.

Cirrhosis, on the other hand, is an irreversible dysfunction of the liver characterized by damage of the parenchyma, alteration of the reticular structure and the connective tissue that sustains the lobules and sinusoids<sup>[2]</sup>. Cirrhosis can progress to hepatic encephalopathy

and to coma<sup>[3]</sup>, but even before these advanced stages, functional alterations of several brain nuclei have been detected during early stages by magnetic transfer ratio, a magnetic resonance imaging technique<sup>[4]</sup>. Cirrhosis could also impact pulmonary function and might be involved in the development of obstructive sleep apnea syndrome (OSAS) in patients with ascitis; however, the early stages have not been associated with OSAS<sup>[5]</sup>. In the advanced stages, cirrhotic patients show an increased frequency of moderate obstructive sleep apnea<sup>[6]</sup>. In addition, cirrhotic patients with metabolic alterations frequently show abnormalities in electroencephalographic recordings<sup>[7]</sup>. Furthermore, patients with severe cirrhosis show a significant increase in power potency associated with the theta frequency and a decrease associated with the  $\alpha$  frequency in the electroencephalogram (EEG)<sup>[8,9]</sup>. Recently, Mostacci *et al.*<sup>[10]</sup> reported significant sleep alterations in 178 patients with cirrhosis when compared to normal control subjects using questionnaires: the basic Nordic sleep and the Epworth Sleepiness Scale. They reported that patients with cirrhosis complained of more daytime sleepiness, because they had fragmented nocturnal sleep caused by frequent nocturnal waking and had difficulties for falling asleep.

Through questionnaires of quality of life, sleep disturbances have been recognized as one of the early signs in patients with cirrhosis and hepatic encephalopathy<sup>[11]</sup>. Sleep disturbances in cirrhosis has not been correlated with clinical parameters or with cognitive impairment. Cirrhotic subjects with unsatisfactory sleep show higher scores for depression and anxiety, raising the possibility that the effects of these chronic emotional alterations might underlie the pathogenesis of sleep disturbances. Moreover, cirrhotic patients show reduced sleep time, increased latencies to sleep and frequent waking. These alterations are not due to previously prescribed medications, but are related to abnormalities of the circadian system<sup>[12]</sup>.

From this evidence we can conclude that hepatic cirrhosis causes a series of cerebral changes, which could modify several behaviors, such as sleep. Alterations of the sleep pattern as hepatic disease develops have not been reported. In addition, chronic administration of CCl<sub>4</sub> in rats induces reactive free radicals that attack membrane components, culminating in cell death<sup>[13]</sup> and promoting fibrosis<sup>[14]</sup>. This is a reliable procedure to induce experimental hepatic cirrhosis. The aim of this study was to analyze the sleep pattern in rats chronically treated with CCl<sub>4</sub> as hepatic damage progresses.

## MATERIALS AND METHODS

### Animals

Ten adult male Wistar rats (250-280 g) were used in this study. The animals were housed in a temperature-controlled room (22°C) and under a 12:12 normal light-dark cycle (light ON at 08:00 am). They were kept in individual clear polycarbonate cages with food and water available *ad libitum*. The experiments were performed following the guidelines of the National Institutes of

Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

### Sleep recording

Under deep general anesthesia induced with a cocktail (ketamine: 3.75 mg/100 g, xylazine: 0.19 mg/100 g, and acepromazine: 0.038 mg/100 g ip), rats were chronically implanted with a standard set of electrodes for sleep recording. Two stainless steel screw electrodes were implanted in the frontal and parietal cortex for the EEG and flexible wires were inserted in the neck muscles to record an electromyogram.

### CCl<sub>4</sub> treatment

One week after surgery, and after 3 d of habituation to the recording conditions, rats ( $n = 10$ ) polygraph recordings were made to obtain their basal sleep parameters. Thereafter, animals received an intraperitoneal (ip) injection, three times a week, containing different dilutions of CCl<sub>4</sub> and mineral oil, always in a total volume of 0.25 mL. Treatment lasted for 11 wk under the following pattern of administration: in the first week the animals received a solution with one part of CCl<sub>4</sub> and six parts of mineral oil (1:6). In the second week, the proportion of the solution changed to 1:5. In the third week the proportion of the solution was 1:4. From week four to week eleven the proportion of the solution was 1:3.

### Polygraph recordings

Polygraph recordings of the rats were obtained for 8 h within the light period, once every week, with the Nihon-Kohden model polygraph. Thus, sleep recordings were done before treatment and after every three injections. In addition to the polygraph recordings, rat behavior was observed during the recording period. The polygraph recordings were scored visually according to standard criteria<sup>[15]</sup>. The frequency and the duration of wakefulness, slow wave sleep (SWS) I, SWS II, and rapid eye movement (REM) sleep were quantified.

### Histological liver slices

Half of the animals died during the treatment (Initial  $n = 10$ ; final  $n = 5$ ). After sleep recordings, animals were killed by an overdose of pentobarbital and their livers were removed for histological examination. Liver slices were stained with hematoxylin-eosin and their morphological characteristics were determined.

### Statistical analysis

All data were expressed as mean  $\pm$  SD and analyzed by SPSS 11.0 software. Data were analyzed using an ANOVA for repeated measurements, followed by Fisher post hoc comparisons to detect significant differences between groups.  $P < 0.05$  was considered statistically significant.

## RESULTS

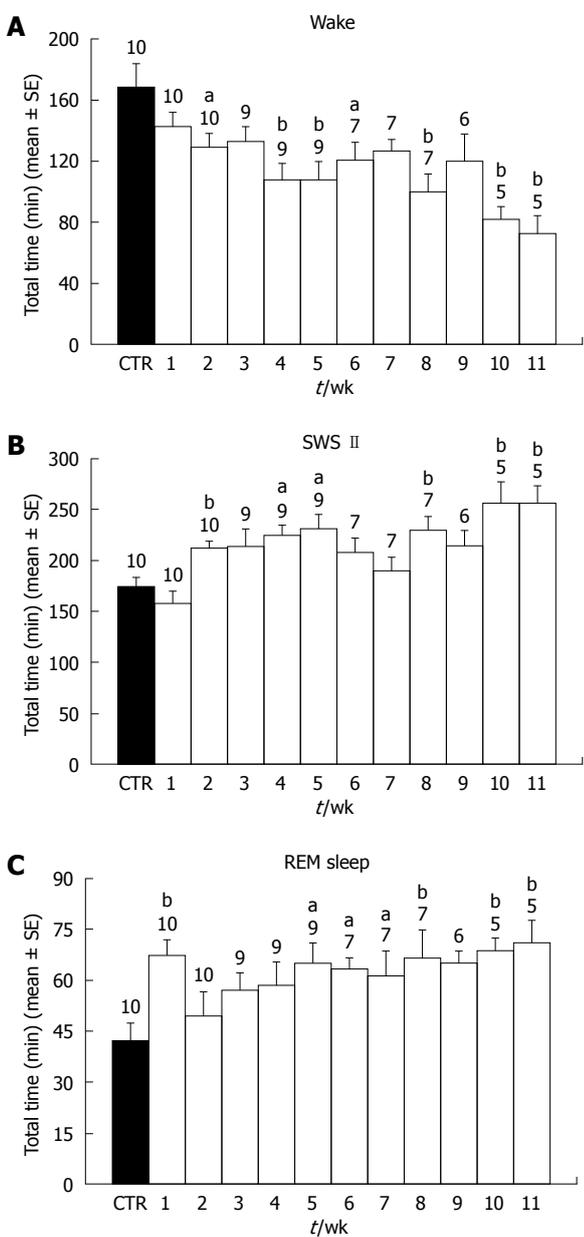
### Sleep pattern

Treatment with CCl<sub>4</sub> induced behavioral changes in all

**Table 1** CCl<sub>4</sub> effect on sleep architecture for 11 wk of treatment (min) (mean ± SE)

Treatment	Wake total time	SWS I total time	SWS II total time	REM sleep total time	Duration of REM sleep epochs	Frequency of REM sleep	Latency of REM sleep	Latency of SWS
Control (n = 10)	169.03 ± 14.94	89.67 ± 9.21	174.82 ± 9.516	42.32 ± 5.47	2.16 ± 0.21	17.3 ± 1.315	48.84 ± 11.54	37.06 ± 6.78
1 wk (n = 10)	142.93 ± 9.21	109.69 ± 12.89	158.40 ± 13.32	67.64 ± 4.89 <sup>b</sup>	2.95 ± 0.27	23 ± 2.27	69.7 ± 26.61	25.60 ± 6.05
2 wk (n = 10)	128.85 ± 9.74 <sup>b</sup>	87.45 ± 8.45	213.62 ± 6.07 <sup>a</sup>	49.52 ± 7.39	2.56 ± 0.14	17.7 ± 1.93	80.27 ± 19.98	26.57 ± 5.58
3 wk (n = 9)	133.40 ± 9.26 <sup>a</sup>	74.36 ± 14.90	214.38 ± 18.94 <sup>a</sup>	57.30 ± 5.44	2.44 ± 0.13	22.22 ± 1.98	51.45 ± 16.44	18.99 ± 4.43
4 wk (n = 9)	107.77 ± 11.07 <sup>b</sup>	80.69 ± 10.27	225.89 ± 10.20 <sup>b</sup>	58.60 ± 7.94 <sup>a</sup>	2.44 ± 0.160	22.22 ± 2.17	63.80 ± 17.81	17.84 ± 3.41
5 wk (n = 9)	107.52 ± 12.58 <sup>b</sup>	74.27 ± 13.55	232.54 ± 14.85 <sup>b</sup>	67.24 ± 5.45 <sup>b</sup>	2.70 ± 0.27	23.55 ± 1.74	73.67 ± 19.52	25.04 ± 4.98
6 wk (n = 7)	121.19 ± 11.64 <sup>b</sup>	86.08 ± 8.86	208.17 ± 16.42	63.64 ± 3.37 <sup>a</sup>	2.72 ± 0.26	22.42 ± 2.01	79.99 ± 18.22	17.58 ± 2.30
7 wk (n = 7)	126.87 ± 7.28 <sup>b</sup>	93.07 ± 10.69	191.5 ± 13.90	61.47 ± 8.03 <sup>a</sup>	2.31 ± 0.21	24.85 ± 3.47	71.49 ± 23.91	19.81 ± 4.54
8 wk (n = 7)	99.64 ± 12.18 <sup>b</sup>	82.11 ± 8.89	230.48 ± 14.96 <sup>b</sup>	66.93 ± 9.00 <sup>b</sup>	1.96 ± 0.23	31.42 ± 2.17 <sup>b</sup>	75.81 ± 11.97	18.08 ± 4.95
9 wk (n = 6)	120.06 ± 17.93 <sup>b</sup>	78.53 ± 7.85	215.29 ± 16.85 <sup>b</sup>	65.41 ± 3.90 <sup>a</sup>	2.56 ± 0.40	24.33 ± 2.78	85.26 ± 32.18	20.03 ± 6.91
10 wk (n = 5)	82.03 ± 8.25 <sup>b</sup>	66.91 ± 13.22	256.29 ± 22.37 <sup>b</sup>	68.89 ± 3.72 <sup>b</sup>	2.60 ± 0.50	26.6 ± 2.56 <sup>b</sup>	60.46 ± 36.58	17.15 ± 7.64
11 wk (n = 5)	72.79 ± 11.77 <sup>b</sup>	77.34 ± 17.61	257.84 ± 18.77 <sup>b</sup>	71.28 ± 7.67 <sup>b</sup>	2.63 ± 0.41	26.4 ± 2.56 <sup>b</sup>	39.64 ± 10.70	17.24 ± 5.22

<sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01 vs control. Repeated measure ANOVA.



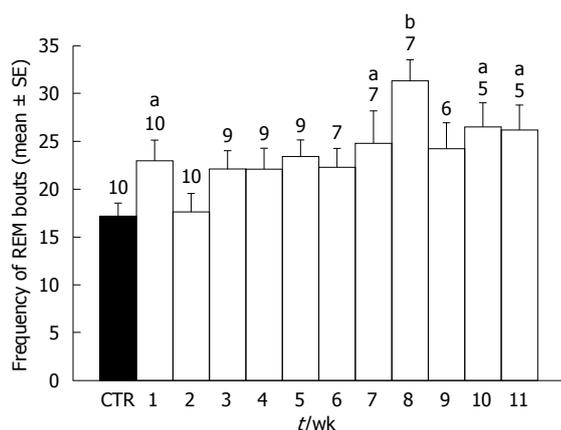
**Figure 1** Percentage of Wakefulness (A), SWS II (B), and REM sleep (C) in weekly 8 h sleep recordings during treatment with CCl<sub>4</sub>. Numbers at the top of the bars express the number of subjects. <sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01 vs control. Repeated measurements were analyzed by ANOVA.

rats, characterized mainly by a progressive decrease in locomotion. During the final 2 wk, four out of the five surviving animals showed ascitis. Five of the animals died before the end of the 11-wk period, so the size of the group decreased as the treatment progressed. Concerning sleep, CCl<sub>4</sub> administration elicited a decrease of wake time throughout the 11 wk of treatment. The decrease was observed from the first week of treatment but only reached statistical significance from the second week. This decrease in wake time grew larger as the treatment progressed, and during weeks 10 and 11, wake time was less than 50% of pretreatment values (Figure 1A). Concerning SWS I, no significant modifications were observed. However, SWS II time showed a significant increase from the second week of treatment. The increase remained constant with only small variations during the 11 wk. Only during weeks six and seven did the increase did not reach statistical significance (Figure 1B). REM sleep showed a significant increase in the first week of treatment (Figure 1C). However, REM sleep duration returned to control levels during the second and third weeks of treatment and thereafter, there was a significant increase that lasted until week eleven. The increase of REM sleep time was due mainly to the duration of each period, because no significant increases in REM sleep frequency were observed during most of the weeks. Only during weeks eight, ten, and eleven were there significant increases in REM sleep frequency (Figure 2).

Table 1 summarizes the data obtained concerning all the parameters recorded during pretreatment and during the 11 wk of recording.

**Histological liver slices**

Histological examination revealed the effects of chronic CCl<sub>4</sub> treatment on liver parenchyma. Figure 3 shows a sample of a liver treated with CCl<sub>4</sub>. A normal liver can be observed in Figure 3A. In Figure 3B, the effects of CCl<sub>4</sub> treatment for 11 wk on liver histological features are shown. Clear indications of cirrhosis were observed (fibrosis, lipidic vacuoles, pycnotic nuclei, and necrosis).



**Figure 2** Frequency of REM sleep bouts observed in weekly eight-hour sleep recordings during treatment with CCl<sub>4</sub>. Numbers at the top of the bars express the number of subjects. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs control. Repeated measurements were analyzed by ANOVA.

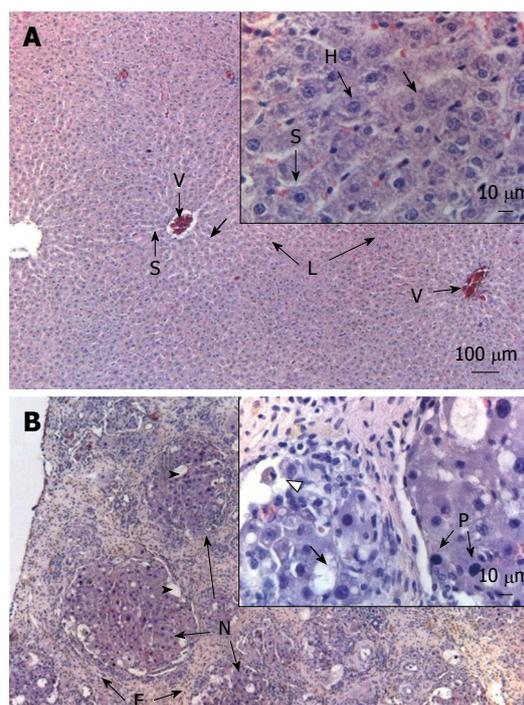
## DISCUSSION

The present results indicate that sleep-wake patterns changed as experimental cirrhosis progressed. REM sleep showed an acute response to the first administrations of CCl<sub>4</sub>. Wake and SWS II time were significantly modified after 2 wk of CCl<sub>4</sub> treatment, and these effects showed a steady and slight increase during the following weeks. These results are consistent with previous reports showing that cirrhotic patients display a reduction in  $\alpha$  activity and an increase in theta and delta activity<sup>[8]</sup>.

Indirect tests, such as actigraphic recordings and questionnaires on sleep, have suggested that cirrhotic patients suffer from unsatisfactory sleep, mainly due to the reduction in the quality of sleep, produced by several awakenings throughout the night<sup>[12]</sup>. However, Steindl *et al.*<sup>[16]</sup> did not find differences in polysomnographic recordings of cirrhotic patients compared to matched healthy controls. However, sleep diaries of these patients indicated more frequent nocturnal awakenings and daytime naps. Moreover, these researchers measured the levels of melatonin and found a significant increase during daytime, when melatonin is normally absent<sup>[16]</sup>. Recently, Velissaris *et al.*<sup>[17]</sup> corroborated these results; they showed that melatonin circadian patterns were altered in cirrhosis patients without clinical encephalopathy. This disruption might reflect changes in the output of the circadian pacemaker located in the suprachiasmatic nucleus of the hypothalamus. It is possible that some of the metabolic disturbances generated by cirrhosis might also alter the function of this biological clock.

On the other hand, several diseases of sleep have been reported, such as the OSAS. OSAS is a frequent disease that has been extensively studied. In patients with advanced liver cirrhosis, OSAS has been reported to be associated with changes in autonomic nervous activities<sup>[18]</sup>.

Recent molecular studies have shown an increased expression of genes associated with monoamine oxidase (MAO-A isoform) and nitric oxide synthase (nNOS isoform) in the brain of cirrhotic patients<sup>[3]</sup>. Moreover, oligodendroglial nodules have been observed in the white



**Figure 3** Effects of repeated administration of CCl<sub>4</sub> on liver morphology in rats after 11 wk of CCl<sub>4</sub> treatment. A: Normal aspect of an untreated liver. In a stain with HE, the typical sinusoidal (S) organization can be observed with lobules (L) surrounding the vessels (V). In a higher magnification, a normal hepatocyte (H) can be distinguished; B: The effects of CCl<sub>4</sub>. A fibrotic process (F) is evident, with hepatocytes grouped in nodules (N) containing vacuoles. In a higher magnification, the bold arrow indicates hepatocytes with lipidic vacuoles can be observed as well as cells with pycnotic nucleus (P) or in necrosis (triangle).

matter associated with CCl<sub>4</sub>-induced liver failure<sup>[19,20]</sup>. In addition, in rats submitted to portacaval anastomosis and in patients with cirrhosis, alterations of the circadian system have been noticed, especially in the rhythm of circadian locomotor activity and in the rhythm of pineal melatonin release<sup>[1,21,22]</sup>. All of these data can account for sleep effects observed in the present study.

Likewise, it has been shown that the activity of the EEG can be modified by several substances, such as ammonium, a potent neurotoxic produced by cirrhotic patients<sup>[3,11,23,24]</sup>. This increase of ammonium affects the homeostasis levels of neurotransmitters and neuropeptides in plasma and in the brain. Thus, the levels of dopamine, acetylcholine, glutamate, nitric oxide, and GABA are modified<sup>[24-26]</sup>. It is possible that, in the present study, the animals treated with CCl<sub>4</sub> have several neurochemical alterations that could modify the levels of different neurotransmitters. These changes, in addition to the alterations in the biological clock, can produce disturbances in some nuclei involved in the regulation of sleep. Further research is needed to elucidate the precise mechanisms of the observed changes.

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**COMMENTS****Background**

Sleep disturbances have been described in patients with advanced cirrhosis. However, the development and mechanisms of these alterations are not known. In this study, the sleep pattern of rats submitted to experimental cirrhosis was analyzed as liver damage progressed. The results showed an early decrease of wake time and sustained increases of slow wave sleep (SWS) and later, of REM sleep. This data suggest that sleep alterations could be the warning signs of liver disease.

**Research frontiers**

This study adds information on the relationship between liver function and cerebral function. The mechanisms through which this reciprocal relationship works remain to be elucidated.

**Innovations and breakthroughs**

The animal model of liver cirrhosis induced by chronic administration of CCL<sub>4</sub> is a suitable model to analyze the relationship between liver failure and brain function disturbances.

**Applications**

This study highlights the need for gastroenterologists to pay attention to sleep disturbances as early signs of liver failure.

**Terminology**

SWS and rapid eye movement sleep and the two major sleep stages in most animal species.

**Peer review**

The content of the article will be interesting not only for the gastroenterologists, but also for other specialists. Further investigations of mechanisms of sleep alterations might yield potentially efficacious approaches to its clinical management.

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## Therapeutic effect of caffeic acid phenethyl ester on cerulein-induced acute pancreatitis

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results and amylase level in the placebo groups were similar to those in the AP group. White blood cell count and TNF- $\alpha$  concentration was nearly the same in the CAPE and placebo groups.

**CONCLUSION:** CAPE may be useful agent in treatment of AP but more experimental and clinical studies are needed to support our observation of beneficial effects of CAPE before clinical usage of this agent.

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**Key words:** Acute pancreatitis; Caffeic acid phenethyl ester; Cerulein

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### Abstract

**AIM:** To evaluate the therapeutic role of caffeic acid phenethyl ester (CAPE) in a rat model of cerulein-induced acute pancreatitis (AP).

**METHODS:** Seventy male Wistar albino rats were divided into seven groups. Acute edematous pancreatitis was induced by subcutaneous cerulein injection (20  $\mu$ g/kg) four times at 1-h intervals. CAPE (30 mg/kg) was given by subcutaneous injection at the beginning (CAPE 1 group) and 12 h after the last cerulein injection (CAPE 2 group). Serum amylase, lipase, white blood cell count, and tumor necrosis factor (TNF)- $\alpha$  levels were measured, and pancreatic histopathology was assessed.

**RESULTS:** In the AP group, amylase and lipase levels were found to be elevated and the histopathological evaluation showed massive edema and inflammation of the pancreas, with less fatty necrosis when compared with sham and control groups. Amylase and lipase levels and edema formation decreased significantly in the CAPE therapy groups ( $P < 0001$ ); especially in the CAPE 2 group, edema was improved nearly completely ( $P = 0001$ ). Inflammation and fatty necrosis were partially recovered by CAPE treatment. The pathological

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### INTRODUCTION

Acute pancreatitis (AP) is a process of acute inflammation in the pancreas, with variable involvement of regional tissues or organ systems. In most patients, acute necrotizing pancreatitis leads to remote organ failure, sepsis and a high death rate<sup>[1]</sup>. Pathophysiology of AP is poorly understood, but interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)- $\alpha$  as pro-inflammatory cytokines, oxidative stress and microvascular ischemia are important factors<sup>[2-4]</sup>. In recent years, pathogenesis-oriented treatments of AP have gained importance. Therefore, new experimental studies have focused on pathophysiological mechanisms such as oxidative stress and inflammatory cytokines<sup>[5,6]</sup>. Propolis is a natural substance that is produced by honeybees from the gum of

various plants. It contains several chemical compounds such as polyphenolic compounds like flavonoids, cinnamic acid derivatives, various steroids, and amino acids<sup>[7,8]</sup>. Caffeic acid phenethyl ester (CAPE) is also a phenolic compound and an active substrate of propolis. Several investigators have shown that CAPE has anti-inflammatory activity by inhibiting the release of arachidonic acid from cell membranes, and suppressing cyclooxygenase (COX)-1 and COX-2 enzyme activity<sup>[9]</sup>, antioxidant activity by lipoxygenase inhibition<sup>[10,11]</sup>, and anti-proliferative, antimutagenic and antitumoral effects by inducing apoptosis in tumor cell lines<sup>[12]</sup>. In addition, CAPE is a potent and specific inhibitor of nuclear factor (NF)- $\kappa$ B and inhibits the activation of NF- $\kappa$ B that is induced by TNF- $\alpha$  and other inflammatory agents<sup>[13]</sup>.

The aim of this study was to investigate the therapeutic efficacy of CAPE in the cerulein-induced acute edematous pancreatitis in rats.

## MATERIALS AND METHODS

### Animals

Seventy male Wistar albino rats, weighing 250-320 g were used from the Physiology Laboratory of Gaziantep University Medical School. The animals were housed under a 12-h light-dark cycle at a temperature of 24°C. Food was withdrawn 12 h before the experiment. All experiments were performed in accordance with the recommendations of the national guidelines for the care and handling of laboratory animals, and followed a protocol approved by the local animal ethics committee.

### Experimental design

Acute edematous pancreatitis was induced by subcutaneous cerulein (Sigma, St Louis, MO, USA) injection (20 g/kg) four times at 1-h intervals<sup>[14]</sup>. Seventy male rats were divided into seven groups of 10.

Group 1 (sham): nothing was applied to the sham group. Group 2 (control): 1 mL saline was given by subcutaneous injecting four times at 1-h intervals, but no medication was applied. Animals were killed 12 h after the last injection. Group 3 (AP group): AP was induced by subcutaneous cerulein injection (20 g/kg dissolved in 1 mL saline) four times at 1-h intervals, but no medication was applied. Animals were killed 12 h after the last injection. Group 4 (CAPE 1) 30 mg/kg CAPE (Sigma) was given by subcutaneous injection at the beginning of the procedure, and at the same time, AP was induced by subcutaneous cerulein injection as described before. Group 5 (CAPE 2): AP was induced in the same way as described above, and CAPE (30 mg/kg) was given at 12 h after the last cerulein injection. Animals were killed 6 h after the CAPE injection. Group 6 (placebo 1): AP was induced by subcutaneous cerulein injection (20  $\mu$ g/kg) four times at 1-h intervals, and 1 mL saline was given at the beginning of the studies. Animals were killed 12 h after the last injection. Group 7 (placebo 2): AP was induced in the same way as described above, and 1 mL saline was given at 12 h after the last cerulein injection. Animals were killed 6 h after the saline injection.

Table 1 Pathological grading system in experimental AP

Edema	0	No edema
	1	Interlobular edema
	2	Moderate interlobular edema + intra-acinar edema
	3	Severe interlobular and intra-acinar edema
Inflammatory infiltration	0	No infiltration
	1	Intravascular margination of granulocytes
	2	Granulocytes present in the perivascular tissue
	3	Diffuse infiltration of entire pancreatic gland
Fat necrosis	0	No necrosis
	1	1-4 necrotic cells (each microscopic area)
	2	5-10 necrotic cells
	3	11-16 necrotic cells

### Assays of treatment efficacy

Under ketamine anesthesia, midline laparotomy was performed on all rats, except Groups 5 and 7, at 15 h (12 h after the last cerulein or saline injection). Groups 5 and 7 were killed 6 h after CAPE injection (Group 5) or saline injection (Group 7). Shortly after the blood specimens were taken from the inferior vena cava, the whole pancreas was extracted quickly and the animals were sacrificed. Blood samples were centrifuged at 3000 rpm for 10 min and the plasma was stored at -70°C until assayed. White blood cell count, amylase, lipase and TNF- $\alpha$  concentrations were measured. Plasma TNF- $\alpha$  concentration was measured by immunoassay kit (Rat TNF- $\alpha$  immunoassay; R&D Systems Inc., Minneapolis, MN, USA), plasma amylase and lipase were measured by commercially available kits from Roche Diagnostics (Mannheim, Germany), using an enzymatic photometric method based on cleavage of the substrate ethylidene-4-nitrophenyl maltoheptaose. Results are expressed as U/L.

### Histopathological scoring

Histopathological evaluation of the pancreas was made in order to understand the extent of the injury. Pancreatic tissue was fixed in formaldehyde solution and embedded in paraffin. Sections were stained with hematoxylin and eosin and were evaluated by light microscopy by two experienced pathologists who were blinded to the experimental treatment groups, according to the Schoenberg grading system<sup>[15]</sup> (Table 1). The tissues were scored using a scale for edema, neutrophil infiltration and fatty necrosis.

### Statistical analysis

Results were given as mean  $\pm$  SD. Comparisons between and among the groups were made using non-parametric test (Mann-Whitney *U* test) and one-way ANOVA. Data were evaluated statistically using SPSS for Windows version 10.0 (Chicago, IL, USA). *P* < 0.05 was taken as significant.

## RESULTS

### Serum amylase, lipase and TNF- $\alpha$ levels

Serum biochemical analysis of amylase, lipase and TNF- $\alpha$  levels and pathological examination results are shown in Table 2. Serum amylase and lipase levels were significantly increased in the cerulein-induced AP group

Table 2 Biochemical, values and pathological scores in cerulein-induced AP (mean  $\pm$  SD)

Groups	TNF- $\alpha$ (pg/mL)	Amylase (U/L)	Lipase (U/L)	White blood cells	Edema	Leukocytic infiltration	Total pathological score	Fat necrosis
Sham	65.14 $\pm$ 1.7	665.14 $\pm$ 54	14.41 $\pm$ 1.7	9279 $\pm$ 1867	0.00	0.00	0.00	0.00
Control	63.29 $\pm$ 3.8	630.20 $\pm$ 64	14.92 $\pm$ 1.7	8755 $\pm$ 1098	0.00	0.00	0.00	0.00
AP	63.83 $\pm$ 3.8	4752 $\pm$ 1328 <sup>b</sup>	112.3 $\pm$ 34.8 <sup>b</sup>	8574 $\pm$ 1437	2.50 $\pm$ 0.5	2.80 $\pm$ 0.42	8.00	0.30 $\pm$ 0.48
CAPE 1	61.55 $\pm$ 8.0	1400 $\pm$ 680 <sup>d</sup>	22.92 $\pm$ 6.9 <sup>d</sup>	8407 $\pm$ 418	1.50 $\pm$ 0.7	2.50 $\pm$ 0.52	4.00 <sup>e</sup>	0.00
CAPE 2	60.52 $\pm$ 6.5	1084 $\pm$ 533 <sup>d</sup>	18.65 $\pm$ 3.7 <sup>d</sup>	8940 $\pm$ 2746	0.50 $\pm$ 0.5 <sup>d</sup>	2.40 $\pm$ 0.51	3.00 <sup>e</sup>	0.00
Placebo 1	62.38 $\pm$ 9.5	4516 $\pm$ 749	49.2 $\pm$ 5.3	9267 $\pm$ 927	2.30 $\pm$ 0.6	2.70 $\pm$ 0.48	8.00	0.30 $\pm$ 0.48
Placebo 2	61.56 $\pm$ 2.5	4219 $\pm$ 235	52.54 $\pm$ 4.8	8544 $\pm$ 895	2.20 $\pm$ 0.6	2.70 $\pm$ 0.48	8.00	0.30 $\pm$ 0.48

<sup>b</sup> $P < 0.001$  vs group 1 and 2; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.001$  vs group 3.

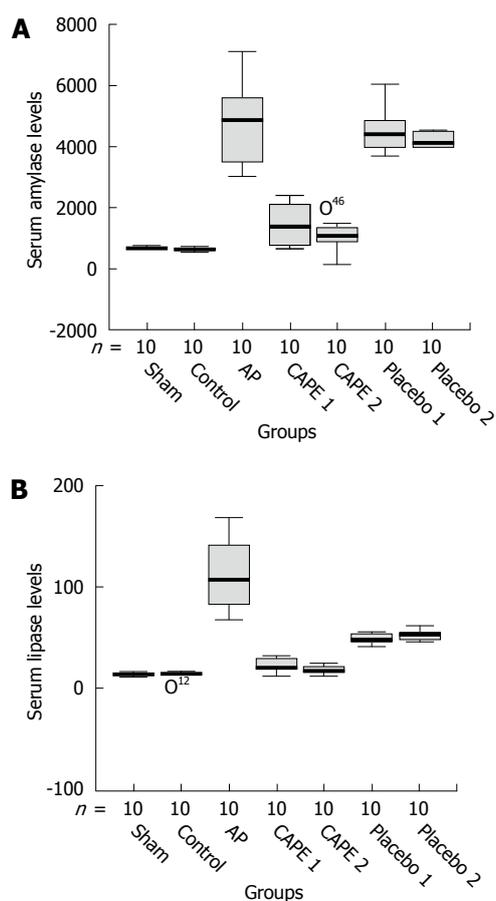


Figure 1 Serum amylase (A) and lipase (B) levels in the experimental groups.

when compared to the control and sham groups ( $P < 0.001$ ). Amylase and lipase levels decreased significantly in the CAPE treatment groups ( $P < 0.001$ ) but the levels were higher than those of the control and sham groups. The levels of amylase and lipase in the placebo groups were similar to those in the AP group (Figure 1A and B). There were no statistically significant differences in serum TNF- $\alpha$  and white blood cell count between the study groups ( $P > 0.05$ , Table 2).

### Pathological examination

In the AP group, histopathological evaluation showed massive edema and inflammation of the pancreas, with less fatty necrosis when compared with the control and sham groups. CAPE treatment significantly decreased edema

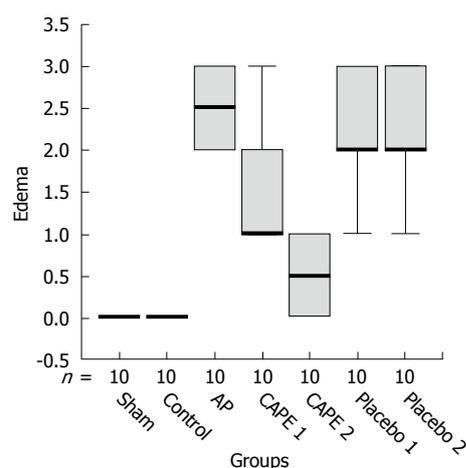
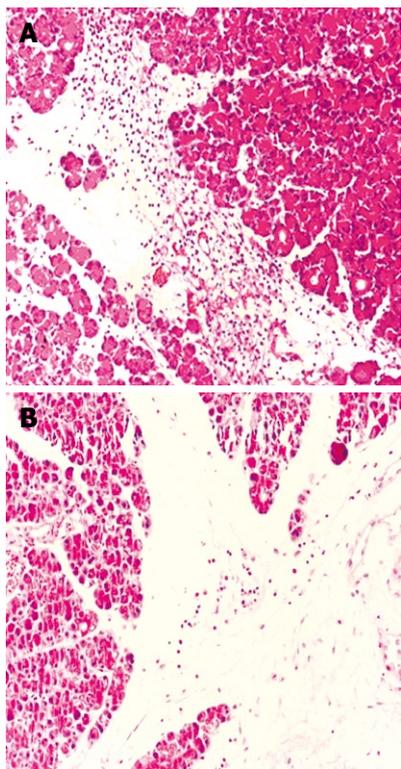


Figure 2 Edema scores in experimental AP groups.

formation, and the most striking finding was that edema was improved nearly completely in the CAPE 2 group ( $P = 0.001$ , Figure 2). Polymorphonuclear leukocytic infiltration was increased in the AP groups ( $P < 0.05$ , Figure 3A). In the therapy groups, inflammation was partially recovered. In the AP groups, fatty necrosis score was  $0.30 \pm 0.48$ . We observed grade 1 fatty necrosis in only three rats in the AP groups. Fatty necrosis was ameliorated in the CAPE treatment groups but this improvement was not statistically significant ( $P > 0.05$ ). The pathological results of the placebo groups were similar to those in the AP groups. After CAPE treatment, the total pathological mean score was decreased significantly ( $P < 0.05$ ) after CAPE treatment (Figure 3B).

## DISCUSSION

Current therapeutic methods are usually insufficient for the treatment of severe AP, despite the development of new diagnostic and therapeutic procedures. Therefore, recently, several experimental studies have focused on the pathogenesis of AP. Several mechanisms, such as oxidative stress, COX-2 and inflammatory cytokines play an important role in the pathogenesis of the disease<sup>[2-4,16]</sup>. CAPE is a phenolic antioxidant, which is an active component of propolis. Previous investigators have demonstrated that CAPE has anti-inflammatory, antioxidant, anti-proliferative and antitumoral effects *in vitro* and *in vivo*<sup>[12]</sup>. In the light of previous findings,



**Figure 3** Histopathological features of cerulein induced AP group and after CAPE therapy. A: Severe edema and leukocytic infiltration of pancreas after cerulein-induced AP (HE,  $\times 200$ ); B: Decreased infiltration in pancreatic tissue after CAPE therapy (HE,  $\times 200$ ).

we investigated the therapeutic role of CAPE as a new agent for the treatment of AP.

TNF- $\alpha$  is a cytokine that plays a central role in the pathogenesis of the disease<sup>[2]</sup>. TNF receptor antagonist observed a reduction in the severity and mortality of experimental pancreatitis<sup>[17]</sup>. Plasma half-life of TNF- $\alpha$  is very short (14-18 min)<sup>[18]</sup>, therefore, we studied TNF- $\alpha$  serum levels in rats, despite this kind of measurement being difficult. We obtained serum at 15 h, and that is probably why the results were low in all groups.

Norman *et al*<sup>[19]</sup> have shown marked amelioration of pancreatic tissue damage and decreased serum amylase and lipase levels after treatment with IL-1 antagonist. Oxidative stress plays an important role in the pathophysiology of AP. For this reason, several studies have reported the therapeutic effect of antioxidant agents. A previous study has disclosed that various antioxidant agents improve pancreatic edema in cerulein-induced pancreatitis, however antioxidants showed no improvement in a sodium-taurocholate model of pancreatitis in rats<sup>[20]</sup>. On the contrary, one recent study in a sodium-taurocholate model of pancreatitis in rats has shown that serum amylase and lipase, edema, leukocytic infiltration, parenchymal necrosis and hemorrhage were significantly decreased by N-acetylcysteine (NAC) treatment. In addition, in the NAC-treated rats, while serum nitrite/nitrate levels were significantly increased, serum concentration of the lipid peroxidation product was significantly decreased. The beneficial effect of NAC may result from its antioxidant activity and the production of and/or inhibition of degradation of nitric oxide<sup>[5]</sup>. In a similar

study, Vaquero *et al*<sup>[21]</sup> have demonstrated that treatment with NAC reduces neutrophil infiltration and mRNA expression for IL-6, cytokines and inducible nitric oxide synthase in pancreatic tissue, by inhibition of NF- $\kappa$ B activity. In conclusion, NF- $\kappa$ B is a key regulator cytokine in induction and oxidative stress in AP. In experimental pancreatitis, the beneficial effect of antioxidants can be explained by inhibition of NF- $\kappa$ B activation. CAPE is a specific and potent inhibitor of NF- $\kappa$ B and causes inhibition of pro-inflammatory cytokine production<sup>[13]</sup>. Likewise, Fitzpatrick *et al*<sup>[22]</sup> have shown that CAPE (30 mg/kg) treatment significantly inhibits NF- $\kappa$ B, and colonic cytokines (TNF- $\alpha$  and IL-1 $\beta$ ) are reduced in experimental colitis in rats.

AP is associated with induction of COX-2 expression. In cerulein-induced pancreatitis, Ethridge *et al*<sup>[16]</sup> have found that COX-2 gene expression is increased in pancreatic tissue. Although serum amylase and lipase are not reduced, the severity of pancreatic necrosis and leukocytic inflammation are significantly decreased by treatment with NS-398 (a COX-2 inhibitor). It has been demonstrated that COX-2 gene expression, activity of COX-1 and COX-2 enzymes, and release of arachidonic acid from cell membranes are inhibited by CAPE<sup>[9]</sup>. In light of this, we investigated the beneficial efficacy of CAPE (which is an antioxidant and anti-inflammatory agent) on the experimental model of cerulein-induced acute edematous pancreatitis in rats. As far as we know, there are no published data on the treatment effect of CAPE in experimental pancreatitis models. In the present study, we showed that CAPE ameliorated the harmful effects in a rat model of cerulein-induced pancreatitis. Serum amylase and lipase levels were decreased by CAPE treatment. In addition, CAPE treatment significantly reduced edema and total pathological mean score. Inflammation and fatty necrosis score were improved but the improvement was not statistically significantly.

There are several models of experimental pancreatitis, such as the cerulein-induced and sodium-taurocholate models. Pancreatic injury is evenly distributed throughout the pancreas in the cerulein-induced models. The reason why we chose the cerulein-induced AP model was that this form of pancreatitis is very similar to that in humans and it occurs within a short time<sup>[23]</sup>. This model is used widely to study potential agents for the treatment of AP<sup>[24]</sup>. In this model, pancreatic inflammation reaches the most severe stage at 12 h, which is why we ended the first part of the study at 12 h after cerulein injection<sup>[25]</sup>. Secondly, we formed a CAPE 2 group to study the effect of CAPE on the most severe stage of pancreatitis at 12 h. Here, our concern was to study the efficacy of the treatment in severe pancreatitis, especially in the full-blown situation. In fact, patients with AP often attend the hospital at an advanced stage, even sometimes with systemic complications.

In conclusion, in the cerulein-induced model of experimental AP, an improvement in the biochemical and histopathological findings were observed in the CAPE treatment groups. CAPE decreased pancreatic tissue injury and this supports the hypothesis that antioxidant

and anti-inflammatory treatment is effective in AP. It is important that CAPE was effective in the CAPE 2 group when AP had already occurred. This will enlighten the following phase 3 and phase 4 studies. Another important step will be to study the efficacy of CAPE in experimental necrotizing pancreatitis. Nevertheless, more experimental and clinical studies are needed to support our observation of the beneficial effects of CAPE before clinical usage of this agent.

## COMMENTS

### Background

Pathophysiology of acute pancreatitis (AP) is poorly understood. Therefore, new experimental therapeutic studies have focused on the pathophysiological mechanisms. The present experimental study investigated the therapeutic role of caffeic acid phenethyl ester (CAPE) as a new agent for the treatment of AP.

### Research frontiers

CAPE is a specific and potent inhibitor of nuclear factor (NF)- $\kappa$ B and causes inhibition of pro-inflammatory cytokine production. CAPE (30 mg/kg) treatment significantly inhibited NF- $\kappa$ B, and colonic cytokines tumor necrosis factor- $\alpha$  and interleukin-1 $\beta$  were reduced in experimental colitis in rats.

### Innovations and breakthroughs

There are no published data on the treatment effect of CAPE in experimental pancreatitis models. In the cerulein-induced model of experimental AP, improvement in biochemical and histopathological findings was observed in the CAPE treatment groups.

### Applications

CAPE may be a useful agent in the treatment of AP but more experimental and clinical studies are needed to support our observation of its beneficial effects.

### Terminology

CAPE is a phenolic compound and an active substrate of propolis. CAPE has anti-inflammatory, antioxidant, anti-proliferative and antitumoral effects *in vitro* and *in vivo*.

### Peer review

This work provides experimental evidence for a protective function of CAPE in a rat model of acute pancreatitis. The study is generally well-designed and controlled. The results show that administration of CAPE reduces serum amylase and lipase levels in cerulein-treated rats and improves pathological scores in pancreatic tissue specimens.

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BRIEF ARTICLE

## CO<sub>2</sub> insufflation for potentially difficult colonoscopies: Efficacy when used by less experienced colonoscopists

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examinations, in addition to insertion to the cecum and withdrawal times.

**RESULTS:** Examination times did not differ, however, VAS scores in the CO<sub>2</sub> group were significantly better than in the air group ( $P < 0.001$ , two-way ANOVA) from immediately after the procedure and up to 2 h later. There were no significant differences between either insufflation method in the EC group ( $P = 0.29$ ), however, VAS scores for CO<sub>2</sub> insufflation were significantly better than air insufflation in the LEC group ( $P = 0.023$ ) immediately after colonoscopies and up to 4 h afterwards.

**CONCLUSION:** CO<sub>2</sub> insufflation reduced patient pain after colonoscopy in potentially difficult cases when performed by LECs.

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**Key words:** CO<sub>2</sub> insufflation; Colonoscopy; Difficult colonoscopy; Experienced colonoscopist; Training

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Uraoka T, Kato J, Kuriyama M, Hori K, Ishikawa S, Harada K, Takemoto K, Hiraoka S, Fujita H, Horii J, Saito Y, Yamamoto K. CO<sub>2</sub> insufflation for potentially difficult colonoscopies: Efficacy when used by less experienced colonoscopists. *World J Gastroenterol* 2009; 15(41): 5186-5192 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5186.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5186>

### Abstract

**AIM:** To clarify the effectiveness of CO<sub>2</sub> insufflation in potentially difficult colonoscopy cases, particularly in relation to the experience level of colonoscopists.

**METHODS:** One hundred twenty potentially difficult cases were included in this study, which involved females with a low body mass index and patients with earlier abdominal and/or pelvic open surgery or previously diagnosed left-side colon diverticulosis. Patients receiving colonoscopy examinations without sedation using a pediatric variable-stiffness colonoscope were divided into two groups based on either CO<sub>2</sub> or standard air insufflation. Both insufflation procedures were also evaluated according to the experience level of the respective colonoscopists who were divided into an experienced colonoscopist (EC) group and a less experienced colonoscopist (LEC) group. Study measurements included a 100-mm visual analogue scale (VAS) for patient pain during and after colonoscopy

### INTRODUCTION

Colonoscopy has a high profile because of its increasingly important role in successfully preventing, detecting and treating colorectal cancer<sup>[1,2]</sup>, however, some patients experience considerable abdominal pain and discomfort when the procedure is performed using air insufflation. In particular, the so-called “difficult colonoscopy” cases<sup>[3-6]</sup>, which involve female patients with a relatively

low body mass index (BMI), patients with a history of abdominal and/or pelvic open surgery and male patients with diverticulosis, often require prolonged insertion to the cecum, thus this procedure can cause increased abdominal pain and discomfort for such patients.

Factors accounting for longer examination times and increased abdominal pain and discomfort can be derived from both a patient's condition and the examining colonoscopist's skill and experience<sup>[7-9]</sup>. Novice and even moderately skilled colonoscopists must improve their technical abilities by gaining experience in successfully handling difficult colonoscopies to become qualified experts, as a suitably high-level colonoscopy training environment has not been established as yet<sup>[10,11]</sup>.

CO<sub>2</sub> insufflation has been reported to reduce patient abdominal pain and discomfort during and after colonoscopies<sup>[12-15]</sup>. Although the safety and efficacy of CO<sub>2</sub> insufflation during colonoscopies have been assessed in earlier studies, air insufflation is still the standard method due to a lack of suitable equipment and inadequate information as to when and on whom CO<sub>2</sub> insufflation should be used during colonoscopy examinations.

We decided to conduct a prospective randomized controlled trial to test the hypothesis that CO<sub>2</sub> insufflation reduces patient abdominal pain and discomfort during and after colonoscopy examinations in potentially difficult cases.

## MATERIALS AND METHODS

### Study protocol

Consecutive patients considered potentially difficult cases for colonoscopic intubation were included in this prospective randomized controlled trial which took place between September 2006 and October 2007. The aim of this study was to clarify the effectiveness of CO<sub>2</sub> insufflation during colonoscopy examinations, with the primary objectives of assessing both patient tolerance and the safety of CO<sub>2</sub> insufflation in these potentially difficult cases. A secondary objective was to clarify any differences between the two insufflation methods in relation to the experience level of the participating colonoscopists. This study was approved by the Ethics Committee at Okayama University Hospital.

### Patients

Patients considered potentially difficult colonoscopy cases, based on published information and clinical experience, were selected, and included females with a relatively low BMI (BMI < 22), patients with a history of abdominal and/or pelvic open surgery, with the exception of low risk procedures for adhesions such as appendectomy or hernia repair, and male patients with previously diagnosed left-side diverticulosis<sup>[3-6]</sup>.

The indications for colonoscopy examination were the standard clinical criteria: colorectal cancer screening, surveillance for polyps, a positive fecal occult blood test, abdominal symptoms or anemia. Exclusion factors included severe heart or lung disease, a prior colorectal

resection, inflammatory bowel disease, severe hematochezia and repeat colonoscopy for therapeutic procedures including polypectomy.

Written informed consent was obtained from each patient and enrolled patients were randomly divided into two groups for colonoscopy examinations using either CO<sub>2</sub> or standard air insufflation. Group allocation for both patients and colonoscopists was performed by specially assigned nurses using standard randomization lists which contained consecutive patient numbers. Each number was linked to one of the two study groups for allocation purposes. These lists were not accessible by the participating colonoscopists.

### Colonoscopy using CO<sub>2</sub> insufflation

Patients underwent bowel preparation with sodium picosulfate the day before their examinations and two liters of polyethylene glycol solution-containing lavage the morning of their colonoscopies. Scopolamine butylbromide (20 mg) was administered intramuscularly to suppress bowel movement, while patients with cardiac disease or benign prostatic hypertrophy received glucagon (1 IU) intramuscularly. Patients were not sedated, although midazolam (2-3 mg, iv) was administered based on the examining colonoscopist's judgment or when requested by the patient due to abdominal pain or distension. Examinations were performed using a pediatric variable-stiffness colonoscope (PVSC) with a distal tip diameter of 11.3 mm (PCF-Q260AI, Olympus Co, Tokyo, Japan).

Procedures were randomly performed by eight colonoscopists who had earlier been divided into two groups according to their colonoscopy experience: four highly experienced colonoscopists (EC) group each of whom had been in colonoscopy practice for over 10 years (TU, JK, KT and SH), and four less experienced colonoscopists (LEC) group with 5-7 years of colonoscopy practice during which each had performed 900-1500 colonoscopies (MK, SI, KH and HF).

If an examining colonoscopist from the LEC group failed to pass through the sigmoid-descending colon junction within 15 min or a patient complained of severe pain, a colonoscopist from the EC group replaced the initial examiner before midazolam was administered and continued insertion to the cecum. When such a case involved a colonoscopist from the EC group as the initial examiner, a more experienced member of the EC group would continue the procedure. After reaching the cecum, the initial examiner proceeded with withdrawal of the colonoscope.

A "complete colonoscopy" was defined as successful insertion to the cecum bottom or terminal ileum. Insertion to the cecum and withdrawal time was recorded for every colonoscopy.

### CO<sub>2</sub> insufflation and monitoring system

CO<sub>2</sub> was administered using a commercial CO<sub>2</sub> regulator (Gas Regulator, Crown, Model FR-IIS-P; Yutaka Engineering, Tokyo, Japan) connected to a CO<sub>2</sub> bottle.

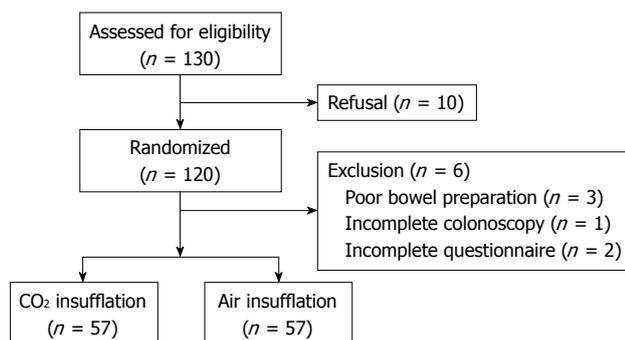


Figure 1 Patient flow chart.

The regulator delivered CO<sub>2</sub> at the rate of 2 L/min. CO<sub>2</sub> and air insufflations were used in a blind fashion both to patients and colonoscopists with full-day colonoscopy sessions randomly assigned CO<sub>2</sub> or air insufflation to avoid unblinding caused by set-up changes between patients.

CO<sub>2</sub> partial pressure was continuously measured using a transcutaneous CO<sub>2</sub> monitoring system (TOSCA 500; Radiometer Basel AG, Switzerland). Processed transcutaneous CO<sub>2</sub> readings (PtcCO<sub>2</sub>) correlate closely with directly obtained arterial blood gas results<sup>[16,17]</sup>. Sensors were attached to a patient's ear lobe with a monitor-specific clip. A colonoscopy assistant recorded readings and an independent observer monitored gas readings to avoid potential serious side effects. CO<sub>2</sub> insufflation was stopped immediately if PtcCO<sub>2</sub> registered > 60 mmHg during any colonoscopy examination.

### Pain and discomfort measurement

A 100-mm visual analogue scale (VAS) consisting of a horizontal line 100 mm in length was used for measuring patient abdominal pain and discomfort (0 mm = painless, 100 mm = extremely painful)<sup>[18]</sup>. Patients recorded the pain level experienced upon reaching the cecum bottom, immediately following their examinations and 30 min, 1, 2, 4 and 6 h afterwards. The VAS score was the distance measured to the nearest millimeter from the left end of the line to the point of the patient's mark.

Another member of the medical staff, who did not know how the procedures were performed, interviewed the patients 30 min after completion of their colonoscopies. A questionnaire was then given to the patients to take home to complete as instructed at intervals of 1, 2, 4 and 6 h and the completed forms were then mailed to the hospital the following day. The completed questionnaires were subsequently mailed to our medical office. No follow-up phone calls were made as 98% of all questionnaires were promptly returned.

### Statistical analysis

A preliminary pilot study was conducted to estimate the SD in pain measurements. With an assumed SD of 19 mm, the study sample size was calculated at 110 patients in order to have an 80% power with two-sided  $\alpha$  levels of 0.05 to detect any differences in VAS scores between

the two insufflation groups ( $\geq 10$  mm was considered clinically important).

The outcomes for our secondary objective to clarify any differences between the two insufflation methods in relation to the experience level of participating colonoscopists were analyzed on an intention-to-treat basis, given the fact that a number of the initial examining colonoscopists were replaced during the insertion phase of the procedure. Statistical comparisons were made using chi-square and Fisher's exact tests. ANOVA was used for repeated measures statistical analysis of pain. Some variables were not distributed normally, thus the Wilcoxon rank sum test was applied for supplementary analysis to compare groups at each measurement point. Statistical analyses were performed using Prism version 5.0 (GraphPad Software, San Diego, CA, USA) and JMP version 6.3 (SAS Institute, Cary, NC, USA). A *P* value < 0.05 was considered significant.

## RESULTS

### Baseline characteristics

A total of 130 patients were asked to participate and 120 consenting patients were randomized into two groups prior to their colonoscopy examinations (Figure 1). Three poor bowel preparation patients were not included and one (0.85%, 1/117) incomplete intubation patient in the air insufflation group with a history of abdominal and pelvic open surgery, whose examination was performed by an EC, was not submitted for consideration. Completed questionnaires were received from 98% of the 116 remaining patients, thus a final total of 114 patients (68% female/32% male) were analyzed in this study. Exactly half or 57 patients were examined using CO<sub>2</sub> insufflation and the other 57 patients were examined with air insufflation. There were no significant differences in baseline patient characteristics including eligibility criteria for potentially difficult cases between the two groups (Table 1).

### Outcome measures comparing CO<sub>2</sub> and air insufflation groups

There were no significant differences in procedure times including intubation, withdrawal and total time between the two groups (Table 2). Midazolam was administered to two patients (4%) in each group. There were no instances of PtcCO<sub>2</sub> > 60 mmHg in the CO<sub>2</sub> insufflation patients or any procedure-related complications in either group.

Figure 2 shows the mean VAS scores during and after colonoscopy examinations. VAS scores in the CO<sub>2</sub> insufflation group were significantly better than those in the air insufflation group (*P* < 0.001, ANOVA for repeated measures). The overall mean difference was 5.3 mm (95% CI: 3.5-7.1, *P* < 0.001). Comparison by nonparametric analysis at each measurement point produced results favoring CO<sub>2</sub> insufflation immediately following the examinations and up to 2 h afterwards. The maximum mean difference of 9.2 mm (95% CI: 0.4-18.0, *P* = 0.0049) was recorded 30 min after the examinations.

	CO <sub>2</sub> group (n = 57)	Air group (n = 57)	P value
Median age, yr (IQR)	65 (59-73)	62 (47-71)	0.107
Females	39 (68)	38 (67)	1.00
Eligibility criteria for difficult cases <sup>1</sup>			
Females with relatively low BMI (< 22)	35 (61)	36 (63)	0.133
Previous abdominal and/or pelvic open surgery	41 (72)	37 (65)	0.546
Males with previously diagnosed left-side diverticulosis	6 (11)	2 (4)	0.271
One or more previous colonoscopies	16 (28)	15 (26)	1.00

<sup>1</sup>Some patients had more than one difficult case factor. IQR: Interquartile range; BMI: Body mass index.

	CO <sub>2</sub> group (n = 57)	Air group (n = 57)	P value
Patients receiving antispasmodic drug (%)	54 (95)	56 (98)	0.616
Median total procedure time, min (IQR)	22.5 (17.9-29.6)	22.3 (16.3-43.9)	0.734
Insertion to cecum	10.3 (6.5-16.6)	9.6 (5.8-16.2)	0.601
Withdrawal	11.9 (10.1-13.6)	12.0 (9.8-14.2)	0.986

	EC group (n = 53)	LEC group (n = 61)	P value
Median total procedure time, min (IQR)	19.5 (15.3-25.8)	23.8 (19.2-34.5)	0.005
Insertion to cecum	7.7 (5.1-13.2)	12.5 (7.0-18.9)	0.036
Withdrawal	10.9 (10.0-13.0)	12.5 (10.2-15.1)	0.003
Examiner replaced during intubation	1	5	0.213

EC: Experienced colonoscopist; LEC: Less experienced colonoscopist.

### Subgroup analysis

Based on the subgroup analysis relative to experience level of the participating colonoscopists, we evaluated 53 patients (46%) in the EC group and 61 patients (54%) in the LEC group. There were no significant differences in eligibility criteria for potentially difficult cases between the two groups, however, the EC group achieved insertion to the cecum significantly faster, while withdrawal and total procedure times were also significantly shorter than those in the LEC group (Table 3). The number of replacements by another colonoscopist was larger in the LEC group (5) than in the EC group (1), however, there was no significant difference between the two groups.

Figure 3 shows the mean VAS scores for 27 CO<sub>2</sub> insufflation patients and 26 air insufflation patients during and following colonoscopy examinations performed by the EC group. There were no significant differences in the mean VAS scores between the two patient groups

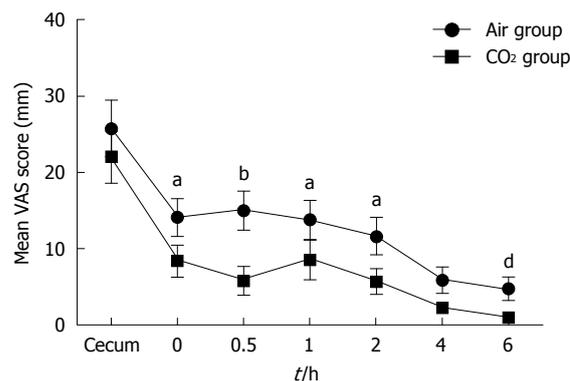


Figure 2 Mean VAS scores at corresponding measurement points during and after colonoscopy examinations in CO<sub>2</sub> and air insufflation groups. VAS scores for CO<sub>2</sub> insufflation were significantly better than those for air insufflation (<sup>a</sup>*P* < 0.001, ANOVA for repeated measures). <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs the CO<sub>2</sub> group at each measurement point by Wilcoxon rank sum test. VAS: Visual analogue scale.

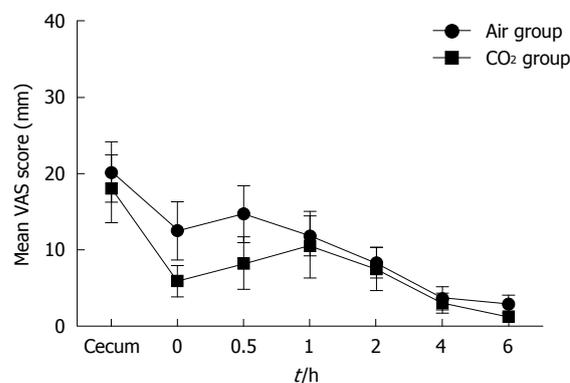


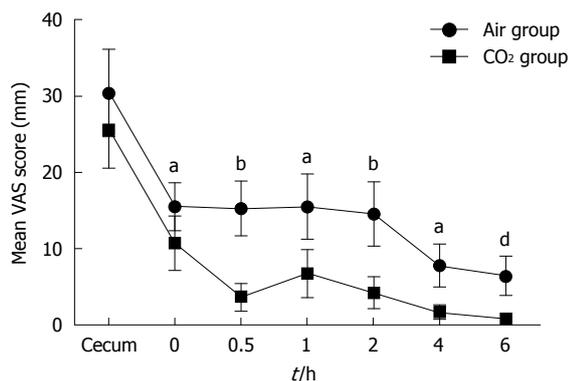
Figure 3 Mean VAS scores at corresponding measurement points during and after colonoscopy examinations for experienced colonoscopists (EC group) in CO<sub>2</sub> and air insufflation groups. There were no significant differences in VAS scores between the two insufflation groups for EC group (*P* = 0.29, ANOVA for repeated measures).

(*P* = 0.29, ANOVA for repeated measures). A comparison of the two patient groups at each measurement point also revealed no significant differences. The maximum mean difference of 6.5 mm (95% CI: -3.7-16.6, *P* = 0.207) occurred 30 min after the examinations.

In the LEC group, 30 CO<sub>2</sub> insufflation patients were evaluated along with 31 air insufflation patients. The mean VAS scores in the CO<sub>2</sub> insufflation group were significantly better than those in the air insufflation group (*P* = 0.023, ANOVA for repeated measures) (Figure 4). The overall mean difference was 7.5 mm (95% CI: 4.9-10.0, *P* < 0.001). A comparison of the two groups by nonparametric analysis at each measurement point produced results favoring CO<sub>2</sub> insufflation from immediately after the examinations up to 4 h later with the maximum mean difference of 11.6 mm (95% CI: 3.4-19.8, *P* = 0.006) occurring 30 min after the examinations.

## DISCUSSION

The increase in patient abdominal pain and discomfort



**Figure 4** Mean VAS scores at corresponding measurement points during and after colonoscopy examinations for less experienced colonoscopists (LEC group) in CO<sub>2</sub> and air insufflation groups. VAS scores for CO<sub>2</sub> insufflation were significantly better compared to air insufflation for LEC group (<sup>d</sup> $P = 0.023$ , ANOVA for repeated measures). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs the CO<sub>2</sub> group at each measurement point by Wilcoxon rank sum test.

often encountered in difficult colonoscopy examination cases is a concern that needs to be satisfactorily resolved by colonoscopists. In this study, we successfully demonstrated the clinical effectiveness of CO<sub>2</sub> insufflation in potentially difficult colonoscopy examination cases. We also clarified the efficacy of CO<sub>2</sub> insufflation for LECs compared to highly ECs.

CO<sub>2</sub> with its characteristic rapid rate of absorption into surrounding tissue has been reported to be more suitable than atmospheric air in various clinical settings<sup>[12-15]</sup>. In fact, several randomized trials have shown that CO<sub>2</sub> insufflation reduced post-colonoscopy abdominal pain and discomfort compared to conventional air insufflation in ambulatory settings. Bretthauer *et al*<sup>[15]</sup> demonstrated that CO<sub>2</sub> insufflation was not only effective, but also safe during colonoscopies in patients receiving conscious sedation. Saito *et al*<sup>[19]</sup> introduced the use of CO<sub>2</sub> insufflation during lengthier colorectal endoscopic submucosal dissections in patients receiving conscious sedation. Their results demonstrated the effectiveness and safety of CO<sub>2</sub> insufflation as well as a resultant reduction in total dosage of midazolam. CO<sub>2</sub> insufflation has also been applied in endoscopic retrograde cholangiopancreatography (ERCP)<sup>[20]</sup> and endoscopic dilatation therapy using a double balloon endoscope<sup>[21]</sup>. There have been few detailed investigative reports on the use of CO<sub>2</sub> insufflation during difficult colonoscopy cases. In addition, the effect of the relative experience of colonoscopists using CO<sub>2</sub> insufflation has not been previously analyzed.

This study validated our theory that CO<sub>2</sub> insufflation is more effective than air insufflation in potentially difficult colonoscopy cases with the comparative difference for the two procedures being particularly discernable between LECs and ECs. Colonoscopy is a technically demanding procedure requiring considerable instruction and on-the-job experience for optimal performance. A suitable training program and sufficient opportunities to improve practical skills in a clinical setting are essential for beginners as well as colonoscopists with a moderate degree of experience<sup>[10,11,22]</sup>.

Difficult colonoscopy examinations performed by LECs require additional time as do ERCP and therapeutic endoscopic procedures, and can cause patient abdominal pain and discomfort both during the procedure and afterwards. The results of our study demonstrated a difference not only in intubation times, but also in withdrawal and overall examination times according to the experience of the participating colonoscopists. Avoiding prolonged insufflation especially during insertion, however, might have led to similar results in the LEC group concerning the clinical effectiveness of CO<sub>2</sub> in reducing patient pain and discomfort.

Lee *et al*<sup>[8]</sup> recommended that trainees perform over 150 examinations in a colonoscopy training program to be technically competent for diagnostic colonoscopy. Our results revealed significant differences in examination times and patient abdominal pain and discomfort after colonoscopy between the EC and LEC groups. The four colonoscopists in the LEC group had each performed a minimum of 900 colonoscopies, thus the question arises as to whether a minimum of 150 cases referred to in the report by Lee above, is sufficient for conducting examinations in potentially difficult colonoscopy cases.

A recent study in Ontario, Canada analyzed factors associated with incomplete colonoscopies based on the following settings: an academic hospital, a community hospital and private medical offices. The incomplete colonoscopy rate was highest in private offices with an odds ratio increase of more than three-fold<sup>[3]</sup>, thus introducing CO<sub>2</sub> insufflation may be particularly useful in reducing patient complaints in non-hospital environments. We refrained from using novice colonoscopists in this study because of the formidable nature of potentially difficult colonoscopy cases. Such novices should only conduct difficult colonoscopies after gaining the necessary experience performing routine colonoscopy examinations.

A number of techniques and devices have reportedly been effective in reducing patient abdominal pain and discomfort during difficult colonoscopies, improving the rate of successful insertion to the cecum, shortening insertion time to the cecum and reducing the dosage of sedatives<sup>[23]</sup> including the use of a pediatric colonoscope<sup>[24]</sup>, variable stiffness colonoscope<sup>[25]</sup>, gastroscope<sup>[26]</sup>, double balloon endoscope<sup>[27]</sup> and hood attached to the top of the colonoscope<sup>[28]</sup>. A PVSC featuring both variable stiffness on demand and a thin diameter was used in our trial. Previously, this instrument was shown not to be superior to adult or standard pediatric colonoscopes<sup>[29-32]</sup>. However, there have been reports that use of the PVSC made it possible to complete colonoscopies that would have been much more difficult or impossible to perform using an adult colonoscope, including patients who had undergone hysterectomies<sup>[31]</sup> and patients with diverticular disease and severe stenosis<sup>[32]</sup>.

There was only one case (0.85%) of incomplete insertion to the cecum in our study and just four (3.5%) patients required sedation. Complete screening colonoscopy without sedation or with on-demand sedation in

academic medical centers has been reported to be in the 88%-99% range<sup>[33-36]</sup>, with the optimum intubation rate obtained using a PVSC. In this study, the PVSC more than likely contributed to the impressive successful intubation rates and reduction in pain during insertion to the cecum achieved in both groups, as well as the favorable intubation times for each group. In several studies performed by ECs at academic medical centers, insertion to the cecum times varied between 7-13 min for colonoscopies performed without sedation or with on-demand sedation<sup>[33-36]</sup>. Our median intubation times of 7.7 and 12.5 min for ECs and LECs, respectively, were in line with these earlier reports.

In conclusion, we clearly demonstrated the clinical effectiveness of CO<sub>2</sub> insufflation in potentially difficult colonoscopy examination cases performed without sedation. We also successfully clarified the efficacy of CO<sub>2</sub> insufflation for LECs.

## COMMENTS

### Background

Colonoscopy is the preferred method for preventing, detecting and treating colorectal cancer, however, prolonged cecal intubation can cause increased patient abdominal pain and discomfort especially in difficult cases, such as female patients with a relatively low body mass index, patients with a history of abdominal and/or pelvic open surgery and male patients with diverticulosis. CO<sub>2</sub> with its rapid rate of absorption has been reported to be more suitable than atmospheric air as an insufflation agent in various clinical settings, although air insufflation is still the standard method due to a lack of suitable equipment and inadequate information as to when and on whom CO<sub>2</sub> insufflation should be used during colonoscopy examinations.

### Research frontiers

This prospective randomized controlled study was conducted to clarify the effectiveness of CO<sub>2</sub> insufflation in potentially difficult cases, particularly in relation to colonoscopist experience level.

### Innovations and breakthroughs

The clinical effectiveness of CO<sub>2</sub> insufflation was clearly demonstrated in potentially difficult colonoscopy examination cases performed without sedation. The procedure that was followed also clarified the efficacy of CO<sub>2</sub> insufflation for less experienced colonoscopists (LEC) particularly in comparison to more experienced colonoscopists.

### Applications

The use of CO<sub>2</sub> insufflation can be incorporated into existing and future colonoscopy training programs in order to further improve the technical skills of colonoscopists.

### Peer review

The authors successfully demonstrated that CO<sub>2</sub> insufflation with its rapid rate of CO<sub>2</sub> absorption and improved efficacy reduced patient pain in potentially difficult cases particularly when colonoscopy examinations were performed by LECs.

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## Crosstalk between angiogenesis, cytokeratin-18, and insulin resistance in the progression of non-alcoholic steatohepatitis

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### Abstract

**AIM:** To elucidate the possible crosstalk between angiogenesis, cytokeratin-18 (CK-18), and insulin resistance (IR) especially in patients with non-alcoholic steatohepatitis (NASH).

**METHODS:** Twenty-eight patients with NASH and 11 with simple fatty liver disease (FL) were enrolled in this study and underwent clinicopathological examination. The measures of angiogenesis, CK-18, and IR employed were CD34-immunopositive vessels, CK-18-immunopositive cells, and homeostasis model assessment of IR (HOMA-IR), respectively. The correlations of these factors with NASH were elucidated.

**RESULTS:** Significant development of hepatic neovascularization was observed only in NASH, whereas almost no neovascularization could be observed in FL and healthy liver. The degree of angiogenesis was almost parallel to liver fibrosis development, and both parameters were positively correlated. Similarly, CK-18

expression and HOMA-R were significantly increased in NASH as compared with FL and healthy liver. Furthermore, CK-18 and HOMA-IR were also positively correlated with the degree of neovascularization.

**CONCLUSION:** These results indicate that the crosstalk between angiogenesis, CK-18, and IR may play an important role in the onset and progression of NASH.

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**Key words:** Angiogenesis; Cytokeratin-18; Fatty liver; Insulin resistance; Non-alcoholic steatohepatitis; Liver fibrosis

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Kitade M, Yoshiji H, Noguchi R, Ikenaka Y, Kaji K, Shirai Y, Yamazaki M, Uemura M, Yamao J, Fujimoto M, Mitoro A, Toyohara M, Sawai M, Yoshida M, Morioka C, Tsujimoto T, Kawaratani H, Fukui H. Crosstalk between angiogenesis, cytokeratin-18, and insulin resistance in the progression of non-alcoholic steatohepatitis. *World J Gastroenterol* 2009; 15(41): 5193-5199 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5193.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5193>

### INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) ranges from simple steatosis to cirrhosis. Fatty liver (FL) has been recognized as a benign and non-progressive condition<sup>[1,2]</sup>. However, non-alcoholic steatohepatitis (NASH) is now widely known as a liver disease which may progress to liver cirrhosis and finally hepatocellular carcinoma (HCC). Fibrosis development is only seen in NASH but not in simple FL. The pathogenesis of NASH is not well understood, but it is unlikely that one of the recognized mechanisms explains all pathogenic processes of NASH. Thereby, NASH may develop as a consequence of a multifactorial process<sup>[2]</sup>.

Angiogenesis i.e. formation of new vessels by sprouting from the pre-existing vasculature, is of central importance for embryonic development and organogenesis. Abnormally pathological angiogenesis is observed in rheumatoid arthritis, psoriasis, diabetic retinopathy, tumor growth, and even in fibrogenesis<sup>[3]</sup>. Although previous studies conducted to determine the molecular process associated with fibrosis and angiogenesis were performed independently, recent studies have revealed that both biological phenomena developed synergistically<sup>[4]</sup>. It was shown that neovascularization significantly increased during the development of liver fibrosis both in human and animal experimental studies<sup>[5,6]</sup>. In the NASH experimental model, we previously demonstrated that angiogenesis plays an important role in the progression of NASH<sup>[7]</sup>. We also reported that neovascularization developed in patients with NASH whereas no marked augmentation was observed in FL or the healthy liver<sup>[8]</sup>.

Recent studies have suggested that increased hepatocyte apoptosis has an important role in progression from simple FL to NASH, and correlates with disease severity and hepatic fibrosis<sup>[9,10]</sup>. During apoptosis, some intracellular proteins are cleaved by caspase. A neoepitope in cytokeratin-18 (CK-18), the major intermediate filament protein in the liver, becomes available at an early caspase cleavage event during apoptosis and is not detectable in the viable or necrotic cells<sup>[11]</sup>. It has been reported that CK-18 fragments were markedly elevated in NAFLD patients as compared with healthy controls, and that the plasma levels of CK-18 correlated with the expression levels in the liver. A significant increase in CK-18 expression could be observed in patients with NASH as compared with the simple FL patients, and the expression level of CK-18 correlated with the presence of fibrosis<sup>[10]</sup>. The same group recently conducted a multicenter validation study which yielded similar results, and CK-18 was identified as an independent predictor of NASH<sup>[12]</sup>.

NASH is well known as a liver disease that frequently complicates insulin resistance (IR) status<sup>[13]</sup>. Current evidence points to IR and subsequent hyperinsulinemia as the key pathogenic factors in NAFLD and progression from simple FL to NASH<sup>[13]</sup>. It has been reported that IR accelerated the progression in a NASH animal model, and that an insulin-sensitizing agent could reverse the underlying pathogenesis involved through improvement in IR<sup>[14]</sup>. We also have demonstrated that IR itself significantly promoted liver fibrosis development in diabetic rats<sup>[15]</sup>. Several studies have shown that insulin-sensitizing agents improved the metabolic and histologic parameters, most notably liver injury and fibrosis, not only in experimental models, but also in patients with NASH<sup>[16,17]</sup>. Collectively, these findings indicate that neovascularization, CK-18 expression, and IR status play pivotal roles in the progression of NASH. We previously demonstrated that angiogenesis and IR play important roles in animal NASH models<sup>[7,15]</sup>. However, no study has been conducted as yet to examine the interaction among these parameters in NASH using human cases.

In the current study, we elucidated the possible

correlation between angiogenesis, CK-18, and IR especially in fibrosis development, which is one of the specific characteristic features of NASH compared with simple FL.

## MATERIALS AND METHODS

### *Patients and methods*

We recruited a total of 39 patients with NAFLD between 2001 and 2007 at Nara Medical University, and three healthy volunteers were enrolled as a control group. Twenty-eight patients with NASH (17 males and 11 females) and 11 patients with simple FL (seven males and four females), diagnosed by histological examination, were enrolled in this study. First, all patients were re-evaluated clinically for evidence of diseases including diabetes mellitus and hypertension. Alcohol-induced hepatitis was excluded according to each patient's self-report and was confirmed by the family. The height and weight were measured, and the body mass index (BMI) was calculated. Hepatitis B virus surface antigen and hepatitis C virus antibody were negative in all patients. The standard liver function tests were performed for all patients. The fasting blood levels of glucose and insulin were assessed, and the homeostasis model assessment parameter of IR (HOMA-IR) was calculated. The serum fibrosis markers, namely, hyaluronic acid, type IV collagen 7S (7S-collagen), and amino-terminal peptide of type-III pro-collagen, were measured by latex agglutination, enzyme immunoassay, and radioimmunoassay, respectively, using routine laboratory methods. The serum levels of leptin and adiponectin were measured by ELISA kit (R&D systems, Tokyo, Japan) according to the manufacturer's instructions as described previously<sup>[18,19]</sup>.

### *Histology and immunohistochemistry*

Liver biopsies obtained for diagnostic purposes were histologically examined. The liver biopsy specimens exceeded 1.5-2 cm in length and 1.4 cm in width, and contained more than five portal areas in each sample that was sufficient to perform the immunohistochemical analysis. In all samples, serial sections were used for analysis. The first section was routinely stained with hematoxylin and eosin for histological examination. Another section was stained with Sirius Red to detect fibrosis development. The fibrosis stages was scored in an F0 to F4 scale, where F0 means absence of fibrosis; F1, portal fibrosis with few septa; F2, few septa; F3, abundant septa with cirrhosis; F4, cirrhosis, as described elsewhere<sup>[20]</sup>. Regarding the 28 patients with NASH, 10 had low-grade fibrosis (F0 and F1), and 18 had high-grade fibrosis (F2 to F4). For determination of neovascularization, we employed immunohistochemical detection of CD34, which is widely used as a marker of neovascularization, using paraffin-embedded sections as described previously<sup>[21]</sup>. We also performed immunohistochemical staining of CK-18 in the liver. Liver tissue samples were fixed in 4% formaldehyde solution and embedded in paraffin. Then, 5  $\mu$ m tissue sections were incubated in 0.1% hydrogen peroxide in 70%

**Table 1** Characteristic features of patients with FL and NASH (mean  $\pm$  SD)

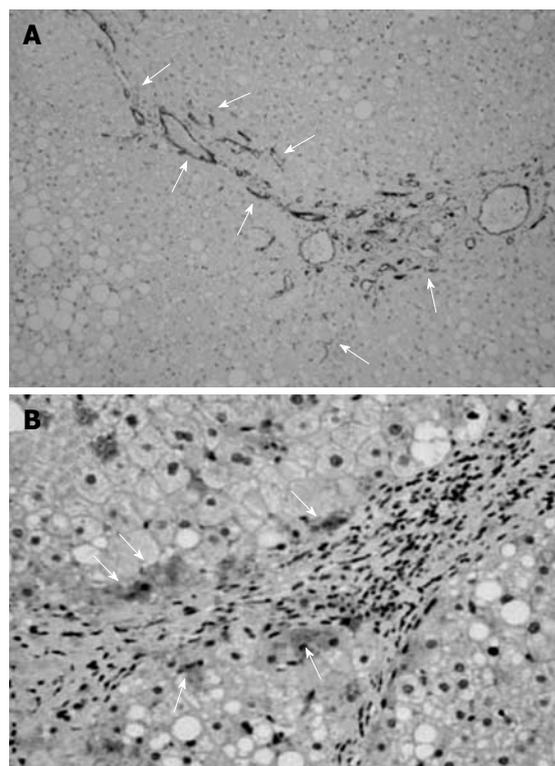
	NASH (n = 28)	FL (n = 11)	P-value
Age (yr)	45.4 $\pm$ 14.7	43.6 $\pm$ 14.1	0.587
Sex (M/F)	17/11	7/4	0.660
BMI (kg/m <sup>2</sup> )	28.5 $\pm$ 6.91	25.8 $\pm$ 1.16	0.042
Total cholesterol (mg/dL)	218.6 $\pm$ 43.3	203.4 $\pm$ 48.3	0.350
Triglycerides (mg/dL)	186.1 $\pm$ 69.3	237.1 $\pm$ 98.2	0.075
FFA (mmol/L)	0.532 $\pm$ 0.207	0.897 $\pm$ 0.804	0.079
Fasting plasma glucose (mg/dL)	113.3 $\pm$ 30.0	98.8 $\pm$ 19.2	0.116
Fasting plasma insulin ( $\mu$ U/mL)	20.8 $\pm$ 11.5	10.0 $\pm$ 3.91	0.018
HOMA-IR	5.79 $\pm$ 4.15	2.26 $\pm$ 0.96	0.038
HbA1c (%)	6.09 $\pm$ 1.29	5.25 $\pm$ 0.53	0.130
Serum albumin (g/dL)	4.57 $\pm$ 0.33	4.54 $\pm$ 0.39	0.774
AST (IU/L)	63.4 $\pm$ 27.0	37.5 $\pm$ 10.0	0.012
ALT (IU/L)	105.5 $\pm$ 65.7	69.8 $\pm$ 31.2	0.035
$\gamma$ -GTP (IU/L)	68.4 $\pm$ 37.1	64.2 $\pm$ 47.8	0.770
ZTT (kU)	7.87 $\pm$ 4.60	5.89 $\pm$ 2.39	0.224
Total bilirubin (mg/dL)	0.80 $\pm$ 0.25	1.00 $\pm$ 0.98	0.305
ICG R15 (%)	10.9 $\pm$ 4.28	9.00 $\pm$ 2.61	0.264
Procollagen-III-peptide (U/mL)	0.83 $\pm$ 0.31	0.96 $\pm$ 0.23	0.454
Collagen IV-7S (ng/mL)	4.46 $\pm$ 1.83	4.40 $\pm$ 1.52	0.762
Hyaluronic acid (ng/mL)	60.4 $\pm$ 57.6	15.0 $\pm$ 9.06	0.036
Platelets (10 <sup>4</sup> / $\mu$ L)	21.2 $\pm$ 8.83	23.2 $\pm$ 4.85	0.470
Leptin (ng/mL)	7.23 $\pm$ 3.98	2.58 $\pm$ 0.43	0.040
Adiponectin (ng/mL)	5.06 $\pm$ 1.90	8.25 $\pm$ 0.95	0.016

FL: Fatty liver; NASH: Non-alcoholic steatohepatitis; M: Male; F: Female.

methanol for 30 min to inhibit the endogenous peroxidase activity. Microwave antigen retrieval was performed at 500 W for 15 min with pH 9 antigen retrieval solution (Nichirei Bioscience Inc., Tokyo, Japan). Then, 10% fetal bovine serum with 0.3% Triton X was used to prevent nonspecific staining. The slides were subsequently incubated overnight at 4°C in humidified chambers with primary rabbit polyclonal anti-CD34 antibody at a dilution of 1:100 and primary rabbit polyclonal anti-CK-18 antibody (DAKO, Kyoto, Japan) at a dilution of 1:100. The sections were rinsed three times in a phosphate-buffered solution and further incubated with a biotinylated secondary antibody for 30 min at room temperature. Antigen-antibody complexes were detected by the avidin-biotin-peroxidase method, using diaminobenzidine as a chromogenic substrate (DAKO, Carpinteria, CA, USA). Finally, the slides were counter-stained with hematoxylin and then examined microscopically. Immunopositive quantitation of CD34 and Sirius Red-positive liver fibrosis areas were evaluated with Adobe Photoshop software and NIH image software as described previously<sup>[22]</sup>. The intensity of CK-18 staining was scored from 0 to 4 as previously described<sup>[8]</sup> with minor modification; 0: no staining; 1: mild (punctured labeling); 2: mild to moderate (dense labeling in a few lesions); 3: moderate; 4: strong (dense and homogeneous labeling in large areas).

### Statistical analysis

All data are expressed as mean  $\pm$  SD. The statistical analysis was performed using the  $\chi^2$  test for independence,



**Figure 1** Representative microphotographs of CD34-positive neovessels and cytokeratin-18 (CK-18)-positive cells in the liver of patients. A: Marked CD34-positive immunopositive vessels could be found in NASH along with liver fibrosis development; B: The immunopositivity of CK-18 was mainly observed in the hepatocytes. The arrows indicate CD34 and CK-18-immunopositivity in A and B, respectively. The original magnification was  $\times$  100.

the two-tailed Student's *t*-test, and simple regression analysis. Correlation between two parameters was tested by the Spearman rank correlation matrix.

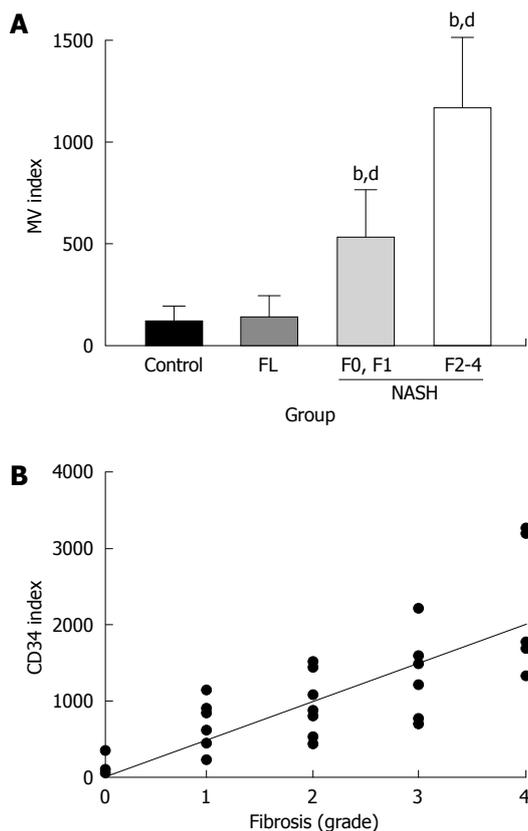
## RESULTS

### Clinical features

The clinical features of both groups are shown in Table 1. Most of the clinical features in the FL and NASH patients were not significantly different except BMI, HOMA-IR, serum aspartate aminotransferase (AST), hyaluronic acid, leptin, and adiponectin. The NASH patients had significantly higher BMI (28.8  $\pm$  4.33 *vs* 25.6  $\pm$  1.20), HOMA-IR (5.79  $\pm$  4.15 *vs* 2.26  $\pm$  0.96), AST (59.4  $\pm$  27.0 *vs* 37.5  $\pm$  10.0), hyaluronic acid (62.0  $\pm$  58.6 *vs* 15.0  $\pm$  9.06), and leptin (7.79  $\pm$  4.47 *vs* 2.58  $\pm$  0.43), than the FL patients. On the other hand, significantly lower serum adiponectin levels were observed in the NASH patients compared with the FL patients (5.31  $\pm$  1.70 *vs* 8.25  $\pm$  0.95).

### Neovascularization

The typical features of CD34-immunopositive neovessels in the liver are shown in Figure 1A. Apparent CD34-positive vessels could be observed neither in the liver of the healthy subjects nor in the FL patients, whereas marked immunopositive vessels could be observed in NASH livers along with the development of liver fibrosis. In the liver of low-grade fibrosis, neovascularization could

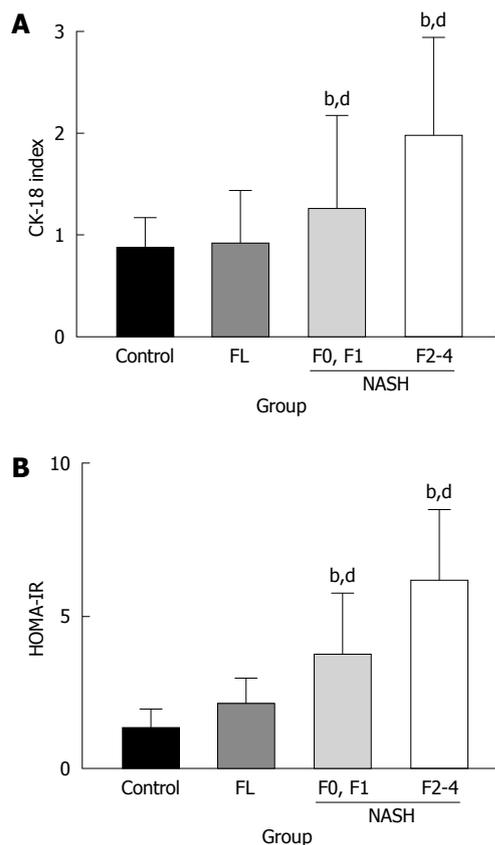


**Figure 2 Semi-quantitative analysis of CD34 and possible correlation with liver fibrosis development.** A: Semi-quantitative analysis of the CD34-positive neovessels in the liver in non-alcoholic fatty liver disease (NAFLD). There was no difference between the control healthy liver and simple FL. In NASH, a marked augmentation of neovascularization was found in the liver of NASH as compared with FL. The magnitude of neovascularization in high-grade (F2 to F4) liver fibrosis was more than in low-grade (F0, F1) fibrosis. The proportional increase in neovascularization was almost parallel to the development of liver fibrosis. <sup>b</sup> $P < 0.01$  vs control group; <sup>d</sup> $P < 0.01$  vs low-grade fibrosis with NASH group. The number of patients in each group was as follows: Control ( $n = 3$ ), FL ( $n = 11$ ), low-grade fibrosis (F0 and F1:  $n = 10$ ), and high-grade fibrosis (F2 to F4:  $n = 18$ ). MV: Microvessel density; B: The relationship between the development of CD-34-positive vessels and fibrosis grade in NAFLD. The degree of angiogenesis correlated with the development of fibrosis. The equation was  $y = 509.82x - 6.7958$ . The correlation coefficient was 0.6288.

be observed around the central vein (zone III). These neovessels progressed to the portal area (zone I) and were also observed along the fibrotic septa in high-grade fibrosis. We next performed a semi-quantitative analysis of the CD34-positive neovessels in conjunction with liver fibrosis development. There was no difference between the normal control liver and the FL (Figure 2A), which conformed with the immunohistochemical features. In NASH, a marked augmentation of neovascularization was found in the liver of NASH cases as compared with the FL. The magnitude of neovascularization in high-grade (F2 to F4) liver fibrosis was more than in low-grade (F0, F1) fibrosis. The increase in neovascularization was almost parallel to the development of liver fibrosis. As shown in Figure 2B, the degree of CD34-positive vessels positively correlated with fibrosis development ( $r^2 = 0.6288$ ).

#### CK-18 and IR

We next examined CK-18 expression in the liver. The

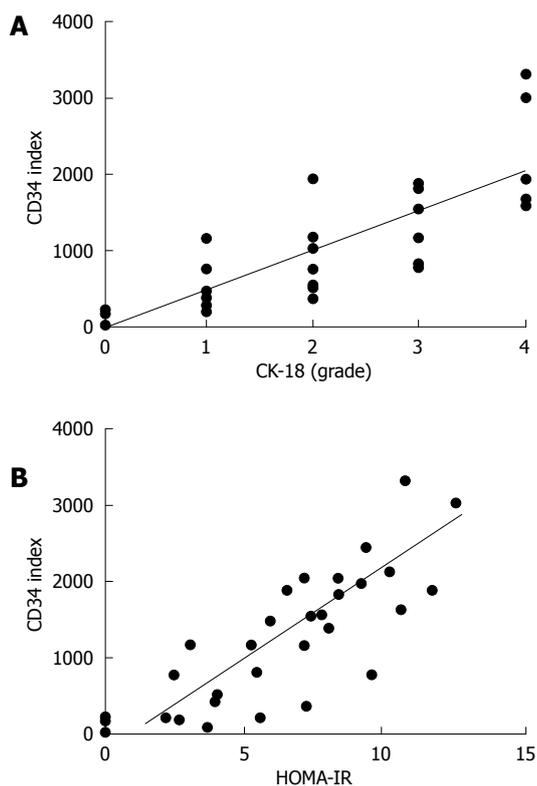


**Figure 3 Semi-quantitative analysis of the CK-18-positive cells in the liver (A) and HOMA-IR (B) in NAFLD.** Similar to the CD34-positive neovascularization, there was no difference between the control healthy liver and FL. In NASH, a marked augmentation of CK-18 (A) and HOMA-IR (B) was found in the liver of NASH as compared with FL. The magnitude of neovascularization in high-grade (F2 to F4) liver fibrosis was more than in low-grade (F0, F1) fibrosis. The number of patients in each group was as follows; C ( $n = 3$ ), FL ( $n = 11$ ), low-grade fibrosis (F0 and F1:  $n = 10$ ), and high-grade fibrosis (F2 to F4:  $n = 18$ ). <sup>b</sup> $P < 0.01$  vs control group; <sup>d</sup> $P < 0.01$  vs low-grade fibrosis with NASH group.

immunopositivity of CK-18 was mainly observed in the hepatocytes (Figure 1B). Similar to CD34, CK-18 expression was significantly increased in the NASH patients as compared with simple FL and healthy liver. Staining of CK-18 was barely visible in the healthy liver and FL, whereas most NASH cases showed positive staining. Among the NASH patients, those with high-grade fibrosis had greater hepatic expression of CK-18 than those with low-grade fibrosis (Figure 3A). We observed that the magnitude of CK-18 expression positively correlated with CD34-positive neovascularization ( $r^2 = 0.6512$ , Figure 4A). We also examined the possible role of IR in progression of NASH, and observed that HOMA-IR was significantly higher in NASH patients than in simple FL and healthy liver. The HOMA-IR also increased with the progression of NASH, and there was a positive correlation between HOMA-IR and hepatic neovascularization (Figure 3B and Figure 4B, respectively).

## DISCUSSION

Recent studies have revealed that angiogenesis plays an important role in many pathological events, including



**Figure 4** The relationship between angiogenesis, CK-18-positive vessels (A), and HOMA-IR (B). The degree of angiogenesis correlated with the CK-18-positive cells (A) and HOMA-IR (B). A: The magnitude of expression of CK-18 correlated positively with CD34-positive neovascularization. The equation was  $y = 500.63x + 4.6606$ . The correlation coefficient was 0.6512; B: Similarly, there was a positive correlation between HOMA-IR and hepatic neovascularization. The equation was  $y = 233.75x - 339.39$ . The correlation coefficient was 0.6184.

liver fibrosis development. Although previous studies conducted to determine the molecular processes associated with fibrosis and angiogenesis were performed independently, recent studies have revealed that both biological phenomena emerged concomitantly<sup>[4]</sup>. Angiogenesis in the liver is characterized by capillarization of the sinusoids<sup>[3]</sup>. It has been shown that capillarization and phenotypic changes of the hepatic sinusoidal endothelial cells (ECs) occur during liver fibrosis development<sup>[23]</sup>. It has been reported that CD34 is not expressed by healthy ECs, but when ECs alter their phenotype, they can express CD34<sup>[24]</sup>. Much attention is focused on a possible association with chronic liver diseases<sup>[25]</sup>. A recent study on chronic hepatitis C (CHC) has shown that the number of CD34-positive new vessels was significantly increased and positively correlated with the fibrosis stage<sup>[26]</sup>. As well as CHC, in the current study we found a significant development of hepatic CD34-positive neovascularization in NASH, whereas almost no development could be observed in FL. The degree of angiogenesis was almost parallel to the development of liver fibrosis in NASH. These results are consistent with our previous experimental finding that angiogenesis increased stepwise during hepatic fibrosis development in several fibrosis models, including the rodent dietary NASH model<sup>[5,7]</sup>. We also observed that HOMA-IR was significantly higher in NASH along with liver

fibrosis development, and that it positively correlated with the development of neovascularization. We previously reported that the IR status, i.e. co-existence of high glucose and insulin, itself significantly promoted liver fibrosis development in rats along with augmentation of neovascularization. Furthermore, high glucose and insulin stimulated *in vitro* neovascularization, and the combination treatment with glucose and insulin significantly promoted the effect as compared with either agent alone<sup>[15]</sup>. Collectively, it is likely that IR-mediated neovascularization is involved in the development and progression of NASH.

Because NAFLD is a common manifestation of metabolic syndromes, various adipocytokines are involved in the progression of NASH<sup>[27,28]</sup>. Among them, adiponectin and leptin are well known to be involved in the pathogenesis of NASH<sup>[27,29,30]</sup>. Adiponectin administration alleviates NAFLD progression in mice, and liver fibrosis is accelerated in adiponectin knockout mice, indicating the protective effect of adiponectin against liver fibrosis development in NASH<sup>[31]</sup>. In the NASH patients, the circulating adiponectin is reportedly decreased<sup>[30]</sup>, and we also observed that the serum level of adiponectin in NASH decreased significantly more than in FL. On the other hand, recent reports have revealed that leptin exerts pro-fibrogenic activity. Hepatic fibrogenesis is impaired in leptin- and leptin receptor-deficient animals<sup>[29,32]</sup>. Leptin also enhances proliferation of activated hepatic stellate cells (HSCs), which play a central role in the development of liver fibrosis<sup>[33]</sup>. In addition to these direct effects on HSC, recent studies have revealed that leptin possesses angiogenic activity<sup>[7,34]</sup>. We previously reported that leptin exerted a potent angiogenic effect, and that leptin-mediated neovascularization played an important role in the development of liver fibrosis in the rat NASH model<sup>[7]</sup>. In the current study, the serum leptin level was significantly higher in NASH than in FL. However, we only measured the serum level of leptin in the current study. The role of leptin in fibrosis development in NASH is still controversial. It has been reported that local leptin plays a more important role than serum leptin in the progression of NASH<sup>[35]</sup>. Further studies are required to elucidate the local leptin and leptin receptor interaction with *in situ* hybridization in the future.

As well as neovascularization, we observed that the hepatocyte apoptosis marker, CK-18, was also significantly increased in NASH as compared with simple FL. This finding was consistent with recent reports suggesting that the CK-18 expression can detect the presence of NASH<sup>[10,12,36]</sup>. Uncontrolled hepatocyte apoptosis proved to be an important event triggering liver fibrogenesis<sup>[9]</sup>. We also observed that the expression of CK-18 was increased along with liver fibrosis development in NASH. Moreover, there was a positive correlation between the CK-18 expression and hepatic neovascularization. These results suggested that there was some crosstalk between CK-18 and angiogenesis in the liver of NASH. As well as being a marker of apoptosis, CK-18 has been found to enhance the migratory and invasive potential of tumor cells<sup>[37]</sup>. Furthermore, it has been reported

that CK-18 expression was significantly higher in HCC than in CHC<sup>[38]</sup>. We previously reported that hepatic neovascularization increased in a stepwise manner during hepatocarcinogenesis, and the increase in angiogenesis was mainly observed in the glutathione-S-transferase placental form (GST-P)-positive pre-neoplastic lesions as compared with the adjacent tissues<sup>[39,40]</sup>. A recent report has shown that CK-18 expression also significantly increased in GST-P-positive lesions<sup>[41]</sup>. These results indicate that, as well as being a marker of apoptosis, CK-18 may have a direct association with hepatic neovascularization.

It is important to elucidate whether the correlation among these factors is a cause or consequence of NASH. However, in this study, we could not identify what factors were the causes or consequences in the progression of NASH. Although the respective factors interact with each other, further sequential studies are required in the future to determine what factors developed prior to other factors during the progression of NASH. Also, the number of patients was not high enough in the current study. We are acquiring a larger number of patients' files and when the sample of patients with NASH and simple FL is adequate, we will perform an advanced analysis of the current parameters.

In conclusion, we have shown that only the liver of NASH cases had marked neovascularization whereas simple FL and healthy livers did not. The hepatic neovascularization was proportional to the increase in grade of liver fibrosis. CK-18 expression and HOMA-IR were also significantly increased in NASH as compared with FL and healthy liver. Furthermore, CK-18 and HOMA-IR also positively correlated with the degree of neovascularization. These results indicate that the crosstalk between angiogenesis, CK-18, and IR may play an important role in the onset and progression of NASH.

## COMMENTS

### Background

It has been reported that angiogenesis, cytokeratin-18 (CK-18), and insulin resistance (IR) play important roles in the development of non-alcoholic steatohepatitis (NASH). The aim of the current study was to elucidate the possible crosstalk between angiogenesis, CK-18, and IR especially in patients with NASH.

### Research frontiers

Significant development of hepatic neovascularization was observed only in NASH, and the degree of angiogenesis was almost parallel to liver fibrosis development. Similarly, CK-18 expression and IR were significantly increased in NASH. Furthermore, CK-18 and IR also positively correlated with the degree of neovascularization.

### Innovations and breakthroughs

Only NASH was associated with marked neovascularization, which was proportional to the increase in grade of liver fibrosis. Moreover, the degree of neovascularization positively correlated with CK-18 and IR in NASH. These results emphasize the new findings that the crosstalk between angiogenesis, CK-18, and IR plays an important role in the onset and progression of NASH.

### Applications

The novel findings may lead to a new alternative therapy for NASH in the near future.

### Peer review

The manuscript is well written and the conclusions are appropriate for the results.

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BRIEF ARTICLE

## Antifibrotic effects of green tea on *in vitro* and *in vivo* models of liver fibrosis

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tea administration can effectively improve liver fibrosis caused by DMN, and may be used as a therapeutic option and preventive measure against hepatic fibrosis.

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**Key words:** Dimethylnitrosamine; Green tea extract; HSC-T6 cell; Liver fibrosis; Rat model; Type 1 collagen

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### Abstract

**AIM:** To examine the protective effect of green tea extract (GT) on hepatic fibrosis *in vitro* and *in vivo* in dimethylnitrosamine (DMN)-induced rats.

**METHODS:** HSC-T6, a rat hepatic stellate cell line, was used as an *in vitro* assay system. Cell proliferation, collagen content, and type 1 collagen expression were examined in activated HSC-T6 cells. Collagen was determined by estimating the hydroxyproline content. In rats with DMN-induced hepatic fibrosis, serum aspartate aminotransferase and alanine aminotransferase concentrations, liver hydroxyproline and lipid peroxides were determined. Pathologic changes were examined by hematoxylin & eosin staining.

**RESULTS:** GT administration prevented the development of hepatic fibrosis in the rat model of DMN-induced liver fibrosis. These results were confirmed both by liver histology and by quantitative measurement of hepatic hydroxyproline content, a marker of liver collagen deposition. Accordingly, inhibition of proliferation, reduced collagen deposition, and type 1 collagen expression were observed in activated HSC-T6 cells following GT treatment. These results imply that GT reduced the proliferation of activated HSC and down regulated the collagen content and expression of collagen type 1, thereby ameliorating hepatic fibrosis.

**CONCLUSION:** This study demonstrates that green

### INTRODUCTION

Hepatic fibrosis is a consequence of severe liver damage and occurs in many forms of chronic liver damage, including virus infection, autoimmune liver diseases and sustained alcohol abuse<sup>[1]</sup>. Hepatic stellate cells (HSC) are recognized as the primary cellular source of matrix components in chronic liver diseases, and therefore play a critical role in the development and maintenance of liver fibrosis<sup>[2]</sup>. The key cellular and molecular events involved in the pathogenesis of liver fibrosis include activation of HSC to a myofibroblast-like phenotype, production of excess matrix proteins, and increased cell proliferation<sup>[2]</sup>. Overproduction of extracellular matrix (ECM) components, particularly collagen, is a characteristic of activated HSC, and activation and proliferation of HSC have been implicated in the pathogenesis of liver fibrosis<sup>[3]</sup>. Therefore, suppression of HSC activation has been proposed as a therapeutic target against hepatic fibrosis<sup>[4]</sup>.

Acetaldehyde, a highly reactive compound produced by alcohol metabolism, stimulates the deposition of ECM proteins. Acetaldehyde also stimulates type 1 collagen synthesis and gene transcription in cultured rat and human HSC<sup>[5]</sup> and in human liver fibroblasts<sup>[6]</sup>.

Several studies have shown that lipid peroxidation stimulates collagen production in fibroblasts and HSC<sup>[7]</sup>, and plays an important role in the development of liver

fibrosis. Lipid peroxidation has been shown to stimulate the expression of collagen gene transcripts<sup>[8]</sup>. It has recently been shown that stellate cells are activated by free radicals as well as by malondialdehyde (MDA), a product of lipid peroxidation<sup>[9]</sup>. In addition, stellate cell activation by type 1 collagen has been shown to be blocked by antioxidants<sup>[9]</sup>, suggesting that lipid peroxidation may play a role in hepatofibrogenesis.

Green tea, which is a widely consumed drink, has received much attention due to its beneficial biological effects. Polyphenols, often collectively referred to as catechins, account for up to 30% of the dry weight and serve as a major effective component of green tea. The effects of green tea have been widely studied and antioxidative, antiallergic, antimutagenic/anticarcinogenic, and antibacterial effects have been documented<sup>[10-12]</sup>. It has been shown that an aqueous extract of polyphenols from green tea (*Camellia sinensis*) reduces liver fibrosis in rats induced by bile duct ligation, and epigallocatechin gallate (EGCG), the major component in green tea, was implicated as the main active ingredient<sup>[13]</sup>. EGCG has been reported to suppress cell proliferation and collagen production in HSC<sup>[14]</sup>. In addition, the hepatoprotective effects of green tea against carbon tetrachloride, cholestasis and alcohol induced liver fibrosis were reported in many studies<sup>[13,15,16]</sup>. However, the hepatoprotective effect of green tea in dimethylnitrosamine (DMN)-induced models has not been studied. The DMN-induced liver fibrosis model can reproduce most of the features observed during human liver fibrosis<sup>[17]</sup>. Furthermore, this model has other advantages such as progressive and remarkable pathological alterations, a high fibrosis reproduction rate, and a low mortality rate in experimental animals<sup>[18]</sup>. This model is also stable even after termination of DMN administration and is a reliable tool for screening antifibrotic agents<sup>[19]</sup>. Therefore, the aim of the present study was to examine the protective effect of green tea extract (GT) on hepatic fibrosis in a rat HSC line and in a rat model of DMN-induced hepatic fibrosis.

## MATERIALS AND METHODS

### Preparation of GT

Green tea, cultivated from Cheju island, Korea, was extracted with 80% methanol and freeze-dried.

### In vitro experiment

**Cell culture:** HSC-T6 cells, an immortalized rat HSC line, were cultured in Dulbecco's minimal essential medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Gibco) and 0.5% antibiotics. Cultures were placed in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C, and the medium was changed twice a week. Acetaldehyde (175 μmol/L) was added to induce collagen type 1 and morphological features of activated stellate cell.

**Cell viability:** HSC-T6 cells were seeded into 96-well plates at a density of  $1.5 \times 10^4$  cells/well until 50% confluence. Cells treated with GT (10, 50, 100 μg/mL)

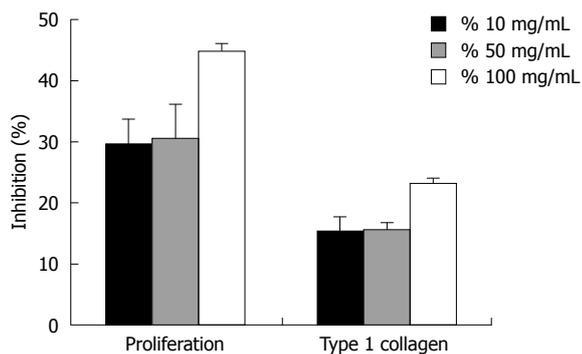
for 48 h were incubated with MTT (1 mg/mL) in a medium for 3 h at 37°C. The supernatant was removed and 100 μL of DMSO was added to each well to dissolve the formazan product. Absorbance at 570 nm was measured using a microplate reader.

**Hydroxyproline content:** Collagen was determined by estimating the hydroxyproline content, an amino acid characteristic of collagen. HSC-T6 cells were lysed after treatment with GT (100 μg/mL) for 24 h. The lysates were hydrolyzed in 6 mol/L HCl for 16 h at 110°C and evaporated to dryness to remove the acid. The residue, dissolved in distilled water, was mixed with 50% isopropanol and chloramine-T solution and left for 10 min at room temperature. Finally, p-dimethylaminobenzaldehyde in 60% perchloric acid was added and heated to 60°C for 25 min. The absorbance was measured at 558 nm.

**Expression of collagen type 1:** The expression of collagen type 1 was observed by ELISA. HSC-T6 cells, seeded on 24-well plates at a density of  $1.5 \times 10^5$  and cultured until 90% confluency, were treated with serum-free DMEM with or without 175 μmol/L acetaldehyde. Ascorbic acid (50 μg/L), and 3-aminopropionitrile fumarate (100 μg/L) were also added to increase the collagen proline hydroxylation and to prevent collagen cross-linking. After 24 h of treatment with GT (100 μg/L), aliquots of medium were transferred into immunowell plates, and glutaraldehyde (0.01%) was added and incubated at room temperature for 1 h. Collagen type 1 antibody (1:4000, Abcam Co., Cambridge, UK) was added and further incubated for 2 h at 37°C. The antigen-coated plates were blocked with casein and incubated with the secondary antibody (1:8000) linked to peroxidase, and subsequently re-incubated with substrate (TMB 10 mg/mL, 3% H<sub>2</sub>O<sub>2</sub>, 50 mmol/L sodium acetate buffer, pH 5.1) for 15 min. The enzymatic reaction was stopped by adding 1 mol/L H<sub>2</sub>SO<sub>4</sub>, and the absorbance at 450 nm was measured with a microplate reader.

### In vivo experiment

**Animals and treatments:** Male albino rats (235-250 g) were purchased from Samtako (Kyunggi-do, Korea) and housed in controlled temperature and relative humidity, and a 12 h light/dark cycle. All experiments were performed according to National guidelines for the use of animals in biomedical research. The rats were randomly assigned to four groups of eight rats each: the normal control group without any treatment (NC), the hepatic fibrosis control group (FC), and hepatic fibrosis with 100 mg/kg GT treated group (FG). Hepatic fibrosis was induced by intraperitoneal injections of 10 mg/kg dimethylnitrosamine (DMN, Sigma, St. Louis, USA) for 3 consecutive days each week over a period of 4 wk. Normal saline was given to NC rats. GT was administered in drinking water which was calculated according to the amount of water consumed the previous day. At the end of the 4 wk experimental period, all rats were killed under ether anesthesia. Blood was obtained from the



**Figure 1** Inhibitory effects of green tea on HSC-T6 cell proliferation and type 1 collagen expression. Green tea suppressed HSC-T6 cell proliferation and type 1 collagen expression in a dose-dependent manner. Data are expressed as mean  $\pm$  SD.

inferior vena cava, and the liver was excised. The liver was immediately frozen for biochemical measurements or fixed in formalin for histochemical examination.

**Hepatotoxicity and lipid peroxidation:** Hepatotoxicity was assessed by quantifying the activities of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using a spectrophotometric diagnostic kit (Youngdong Pharmaceutical Co., Korea). Lipid peroxidation in the liver and serum were determined by measuring the levels of MDA, an end product of lipid metabolism. For the serum sample, 3 mol/L sulfuric acid and 100 g/L phosphotungstic acid were added and incubated at room temperature for 10 min, and then centrifuged. For the liver sample, homogenates of liver in potassium phosphate buffer were prepared. MDA contents in the serum and liver samples were determined using a colorimetric reaction with thiobarbituric acid.

**Hepatic hydroxyproline content:** A portion of liver tissue (200 mg) was homogenized in 10 volumes of 0.5 mol/L potassium phosphate (KP) buffer and hydroxyproline content was measured as described above.

**Histology of liver:** Liver tissues were fixed in 10% neutral buffered formalin, dehydrated with 50%-100% ethanol, and embedded in paraffin. Five micrometer sections were cut and stained with hematoxylin-eosin.

### Statistical analysis

All data were analyzed and expressed as mean  $\pm$  SD. Comparisons were performed by Student's *t*-test to detect differences between the groups. A level of  $P < 0.05$  was considered statistically significant.

## RESULTS

### Effects on cell proliferation and collagen production

The anti-proliferative activity in HSC-T6 cells was determined by cell viability using the MTT assay. As shown in Figure 1, HSC-T6 cell proliferation was dose-dependently inhibited by GT. GT at 10, 50 and 100  $\mu$ g/mL caused

**Table 1** Relative organ weights and serum enzyme levels in rats with DMN-induced hepatic fibrosis ( $n = 10$ ) (mean  $\pm$  SD)

Group	NC	FC	FG
Liver index	3.1 $\pm$ 0.2	2.9 $\pm$ 0.2	2.8 $\pm$ 0.3
Spleen index	0.2 $\pm$ 0.03 <sup>a</sup>	0.7 $\pm$ 0.1 <sup>c</sup>	0.4 $\pm$ 0.1
ALT (IU/mL)	254.2 $\pm$ 26.3 <sup>a</sup>	572.4 $\pm$ 83.9 <sup>c</sup>	312.8 $\pm$ 29.3
AST (IU/mL)	88.2 $\pm$ 12.1 <sup>a</sup>	451.2 $\pm$ 23.1 <sup>c</sup>	224.4 $\pm$ 24.4

NC: Normal control group; FC: Hepatic fibrosis control group; FG: Hepatic fibrosis with green tea extract (100 mg/kg) treated group. Liver index: Liver weight/body weight  $\times$  100; Spleen index: Spleen weight/body weight  $\times$  100; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase. <sup>a</sup> $P < 0.05$  vs FG; <sup>c</sup> $P < 0.05$  vs FG.

dose-dependent inhibition of HSC-T6 cell proliferation by 29.5%  $\pm$  4.2%, 30.6%  $\pm$  5.6%, and 44.8%  $\pm$  1.2 %, respectively ( $P < 0.05$ , Figure 1). The antiproliferative effects were not related to the nonspecific cytotoxic effects of green tea because cells showed normal morphology (data not shown).

To assess the effect of GT on ECM production, hydroxyproline content and type 1 collagen expression, assessed by ELISA, were examined in activated HSC-T6 cells. Serum-starved HSC-T6 cells were cultured with acetaldehyde and GT treatment for 24 h. Treatment with 100  $\mu$ g/mL GT significantly reduced cell hydroxyproline content by 23.0%  $\pm$  2.1% compared to the control group. Furthermore, the expression of type 1 collagen was up-regulated by acetaldehyde stimulation, and GT markedly reduced collagen type 1 expression in a dose-dependent manner. Acetaldehyde at a concentration of 175  $\mu$ mol/L induced collagen type 1 expression by 17.4%  $\pm$  0.1%, and 10, 50 and 100 mg/mL GT reduced collagen type 1 expression by 15.2%  $\pm$  2.2%, 15.5%  $\pm$  1.3%, and 23.0%  $\pm$  1.1%, respectively (Figure 1).

### Effects on organ weights

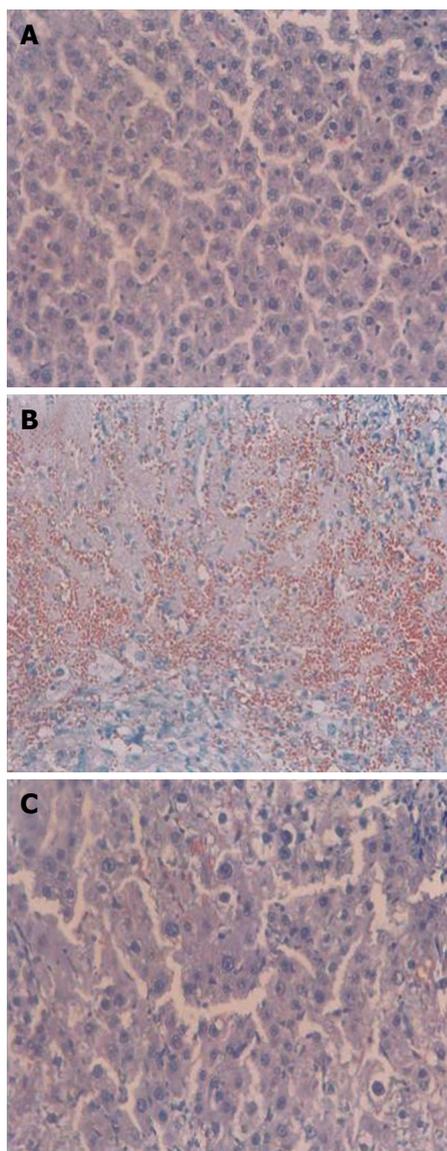
As shown in Table 1, the liver index, which is the percent of liver weight at final body weight, was not significantly different among the experimental groups. In contrast, relative spleen weight was increased 3.5-fold by DMN treatment, and GT administration restored the relative spleen weight ( $P < 0.05$ ).

### Serum biochemical analysis

AST and ALT concentrations in serum were used as biochemical markers to evaluate hepatic injury. ALT is a cytosolic enzyme, primarily present in the liver. An increase in plasma ALT indicates liver damage more specifically than AST. AST, which is a mitochondrial enzyme present in large quantities in the heart, liver, skeletal muscle, and kidney, in part indicates liver injury. Serum activities of ALT and AST were markedly increased with DMN treatment and GT supplementation attenuated the elevation of AST and ALT activities (Table 1).

### Histology and fibrosis marker

Liver fibrosis was evaluated by hematoxylin & eosin staining. The control group showed normal architecture



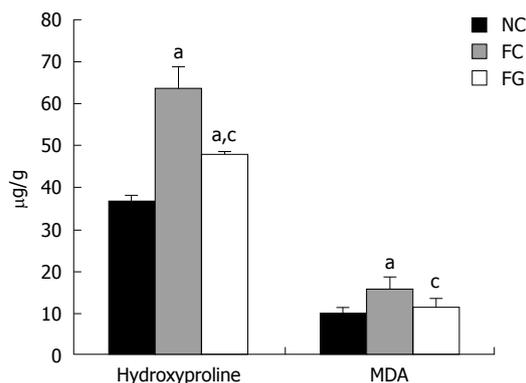
**Figure 2** Effects of green tea extract on liver tissue morphology in DMN-induced fibrosis model. Representative pictures of hematoxylin and eosin-stained sections of liver tissue from normal control rat (A), DMN-treated control (B), DMN-treated + green tea extract (100 mg/kg) group (C). Original magnification,  $\times 200$ .

(Figure 2A), whereas the DMN-treated group exhibited necrosis, congestion, hemorrhage, and destruction of the lobular architecture (Figure 2B). Red blood cells from blood vessels were found in liver tissue due to the collapse of the matrix structure. GT administration exhibited notable recovery effects (Figure 2C).

#### Hydroxyproline and lipid peroxide content in liver

The histological findings were corroborated by biochemical parameters of liver tissue collagen content determined by hydroxyproline, and lipid peroxide determined by MDA.

Hydroxyproline, a product of collagen metabolism, is an amino acid characteristic of collagen. The total collagen present in liver was, therefore, determined by estimating the hydroxyproline content. As shown in Figure 3, hydroxyproline content was significantly in-



**Figure 3** Effects of green tea on hydroxyproline and lipid peroxide (MDA) levels in DMN-treated rat liver. Data are expressed as mean  $\pm$  SD. MDA: Malondialdehyde. <sup>a</sup> $P < 0.05$  vs normal control (NC), <sup>c</sup> $P < 0.05$  vs fibrosis control (FC).

creased following DMN treatment (FC), indicating that the liver fibrosis model was successfully established. GT administration (FG, 100 mg/kg) restored the hydroxyproline content in fibrotic liver. Lipid peroxides, measured in terms of the formation of MDA, were significantly increased in DMN-induced rat liver. GT administration significantly reduced the lipid peroxide level.

## DISCUSSION

Hepatic fibrosis is characterized by an abnormal accumulation of ECM proteins, particularly collagen<sup>[3,4]</sup>. When hepatic fibrosis occurs, collagen proliferation, mainly collagen type 1 and 3, accounts for 50% of the total protein in fibrotic liver<sup>[20]</sup>, and collagens are the main components of ECM. Therefore, collagen type 1 is an important parameter reflecting the metabolism of collagen in liver. The main collagen producing cells in the liver are HSC, which proliferate and undergo a process of activation during the development of fibrosis resulting in increased capacity for collagen synthesis<sup>[21]</sup>. Changes in hydroxyproline content in the liver are considered an index for collagen metabolism and provide valuable information on the biochemical and pathological states of liver fibrosis. The present study demonstrated that consumption of GT prevented the development of hepatic fibrosis in a rat model of DMN-induced liver fibrosis. The results were confirmed both by liver histology and by quantitative measurement of hepatic hydroxyproline content, a marker of collagen deposition in liver.

Accordingly, inhibition of proliferation, reduced collagen content, and type 1 collagen expression were observed in activated HSC-T6 cells following GT treatment. Activated HSC are the main source of ECM when liver fibrosis occurs<sup>[22]</sup>. Therefore, these results imply that GT inhibit the proliferation of activated HSC and down regulate the collagen content and expression of collagen type 1, thereby inhibiting hepatic fibrosis. The results of the present study are consistent with previous observations showing that EGCG, the major component in green

tea, suppresses collagen production<sup>[23]</sup>, and proliferation<sup>[24]</sup> in HSC.

Oxidative stress resulting from the increased production of reactive oxygen species and lipid peroxides is suggested to be associated with the proliferation and activation of stellate cells either directly or through paracrine stimulation of neighboring cells including injured hepatocytes<sup>[25]</sup>. Furthermore, oxidative stress has been shown to modulate collagen gene expression<sup>[7]</sup>. Therefore, a number of studies have focused on the pathogenetic significance of oxidative stress in liver injury, as well as on the therapeutic intervention of this process with antioxidant and metabolic scavengers. GT administration resulted in a reduction of lipid peroxide in HSC-T6 cells and DMN-treated fibrotic liver. Chen *et al*<sup>[26]</sup> have also shown that a single-dose of EGCG improved hepatic injury in rats induced by CCl<sub>4</sub> administration through the inhibition of lipid peroxidation.

In conclusion, this study demonstrates that green tea administration can effectively improve liver fibrosis caused by DMN as shown by reduced levels of collagen, lipid peroxidation, HSC proliferation, and type 1 collagen expression in the liver. Therefore, green tea may protect liver cells and reduce the deposition of collagen fibers in the liver. Green tea provides a safe and effective strategy for improving hepatic fibrosis.

## COMMENTS

### Background

Hepatic stellate cells (HSC) are recognized as the primary cellular source of matrix components in chronic liver diseases, and therefore play a critical role in the development and maintenance of liver fibrosis. Overproduction of extracellular matrix components, particularly collagen, is a characteristic of activated HSC, and activation and proliferation of HSC have been implicated in the pathogenesis of liver fibrosis.

### Research frontiers

Hepatoprotective effects of green tea against carbon tetrachloride, cholestasis and alcohol induced liver fibrosis were reported in many studies. However, the hepatoprotective effect of green tea in dimethylnitrosamine (DMN)-induced models has not been studied.

### Innovations and breakthroughs

The present study demonstrates that consumption of green tea prevents the development of hepatic fibrosis in a rat model of DMN-induced liver fibrosis. These results were confirmed both by liver histology and by quantitative measurement of hepatic hydroxyproline content, a marker of collagen deposition in the liver. Accordingly, inhibition of proliferation, reduced collagen content, and type 1 collagen expression were observed in activated HSC cells following green tea treatment.

### Applications

This study demonstrates that green tea may protect liver cells and reduces the deposition of collagen fibers in the liver. Green tea provides a safe and effective strategy for improving hepatic fibrosis.

### Terminology

HSC are the main collagen producing cells in the liver, which proliferate and undergo a process of activation during the development of fibrosis resulting in an increased capacity for collagen synthesis.

### Peer review

This study examined the protective effect of green tea extract on hepatic fibrosis in a HSC line and in a rat model of DMN-induced liver fibrosis. Green tea administration prevents the development of hepatic fibrosis in the rat model of liver fibrosis. Furthermore, inhibition of proliferation, reduced collagen deposition, and type 1 collagen expression were observed in activated HSC cells following green tea treatment. The results imply that green tea may protect

liver cells and reduce the deposition of collagen fibers in the liver. Green tea provides a safe and effective strategy for improving hepatic fibrosis.

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BRIEF ARTICLE

## Factors relating to the short term effectiveness of percutaneous biliary drainage for hilar cholangiocarcinoma

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cutaneous biliary drainage was related to patient's prothrombin time or the extent of tumor involvement. It, however, had no impact on survival.

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**Key words:** Cholangiocarcinoma; Percutaneous biliary drainage; Treatment effectiveness

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### Abstract

**AIM:** To identify factors that were related to the short term effectiveness of percutaneous transhepatic biliary drainage in cholangiocarcinoma patients and to evaluate the impact of palliative drainage on their survival.

**METHODS:** Seventy-four patients with hilar cholangiocarcinoma who underwent percutaneous biliary drainage were enrolled in the study. The demographic and laboratory data as well as the imaging characteristics were retrospectively analyzed to correlate with the bile output and reduction rate of serum bilirubin 1 wk after drainage.

**RESULTS:** Patients with more bile duct visualized on percutaneous transhepatic cholangiography or absence of multiple liver metastases on imaging studies had more bile output after biliary drainage [odds ratio (OR): 8.471,  $P = 0.010$  and OR: 1.959,  $P = 0.022$ , respectively]. Patients with prolonged prothrombin time had a slow decrease in serum bilirubin (OR: 0.437,  $P = 0.005$ ). The median survival time was not significantly different in patients with low or high bile output (75 d vs 125 d,  $P = 0.573$ ) or in patients with slow or rapid reduction of serum bilirubin (88 d vs 94 d,  $P = 0.576$ ).

**CONCLUSION:** The short term effectiveness of per-

### INTRODUCTION

Cholangiocarcinoma is the second most common primary liver cancer, after hepatocellular carcinoma and the incidence is increasing<sup>[1,2]</sup>. Hilar cholangiocarcinoma with the tumor involving the biliary confluence or the right or left intrahepatic ducts is most common and accounts for 40%-60% of all cholangiocarcinomas<sup>[3]</sup>. About 80% of patients with hilar cholangiocarcinoma are unsuitable for curative surgical resection due to severe comorbidity for major surgery, metastases or advanced loco-regional disease<sup>[4]</sup>. Percutaneous or endoscopic biliary drainage is usually performed as a palliative treatment to relieve these patients from jaundice, pain, and cholangitis<sup>[5]</sup>.

A fluent bile output after percutaneous transhepatic biliary drainage (PTBD) reduces the biliary pressure and therefore, alleviates the cholangitis and pain of patients. The reduction of serum bilirubin is usually the hallmark of successful biliary drainage. Nonetheless, despite the drainage catheter being correctly positioned in the bile duct, there are still some patients who have scanty bile output and persistent elevation of serum bilirubin<sup>[6]</sup>. We retrospectively analyzed the clinical and imaging characteristics of these patients in an attempt to identify the factors related to bile output and reduction of serum bilirubin after PTBD. In addition, we also compared

the survival of patients with different bile output and reduction rates of bilirubin after PTBD to investigate if the short term effectiveness of biliary drainage had any impact on the long term survival.

## MATERIALS AND METHODS

### Patients

From January 1998 to June 2007, 74 consecutive patients with hilar cholangiocarcinoma who underwent PTBD in our hospital, a tertiary transferring center, were enrolled. The diagnosis of cholangiocarcinoma was confirmed either by pathologic diagnosis ( $n = 39$ ) or by imaging studies plus clinical follow-up which illustrated further tumor progression ( $n = 35$ ). These patients included 39 males and 35 females with a mean age of  $66.1 \pm 12.8$  and  $66.5 \pm 12.5$  years, respectively. All of the patients, irrespective of future treatment modality, underwent PTBD due to the presence of jaundice and a dilated biliary system and were further observed for at least 1 wk to evaluate the effectiveness of PTBD. Of them, 54 patients were unsuitable for surgical intervention on the basis of their comorbidity and/or tumor extent and were enrolled for survival analysis.

### Clinical characteristics

The medical records of each patient were retrospectively reviewed. Data collected from all patients on the day of, or 1 d before, biliary drainage included the initial serum levels of albumin, bilirubin, alanine aminotransferase (ALT), alkaline phosphatase and prothrombin time. The average daily bile output and the serum total bilirubin level 1 wk after drainage were recorded for the evaluation of drainage effectiveness. The overall survival time of patients who did not undergo surgical resection was also checked.

### Imaging characteristics analysis

Percutaneous transhepatic cholangiography (PTC), contrast enhanced computed tomography (CT) and/or magnetic resonance (MR) imaging were reviewed for imaging characteristics analysis. The liver tumors were classified as type I, II, IIIa, IIIb, and IV according to the system of Bismuth *et al.*<sup>71</sup>. The maximal diameter of the tumors was estimated on CT or MR imaging either by direct measurement of the tumor size if its margin could be clearly defined or indirect measurement of the distance between dilated ducts if the tumor was difficult to visualize. The maximal diameter of the intrahepatic duct punctured for biliary drainage was measured on CT, MR, or PTC imaging. The number of visualized intrahepatic bile ducts (first branches of right and left intrahepatic ducts) was counted later on a follow-up PTC as it may depend on the injection pressure of contrast and the radiologist usually injected less contrast media to avoid risk of cholangitis when performing PTBD. A follow-up PTC taken several days after the biliary drainage, although it may still have some limitation, should carry a lower risk of cholangitis and allow more contrast injection. In addition, CT or MR

imaging was checked for multiple liver metastases, locoregional lymphadenopathy and peritoneal involvement.

### Percutaneous transhepatic biliary drainage procedure

Before PTBD, the bleeding profile was checked and treated if abnormal, and antibiotic therapy was commenced. If CT or MR images showed dilated ducts were confined to a single lobe, PTBD was performed *via* that lobe. If the ducts of both lobes were dilated, our radiologists preferred to approach from the left side unless it was atrophied due to tumor invasion of the ipsilateral portal vein. The duct selected for drainage depended on the decision of the radiologist, who selected the most feasible duct to approach. PTBD was performed as a standard procedure. In brief, the biliary system was punctured and a guide wire (0.035 inch) was introduced into the bile duct. Through the guide wire, the puncture tract was dilated using a bougie, followed by the insertion of an 8-French pigtail catheter.

### The effectiveness of biliary drainage

The average daily bile output during the first week after PTBD was calculated. The reduction rate of serum bilirubin was calculated by dividing the reduction of total bilirubin level after 1 wk of drainage by the original level. For differentiating the effectiveness of biliary drainage, an average bile output of more or less than 300 mL/d and a reduction rate of serum bilirubin of higher or lower than 20% (i.e. the integer of median value of patient distribution) were chosen to further divide patients into two groups with similar sample size.

### Statistical analysis

Except for patient survival which was presented as median survival days, all the other data were expressed as mean  $\pm$  SD. The differences in demographic, biochemical, and imaging characteristics between patient groups were compared by Independent-Samples *t* test or  $\chi^2$  test as appropriate. Variables that achieved statistical significance ( $P < 0.05$ ) or were close to significance ( $P < 0.1$ ) in the univariate analysis were subsequently included in a multivariate analysis using a stepwise forward logistic regression. The survival of patients with different effectiveness of biliary drainage was compared by using the log-rank test. The statistical calculations were computed using the SPSS 12.0 program for Windows (SPSS Inc., Chicago, IL, US).

## RESULTS

### Events associated with the biliary drainage

There was no procedure associated mortality or significant morbidity, except two cases of transient hemobilia and two cases of cholangitis. Most of our patients who did not undergo surgical intervention were maintained on PTBD and only 12 (16.2%) of them were switched to external-internal biliary drainage. The drainage was further revised if the initial PTBD drainage had unsatisfactory function during the follow up. Of the 26 patients

**Table 1** The clinical characteristics of patients with high and low bile output (mean  $\pm$  SD)

	Bile output		P-value <sup>1</sup>
	> 300 mL (n = 33)	< 300 mL (n = 41)	
Age (yr)	64.5 $\pm$ 10.3	67.7 $\pm$ 14.2	0.25
Sex (male:female)	20:13	19:22	0.35
Biochemical data			
Albumin (g/dL)	3.4 $\pm$ 0.6	3.0 $\pm$ 0.5	0.01
Total bilirubin (mg/dL)	12.2 $\pm$ 7.4	12.0 $\pm$ 8.5	0.92
ALT (U/L)	132.5 $\pm$ 184.5	142.6 $\pm$ 115.2	0.77
Prolonged prothrombin time (s)	1.0 $\pm$ 1.1	1.2 $\pm$ 1.3	0.42
Bilirubin reduction rate (%)	22.5 $\pm$ 41.2	13.6 $\pm$ 29.7	0.31
Alkaline phosphatase (U/L)	463.9 $\pm$ 301.8	552.9 $\pm$ 373.8	0.297
Imaging characteristics			
Visualized bile ducts on PTC	2.7 $\pm$ 1.2	1.7 $\pm$ 1.0	0.001 <sup>2</sup>
Tumor size (cm)	5.3 $\pm$ 2.9	4.6 $\pm$ 2.5	0.31
Diameter of bile duct (mm)	10.5 $\pm$ 6.1	11.1 $\pm$ 5.9	0.65
Approach side (right:left)	9:24	10:31	0.79
Bismuth type I / II / IIIa / IIIb/IV <sup>3</sup>	8/6/5/3/7	2/6/11/11/5	0.03
Lymphadenopathy	10	9	0.59
Liver metastasis	4	14	0.03 <sup>2</sup>
Peritoneal involvement	7	8	1.00

<sup>1</sup>By Independent-Samples *T* test and  $\chi^2$  test as appropriate; <sup>2</sup>Only the absence of multiple liver metastases and more intrahepatic bile ducts visualized on PTC were statistically significant in multivariate analysis; <sup>3</sup>Eleven patients with ambiguous imaging that cannot be confidently classified were not included for analysis. PTC: Percutaneous transhepatic cholangiography.

who underwent palliative treatment only and survived longer than 90 d, 20 patients (77%) received at least one drainage revision.

### Bile output after drainage

There were 33 patients with and 41 patients without an average bile output of more than 300 mL/d. As shown in Table 1, among the biochemical data, only a higher serum albumin concentration was associated with higher bile output in patients undergoing PTBD. Higher bile output was, however, not associated with a more rapid reduction of serum bilirubin as it was not significantly different between both groups. Patients who had fewer intrahepatic bile ducts shown on PTC, hence a more advanced Bismuth classification, or multiple liver metastases had lower bile output after biliary drainage. The size of tumor, the diameter of bile duct, the approach side of PTBD, and the presence of lymphadenopathy or peritoneum involvement were all unrelated to the amount of bile output.

In multivariate analysis, the absence of liver metastases and a greater number of intrahepatic bile ducts visualized on PTC were still significantly associated with a higher bile output. The odds ratios (ORs) were 8.471, (95% CI: 1.675-42.835,  $P = 0.010$ ) for negative metastasis and 1.959, (95% CI: 1.103-3.481,  $P = 0.022$ ) for more intrahepatic bile ducts on PTC.

### Reduction rate of serum bilirubin after drainage

Of the 70 patients available for analysis of bilirubin

**Table 2** Clinical characteristics between the patients who had rapid and slow bilirubin reduction rates after drainage (mean  $\pm$  SD)

	Reduction		P-value <sup>1</sup>
	> 20% (n = 37)	< 20% (n = 33)	
Age (yr)	67.6 $\pm$ 12.5	65.4 $\pm$ 12.3	0.515
Sex (male:female)	19:18	18:15	1.000
Biochemical data			
Albumin (g/dL)	3.4 $\pm$ 0.5	3.0 $\pm$ 0.6	0.016
Total bilirubin (mg/dL)	10.6 $\pm$ 5.9	15.0 $\pm$ 5.1	0.022
ALT (U/L)	172.3 $\pm$ 190.2	102.1 $\pm$ 70.0	0.048
Prolonged prothrombin time (s)	0.7 $\pm$ 0.7	1.7 $\pm$ 1.4	0.001 <sup>2</sup>
Alkaline phosphatase (U/L)	499.5 $\pm$ 268.6	543.8 $\pm$ 428.8	0.630
Imaging characteristics			
Visualized bile ducts on PTC	2.2 $\pm$ 1.2	2.1 $\pm$ 1.2	0.815
Tumor size (cm)	4.9 $\pm$ 2.6	4.9 $\pm$ 3.0	0.968
Diameter of bile duct (mm)	11.0 $\pm$ 5.9	10.4 $\pm$ 5.9	0.670
Approach side (right:left)	10:27	8:25	1.000
Bismuth type I / II / IIIa / IIIb/IV <sup>3</sup>	5/7/10/5/6	5/5/6/9/5	0.657
Lymphadenopathy	11	7	0.583
Liver metastasis	12	5	0.159
Peritoneal involvement	10	5	0.379

<sup>1</sup>Abbreviations as in Table 1; <sup>2</sup>In multivariate analysis, only the prothrombin time was significantly associated with bilirubin reduction; <sup>3</sup>Abbreviations as in Table 1.

reduction (four patients did not have a serum bilirubin check 1 wk after drainage), 37 patients had a bilirubin reduction of more than 20% after 1 wk of drainage and 33 patients did not. The reduction rate of bilirubin was significantly more rapid in patients who had a less prolonged prothrombin time or a better serum biochemical profile including higher serum albumin, lower serum bilirubin or ALT (Table 2). None of the imaging characteristics such as tumor size, Bismuth classification, liver metastasis and lymphadenopathy had an impact on the rate of serum bilirubin reduction after biliary drainage.

In multivariate analysis, only prolonged prothrombin time was significantly associated with a slower reduction rate of serum bilirubin. The OR was 0.437 (95% CI: 0.245-0.780,  $P = 0.005$ ), compared to those with normal prothrombin time.

### Short term effectiveness of biliary drainage and patients' survival

Most of the patients who were unsuitable for surgical resection died of cholangitis and/or liver failure. Only one patient was still alive at the time of analysis. The median survival time of these patients was 94 d. Although 12 patients received additional drainage due to scanty biliary output or persistent hyperbilirubinemia, the median survival of patients with bilateral biliary drainage was similar to that of patients who received only unilateral drainage (66 d vs 94 d,  $P = 0.358$ ). In addition to biliary drainage, 10 of the 54 patients who had unresectable tumors also underwent external beam radiation therapy. The median survival was, however, not significantly longer

in these patients (103 d) than in those who received drainage only (88 d,  $P = 0.493$ ). These patients were therefore, combined for survival analysis.

The serum bilirubin reduction rate, whether it was more rapid or slower than 20% did not affect the median survival time of patients (94 d *vs* 88 d,  $P = 0.576$ ). The median survival time was also similar between patients with a bile output less than 300 mL (75 d) and those with a bile output more than 300 mL (125 d,  $P = 0.573$ ).

## DISCUSSION

As shown in our previous and current studies, bile output was not correlated with the reduction rate of bilirubin<sup>[8]</sup>. Both liver cells and ductular cells contribute to the formation of bile<sup>[9]</sup>. Biliary obstruction results in proliferation of bile ducts and ductules. The increased amount of bile after relief of obstruction can be caused by excretion of water and electrolytes from the proliferated biliary epithelial cells (i.e. the secretin choleresis)<sup>[9]</sup>. Nevertheless, the reduction of serum bilirubin is dependent on bilirubin excretion by liver cells which can be impaired as a result of cholestasis<sup>[10]</sup>. Prolonged biliary obstruction in these patients results in bile duct proliferation and hepatocyte damage, which explains the discrepancy between bile output and bilirubin reduction rate. A high bile output is therefore, not associated with a rapid reduction in serum bilirubin if most of the bile comes from the bile ducts rather than liver cells.

Cholangiocarcinoma with more advanced Bismuth classification implies a complicated biliary obstruction and is frequently found to have fewer intrahepatic bile ducts depicted on PTC<sup>[7]</sup>. Liver metastases also cause biliary obstruction by compressing the bile ducts externally. The presence of multi-site biliary obstruction, either caused by intra-luminal obstruction or external compression of the tumor, leads to fewer sources of bile flowing into the drained bile duct and was therefore, associated with less bile output after PTBD in our observations.

Accumulation of bile salts within the liver can cause necrosis and apoptosis of liver cells<sup>[10-13]</sup>. The liver may need more time to recover in cases with long lasting biliary obstruction. Prolonged prothrombin time in cholangiocarcinoma patients can be due to either vitamin K deficiency or impaired liver synthesis of coagulation factors, both sequelae of prolonged cholestasis. Weston reported that patients with prolonged prothrombin time took longer for bilirubin reduction after endoscopic biliary stenting<sup>[14]</sup>. Similarly, patients with a prolonged prothrombin time in our study also had a slow reduction of serum bilirubin.

Surgical resection is the standard treatment for intrahepatic cholangiocarcinoma<sup>[15]</sup>. Patients unsuitable for surgical resection live a significantly shorter time than those undergo curative tumor resection. Previous reports indicate that the method of drainage has no impact on patient survival<sup>[4]</sup>. Our data further showed that patient's survival was not related to the bile output

or the rate of bilirubin reduction after biliary drainage. This may be because even though the drainage provides good short term success in palliating symptoms, it is however associated with significant morbidity in the long term follow up including catheter clogging, catheter dislodgement, cholangitis, and liver failure<sup>[16]</sup>. As seen in our study, most of the patients who survived more than 90 d received multiple drainage revisions and as a consequence of tumor progression, most of them still died of cholangitis and liver failure. Therefore, the short term effectiveness of PTBD adds little benefit to survival unless other effective methods such as photodynamic therapy are available to retard the tumor progress and keep the biliary systems patent<sup>[17,18]</sup>.

In conclusion, our study identified factors that were related to the short term effectiveness of PTBD in patients with hilar cholangiocarcinoma. After biliary drainage, patients may have less bile output in the presence of multiple sites of biliary obstruction, and slower reduction of serum bilirubin if the prothrombin time is prolonged. A higher bile output was not associated with a more rapid bilirubin reduction. Although we only observed the drainage effect at 1 wk which may not reflect the ultimate result of drainage, we did find that a short term relief of biliary obstruction by PTBD was not associated with a better survival in patients with unresectable cancer.

## COMMENTS

### Background

Biliary drainage is performed as a palliative treatment of hilar cholangiocarcinoma. The reduction of serum bilirubin is usually the hallmark of successful biliary drainage. However, some patients may have persistent jaundice or scanty bile output after biliary drainage.

### Research frontiers

This paper analyzed factors relating to bile output and reduction of serum bilirubin after percutaneous biliary drainage. Furthermore, the impact of short term effectiveness of biliary drainage on long term survival was investigated.

### Innovations and breakthroughs

After percutaneous biliary drainage, high bile output was not associated with a rapid reduction of serum bilirubin. The bilirubin reduction was related to patient's prothrombin time and the bile output, to the extent of tumor involvement. Neither the amount of bile output nor the rate of bilirubin reduction had an impact on survival.

### Applications

The effectiveness of percutaneous biliary drainage can be properly estimated before the procedure. Patient should be observed for daily bile output as well as the reduction of serum bilirubin. An initially well functioning biliary drainage does not link to a longer survival of patients and further efforts to maintain biliary patency are required.

### Peer review

Chen *et al* found that the short term effectiveness of percutaneous biliary drainage was related to patient's prothrombin time or the extent of tumor involvement, but it had no impact on survival. The paper is informative.

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## Effect of electro-acupuncture on substance P, its receptor and corticotropin-releasing hormone in rats with irritable bowel syndrome

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### Abstract

**AIM:** To investigate the effect and mechanism of electro-acupuncture (EA) at ST25 and ST37 on irritable bowel syndrome (IBS) of rats.

**METHODS:** A total of 21 male Sprague-Dawley rats were randomly divided into normal group, model group and EA group. A rat model of IBS was established by constraining the limbs and distending the colorectum of rats. Rats in EA group received bilateral EA at ST25 and ST37 with a sparse and intense waveform at a frequency of 2/50 Hz for 15 min, once a day for 7 d as a course. Rats in normal and model groups were stimulated by distending colorectum (CR). An abdominal withdrawal reflex (AWR) scoring system was used to evaluate improvements in visceral hypersensitivity. Toluidine blue-improved method, immunohistochemistry and radioimmunoassay were used to observe mucosal mast cells (MC), changes of substance P (SP) and substance P receptor (SPR) in colon and change of corticotropin-releasing hormone (CRH) in hypothalamus.

**RESULTS:** The threshold of visceral sense was significantly lower in model group than in normal group,

and significantly higher in EA group than in model group. The number of mucosal MC was greater in model group than in normal group and significantly smaller in EA group than in model group. The CRH level in hypothalamus of rats was significantly higher in model group than in normal group, which was remarkably decreased after electro-acupuncture treatment. The SP and SPR expression in colon of rats in model group was decreased after electro-acupuncture treatment.

**CONCLUSION:** EA at ST25 and ST37 can decrease the number of mucosal MC and down-regulate the expression of CRH in hypothalamus, and the expression of SP and SPR in colon of rats with IBS.

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**Key words:** Electro-acupuncture; Corticotropin-releasing hormone; Irritable bowel syndrome; Substance P; Substance P receptor

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### INTRODUCTION

Irritable bowel syndrome (IBS) is a common bowel disorder characterized by recurrent abdominal pain or discomfort associated with altered bowel habits in the absence of structural pathology<sup>[1]</sup>. Since IBS is diagnosed based on its symptoms and its pathophysiology is unclear, its treatment outcome remains unsatisfactory<sup>[2,3]</sup>. Our previous study showed that electro-acupuncture (EA) is effective against IBS<sup>[4]</sup>. However, its mechanism of action needs to be further studied.

IBS patients often describe a correlation between

stressful life events and the onset or exacerbation of their gastrointestinal symptoms, and seem more susceptible to stressful events in daily life<sup>[5]</sup>. The central nervous system response to stressful events modulates the autonomic nervous system outflow and activates the hypothalamic-pituitary-adrenal axis<sup>[6]</sup>. Dysfunction of these systems has been proposed to be an etiological factor for IBS<sup>[7,8]</sup>. It has been reported that there is a difference in hormone level involving stress response between IBS patients and healthy subjects<sup>[8]</sup>. Central release of corticotropin-releasing hormone (CRH) plays an important role in the stress response<sup>[9]</sup>, inducing a higher adrenocorticotropic hormone (ACTH) level and a more profound enhancement of colonic motility in IBS patients than in healthy controls<sup>[10]</sup>. It has been shown that CRH increases rectal sensitivity<sup>[11]</sup>. Thus, alterations in neuroendocrine response to stress may be of importance in the pathophysiology of IBS<sup>[12]</sup>.

Visceral hypersensitivity is highly prevalent in IBS patients, and activation of intestinal mast cells (MC) may play a role in visceral hypersensitivity because they are in close proximity to gastrointestinal mucosal sensory nerve terminals containing neuropeptides, and a bidirectional pathway connecting the central nervous system, gut and MC have been demonstrated. MC at the ileocecal junction may be a mediator of the gut and nervous system in IBS<sup>[13]</sup> and substance P (SP) is a gastrointestinal peptide hormone. Both of them reside in the gastrointestinal tract and central nervous system. SP is also an interactive signaling molecule between the nervous and immune systems<sup>[14]</sup> and can modulate the function of intestinal mucosal MC by regulating neurosecretion and paracrine secretion.

This study was to explore the effect of EA at ST25 and ST37 on IBS by observing the MC count, the CRH level in hypothalamus, and the expression of SP and SPR in colon of rats.

## MATERIALS AND METHODS

### Animals

Twenty-one male Sprague-Dawley rats (SPF class), weighing 185-215 g, were supplied by Experimental Animal Center of Shanghai University of TCM, and randomly divided into normal group, model group and EA group according to their weights, 7 in each group. All rats were housed at a constant temperature and a humidity environment with free access to food and water. All studies were performed in accordance with the proposals of the Committee for Research and Ethical Issues of the International Association and approved by the Committee on the Use of Human and Animal Subjects in Teaching and Research, Shanghai University of TCM.

### Establishment of rat model of IBS

An experimental rat model of IBS was established as previously described<sup>[15,16]</sup>. On the second day after the rats were fasted, experiment was begun. Rats in the

Table 1 AWR<sup>[14]</sup> scoring criteria

Score 0	No behavioral response to CRD
Score 1	Immobile during distension of CR and occasional clicking the head at onset of the stimulus
Score 2	A mild contraction of abdominal muscles, but no lifting of abdomen off the platform
Score 3	A strong contraction of abdominal muscles and lifting of abdomen off the platform, no lifting of pelvic structure off the platform
Score 4	Arching body and lifting of pelvic structure and scrotum

AWR: Abdominal withdrawal reflex; CRD: Colorectal distension; CR: Colorectum.

normal group were given grabbing around the anus, while rats in the other two groups were stimulated by distending colorectum (CR). Limbs of the rats were fixed with medical adhesive tapes to limit their movements. The fixed rats could crawl without using their rear limbs. CR was distended before the limbs of rats were constrained and after the medical adhesive tapes were removed. The finger of a disposable rubber glove was tightly fixed onto the end of a polyethylene tube with 4 holes (0.5 cm apart) using medical silk thread as a 4 cm-long balloon. The other end of the tube was connected to a 10 cm-long rubber tube with a tri-channel valve connected to a syringe and sphygmomanometer. Vaseline was smeared on surface of the balloon which was slowly inserted into 5 cm of the rat anus along the physical curve of CR. The fixed time was 2 h each day, and CR was distended for 3 min, once every other day for 8 d. The whole modeling time was 15 d.

### Treatment

Rats in the EA group were treated with EA at bilateral ST 25 and ST 37, once a day for 7 d as a course. Needles were pricked 0.3 cm in depth with a dense-sparse waveform at a frequency of 2/50 Hz and retained for 15 min. Rats in the normal and model groups received no EA treatment.

### Contraction reaction in rat abnormal scoring test

The abdominal withdrawal reflex (AWR) scoring criteria<sup>[14]</sup> are shown in Table 1. Rats in the model group were fasted in afternoon of the previous day. Vaseline was smeared on surface of the balloon which was slowly inserted into 5 cm of the rat anus according to the physical curve of CR and retained for 5 min. The test was begun when the rats became adapted.

After air was added into the balloon with a syringe, the rat rectum was stimulated and different degrees of contraction reaction were observed. The pressure (mmHg) during behavior response scored as 1, 2, 3, and 4 was recorded and expressed as the threshold of sensitivity. Each score was tested three times, and each rat was tested by two persons not participating in this research. Means were calculated (6 values in total). Three-minute intervals were set between each two tests for the full adaptation of rats.

Table 2 Threshold pressure of rat contraction reaction in different groups ( $n = 7$ ) (mean  $\pm$  SD)

Group	Threshold pressure (mmHg)			
	Score 1	Score 2	Score 3	Score 4
Normal group	23.38 $\pm$ 3.15	41.26 $\pm$ 3.58	68.00 $\pm$ 8.97	86.35 $\pm$ 10.01
Model group	15.50 $\pm$ 3.25 <sup>b</sup>	23.76 $\pm$ 3.91 <sup>b</sup>	37.43 $\pm$ 6.75 <sup>b</sup>	57.95 $\pm$ 5.45 <sup>b</sup>
EA group	21.81 $\pm$ 1.93 <sup>d</sup>	34.02 $\pm$ 3.87 <sup>b,d</sup>	50.50 $\pm$ 7.28 <sup>b,d</sup>	63.23 $\pm$ 6.24 <sup>b,d</sup>

<sup>b</sup> $P < 0.01$  vs normal group; <sup>d</sup> $P < 0.01$  vs model group.

Table 3 Corticotropin-releasing hormone level in hypothalamus of rats and MC count in colonic membrane of rats in different groups ( $n = 7$ ) (mean  $\pm$  SD)

Group	CRH level (pg/mg)	MC count in each visual field
Normal group	42.68 $\pm$ 4.39	2.19 $\pm$ 0.31
Model group	66.63 $\pm$ 18.19 <sup>a</sup>	10.0 $\pm$ 1.21 <sup>a</sup>
EA group	42.81 $\pm$ 7.44 <sup>c</sup>	4.81 $\pm$ 0.63 <sup>a,c</sup>

<sup>a</sup> $P < 0.05$  vs normal group; <sup>c</sup> $P < 0.05$  vs model group. EA: Electro-acupuncture; MC: Mast cells.

### Observation using toluidine blue-improved method

Samples were taken from the descending colon (5 cm above anus) and cecum, cleaned with normal saline, fixed with 10% formalin, dehydrated, paraffin-embedded, cut into sections and stained with toluidine blue-improved (TBI) method, deparaffinized and rehydrated, dipped in toluidine blue for 30 min. Two or three drops of glacial acetic acid were added into the samples until the presence of pretty clear nuclei and granulation. The samples were dried with cold air, cleaned in xylene, mounted onto Permount or Histoclad, and observed under a microscope (Olympus-BH2,  $\times 100$  and  $\times 400$ ). Three high-power fields ( $\times 400$ ) were randomly selected and the number of MC was counted and expressed as mean.

### Radioimmunoassay for CRH

**Sample preparation:** All rats were killed by dislocating cervical vertebra, with their brain taken out and hypothalamus isolated in ice bath. The hypothalamus was rinsed with 0.9% sodium chloride and restored in a liquid nitrogen container. The hypothalamus was taken out from the liquid nitrogen container, weighed and labeled. One milliliter 1 mol/L glacial acetic acid was added and homogenized for 100 min, then 0.8 mL 1 mol/L NaOH was added and centrifuged for 20 min at 4000 r/min. The supernatant was stored at  $-20^{\circ}\text{C}$  for radioimmunoassay. The sample (50  $\mu\text{L}$ ) was incubated for 24 h at  $4^{\circ}\text{C}$ . Then, 500  $\mu\text{L}$  separating-medium was added into each tube, incubated at room temperature for 45 min, centrifuged for 20 min at 4000 r/min. The supernatant was aspirated and the results were calculated.

**Immunohistochemistry for SP/SPR:** Sample sections were deparaffinized in xylene for 10 min, and dehydrated in 95%, 90%, 70% ethanol for 2 min. Primary antibody was bound to the specific rabbit anti-rat antigen diluted

at 1:150, at  $4^{\circ}\text{C}$  for 18 h. The samples stained with the envision immunohistochemistry method served as a positive control, while PBS-alternated primary antibody served as a negative control. Brown and dark brown granulation was observed with a background of purple blue. The positive expressing areas of SP and SPR under three fields were averaged.

### Statistical analysis

Experimental data were expressed as mean  $\pm$  SD. Statistical analyses were performed using SPSS 13.0 (SPSS Inc. Wacker Drive, Chicago, Illinois). Differences in mean were compared by one way ANOVA.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Contraction reaction in rat abnormal scoring test

The threshold pressure was remarkably lower in model group than in normal group, and obviously higher in EA group than in model group ( $P < 0.01$ , Table 2).

### Effect of EA on CRH in hypothalamus of rats

The CRH level was significantly higher in hypothalamus of rats in model group than in normal group ( $P < 0.05$ ), which was significantly decreased after EA treatment ( $P < 0.05$ ). No significant difference was found in CRH level between normal and EA groups (Table 3).

### MC in rat colonic membrane

The number of MC was greater in model group than in normal group ( $P < 0.05$ , Table 3) and smaller in EA group than in model group ( $P < 0.05$ ). The plasma of MC was stained purple, while nuclei were stained dark blue, scattered in mucous and submucous layers, or gathered into groups or lined up. The cells were round, oval, shuttle-like, and arose in shape. Small cells had little plasma and were clear in shape, while big cells had more plasma and were unclear in shape.

### SP and SPR expression in colon tissue of rats

The expression level of SP and SPR was higher in model group than in normal group ( $P < 0.05$ ), which was decreased after EA treatment ( $P < 0.05$ , Table 4, Figure 1).

## DISCUSSION

IBS is a prevalent functional gastrointestinal (GI) disorder

Table 4 SP and SPR expression in colonic membrane of rats in different groups ( $n = 7$ ) (mean  $\pm$  SD)

Group	SP expression		SPR expression	
	Optical density	Expressing area ( $\mu\text{m}^2$ )	Optical density	Expressing area ( $\mu\text{m}^2$ )
Normal group	14.21 $\pm$ 0.64	1772.77 $\pm$ 176.34	14.86 $\pm$ 0.48	362.65 $\pm$ 41.96
Model group	18.21 $\pm$ 1.07 <sup>a</sup>	3157.31 $\pm$ 304.95 <sup>a</sup>	16.36 $\pm$ 1.14 <sup>a</sup>	532.83 $\pm$ 105.60 <sup>a</sup>
EA group	16.29 $\pm$ 0.95 <sup>a,c</sup>	2020.09 $\pm$ 116.31 <sup>a,c</sup>	13.71 $\pm$ 0.70 <sup>a,c</sup>	340.02 $\pm$ 29.61 <sup>c</sup>

<sup>a</sup> $P < 0.05$  vs normal group; <sup>c</sup> $P < 0.05$  vs model group. SP: Substance P; SPR: Substance P receptor.

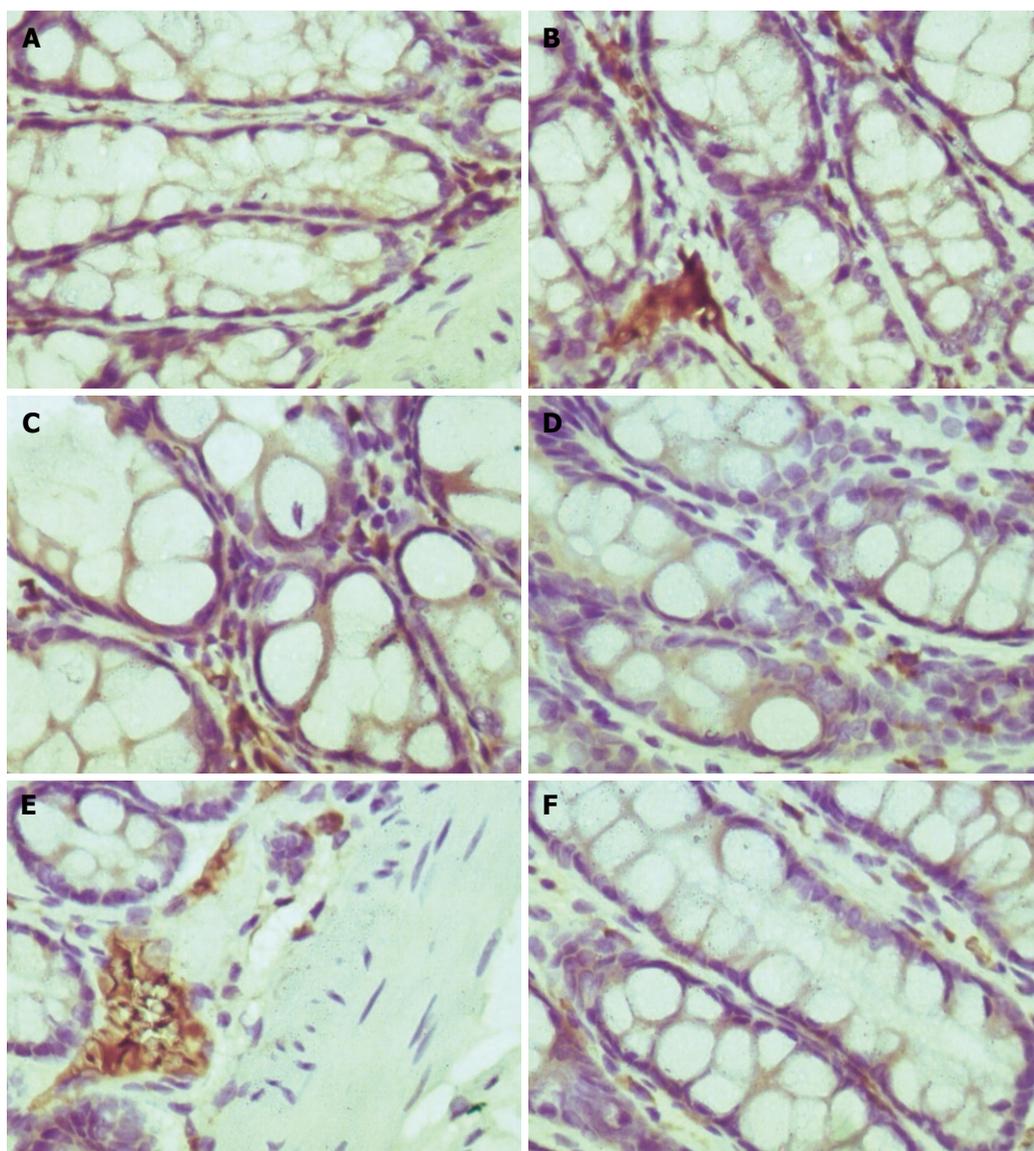


Figure 1 Expression of substance P and its receptor in colonic tissue of rats in normal group (A, D), model group (B, E) and EA group (C, F) ( $\times 400$ ).

characterized by chronic or recurrent abdominal pain or discomfort associated with altered bowel habits<sup>[6]</sup>.

IBS is presumed to be a disorder of the brain-gut link<sup>[17]</sup>. Psychological stress induces colonic segmental contractions which are exaggerated in IBS patients<sup>[18,19]</sup>. Stress can alter GI function. However, the mechanism underlying stress-induced intestinal response is still unclear. Epidemiological data show that psychological stress is one of the most important etiological factors for IBS. Mental stress is one of the factors for the in-

duction or aggravation of the symptoms of IBS<sup>[20]</sup>. Visceral hypersensitivity and dysregulation of central pain perception in the brain-gut axis play a pivotal role in the pathophysiology of IBS.

The central nervous system response to stress modulates the autonomic nervous system outflow and activates the hypothalamic-pituitary-adrenal axis<sup>[6]</sup>. Dysfunction of these systems has been proposed to be an etiological factor for IBS<sup>[7,8]</sup>. In addition, CRH, which plays an important role in the stress response<sup>[9]</sup>, induces

a higher adrenocorticotrophic hormone (ACTH) level and a more profound enhancement of colonic motility in IBS patients than in healthy controls<sup>[10]</sup>. It has been shown that CRH increases rectal sensitivity<sup>[11]</sup>. Thus, alterations in neuroendocrine response to stress may be of importance in the pathophysiology of IBS<sup>[12]</sup>.

It was reported that CRH injected into the intracerebral ventricle of rats exerts a stimulatory effect on colonic motor function by inducing spike burst activities in the proximal colon, accelerating transit, and inducing defecation<sup>[21-23]</sup>. Use of nonselective CRH1 (NBI-359565) receptor antagonists showed that colonic motor function induced by CRH is delayed in rats, suggesting that central CRH combined with CRH1 receptor can regulate the colon function<sup>[23]</sup>. In the study by Fukudo *et al.*<sup>[10]</sup>, the descending colon motility induced by CRH was greater in IBS patients than in healthy subjects, CRH produced duodenal phase III motor activities in 80% of healthy subjects and duodenal dysmotility in 40% of IBS patients, the time of abdominal symptoms evoked by CRH was significantly longer in IBS patients than in healthy subjects, the plasma ACTH level induced by CRH was significantly higher in IBS patients than in healthy subjects, indicating that human intestinal motility is probably modulated by exogenous CRH. The brain-gut in IBS patients may have an exaggerated response to CRH. Intravenous injection of CRH can promote the viscera sensibility in rats, which can be inhibited by CRH1 receptor antagonists<sup>[23]</sup>. This study showed that the CRH expression level in hypothalamus of rats was significantly higher in model group than in normal group ( $P < 0.05$ ), which was remarkably decreased ( $P < 0.05$ ) after EA treatment. No distinct difference in CRH expression was found between normal and EA groups, suggesting that EA therapy can inhibit the expression of CRH in hypothalamus of rats.

Recently, the role of probiotics in intestinal ecosystems has received great attention because of their beneficial effects on human and animal gut health<sup>[24]</sup>. It has been shown that probiotics can improve inflammation in some IBS patients and alleviate IBS symptoms such as pain<sup>[25]</sup>. It has been demonstrated in animal studies that neonatal intervention with probiotics can protect against short and long term consequences of impaired intestinal barrier function and gut-associated immune dysfunction induced by neonatal stress, reduce elevated corticosterone levels in pups with early psychological trauma (maternal deprivation), suggesting that normalization of HPA-axis activity is mediated by the effect of probiotics on gut function<sup>[26-30]</sup>. Further study is needed to explore the relation between acupuncture and probiotics used in treatment of IBS.

The pathological mechanism of IBS is not clear, but it is believed to be associated with alterations in mentality, GI motility, and visceral sensitivity, *etc.* Recently, researchers have suggested the role of inflammatory cells in the pathogenesis of IBS<sup>[31]</sup>. Mucosal MC are located throughout the gut in close proximity to enteric nerves, and secrete numerous inflammatory substances including

histamine, cytokines, proteases, and eicosanoids that are known to sensitize visceral sensory nerve fibers. That is why some researchers have become interested in them.

SP is closely related with the pathological change in IBS, which plays a role in stress, intestinal infection, and visceral hypersensitivity in the development of IBS<sup>[31,32]</sup>. Meanwhile, SP is a gastrointestinal peptide hormone existing in the central nervous system and gastrointestinal tract, and a signaling molecule connecting the nervous system to the immune system. Wang *et al.*<sup>[33]</sup> reported that the expression of SP and c-fos protein in the enteric and central nervous systems of the rat model of constipation-predominant IBS is abnormal, suggesting that abnormal changes in SP may be involved in the pathogenesis of IBS, and SP containing the neural pathway may be one of the neural pathways that play an important role in the regulation of gastrointestinal function.

SP in the intestinal tract is mainly produced by nerve terminal and endocrine cells such as MC. SP in combination with its receptor exerts its effect on the homologous effector cells of stomach and intestine, leading to complicated physiologic functions such as gastrointestinal motility, sensibility, secretion and absorption. In the enteric nervous system, SP, as an enteric nervous system of neurotransmitters, can increase gastrointestinal motility, promote contraction of alimentary tract smooth muscle, reinforce colon progradation, and stimulate water and electrolyte secretion in small intestine and colon. Some researchers believe that mucosal MC can restore the function and paresthesia of intestinal tract, while others hold that there is an amplifying ring among SP, MC and sensory neurofibra. Releasing of neuropeptides from sensory nerve ending, such as SP, has a direct effect on target organs. SP in combination with its special receptor on the surface of mucosal MC can activate and degranulate MC, releasing histamine and influencing sensorineural function, which promotes SP and local blood vessels to release nerve growth factor. In this study, the increased expression of SP was closely related with the number of MC in lamina propria of rats with IBS. It was reported that MC are associated with neurofibra by membrane-membrane touch<sup>[34]</sup>. The number of MC and degranulated MC is greater in IBS patients than in healthy subjects and the activated MC are adjacent to the inner-intestinal neuroplexus<sup>[13,35]</sup>. Our previous study showed that MC in colonic mucosa and c-fos positive cells are significantly increased, EA at ST-25 and Tegaserod injected into stomach can inhibit the proliferation and activation of MC in the colon, and regulate the secretion of SP, SPR, VIP, and VIPR, but the effect of EA is obviously better than that of Tegaserod<sup>[36]</sup>. In this study, the number of MC, the optical density and positive expressing areas of SP, SPR were greater in rats with IBS than in normal rats, indicating that MC, SP and SPR are closely related with the development of IBS ( $P < 0.05$ ). However, MC, SP and SPR were decreased after EA treatment ( $P < 0.05$ ), suggesting that EA at ST25 and ST37 can effectively

adjust the dysfunction of MC and down-regulate the expression of SP and SPR.

In conclusion, dysfunction of the central and enteric nervous systems leads to IBS. EA at ST25 and ST37 can decrease the number of MC, the expression of SP and SPR in colon, and the CRH level in hypothalamus of rats.

## COMMENTS

### Background

Irritable bowel syndrome (IBS) is a common disorder in clinical practice, but its pathophysiology has not been completely elucidated, which makes its treatment difficult. The authors' previous study showed that the general therapeutic rate of electro-acupuncture (EA) at ST-25 for IBS is 84.90%. However, the regulatory effect of EA on IBS is still unknown.

### Research frontiers

More and more data show that IBS is closely related with the brain-gut axis, which has becoming a hot spot of study.

### Innovations and breakthroughs

The results of the authors' study have proved that EA at ST25 and ST37 is effective against IBS. EA at ST25 and ST37 exerts its effect on IBS by decreasing the number of mucosal mast cells and down-regulating the expression of substance P, substance P receptor in colon and corticotropin-releasing hormone in hypothalamus of rats.

### Applications

The experimental and clinical data can be used in further study on EA in treatment of IBS.

### Peer review

This study is interesting. Its findings are useful for the treatment of IBS.

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CASE REPORT

## Biloma: An unusual complication in a patient with pancreatic cancer

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### Abstract

The term biloma describes an encapsulated collection of bile within the abdomen, usually secondary to bile duct disruption. The commonest causes reported in the literature are iatrogenic (secondary to hepatobiliary surgery), trauma or complications due to choledocholithiasis. A few cases have been reported as complications of cholangiocarcinoma or acute cholecystitis. We report the case of a 64-year-old man initially diagnosed with a non-obstructive malignancy of the pancreas, who developed a spontaneous intrahepatic biloma 8 mo later. This was identified following a 1-wk history of fever, rigors and icterus. The biloma was identified on computed tomography and subsequently drained under ultrasound guidance. Forty-eight hours later, a stent was inserted endoscopically into his common bile duct and he made an uneventful in-hospital recovery. We believe this is the first documented case of spontaneous intrahepatic biloma to occur secondary to pancreatic malignancy.

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**Key words:** Obstructive jaundice; Endoscopic retrograde cholangiopancreatography; Computed tomography; Choledocholithiasis; Bile duct diseases

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### INTRODUCTION

In 1979, Gould and Patel<sup>[1]</sup> described the case of a 32-year-old man who was found to have a large encapsulated collection of bile outside the biliary tree, secondary to a tear in the right hepatic duct. This followed a traumatic insult to the upper abdomen. The patient proceeded to have this collection drained and a T-tube was inserted to permit bile drainage. Since the initial description, there have been a few documented cases of spontaneous biloma formation, usually in the context of choledocholithiasis. The current case is sufficiently exceptional with regard to location, etiology and mode of presentation to merit a report.

### CASE REPORT

A 64-year-old man was referred to the gastroenterology clinic by his general practitioner (GP) with constant upper abdominal pain that was worse after eating. In addition, the patient had been suffering from alternating constipation and loose stools. He denied the passage of blood in his motions or any weight loss.

A full blood count revealed normocytic anemia (hemoglobin 123 g/L; mean corpuscular volume: 88.4 fL). Renal and liver function tests were normal. Given the features on presentation, a colonoscopy was suggested, however, he was reluctant to go ahead with the procedure. As an alternative, a computed tomography (CT) pneumocolon was arranged.

Although no significant polyps or other colonic abnormalities were seen, there was an irregular 3 cm × 4.5 cm mass arising from the neck/body of the pancreas, with multiple lymph nodes in the peripancreatic region. Further assessment with a dual-phase CT scan of the pancreas was performed (Figure 1). As a result of encasement



**Figure 1** CT pneumocolon, which suggested the presence of advanced pancreatic malignancy.



**Figure 2** Large intrahepatic cystic lesion with bile duct compression caused by enlargement of the pancreatic neoplasm.

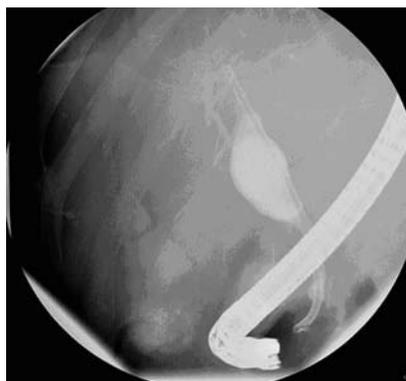
of the superior mesenteric artery and portal veins, the tumor was deemed inoperable. An ultrasound-guided biopsy of the lesion was undertaken, and histology confirmed adenocarcinoma of the pancreas.

The patient went on to complete three courses of gemcitabine chemotherapy but further staging CT demonstrated slowly progressive disease over a 3-mo period.

Six months later, the patient was referred by his GP to the on-call medical team with a week-long history of nausea, anorexia and new-onset jaundice. Upon examination, he was tender in the epigastrium and right upper quadrant, with no overt peritonism. His blood tests were as follows: bilirubin, 164  $\mu\text{mol/L}$ ; alanine aminotransferase, 84 IU/L; alkaline phosphatase, 5567 IU/L; albumin, 35 g/L; C-reactive protein (CRP), > 160 mg/L; prothrombin time, 16.3 s; and white blood cell count,  $12.9 \times 10^9/\text{L}$ .

Repeat CT of the pancreas was undertaken, which demonstrated a dilated common bile duct (CBD) with external compression from enlargement of the pancreatic tumor, and a large, intrahepatic cystic lesion that measured 12 cm  $\times$  9 cm, adjacent to the gallbladder (Figure 2).

Ultrasound-guided drainage was performed and yielded frank bile in keeping with an intrahepatic biloma. Microbiological analysis of the fluid did not reveal the presence of any pathogenic organisms. Endoscopic retrograde cholangiopancreatography (ERCP) demonstrated



**Figure 3** ERCP demonstrating a smooth stricture in the distal CBD, with dilated common hepatic ducts above. Contrast material was seen to flow within the biloma collection, which implied communication with the intrahepatic ducts.

a smooth stricture of the distal CBD and a dilated common hepatic duct above. Contrast material was seen to be flowing into the biloma, which implied a connection with the intrahepatic ducts (Figure 3). The stricture was stented and drainage from the biloma ceased within 48 h following insertion.

## DISCUSSION

The first reported case of a biloma was reported by Gould and Patel in 1979<sup>[1]</sup>. They reported extrahepatic bile leakage post trauma to the right upper quadrant of the abdomen. The bile accumulated in an encapsulated form. Although originally described as a bilious collection outside the liver, the term biloma has been extended to include any such lesion that may be intrahepatic but anatomically outside the biliary tree<sup>[2]</sup>. The majority of bilomas are iatrogenic and follow transhepatic cholangiography, liver biopsy, ERCP or cholecystectomy. Biloma also has been recognized to arise following external trauma<sup>[3]</sup>. Spontaneous biloma is exceedingly rare, and the majority occur secondary to choledocholithiasis or cholangiocarcinoma<sup>[4]</sup>. Rarer causes have been reported in the context of sickle cell disease<sup>[5]</sup> or as a complication of hepatic infarction and abscess formation. To the best of our knowledge, biloma that occurs secondary to primary pancreatic malignancy has not been reported previously in the literature.

As diagnostic techniques continue to evolve, an increasing number of cases have been identified, but the exact mechanism behind spontaneous biloma formation is still unknown. Postulated pathogenic mechanisms are Sphincter of Oddi spasm, CBD tumor or calculus obstruction that results in increased intraductal pressure, bile duct necrosis and rupture of the bile duct. As a result of the relatively slow onset of ductal obstruction that occurs in the context of a pancreatic neoplasm, such an acute elevation in biliary pressure is unusual (cf. impacted CBD stone). The size and location of biloma is influenced by the cause of rupture, location and size of bile leak, and rate of absorption by the peritoneum.

Most bilomas are secondary to CBD rather than hepatic duct perforation<sup>[6]</sup>.

There is no difference in the incidence between males and females, but the condition is found more often in the sixth to seventh decades of life. The age predominance may reflect that of the underlying etiological factor rather than that of developing the complication. Presentation is nonspecific, with abdominal pain, usually in the right upper quadrant (although a few reported cases of bile migration to the left subphrenic space have been documented, which has given rise to a predominance of pain on the left side). Fever may be accompanied by jaundice and abdominal distension. Extreme cases that result in bilious ascites also have been reported<sup>[7]</sup>. In our case, there was no history of recent hepatobiliary intervention (pancreatic biopsy had been performed 6 mo prior to presentation, but no evidence of biloma was seen on interval scanning). There were complaints of fever, rigors, anorexia and scleral icterus. Blood tests may show neutrophil leukocytosis, elevated CRP and obstructive liver function tests. Blood cultures may show Gram-negative bacteremia. Biloma can be picked up on ultrasound, CT or magnetic resonance imaging. Despite advancing imaging modalities, biloma may be difficult to differentiate from large cystic metastasis, seroma, angioma or lymphocele. Ultrasound becomes useful in this situation, with a definitive diagnosis being made following radiologically guided aspiration. Once fluid is obtained, microbiological testing is mandatory to exclude the presence of coexisting infection. ERCP is particularly useful in diagnosing an active leak; this may also allow therapeutic intervention<sup>[8]</sup>. Precise location of the biloma allows for percutaneous drainage, which negates the need for surgical intervention. Endoscopic intervention includes sphincterotomy with stone extraction, if appropriate, to lower biliary pressure. Placement of a stent in more distal lesions is an option, as this reduces

the pressure gradient into the duodenum and facilitates forward flow of bile. This also relieves obstruction from lesions that narrow the biliary tree. In our case, the latter approach was used to overcome the obstruction caused by the large pancreatic tumor.

Surgical management remains contentious but can be useful in cases of ongoing leakage despite endoscopic therapy. The goals are to halt abdominal contamination from bile by means of peritoneal drainage, surgical closure of active leaks, and T-tube drainage<sup>[7,9]</sup>.

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## Ectopic papilla of Vater in the pylorus

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### Abstract

The major papilla of Vater is usually located in the second portion of the duodenum, to the posterior medial wall. Sometimes the mouth of the biliary duct is located in other areas. Drainage of the common bile duct into the pylorus is extremely rare. A 73-year old man, with a history of duodenal ulcer, was admitted to hospital with the diagnosis of cholangitis. Dilatation of the extrahepatic biliary duct was observed by abdominal ultrasonography, and endoscopic retrograde cholangiopancreatography (ERCP) was performed. No area suggesting the presence of the papilla of Vater was found within the second duodenal portion. Finally the major papilla was located in the theoretical pyloric duct. Cholangiography was performed and choledocholithiasis was found in the biliary tree. The patient underwent dilatation of the papilla with a balloon tyre and removal of a 7 mm stone using a Dormia basket, which solved the problem without further complications. This anomaly increased the difficulty of performing therapeutic interventions during ERCP. This alteration in anatomy may increase the risk of complications during papillotomy, with a theoretically higher risk of perforation. Dilatation using a balloon was the chosen therapeutic technique both in our case and in the literature, due to its low rate of complications.

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**Key words:** Ectopic common bile duct; Endoscopic dilatation; Endoscopic retrograde cholangiopancreatography; Papilla of Vater; Papillotomy; Pyloric drainage

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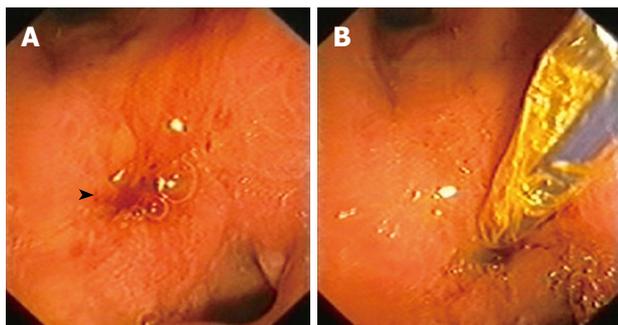
Guerra I, Rábago LR, Bermejo F, Quintanilla E, García-Garzón S. Ectopic papilla of Vater in the pylorus. *World J Gastroenterol* 2009; 15(41): 5221-5223 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5221.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5221>

### INTRODUCTION

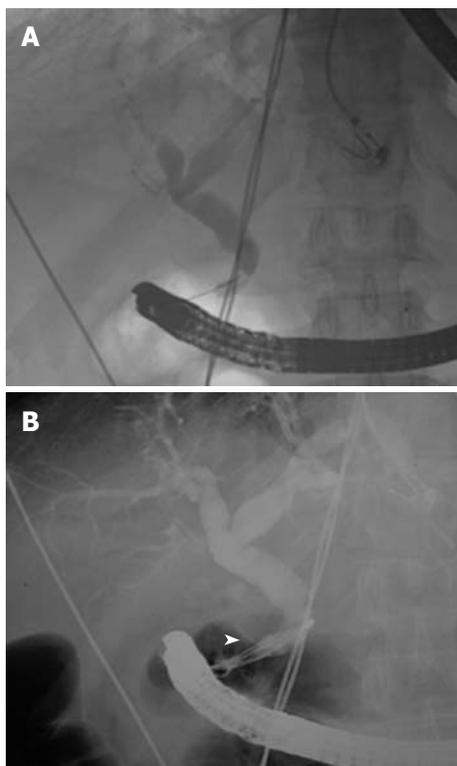
The major papilla of Vater is usually located in the second portion of the duodenum, to the posterior medial wall. Both the common bile duct and the main pancreatic duct empty into it. Sometimes the mouth of the biliary duct is located in other areas along the duodenum, mainly within the third or the fourth portion<sup>[1-3]</sup>, although it has also been found with a much lower frequency in the duodenal bulb<sup>[4-6]</sup>. In such cases it is common to find a previous duodenal ulcerous pathology<sup>[7]</sup>. Very rarely has the papilla been found in the stomach<sup>[8-10]</sup>, although the frequent use of endoscopic retrograde cholangiopancreatography (ERCP) has increased its recognition. In the literature we found a case in the 1930's describing common bile duct drainage into the pylorus<sup>[2]</sup>, and another more recent case of drainage into the duodenal wall adjacent to the pylorus<sup>[11]</sup>, however, no more cases have been published. We describe a case in which the mouth of the biliary duct was found in the pyloric channel.

### CASE REPORT

A 73-year-old patient, with a history of digestive bleeding secondary to duodenal ulcer 10 years previously, presented to hospital due to high temperature and pain in the right hypochondrium. His C-reactive protein level was 190 mg/dL, alanine-aminotransferase 40 IU/L, leucocytes 15 500/mm<sup>3</sup> and total bilirubin 2.62 mg/dL. Abdominal ultrasonography showed dilatation of the extrahepatic biliary duct, with no lithiasis. After the diagnosis of cholangitis with increased bilirubin and dilatation of the biliary duct was made, ERCP was performed. No area suggesting the presence of the papilla of Vater was found within the second duodenal portion. Finally the major papilla was located in the theoretical pyloric duct (Figure 1), since the patient showed a duodenal bulb which was deformed and post-ulcerous, having disappeared almost completely. The papilla did not show clear anatomical signs which made it inadvisable to perform a papillotomy. Cholangiography was carried out and choledocholithiasis



**Figure 1** Endoscopic retrograde cholangiopancreatography. A: View of the papilla located in the pylorus (arrow head); B: Piping of the papilla.

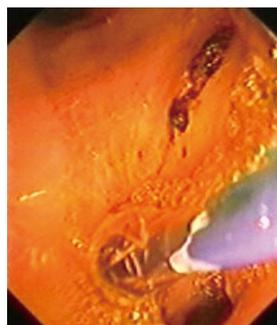


**Figure 2** Cholangiography. A: Common bile duct ending in a hook shape, common in proximal drainage of the papilla; B: Removal of choledocholithiasis with Dormia basket (arrow head).

was found in the biliary tree (Figure 2A). The patient underwent dilatation of the papilla with a balloon tyre and removal of a 7 mm stone using a Dormia basket, which solved the problem without further complications (Figures 2B and 3).

## DISCUSSION

The papilla of Vater is a bulge in the duodenal mucosa into which both the common bile duct and the Wirsung empty, sometimes shaping into a Y, or they can be separated by a mucosa layer making them independent. The most common location of the papilla of Vater is within the posterior medial wall of the second portion of the duodenum. The frequent use of ERCP has allowed better observation of papillae in an ectopic location.



**Figure 3** Endoscopic retrograde cholangiopancreatography. Balloon dilatation of the ectopic papilla.

Although the diagnosis of ectopic papillae have been described by other types of radiological studies such as computed tomography<sup>[4]</sup>, X-ray of the esophagus-gastrointestinal tract<sup>[12]</sup>, intraoperative cholangiography<sup>[11]</sup> and echoendoscopy<sup>[5]</sup>, most ectopic papillae are identified by ERCP. An ectopic location distal to the second duodenal portion, within the third and fourth duodenal portions, has been described frequently, and has a frequency rate of 5.6% to 23%<sup>[13]</sup>. Ectopic papillae are much less frequent in a proximal location, and a few cases have been located in the gastric, pylorus and duodenal bulb areas<sup>[14]</sup>. Filippini in 1931 described the first case of papilla located in the pylorus<sup>[2]</sup>, although Quintana and Labat<sup>[2]</sup> mentioned 3 cases of dual drainage in the duodenum and pylorus. Another recent case has been described where the papilla was located in the posterior duodenal wall below the pylorus<sup>[11]</sup>. In our subject the papilla was located in the pylorus, with a duodenal bulb which was deformed due to previous ulcerous pathology.

A theory on the origin of ectopic papillae has been suggested which describes their occurrence during embryonic formation. The liver originates in the hepatic diverticulum, which is divided into the hepatic pars and the cystic pars during embryogenesis. The hepatic pars then develops into both the liver and the hepatic ducts, while the cystic pars develops into the gall-bladder and the cystic duct. The common bile duct originates in the hepatic antrum, which is the common area of the hepatic diverticulum. Boyden<sup>[15]</sup> claimed that an earlier subdivision of the hepatic diverticulum could cause the common bile duct to empty into different locations other than the usual location.

Ectopic papillae located in the bulb may be secondary to an ulcerous duodenal pathology, which could cause, due to contiguity, anomalous drainage in the duodenum<sup>[11]</sup>. In our patient the location of the papilla in the pyloric channel might be related to the patient's previous ulcerous duodenal pathology. Nevertheless, since we did not find signs of the papilla in the second duodenal portion, his condition was probably due to a congenital malformation with biliary and pancreatic drainage in the posterior pyloric area, which could have caused the later development of a duodenal ulcer. In our subject we observed the diagnosis requirements for an ectopic papilla as described by Lee *et al*<sup>[14]</sup>, for a location in the bulb which were: (1) an orifice was observed in the bulb by duodenoscopy or upper endoscopy, and the bile duct and/or the pancreatic duct were directly visualized radiographically, when contrast

was injected *via* this opening; (2) there was direct drainage of the common bile duct into the duodenal bulb without evidence of any other drainage into the duodenum on cholangiography; and (3) there was no evidence of a papilla-like structure in the second or third duodenal portion on duodenoscopic examination. Fistula secondary to ulcer disease or choledocholithiasis, spontaneous or iatrogenic surgical fistula, and surgical choledochenteric diversion should be included in the differential diagnosis<sup>[8]</sup>.

The clinical importance of the ectopic location of the papilla means that there is a tendency for the development of choledocholithiasis through anomalous bile drainage, due to the lack of a sphincter mechanism. Likewise, it can also lead to mucosal damage in the area, with swelling and ulcer formation, due to the action of biliary pancreatic secretion<sup>[14]</sup>. The clinical symptoms may include recurrent abdominal pain, which could explain the high percentage of patients undergoing cholecystectomy described in some series<sup>[13]</sup>. The absence of a sphincter would allow passage of the gastroduodenal contents into the main bile duct, possibly causing cholangitis in association with biliary obstruction<sup>[7,13,14]</sup>. In a recent study by Disibeyaz *et al*<sup>[13]</sup> on 39 patients where the papilla was located in the bulb, they detected episodic abdominal pain in 95% of patients and cholangitis in 59% of patients. The predominance of male sex and an association with ulcerous duodenal pathology in 61.5% of the patients should be emphasized. In our case, the patient had a history of duodenal ulcer and was admitted to hospital suffering from cholangitis, which led us to think that this patient had a papilla in an ectopic location when we failed to find it in its usual location, making it necessary to check both the duodenal bulb and the stomach.

This anomaly increased the difficulty of performing therapeutic interventions during ERCP. This alteration in anatomy, when there are no clear anatomic signs, may increase the risk of complications during papillotomy, with a theoretically higher risk of perforation, as has been described in the literature<sup>[13]</sup>. Balloon dilatation may be the chosen technique for these patients when there is a biliary obstruction. Its effectiveness has recently been suggested, especially when gallstones need to be removed, without relevant complications<sup>[13]</sup>. Stent installation may be necessary when balloon dilatation is unsuccessful and the patient has comorbidities which make surgery inadvisable. The surgical option should only be taken in these cases when endoscopic treatment is not effective.

In conclusion, ectopic location of the papilla is a rare finding, however, the frequent use of ERCP may increase the number of cases diagnosed. Although a distal location is found most frequently, a proximal location must be taken into account, especially in patients with a history of recurrent abdominal pain, cholangitis, ulcerous duodenal pathology and biliary obstruction in whom the papilla cannot be found in its usual location during ERCP.

Although further studies are necessary, balloon dilatation was the chosen therapeutic technique both in our case and in the literature, due to its low rate of complications. Technical difficulty and a higher probability of perforation when performing a papillotomy, due to the lack of anatomic reference, make its use inadvisable.

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CASE REPORT

## Gastrointestinal stromal tumor causing small bowel intussusception in a patient with Crohn's disease

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### INTRODUCTION

Intussusception, defined as the telescoping of a segment of the gastrointestinal tract into an adjacent one, is extremely rare in the stomach, but more common in the small intestine, ileocecal junction and colon. It is the leading cause of intestinal mechanical obstruction and the second most common surgical emergency in children<sup>[1]</sup>. However, it is rather infrequent in adults, accounting for 0.1% of all surgical admissions and 1%-5% of mechanical bowel obstructions<sup>[2]</sup>. In these cases, it is frequently related to malignancy.

Gastrointestinal stromal tumors (GISTs) are a subset of mesenchymal tumors of varying differentiation. They are rare clinical entities, constitute less than 3% of all gastrointestinal malignant neoplasms and represent only 20% of small-bowel malignant neoplasms (excluding lymphoma)<sup>[3]</sup>. With the improvement on immunohistochemical staining techniques and ultrastructural evaluation, GISTs are recognized as a distinct group of mesenchymal tumors now<sup>[4]</sup>.

In the current report, we present a case of a 45-year-old man with Crohn's disease complaining of intermittent vague abdominal pain for a period of 4 mo. Enteroclysis, computer tomography (CT) and magnetic resonance imaging (MRI) abdominal scans showed small bowel intussusception, a diagnosis which was confirmed after surgical exploration of the abdomen where a tumor causing a jejunoileal intussusception was identified. Pathological examination of the surgical specimen revealed a gastrointestinal stromal tumor as well as inflammation and aphthous ulcerations, which were characteristic of Crohn's disease involving small bowel. Coexistence of these clinical entities resulting in intussusception has never been reported in the literature.

### Abstract

We report a case of jejunoileal intussusception in a 42-year-old patient with Crohn's disease caused by a gastrointestinal stromal tumor. The patient complained of vague diffuse abdominal pain for a period of 4 mo. Intussusception was suspected at computer tomography and magnetic resonance imaging scans. Segmental resection of the small intestine was performed. Pathological examination of the surgical specimen revealed a gastrointestinal stromal tumor as well as aphthous ulcerations and areas of inflammation, which were characteristic of Crohn's disease. This is the first report of small bowel intussusception due to a gastrointestinal stromal tumor coexisting with Crohn's disease.

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**Key words:** Gastrointestinal stromal tumor; Crohn's disease; Intussusception

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## CASE REPORT

A 45-year-old man with symptoms of bowel obstruction was admitted to the surgical department of our hospital in April, 2008. He complained of diffuse blunt abdominal pain preceded by two episodes of vomiting and obstipation. The patient reported similar attacks of milder episodes, consisting of intermittent abdominal pain and bloating for the past 4 mo. He was diagnosed as Crohn's disease following gastroenterological evaluation with colonoscopy and terminal ileum biopsies at sites of ileitis 1 year ago on the grounds of chronic diarrhea, cramp abdominal pain attacks and mild anemia. The patient did not receive any regular medication. Physical examination showed mild abdominal distention with slight tenderness and hypoactive bowel sounds. No mass was palpated and rectal examination did not reveal any blood or malignancy. Routine laboratory tests and chest films were normal. However, abdominal plain radiographs showed some small bowel air-fluid levels. The patient underwent a CT scan with *po* and intravenous contrast of 5-mm thick slice, which showed thickening of the distal ileum wall and an intraluminal mass resulting in partial obstruction of the small intestine. Sagittal fast spin echo (FSE) T2, FSE T2 with fat suppression and axial FSE T1 with 5-mm thick slice abdominal MRI (Figure 1) fortified the suspicion of intussusception since it showed a pathognomonic bowel within bowel configuration. In order to secure the diagnosis, an enteroclysis was performed, which showed a jejunoileal stricture caused by invagination of the jejunum into the ileum and proximal to the stenosis bowel dilatation (Figure 2).

Taking into account the high possibility of a malignant mass causing bowel intussusception in an adult patient, laparoscopic approach was not considered in this case, in order to achieve the best possible oncologic clearance at the surgical intervention that followed. So, the patient underwent a laparotomy which identified a jejunoileal intussusception (Figure 3). An approximately 20-cm partial small bowel resection including wide margins and a wedge resection of the respective mesentery up to the beginning of the feeding vessels were performed. The bowel lumen was longitudinally opened immediately following removal of the specimen and an exophytic round-shaped mass of smooth circumference approximately 6 cm in maximum diameter, which was hard at palpation, was easily appreciated. Pathological examination of the surgical specimen proved the lesion to be a stromal tumor with an immunohistochemical profile of c-kit (+), S-100 (-), SMA (-), desmin (-), EMA (-), CD34 (-), AE1/AE3 CK8.18 (-), NSE (-), and synaptophysin (-). The tumor was invading the muscularis propria layer at the point of the intussusception (Figure 4A and B). No lymph node infiltration was identified. Microscopic examination of the remaining specimen disclosed findings consistent with Crohn's disease. The lesion contained aphthous ulcerations and showed severe polymorphonuclear and lymphocytic infiltration as well as eosinophils and intraluminal abscesses.



Figure 1 Abdominal MRI scan (Sagittal fast spin echo T2) revealing the jejunoileal intussusception.



Figure 2 Enteroclysis showing jejunoileal stricture caused by invagination of the jejunum into the ileum and proximal to the stenosis bowel dilatation.

The continuity of the small bowel was restored with a side-to-side entero-enteral anastomosis. The patient, discharged on the 7th postoperative day, following an uncomplicated recovery, was subjected to follow-up with clinical examinations and CT scans at 6-mo intervals and had no clinical or radiologic recurrence at the time when we wrote this paper.

## DISCUSSION

Tumors of the small intestine are rare and usually benign. The majority of them are leiomyomas, but adenocarcinomas and GISTs, although uncommon, may also appear. Conventional histologic methods could not produce correct diagnoses and most tumors referred to as leiomyomas and leiomyosarcomas in the older medical literature are actually GISTs. On the basis of immunohistochemical and ultrastructural studies, GISTs are mesenchymal tumors of the gastrointestinal tract that may show myogenic and/or neurogenic characteristics<sup>[5]</sup>, and are usually asymptomatic, but they have been reported to present with bleeding, obstruction or perforation in some cases<sup>[6]</sup>.

Intussusception is the invagination of one bowel loop and its mesentery into the bowel lumen distal to it. It occurs when a proximal segment of intestine (intussusceptum) telescopes into the intestinal segment distal to it (intussuscipiens), and is the second most common complication when tumors locate in the ileum<sup>[7]</sup>. Invaginations of the lumen may also cause gastrointestinal

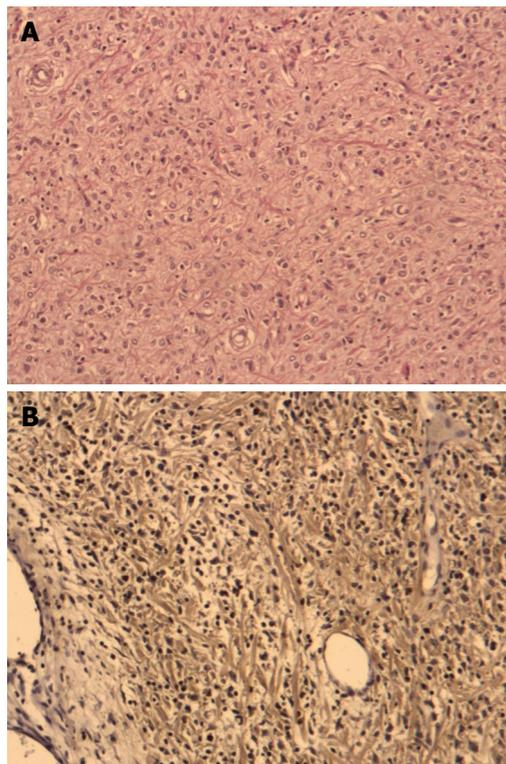


**Figure 3** Intraoperative appearance of the jejunoleal intussusception.

bleeding or necrosis of the tumor. Usually patients have slightly enlarging tumors with no rapid onset. Their complaints are usually non specific and it is finally risky to miss the diagnosis. Our patient experienced vague abdominal pain for a period of 4 mo which led him to undergo a colonoscopy that came up normal.

Intussusception is correctly diagnosed preoperatively in only one-third of cases<sup>[7]</sup>. An accurate diagnosis of intussusception should include a good and specific medical history, a thorough physical examination, radiography, CT, MRI and enteroclysis or even an endoscopic ultrasound or capsule endoscopy. Ultrasound is useful in confirming obstruction and may sometimes identify the cause<sup>[8]</sup>. However, it is highly operator-dependent. Taking into account the rarity of such findings, it requires an experienced radiologist. In our case, ultrasound was not diagnostic of intussusception. It has been recently reported that CT and MRI offer great help in establishing the preoperative diagnosis of intussusception<sup>[9]</sup>. On CT, one can diagnose a bowel intussusception within its configuration, thickening of the wall and compressed mesenteric fat and vessels<sup>[9]</sup>. Although not routinely reported in such cases, an abdominal MRI, like in our report, can aid to establish the preoperative diagnostic suspicion of intussusception.

In contrast to childhood where intussusception is idiopathic in 90% of cases and the basic underlying cause of intussusception is the hypertrophy of Payer's patches activity, adult intussusception has a definable pathologic lesion in over 90% of cases, with neoplasms considered to be the cause in 65% of them<sup>[10]</sup>. Any intraluminal lesion, especially polyps, which irritates and alters normal peristaltic activity, is able to trigger an intraluminal invagination finally causing an intussusception. Subsequent peristaltic bowel activity produces an area of sequence constriction and relaxation, thus telescoping the leading point through the distal bowel lumen<sup>[11]</sup>. The malignancy is more likely to be located in the colon rather than in the small bowel. Less common etiologies of intussusception in adults include postoperative factors (adhesions, suture lines, *etc.*), polyps, Meckel's disease, sprue, cecal duplication and intramural hematoma<sup>[1]</sup>. The presentation of adult intussusception is usually subacute or chronic<sup>[2]</sup>. The most common symptoms are crampy abdominal pain, nausea, vomiting, abdominal distention



**Figure 4** Histological examination (A) (HE,  $\times 100$ ) and c-kit immunohistochemical positive staining (B) (HE,  $\times 40$ ) of the gastrointestinal stromal tumor.

or tenderness. Only up to 20% of all cases present with complete bowel obstruction and acute onset<sup>[10]</sup>. Moreover, it has been reported that a palpable mass is present in 7%-42% of the cases<sup>[11]</sup>.

In the literature, five cases of small bowel intussusception from a stromal tumor in adults have been described<sup>[5,6,10,12,13]</sup>. The most recent one was an intraluminal leiomyoma of the small intestine in a 72-year-old female patient, which resulted in invagination and partial obstruction of the jejunum<sup>[12]</sup>. Another recent report presented a case of a 60-year-old woman with a jejunoleal intussusception due to a myxoid stromal tumor<sup>[10]</sup>. Others described a GIST as the cause of intussusception in a 32-year-old man and emphasized the role of ultrasound in preoperative diagnosis of the disease<sup>[13]</sup>. In an older report<sup>[5]</sup>, a case of a 42-year-old patient with intussusception of the lower jejunum was presented and the patient was admitted with progressive anemia, massive melena and lower abdominal pain. Finally, a malignant GIST of the small intestine causing small bowel obstruction due to ileal invagination has also been described<sup>[6]</sup>.

Coincidence of Crohn's disease and gastrointestinal stromal tumor is extremely rare and only two cases have been reported to date<sup>[14,15]</sup>. The first one described a polypoid tumor within Meckel's diverticulum in an 81-year-old male patient with Crohn's disease and the tumor was immunohistochemically proved to be a GIST<sup>[14]</sup>. The second report presented a 51-year-old patient with a high risk GIST of the terminal ileum within an area of Crohn's ileitis<sup>[15]</sup>.

Conclusively, in the present report we describe a case of a 42-year-old man with no significant medical history other than a 4-mo intermittent dull abdominal pain who presented with symptoms of acute bowel obstruction. Laparotomy revealed a jejunoileal intussusception and histological examination of the surgical specimen indicated a GIST tumor causing the intussusception as well as patchy areas of inflammation and erosions similar to those identified in Crohn's disease. Coexistence of these pathologically distinct and extremely rare entities has not been reported and represents a unique finding.

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CASE REPORT

## A case of advanced intrahepatic cholangiocarcinoma successfully treated with chemosensitivity test-guided systemic chemotherapy

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### Abstract

Intrahepatic cholangiocarcinoma (ICC) is a relatively rare and highly fatal neoplasm that arises from the biliary epithelium. Prognosis is generally poor and survival is limited to a few months. Here we present a case of advanced ICC successfully treated by chemosensitivity test-guided systemic chemotherapy combining S-1 and cisplatin (CDDP). A 65-year-old woman with a liver tumor was referred to our hospital on November 21, 2007. Abdominal ultrasonography and computed tomography (CT) showed low-density masses of 50 and 15 mm in diameter, respectively in segment VIII of the liver and in the enlarged lymph node in the para-aorta. Ultrasonography-guided fine needle biopsy diagnosed the tumors as ICC. Since the patient was inoperable for lymph node metastasis, she underwent systemic chemotherapy with gemcitabine. Six months after initiation of chemotherapy, CT revealed ICC progression in the liver and pleural dissemination with pleural effusion. The patient was admitted to our hospital for anticancer drug sensitivity testing on June 9, 2008. Based on the sensitivity test results, we elected to administer systemic chemotherapy combining S-1 and CDDP. Two months into the second chemotherapy treatment, CT revealed a reduction of the tumors in the liver and lymph node and a decrease in pleural effusion.

After eight cycles of the second chemotherapy, 17 mo after ICC diagnosis, she is alive and well with no sign of recurrence. We conclude that chemosensitivity testing may effectively determine the appropriate chemotherapy regimen for advanced ICC.

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**Key words:** Chemosensitivity testing; Cholangiocarcinoma; Cisplatin; Liver neoplasms; Gemcitabine; S-1; Systemic chemotherapy

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Abe K, Wakatsuki T, Katsushima F, Monoe K, Kanno Y, Takahashi A, Yokokawa J, Ohira H. A case of advanced intrahepatic cholangiocarcinoma successfully treated with chemosensitivity test-guided systemic chemotherapy. *World J Gastroenterol* 2009; 15(41): 5228-5231 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5228.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5228>

### INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC) is a relatively rare and highly fatal neoplasm that arises from the biliary epithelium. Radical surgery is currently the optimal therapy for ICC with curative potential. However, most patients present with advanced disease at the time of diagnosis. Prognosis in these patients is poor and survival is limited to a few months<sup>[1]</sup>.

Biliary tract carcinoma (BTC) has traditionally been divided into cancers of the gallbladder, the extrahepatic bile ducts, ampulla of Vater, whereas ICC has been classified as liver cancer. Lately, however the term BTC has been used to include the gallbladder, the extrahepatic bile ducts, ICC and the ampulla of Vater.

Chemotherapy has been performed in cases of unresectable advanced ICC and postoperative recurrence of ICC. However, a standard chemotherapeutic regimen has not yet been established for ICC. There are phase II

trials that support the following combinations: gemcitabine/cisplatin (CDDP), gemcitabine/oxaliplatin, gemcitabine/capecitabine, and 5-fluorouracil in unresectable or metastatic ICC<sup>[2]</sup>.

Chemosensitivity testing using surgical material is an established method to evaluate tumor response prior to chemotherapy<sup>[3-5]</sup>. Sensitivity, specificity and accuracy of chemosensitivity testing were reportedly 82.7%, 70.7% and 73.6%, respectively<sup>[6]</sup>. Several methods are established to measure cancer cell viability<sup>[7]</sup>. Recently, chemosensitivity testing for gastric cancer treatment has been approved in Japan. However, it has seldom been performed for ICC, since ICC occurs more rarely than other gastrointestinal malignancies. The adenosine triphosphate (ATP) assay is a highly sensitive and precise method for measuring cell viability, and only a few dozen cells are necessary for the ATP assay<sup>[8,9]</sup>. Very few reports of chemosensitivity testing used surgical material from ICC. This is the first report concerning chemosensitivity testing using the ATP assay for patients with unresectable ICC. We present a case of advanced ICC successfully treated by chemosensitivity test-guided systemic chemotherapy combining S-1 and CDDP.

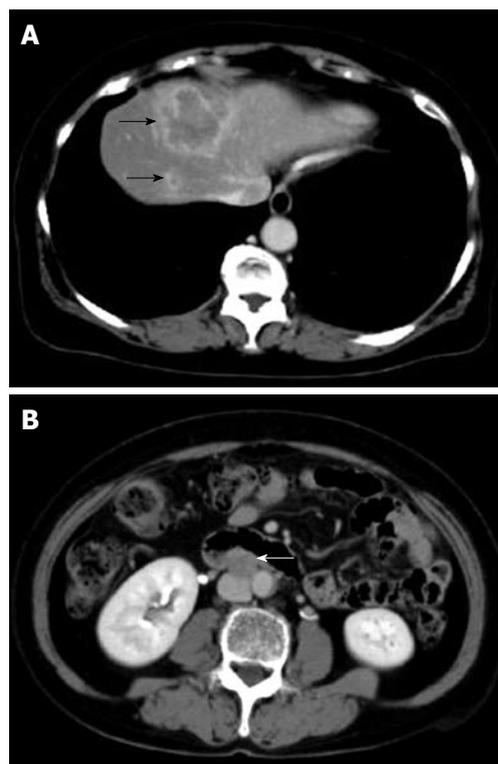
## CASE REPORT

A 65-year-old woman was examined in a follow-up visit at a local hospital for fatty liver 1 year post-diagnosis on October 30, 2007. Although she exhibited no symptoms, abdominal ultrasonography revealed tumors in the liver, and she was referred to our hospital on November 21, 2007. Her past medical and family histories were not remarkable. She did not consume alcohol. On admission, her conjunctivae were not jaundiced, and heart and respiratory sounds were normal. The liver, spleen and tumor were not palpable.

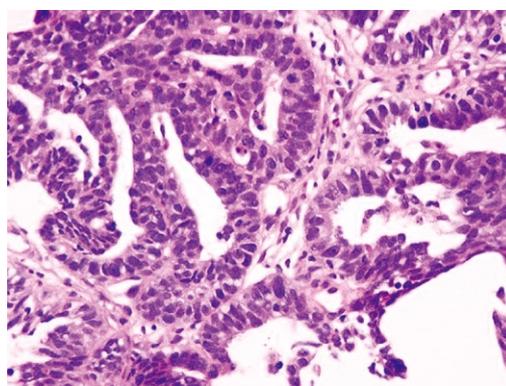
Laboratory findings on admission were as follows: aspartate aminotransferase 24 IU/L (normal, 13-33 IU/L), alanine aminotransferase 39 IU/L (normal, 6-27 IU/L),  $\gamma$ -glutamyl transpeptidase 26 IU/L (normal, 10-47 IU/L) and alkaline phosphatase 240 IU/L (normal, 115-359 IU/L). Assays for hepatitis B surface antigen and hepatitis C virus antibody were negative. All tumor markers tested showed normal values: specifically, 1.8 ng/mL for CEA (criterion, < 5.0), 5.8 U/mL for CA 19-9 (criterion, < 37), 6.2 ng/mL for AFP (criterion, < 10.0), 10 mAU/mL for PIVKALII (criterion, < 40).

Abdominal ultrasound and computed tomography (CT) scan with contrast enhancement revealed a low-density mass, measuring 50 and 15 mm in diameter, in segment VIII of the liver. The tumor did not show enhancement during the arterial phase but did show peripheral rim enhancement during the portal phase. The CT scan also revealed an enlarged lymph node in the para-aorta (Figure 1). The portal vein was intact until the third branch. Chest X-ray and a CT scan revealed no pleural metastasis.

Ultrasonography-guided fine needle biopsy was performed at the main tumor of the liver on November 22, 2007. Microscopic examinations showed tubular adenocarcinoma (Figure 2). Gastroendoscopy and colonoscopy did not show any other malignancy. Subsequently, the tumor was diagnosed as ICC with intrahepatic metastasis and



**Figure 1** Computed tomography (CT) of the tumor on first admission. CT shows a low-density lesion with rim enhancement in segment VIII of the liver (black arrows) (A) and enlarged lymph node in the para-aorta (white arrow) (B).



**Figure 2** Hematoxylin-eosin staining. Cuboidal cancer cells with chromatin-rich nuclei had proliferated invasively, forming indistinct glandular structures.

lymph node metastasis. Since the patient was inoperable for lymph nodes metastasis, she underwent systemic chemotherapy with gemcitabine (800 mg/m<sup>2</sup>, 30 min iv infusion), which was administered on days 1, 8 and 15, and repeated every 4 wk. Six months after initiation of chemotherapy, CT revealed ICC progression in the liver and pleural dissemination with pleural effusion (Figures 3 and 4). The patient was admitted to our hospital for anticancer drug sensitivity testing on June 9, 2008. Cultures and ATP assays were performed as described previously<sup>[5]</sup>. Cell viability was evaluated by measuring the intracellular ATP level using bioluminescence as described by Kangas *et al*<sup>[10]</sup>. Based on the sensitivity test results, we elected to administer systemic chemotherapy combining S-1 and CDDP (S-1 80 mg/m<sup>2</sup> per day orally administered for 3 wk,

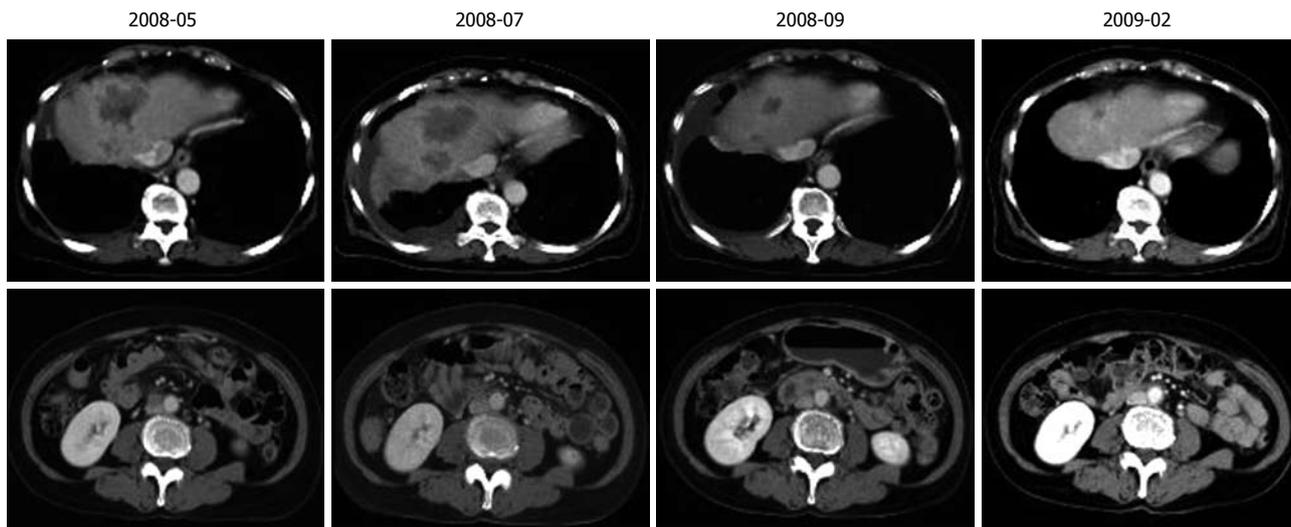


Figure 3 Reduction in tumors in the liver and lymph node following chemotherapy.

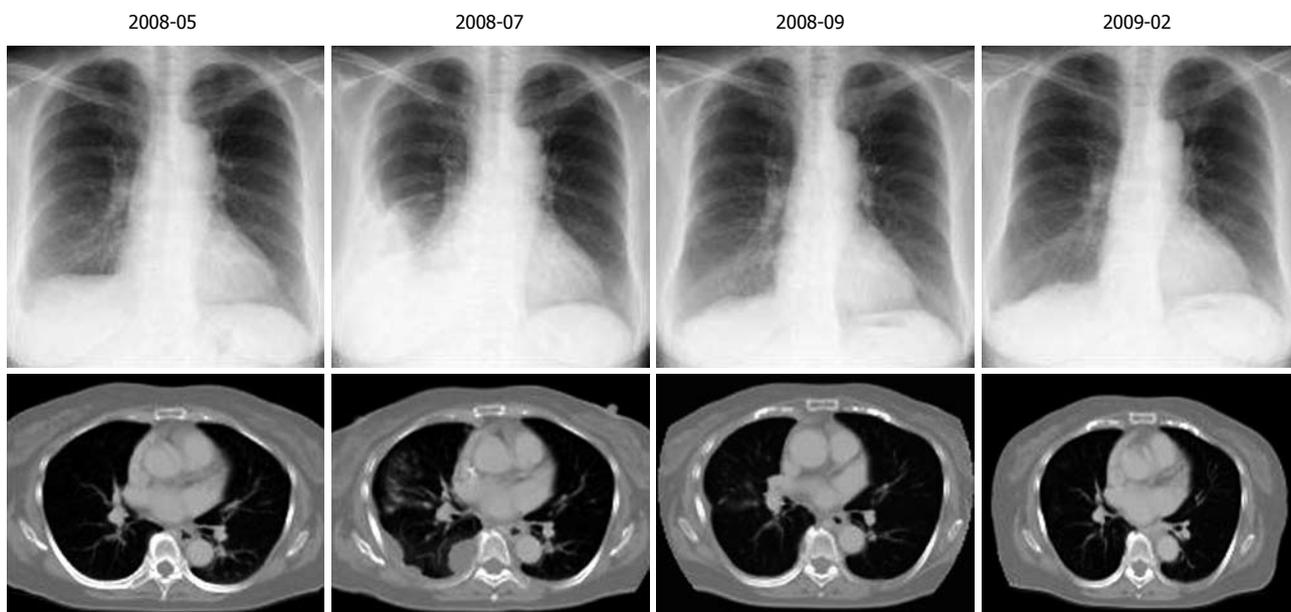


Figure 4 Decreased pleural metastases following chemotherapy.

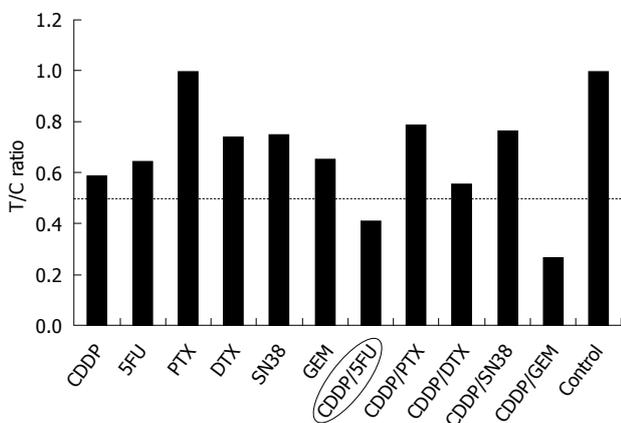


Figure 5 Cell viability evaluated by measuring the intracellular ATP level using bioluminescence. The T/C (treated/control) ratio, or the ratio of ATP quantity of a tumor sample treated with anticancer drugs to that of the control, was used as the index of chemosensitivity.

CDDP 60 mg/m<sup>2</sup> iv infusion administered on day 8, and repeated every 5 wk) (Figure 5). She had symptoms of cough in July 2008. Two months into the second chemotherapy treatment, she had no symptoms and CT revealed a reduction in the tumors of the liver and lymph node and a decrease in pleural effusion (Figures 3 and 4). Side effects and complications of chemotherapy were tolerable, and she experienced only grade-1 nausea. CDDP added to S-1 might worsen nausea. After eight cycles of the second chemotherapy regime, 17 mo after ICC diagnosis, she is alive and well with no sign of recurrence.

## DISCUSSION

Although radical surgery is considered the most effective therapy for ICC, only 20% of patients present with resectable disease<sup>[11]</sup>. Without surgery, ICC is a rapidly fatal disease with a 5-year survival rate of less than 5%<sup>[12]</sup>, while

in curative resections the 5-year survival rate approaches 20%-35% in cases with negative surgical margins<sup>[13]</sup>. In the present case, to our surprise, the patient with unresectable ICC survived more than 17 mo with chemotherapy. The role of systemic chemotherapy in unresectable ICC is undefined. Although chemotherapy has been reported to be more beneficial than the best supportive care<sup>[14]</sup>, no standard chemotherapy regimen has yet been identified. Most promising approaches involve the use of single agents such as gemcitabine, which has been shown to be an effective therapy for BTC in phase II trials<sup>[15,16]</sup>. Response rates for gemcitabine ranged from 8% to 36% and overall survival times from 6.3 to 16 mo. S-1 is a novel oral fluoropyrimidine agent, which contains tegafur, gimeracil and oteracil potassium. Gimeracil is a competitive inhibitor of dihydropyrimidine dehydrogenase and achieves higher concentrations of 5-fluorouracil in plasma and tumor tissues<sup>[17]</sup>. Fluoropyrimidines have known synergistic effects with CDDP<sup>[18]</sup>, and combinations of S-1 and CDDP are reportedly effective therapies for patients with advanced BTC in phase II trials<sup>[19]</sup>. In the present case, we selected gemcitabine as first line chemotherapy, because gemcitabine has been extensively evaluated in patients with metastatic BTC.

Chemosensitivity testing using surgical material is an established method for evaluation of tumor response prior to chemotherapy. Several methods have been established for measurement of cancer cell viability. However, it has seldom been performed in ICC, since ICC occurs more rarely than gastrointestinal malignancies and the prognosis of ICC is poor. There have been few reports of chemosensitivity testing using surgical material for ICC without the ATP assay<sup>[20-22]</sup>. The ATP assay is a highly sensitive and precise method used to measure cell viability. The ATP assay has been shown to be a good predictor of response to chemotherapy in other tissue types, such as breast cancer, ovarian cancer, colorectal adenocarcinoma, melanoma and lung cancer. The assay predicts that anticancer drugs with a lower treated/control (T/C) ratio (< 0.6) are more sensitive. In the present case, based on the results of chemosensitivity testing, we selected systemic chemotherapy combining S-1 and CDDP. Ultimately, *in vitro* ATP chemosensitivity testing was useful. Since 5-fluorouracil has considerable toxic effects and entails the inconvenience of continuous iv infusions, we selected S-1 as an alternative to 5-fluorouracil. Although the T/C ratio combining gemcitabine and CDDP was more sensitive than that combining S-1 and CDDP, we did not select the former combination since gemcitabine administration on its own did not halt disease progression.

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CASE REPORT

## Recurrent massive bleeding due to dissecting intramural hematoma of the esophagus: Treatment with therapeutic angiography

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### Abstract

Spontaneous or traumatic intramural bleeding of the esophagus, which is often associated with overlying mucosal dissection, constitutes a rare spectrum of esophageal injury called dissecting intramural hematoma of the esophagus (DIHE). Chest pain, swallowing difficulty, and minor hematemesis are common, which resolve spontaneously in most cases. This case report describes a patient with spontaneous DIHE with recurrent massive bleeding which required critical management and highlights a potential role for therapeutic angiography as an alternative to surgery.

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**Key words:** Esophagus; Intramural hematoma; Therapeutic angiography

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dissecting intramural hematoma of the esophagus: Treatment with therapeutic angiography. *World J Gastroenterol* 2009; 15(41): 5232-5235 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5232.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5232>

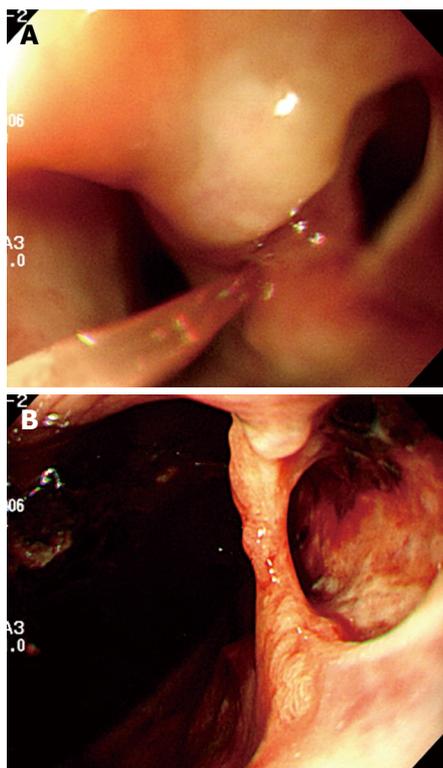
### INTRODUCTION

Dissecting intramural hematoma of the esophagus (DIHE) is a rare but well-known esophageal injury<sup>[1,2]</sup>. It is characterized by a concentric or eccentric intramural hematoma associated with dissection of the esophageal wall<sup>[2]</sup>. Forceful vomiting, mechanical insult, and underlying coagulopathy are common causes. It can also occur spontaneously without any evident cause<sup>[3]</sup>. DIHE is considered a benign disease. The hemorrhage from DIHE does not have clinically significant consequences, although in rare cases massive bleeding with hypovolemic shock can occur. We report a case of DIHE presenting as recurrent massive intraluminal bleeding that was treated by transarterial embolization.

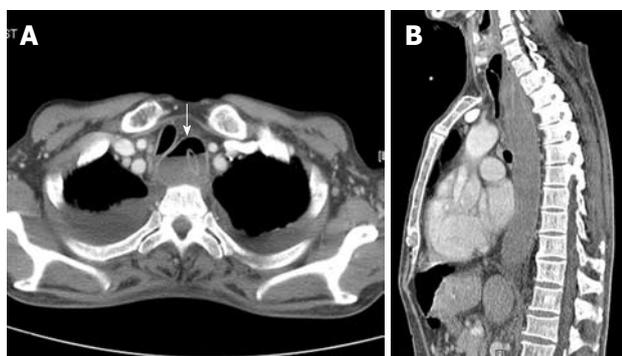
### CASE REPORT

A 57-year-old man visited the emergency room complaining of a sore throat and difficulty swallowing for 5 d. He had a history of alcoholic liver cirrhosis (Child-Pugh class B), but he continued drinking. Laryngoscopy and cervical computed tomography (CT) revealed severe laryngopharyngitis. Upper gastrointestinal endoscopy showed marked laryngeal edema and a small ulceration in the upper esophageal sphincter. There was no intraluminal mass, except for small varices in the distal esophagus. The gastric mucosa was moderately congested. The patient denied any history of cervical trauma or instrumentation. He was treated medically with antibiotics, and his initial symptoms subsided within 5 d.

One week later, the patient complained that he regurgitated blood from the throat. He continued to spit up small volumes of fresh blood repeatedly. His hemoglobin dropped from 13.9 to 6.5 g/dL, and 4 U of packed red cells were transfused. Emergency endoscopy revealed a small opening in the cervical esophagus and



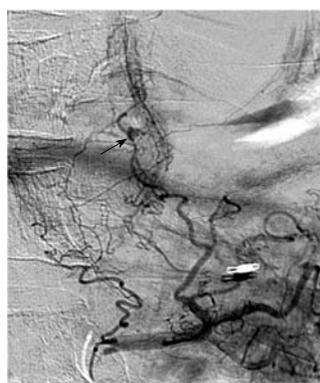
**Figure 1 Endoscopic view.** A: A small opening in the cervical esophagus; B: Mucosal bridging with a large mucosal defect in the esophagogastric junction.



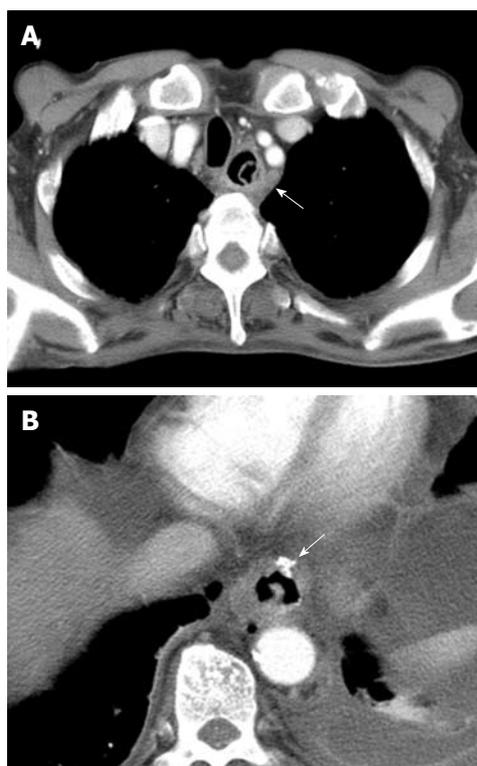
**Figure 2 Chest CT images.** A: The transverse view of the proximal esophagus shows a concentric intramural hematoma and mucosal dissection with an air-fluid level in the false lumen (arrow). Bilateral pleural effusions are seen; B: The sagittal view shows an extensive intramural hematoma of the esophagus.

mucosal bridging with a large mucosal defect around the esophagogastric junction (Figure 1). Active bleeding was detected from a vessel exposed on the ulcer base in the cardia. After hemostasis with endoscopic clipping, his vital signs and hemoglobin stabilized. Chest CT performed because of the esophageal lesions revealed dissection of the wall and a large circumferential intramural hematoma in the esophagus (Figure 2). Based on the endoscopy and chest CT findings, DIHE with cardiac ulcer bleeding was diagnosed. The patient was treated conservatively without oral intake.

Eight days later, the hematemesis recurred. Conservative treatment with massive transfusion of packed red cells and fresh frozen plasma was ineffective. Endoscopy



**Figure 3 Celiac angiography** showing a hyperstaining pseudoaneurysmal lesion (arrow) in an esophageal branch of the left gastric artery. The branch was embolized using glue and lipiodol.



**Figure 4 Follow-up chest CT.** A: The transverse view of the proximal esophagus showing a resolved intramural hematoma and improved dissection (arrow); B: The embolized lesion is seen as focal lipiodol uptake in the distal esophagus (arrow).

showed no bleeding at the previously treated site. However, continuous oozing of blood from the distal esophagus was observed. His vital signs were unstable.

Since surgery was very risky due to underlying liver cirrhosis, less invasive celiac angiography was performed for hemostasis. A small hyperstaining pseudoaneurysmal lesion was observed at an esophageal branch of the left gastric artery (Figure 3). The branch was embolized using glue and lipiodol. After the procedure, the patient's vital signs and hemoglobin stabilized. However, he experienced a few bouts of minor hematemesis 10 d later. Transarterial embolization using glue and coils was reattempted, and no further bleeding occurred. Follow-up chest CT 4 wk later showed a resolving hematoma and double lumens separated by the dissected mucosa. The embolization site was seen as focal lipiodol uptake in the distal esophagus (Figure 4). Endoscopic examination

Table 1 Summary of 11 patients with dissecting intramural hematoma of the esophagus presenting major hemorrhage

Ref.	Sex/age (yr)	Underlying diseases	Precipitating factor	Primary symptoms	Treatment	Outcome
Atefi <i>et al</i> <sup>[9]</sup>	M/29	Diabetes Pneumonia Hemodialysis	Retching	Hematemesis	Conservative	Died
Kerr <sup>[15]</sup>	F/72	Hypertension	None	Epigastric pain	Conservative	Resolved
Ortiz <sup>[16]</sup>	F/80	None	Food (rice)	Chest pain, dysphagia	Surgical	Died
Freeman <i>et al</i> <sup>[14]</sup>	F/59	None	None	Chest pain	Conservative	Resolved
de Vries <i>et al</i> <sup>[12]</sup>	M/88	None	Minor head trauma	Retrosternal pain	Conservative	Resolved
Folan <i>et al</i> <sup>[13]</sup>	M/58	Alcoholism	Vegetable (broccoli)	Dysphagia	Surgical	Resolved
Takaoka <i>et al</i> <sup>[17]</sup>	F/87	Suppurative cholangitis	Nasobiliary catheter	Hematemesis	Conservative	Resolved
Cullen <i>et al</i> <sup>[11]</sup>	F/74	Hypertension	Heparin IV	Chest pain, dysphagia	Conservative	Resolved
Yamashita <i>et al</i> <sup>[18]</sup>	F/67	Cerebral aneurysm	Heparin IV Retching	Hematemesis	Conservative	Resolved
Bandyopadhyay <i>et al</i> <sup>[10]</sup>	M/86	Hypertension Ischemic heart disease	Warfarin	Hematemesis	Conservative	Died
Present case	M/57	Liver cirrhosis	None	Hematemesis	Therapeutic angiography	Resolved

was declined. Supportive care with parenteral nutrition was maintained. The patient was discharged without complications after 6 wk.

## DISCUSSION

The esophagus is susceptible to various extrinsic injuries (from ingested foods, instruments, and bougienage) or intrinsic sheering forces induced by retching, vomiting, or coughing. DIHE lies in the spectrum of esophageal injuries between a mucosal tear (Mallory-Weiss syndrome) and a transmural laceration (Boerhaave's syndrome)<sup>[2,4]</sup>. Although these syndromes are usually associated with severe vomiting, DIHE is not always caused by emesis<sup>[4]</sup>. One out of five patients reports no history of trauma. However, an underlying coagulopathy is found in many patients with so-called spontaneous DIHE<sup>[1]</sup>. Portal hypertension and endoscopic variceal sclerotherapy are also associated with DIHE in cirrhotic patients<sup>[5]</sup>. Although the direct cause was not clear in this present case, the cervical esophageal ulcer and underlying portal hypertension may have been precipitating factors.

Acute chest pain is a common presenting symptom and should be differentiated from acute myocardial infarction and aortic dissection<sup>[6]</sup>. Hematemesis and difficulty swallowing may ensue, and these are helpful for differentiating from other critical diseases. The typical triad of DIHE (chest pain, dysphagia, and hematemesis) is evident only in one third of cases<sup>[1]</sup>. Therefore, the rarity of DIHE and atypical symptoms can delay correct diagnosis. In our case, repeated hematemesis with hypovolemia was the only clinical feature.

DIHE is generally benign. Most patients recover fully with conservative management<sup>[1]</sup>. Esophageal obstruction and major bleeding constitute two major complications of DIHE. Esophageal obstruction by the hematoma may cause or aggravate difficulty swallowing. Successful endoscopic decompression or surgical treatment have been reported in such cases<sup>[7,8]</sup>.

A major intraluminal hemorrhage seems to be caused by overlying mucosal rupture and evacuation of

the hematoma, however, the exact mechanism remains unclear. Although half of patients are reported to experience hematemesis, the volume of bleeding is usually small, and major bleeding with hypovolemic shock is uncommon.

We reviewed 119 patients who were presented in 87 English-language reports since 1968. Major bleeding was defined as more than 500 mL hematemesis with hypovolemic shock or bleeding that required transfusion of at least 4 U. Eleven cases (9.2%), including the present case, were collected and summarized in Table 1<sup>[9-18]</sup>. Seven cases were elderly patients (five females and two males). The bleeding was attributed to anticoagulation therapy in three cases<sup>[10,11,18]</sup>. The majority were treated conservatively. However, this type of treatment was not always effective. An elderly patient who was treated conservatively died after 5 d<sup>[10]</sup>. Two patients were treated with surgery<sup>[13,16]</sup>; one of them had a 5-cm longitudinal tear with a pulsatile bleeding vessel between 25 and 30 cm from the alveolar ridge on endoscopy, and the active arterial bleeder was identified and treated with an emergency thoracotomy<sup>[13]</sup>. In our case, angiography located and treated the bleeding site at an esophageal branch of the left gastric artery. Extensive DIHE and major bleeding might be caused by a small bleeding artery. In this situation, therapeutic angiography may be effective.

Therapeutic angiography has been used to control major non-variceal gastrointestinal bleeding and has been reported to be safe, effective, and durable<sup>[19]</sup>. It is usually considered when endoscopic therapy has failed or when emergency surgery carries a high risk of mortality. It has also been effective in the hemostasis of uncontrolled Mallory-Weiss syndrome<sup>[20]</sup> and in the treatment of a giant gastric intramural hematoma<sup>[21]</sup>.

In conclusion, massive intraluminal bleeding can be a complication of DIHE. Although most bleeding in DIHE can be managed medically, prompt, appropriate treatment should be attempted in hemodynamically unstable patients. In such cases, therapeutic angiography may be a useful treatment alternative to surgery.

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CASE REPORT

## Successful endoscopic removal of a giant upper esophageal inflammatory fibrous polyp

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**Author contributions:** Zhang J and Zhang ST contributed equally to this article; Zhang J directly participated in the operation, collected the clinical data and wrote the paper; Zhang ST designed the research; Hao JY also directly participated in the study, including acquisition of data; Li SWH revised the manuscript for intellectual content.

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### Abstract

Giant esophageal inflammatory fibrous polyp (especially > 17 cm in size) is seen rarely. Endoscopic removal has been reported rarely because the procedure is technically demanding and the hemostasis is difficult to ascertain. Here, we describe a case of a giant upper esophageal inflammatory fibrous polyp that was resected successfully by endoscopy.

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**Key words:** Digestive system endoscopic surgery; Polyps; Endosonography; Esophageal neoplasms; Hemostasis; Endoscopic; Middle aged

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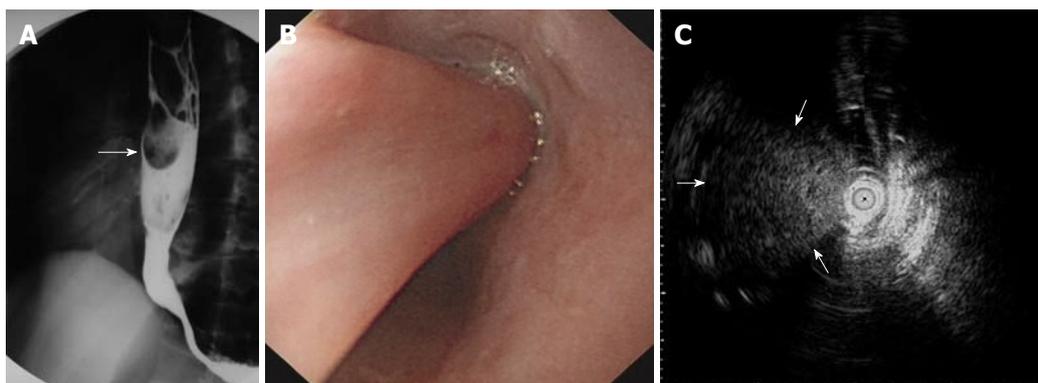
### INTRODUCTION

Giant esophageal inflammatory fibrous polyp (especially > 17 cm in size) is seen rarely<sup>[1]</sup>. Surgical excision is usually advised<sup>[2-5]</sup>. Endoscopic removal has been reported rarely<sup>[6]</sup> because the procedure is technically demanding and hemostasis is difficult to ascertain. Here, we described a giant upper esophageal inflammatory fibrous polyp that was resected successfully by endoscopy.

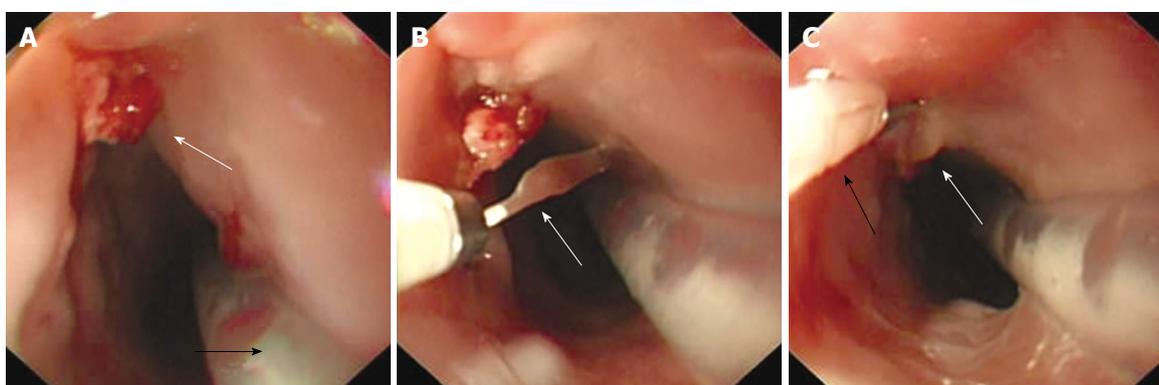
### CASE REPORT

A 50-year-old man presented to our hospital with a 6-mo history of progressive difficulty in swallowing, initially, solid and then liquid food for the past 2 mo. Physical examination and blood tests, including coagulation parameters, were normal. Barium swallow revealed a 17-cm polypoid filling defect inside the esophagus that moved with deglutition (Figure 1). Contrast thoracic computed tomography (CT) revealed a localized isodense upper esophageal lesion, which was free from the lower part of the esophagus. The esophageal wall was intact and there was no evidence of infiltration into adjacent organs or any mediastinal lymphadenopathy. Gastroscopy showed a giant polyp with smooth overlying mucosa, extending from 18 to 35 cm (measured from the incisors), and endoscopic ultrasonography (EUS) revealed well-distributed, low-echo-level lesions at the root of the polyp, excluding echoless lumen-like structures (no large blood vessels inside the lesions) (Figure 1). Therefore, the esophageal polyp was a benign lesion. Endoscopic removal of the polyp with an electro-surgical snare was attempted, using the ICC 200 (ERBE, Tubingen, Germany). The procedure was performed under general anesthesia with elective intubation and airway protection. Equipment for hemostasis, including hemoclips, Coagrasper, injection needle and adrenaline for injection was prepared before the procedure.

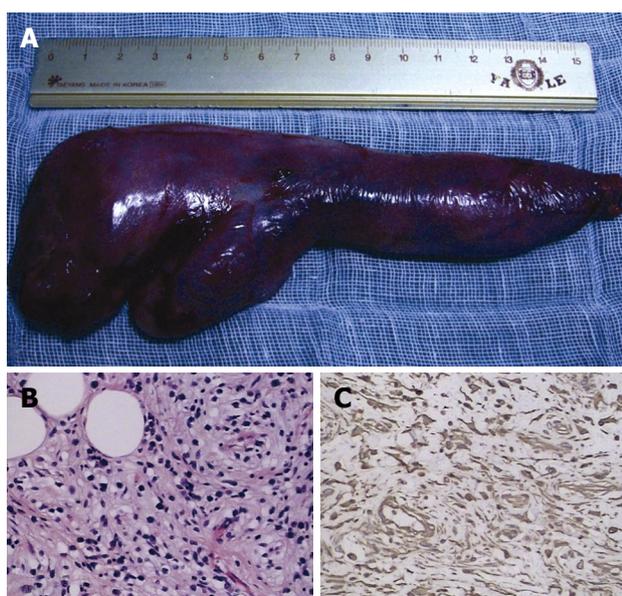
Firstly, we inserted a detachable loop of appropriate stiffness and size through the biopsy channel. Then, the detachable loop was placed from the base to the neck of the esophageal polyp. The loop was closed gradually and the polyp was lifted away from the esophageal wall. Polypectomy was performed and air was inflated to distend the esophagus. The setting of the electro-surgical units was as follows: COAG mode (effect 350 W) was first applied for 2 s, so that the small blood vessels over



**Figure 1 Imaging of esophageal polyp.** A: Barium swallow showed a giant esophageal polyp (arrow), which was free from the middle and lower esophageal wall; B: Gastroscopy showed the polyp with smooth overlying mucosa (at 18 cm from the incisors); C: EUS revealed well-distributed, low-echo-level lesions at root of the polyp (white arrows), excluding echoless lumen-like structures.



**Figure 2 Esophageal polyp removal.** A: Residual polyp (white arrow) after polypectomy at the left wall of the esophagus (18 cm from incisor). A nasogastric tube (black arrow) used for suction (size 16 Fr) was placed over the right wall of the esophagus; B: Hemoclips (white arrow) were applied to the residual polyp; C: After application of the hemoclips (black arrow) to the residual polyp (white arrow).



**Figure 3 Polypectomy specimen and its pathology.** A: The resected esophageal lesion measured 17 cm in length, and the neck, body and tail of the lesion measured 1, 3 and 5 cm, respectively; B: HE staining (original magnification,  $\times 200$ ) showed the presence of lots of fibroblasts, with some acidophilic cells, plasma cells and adipose cells; C: Immunostaining (original magnification,  $\times 200$ ) for vimentin showed strong staining of the cells.

the mucosa were coagulated. Then, we employed the endo-cut mode (effect 380 W) to CUT. Near the end of polypectomy, COAG mode was resumed to minimize bleeding from the central blood vessel. Adequate air insufflation and distension of the esophagus minimized the risk of burning and esophageal perforation. Only minor bleeding was noted at the polypectomy site. The hemostasis was achieved securely with 1:10 000 adrenaline injection and application of two hemoclips over the polypectomy site (Figure 2).

In addition, a 16-Fr nasogastric tube was placed to remove all the blood and gastric fluid during the procedure (Figure 2). If the esophageal polyp failed to be removed or there was any complication, an on-site thoracic surgeon assessed the patient urgently for operation.

The resected esophageal polyp specimen (Figure 3) was retrieved with the snare and sent to the pathology laboratory for pathological examination. Hematoxylin and eosin (HE) staining of the removed esophageal specimen showed the presence of lots of fibroblasts, with some adipocytes, plasma cells and acidophilic cells (Figure 3). Immunostaining revealed CD 117 (-), CD 34 (-), S-100 (-), desmin (-), actin (-), cytokeratin (-), HMB45 (-) and vimentin (+) (Figure 3). The histological diagnosis was

inflammatory fibrous polyp. The patient remained well and was discharged 1 wk after the procedure. Follow-up gastroscopy 4 wk later was normal.

## DISCUSSION

Inflammatory fibrous polyp, also known as inflammatory pseudotumor, is a benign intraluminal tumor that consists of a mixture of inflamed fibrous, granulation tissue and lipomatous elements, which is covered by normal squamous epithelium<sup>[7]</sup>. Giant esophageal inflammatory fibrous polyp (especially > 17 cm in size) is seen rarely<sup>[1,8]</sup>. The presenting symptoms of dysphagia and sensation of a mass in esophageal inflammatory fibrous polyp are the same as in other esophageal tumors (such as leiomyoma and gastrointestinal stromal tumor), unless there is development of regurgitation of the polyp through the mouth or asphyxiation<sup>[9]</sup>. Some cases may stay asymptomatic or with heartburn for a long time<sup>[10,11]</sup>. Inflammatory fibroid polyps should be considered in the differential diagnosis of submucosal and polypoid esophageal masses, although distinctive radiographic features are not found<sup>[12]</sup>. Usually, the diagnosis is made by imaging or endoscopic studies. Barium-enhanced contrast of the esophagus usually shows a sausage-shaped mass with multiple filling defects, which originates in the cervical esophagus and extends to the lower esophagus<sup>[10]</sup>. Endoscopy usually shows an intraluminal mass that is mobile and covered with normal mucosa. Careful examination of the upper esophageal sphincter may reveal the stalk of the pedunculated mass. It is not difficult to distinguish esophageal inflammatory fibrous polyp from leiomyoma, which is usually relatively flat, and non-pedunculated intramural lesions in the middle and lower third of the esophagus<sup>[13]</sup>. EUS has been reported as a method to demonstrate the submucosal origin of anal polyps<sup>[14,15]</sup>.

As a result of its special origin, there may be uncontrollable bleeding during endoscopic resection. Endoscopic removal of giant upper esophageal lesions requires thorough assessment before the procedure. Therefore, multiple modalities (barium, CT, EUS and gastroscopy) are important to delineate the nature and origin of the lesion. EUS provides information on the diameter of the polyp as well as its vascularity and insertion point<sup>[6]</sup>. Special precautions during hemostasis and elective airway protection are necessary to prevent bleeding and aspiration. The placement of the nasogastric tube, adequate insufflation and distension of the esophagus can minimize the risk of burning and esophageal perforation. The site of snare polypectomy should be kept away from the base of stalk to prevent esophageal perforation. Advances in the techniques of endoscopic treatment and the improvement

of endoscopic accessories make endoscopic removal of giant esophageal polyps feasible. Post-polypectomy hemostasis can be achieved with adrenaline injection and hemoclips. If hemostasis fails, one can use Coagrasper to coagulate even a small area of bleeding.

In conclusion, with thorough assessment with multiple imaging modalities, and the availability of good endoscopic accessories, giant upper esophageal inflammatory fibrous polyp can be resected by endoscopy safely and successfully.

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## Pedunculated hepatocellular carcinoma and splenic metastasis

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### Abstract

Only a few cases of pedunculated hepatocellular carcinoma (P-HCC) have been reported in the literature. The common sites of extrahepatic metastases in patients with HCC are the lungs, regional lymph nodes, kidney, bone marrow and adrenals. Metastasis to spleen is mostly *via* hematogenous metastasis, direct metastasis to spleen was very rare. We report a case of P-HCC presenting as a left upper abdominal lesions which involved the spleen that was actually a P-HCC with splenic metastasis. This case is unique as P-HCC directly involved the spleen which is not *via* hematogenous metastasis.

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**Key words:** Pedunculated hepatocellular carcinoma; Splenic metastasis; Hematogenous metastasis; Direct metastasis; Splenectomy

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### INTRODUCTION

The pedunculated hepatocellular carcinoma (P-HCC) which protrudes from its pedicel or presents as epibiotic mass almost making no invasion into the liver, is a rare exception to the gross type<sup>[1,2]</sup>. To date, only a few cases have been reported<sup>[2-4]</sup>. The common sites of extrahepatic metastases in patients with hepatocellular carcinoma (HCC) are the lungs, regional lymph nodes, kidney, bone marrow and adrenals, which is *via* hematogenous metastasis. The P-HCC directly invading the spleen not *via* hematogenous metastasis is extremely rare. In this report, we describe a case of P-HCC which directly involved the spleen.

### CASE REPORT

A 68-year-old man with HBV-related cirrhosis was admitted to our hospital because of left flank pain and loss of weight for a forty-day duration. A mass lesion could be touched in left upper abdomen. AFP level was 166.02 ng/mL, CA125, CA199 and CEA were negative. HBVDNA level was  $1.51 \times 10^4$  copies/mL. Sonographic and CT scan showed a 17 cm  $\times$  14 cm  $\times$  10 cm tumor between left hepatic lobe and spleen, which also involved the upper pole of spleen and almost made no invasion into the liver (Figure 1). Celiac and hepatic arteriography displayed mass lesions taking blood from left hepatic artery, splenic artery and left inferior phrenic artery, and transarterial chemoembolization was performed (Figure 2). Image-guided biopsy of tumor was consistent with HCC.

At operation, mild cirrhosis was found in the liver, a large tumor lied in the left upper abdomen between left hepatic lobe and spleen. The upper pole of spleen was involved, almost making no invasion into the liver, gastrointestinal and pancreas (Figure 3). He underwent spleen, tumor and partial left hepatic lobe resection in January 2008. The loss of blood was 1000 mL in total. HCC and splenic metastasis were confirmed by pathological examination (Figure 4). The postoperative clinical course was uneventful, with a negative follow-up for clinical and radiological investigation at 17 mo after surgery.

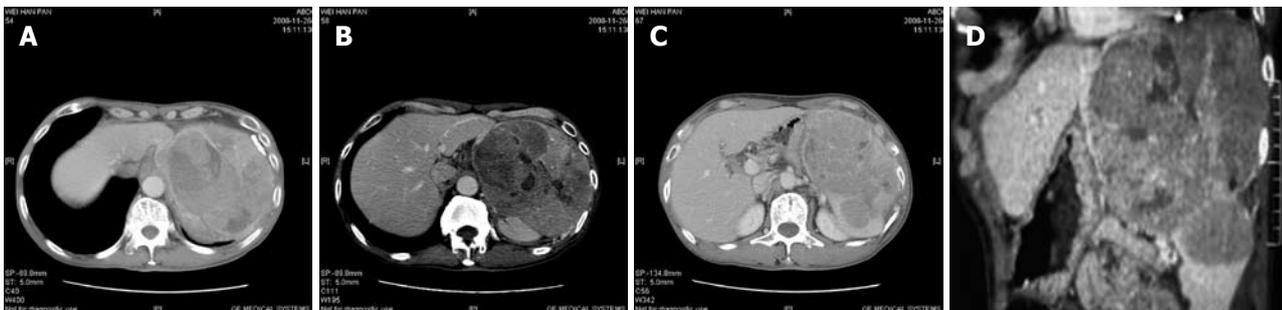
### DISCUSSION

The P-HCC has been reported to occur in 0.24%-3.0% of all HCC patients<sup>[5]</sup>. Hematogenous metastasis to spleen

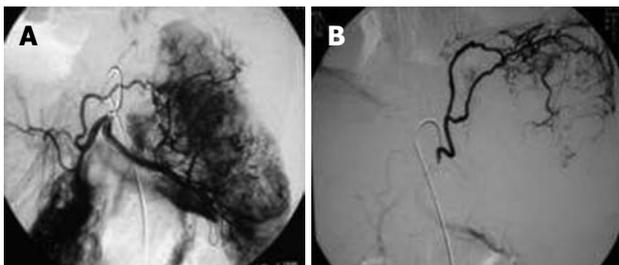
**Table 1** Previous cases reported in literature with HCC and splenic metastasis

Authors	Age (yr)/sex	Metastasis type	Clinical manifestations	Intrahepatic metastasis at time of splenic metastasis	Metastasis to other organs
Filik <i>et al</i> <sup>[6]</sup>	62/W	H	Severe ascites and abdominal pain	Multiple HCC	None
	47/M	H	Right flank of pain	HCC	None
Hanada <i>et al</i> <sup>[7]</sup>	59/M	H	Asymptomatic	Multiple HCC	Adrenal gland
	69/W	H	Not described	None	None
	67/M	H	Not described	None	Lung
Yamamoto <i>et al</i> <sup>[11]</sup>	68/W	H	Abdominal fullness	Single HCC	None
	61/M	H	Swelling cervical lymph nodes	HCC	Cervical lymph nodes
Fujimoto <i>et al</i> <sup>[12,13]</sup>	62/M	H	LUQ mass	None	None
	62/M	H	Spontaneous rupture of spleen	HCC	None
Horie <i>et al</i> <sup>[14]</sup>	62/W	H	Spontaneous rupture of spleen	HCC	None
Kato <i>et al</i> <sup>[17]</sup>	55/M	H	Not described	HCC	None
Iwaki <i>et al</i> <sup>[8]</sup>	60/M	H	Asymptomatic	Multiple HCC	Lung and jejunal
Sumiya <i>et al</i> <sup>[16]</sup>	78/M	H	Spontaneous rupture of spleen	None	Lung
Hayashi <i>et al</i> <sup>[15]</sup>	76/M	H	Asymptomatic	None	None
Hama <i>et al</i> <sup>[9]</sup>	61/M	H	Asymptomatic	Multiple HCC	Lung
Nakamura <i>et al</i> <sup>[10]</sup>	54/M	H	Asymptomatic	Multiple HCC	Lung

HCC: Hepatocellular carcinoma; H: Hematogenous metastasis; LUQ: Left upper quadrant.



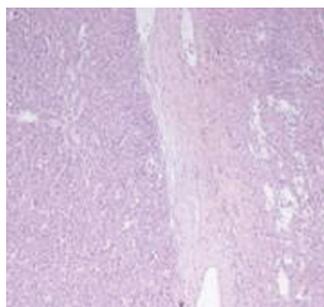
**Figure 1** CT scan in abdomen showing a mass tumor between left hepatic lobe and spleen directly involving the upper pole of spleen and almost making no invasion into the liver (A-D).



**Figure 2** Celiac and hepatic arteriography confirmed the mass lesions taking blood from left hepatic artery and splenic artery (A) and inferior phrenic artery (B).



**Figure 3** Postoperative photography showing the lesions directly involving the upper pole of spleen.



**Figure 4** Histopathology showing the splenic metastasis of hepatocellular carcinoma (HE, × 40).

is very rare with a reported prevalence of 0.7%-0.8% in HCC patients<sup>[6,7]</sup>, but it is probably more common than direct metastasis.

Preoperative differential diagnosis between metastatic or primary splenic tumors is difficult. High levels of AFP (> 1210 ng/mL) may contribute to the diagnosis of P-HCC. With improvement in diagnostics such as angiography and CT scan, the preoperative diagnosis is feasible in patients with negative or mild increase of AFP level. In this patient, selective celiac arteriography showed a tumor fed by hepatic artery, splenic artery and left inferior phrenic artery, from which we can judge the blood

supply and diagnose the tumor. Image-guided biopsy of tumor was utilized to confirm the presence of HCC when the imaging study could not draw a conclusion.

The intrahepatic metastasis from HCC occurs mostly commonly *via* the portal vein, which is followed by hematogenous metastasis to the lungs and bone, lymph node metastasis, direct metastasis and peritoneal metastasis. Previous cases in the literature with HCC and splenic metastasis are summarized in Table 1<sup>[6-17]</sup>. Metastasis to spleen occurred hematogenously in previous cases. In the present case, the splenic metastasis occurred directly. The cumulative survival rates of extrahepatic metastasis of HCC were very poor. Such lesions in the case may not represent remote metastases, but they are actually HCC with extended invasion to the spleen. Whether splenic metastasis happens directly or hematogenously should be distinctive and the resection of P-HCC and splenic metastasis can be curative in the former. The distinction between the two is important, as it affects the stage, prognosis and management of the patient. Although the long-term outcome of resection for such splenic metastasis is unknown, direct splenic metastasis of P-HCC can be easily controlled to obtain gross disease clearance and may achieve better long-term survival.

In conclusion, splenic metastases of P-HCC are difficult to distinguish from primary splenic tumors, even with modern imaging studies. The treatment involves resection and surgical exploration, whenever possible.

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## Vascular tumors and malformations of the colon

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### Abstract

The term "hemangioma" refers to the common tumor of infancy that exhibits rapid postnatal growth and slow regression during childhood. It may cause confusion with venous malformations that are often incorrectly called "cavernous hemangioma". Venous malformations comprise abnormally formed channels that are lined by quiescent endothelium. Accurate diagnosis is required for selecting the appropriate treatment.

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**Key words:** Hemangioma; Venous malformations; Surgery; Sclerotherapy; Colon

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### TO THE EDITOR

With regard to the article entitled "Large cavernous hemangioma in the cecum treated by laparoscopic ileocecal resection" by Huh *et al*<sup>[1]</sup> published recently on the *World Journal of Gastroenterology*, there are some pertinent considerations. In 1982, Mulliken and Glowacki<sup>[2]</sup> classified vascular lesions into vascular tumors (infantile hemangioma, rapidly involuting congenital hemangioma, non-involuting congenital hemangioma, kaposiform

hemangioendothelioma and tufted angioma) and vascular malformations (arteriovenous malformation, venous malformation, lymphatic malformation, lymphatic-venous malformation, and capillary malformation). In 1996, the International Society for the Study of Vascular Anomalies approved this classification system to establish a common language for the many different medical specialists who are involved in the management of these lesions. A great variety of vascular anomalies is incorrectly referred to as "hemangiomas" in the medical literature and a significant number of patients receive ineffective and potentially harmful treatment based on misclassification. Hemangiomas are usually not present at birth; they proliferate during the first year of life; and then they involute. They are composed of proliferating endothelial cells. Venous malformations consist of dysplastic vessels and are present on a lifelong basis. Unlike hemangiomas, there is no proliferation phase. They seem to grow because the vessels progressively dilate and they do not present a regression phase<sup>[3]</sup>. Histological findings in venous malformations consist of large, dilated, blood-filled vessels lined by flattened endothelium as reported in the article.

After close examination of the reported case of "cavernous hemangioma", we support the diagnosis of venous malformation in the cecum. Colonic venous malformations are often mistaken for tumors because of a similar presentation (from a vague blue patch to a soft blue mass as described in the article) and improper nomenclature. The term "cavernous hemangioma" is frequently used to name a venous malformation. These lesions may cause chronic and acute gastrointestinal hemorrhage.

Sclerotherapy of venous malformations is an effective treatment and seems to be the best therapeutic option, although the surgical resection is preferred for venous malformations localized in the colon in case of bleeding. Although these vascular malformations generally are incompletely resectable because of diffuse pelvic and mesenteric involvement, the goal is to abate bleeding by excluding the lesion from the gastrointestinal lumen. The minimally invasive surgical procedure performed by the authors of this article seems to be appropriate since the venous malformation was removed from the lumen. Other procedures like colectomy with mucosectomy and endorectal pull-through should be considered for diffuse venous malformations of the colorectum<sup>[4]</sup>.

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## Meetings

### Events Calendar 2009

January 12-15, 2009  
Hyatt Regency San Francisco, San Francisco, CA  
Mouse Models of Cancer

January 21-24, 2009  
Westin San Diego Hotel, San Diego, CA  
Advances in Prostate Cancer Research

February 3-6, 2009  
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)  
Second AACR Conference  
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009  
Hyatt Regency Boston, Boston, MA  
Translation of the Cancer Genome

February 8-11, 2009  
Westin New Orleans Canal Place, New Orleans, LA  
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009  
Hong Kong Convention and Exhibition Centre, Hong Kong, China  
19th Conference of the APASL  
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009  
Orlando, Florida  
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009  
Vienna, Austria  
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease  
[www.easl.ch/vienna2009](http://www.easl.ch/vienna2009)

March 13-14, 2009  
Phoenix, Arizona  
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009  
Marriott Wardman Park Hotel  
Washington, DC  
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009  
Glasgow, Scotland  
British Society of Gastroenterology (BSG) Annual Meeting  
Email: [bsg@mailbox.ulcc.ac.uk](mailto:bsg@mailbox.ulcc.ac.uk)

April 8-9, 2009  
Silver Spring, Maryland  
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009  
Colorado Convention Center, Denver, CO  
AACR 100th Annual Meeting 2009

April 22-26, 2009  
Copenhagen, Denmark  
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)  
<http://www.easl.ch/>

May 17-20, 2009  
Denver, Colorado, USA  
Digestive Disease Week 2009

May 29-June 2, 2009  
Orange County Convention Center  
Orlando, Florida  
45th ASCO Annual Meeting  
[www.asco.org/annualmeeting](http://www.asco.org/annualmeeting)

May 30, 2009  
Chicago, Illinois  
Endpoints Workshop: NASH

May 30-June 4, 2009  
McCormick Place, Chicago, IL  
DDW 2009  
<http://www.ddw.org>

June 17-19, 2009  
North Bethesda, MD  
Accelerating Anticancer Agent Development

June 20-26, 2009  
Flims, Switzerland  
Methods in Clinical Cancer Research (Europe)

June 24-27 2009  
Barcelona, Spain  
ESMO Conference: 11th World Congress on Gastrointestinal Cancer  
[www.worldgicancer.com](http://www.worldgicancer.com)

June 25-28, 2009  
Beijing International Convention Center (BICC), Beijing, China  
World Conference on Interventional Oncology  
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009  
Snowmass, CO, United States  
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009  
Aspen, CO, United States  
Molecular Biology in Clinical Oncology

August 1-7, 2009  
Vail Marriott Mountain Resort, Vail, CO, United States  
Methods in Clinical Cancer Research

August 14-16, 2009  
Bell Harbor Conference Center, Seattle, Washington, United States  
Practical Solutions for Successful Management  
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009  
Beijing International Convention Center (BICC), Beijing, China  
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists (IASGO)  
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009  
Taipei, China  
Asian Pacific Digestive Week  
<http://www.apdwcongress.org/2009/index.shtml>

October 7-11, 2009  
Boston Park Plaza Hotel and Towers, Boston, MA, United States  
Frontiers in Basic Cancer Research

October 13-16, 2009  
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States  
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009  
Versailles, France  
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009  
Boston, MA, United States  
The Liver Meeting

November 15-19, 2009  
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States  
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009  
London, UK  
Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

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Data that are not statistically significant should not be noted.  $^aP < 0.05$ ,  $^bP < 0.01$  should be noted ( $P > 0.05$  should not be noted). If there are other series of *P* values,  $^cP < 0.05$  and  $^dP < 0.01$  are used. A third series of *P* values can be expressed as  $^eP < 0.05$  and  $^fP < 0.01$ . Other notes in tables or under illustrations should be expressed as  $^1F$ ,  $^2F$ ,  $^3F$ ; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, etc., in a certain sequence.

#### Acknowledgments

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#### Format

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*English journal article (list all authors and include the PMID where applicable)*

- 1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

*Chinese journal article (list all authors and include the PMID where applicable)*

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

*In press*

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of

balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

#### Organization as author

- 4 **Diabetes Prevention Program Research Group.** Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

#### Both personal authors and an organization as author

- 5 **Vallancien G,** Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

#### No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

#### Volume with supplement

- 7 **Geraud G,** Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

#### Issue with no volume

- 8 **Banit DM,** Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

#### No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

### Books

#### Personal author(s)

- 10 **Sherlock S,** Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

#### Chapter in a book (list all authors)

- 11 **Lam SK.** Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

#### Author(s) and editor(s)

- 12 **Breedlove GK,** Schorfheide AM. Adolescent pregnancy. 2nd ed. Wicczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

#### Conference proceedings

- 13 **Harnden P,** Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

#### Conference paper

- 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

#### Electronic journal (list all authors)

- 15 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>

#### Patent (list all authors)

- 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

### Statistical data

Write as mean  $\pm$  SD or mean  $\pm$  SE.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as  $\chi^2$  (in Greek), related coefficient as *r* (in italics), degree of freedom as  $\nu$  (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose)  $6.4 \pm 2.1$  mmol/L; blood CEA mass concentration, *p* (CEA) =  $8.6 \pm 2.5$   $\mu$ g/L; CO<sub>2</sub> volume fraction, 50 mL/L CO<sub>2</sub>, not 5% CO<sub>2</sub>; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23243641.

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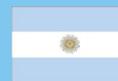
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## Potential role of chitinase 3-like-1 in inflammation-associated carcinogenic changes of epithelial cells

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presumably by activating the mitogen-activated protein kinase and the protein kinase B signaling pathways. Anti-CHI3L1 antibodies or pan-chitinase inhibitors may have the potential to suppress CHI3L1-mediated chronic inflammation and the subsequent carcinogenic change in epithelial cells.

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### Abstract

The family of mammalian chitinases includes members both with and without glycohydrolase enzymatic activity against chitin, a polymer of N-acetylglucosamine. Chitin is the structural component of fungi, crustaceans, insects and parasitic nematodes, but is completely absent in mammals. Exposure to antigens containing chitin- or chitin-like structures sometimes induces strong T helper type-I responses in mammals, which may be associated with the induction of mammalian chitinases. Chitinase 3-like-1 (CHI3L1), a member of the mammalian chitinase family, is induced specifically during the course of inflammation in such disorders as inflammatory bowel disease, hepatitis and asthma. In addition, CHI3L1 is expressed and secreted by several types of solid tumors including glioblastoma, colon cancer, breast cancer and malignant melanoma. Although the exact function of CHI3L1 in inflammation and cancer is still largely unknown, CHI3L1 plays a pivotal role in exacerbating the inflammatory processes and in promoting angiogenesis and remodeling of the extracellular matrix. CHI3L1 may be highly involved in the chronic engagement of inflammation which potentiates development of epithelial tumorigenesis

### INTRODUCTION

Chitin, the linear polymer of  $\beta$ -1,4-linked N-acetylglucosamine (GlcNAc), is a structural component of the cell walls and coatings of many organisms, and represents the second most abundant polysaccharide in nature after cellulose. Chitin efficiently protects crustaceans, insects, parasites, fungi, and other pathogens from the harsh adverse effects of their environments and/or hosts<sup>[1]</sup>. Although chitin has not been found in mammals, several mammalian proteins with homology to fungal, bacterial, or plant chitinases have been identified<sup>[2-6]</sup>. Chitotriosidase (CHIT1), and acidic mammalian chitinase (AMCase) are the only 2 of these proteins demonstrating chitinolytic (glycohydrolase) activity, while none of the other mammalian chitinases show enzymatic activity despite the retention and conservation of the substrate-binding cleft of the chitinases<sup>[7-9]</sup>. Therefore, the latter chitinases are called chitinase-like-lectins (Chi-lectins). Only recently, our group and others have identified the important biological roles of mammalian chitinases in chronic inflammatory conditions including inflammatory bowel disease (IBD), type 2 diabetes, proliferative dermatitis and allergic bronchial asthma<sup>[10-16]</sup>. One mammalian chitinase, chitinase

3-like-1 (CHI3L1, also known as YKL-40 or HC-gp39) is overexpressed in many pathological conditions including fibroblastic change in liver cirrhosis, increased deposition of connective tissue components and hyperplastic synovium in rheumatoid arthritis, and increased cellular infiltration as well as epithelial proliferation in chronic colitis. CHI3L1 is difficult to detect in the body of normal individuals and the biological role of CHI3L1 in embryonic development or distribution of this molecule in normal tissues is still largely unclear. Interestingly, significantly high amounts of CHI3L1 are detected in the involuting mammary gland upon cessation of lactation<sup>[2]</sup>, however the exact biological role of CHI3L1 in this process has not yet been elucidated. CHI3L1 can also directly regulate the critical processes of adhesion and migration in vascular smooth muscle cells *in vitro*<sup>[17]</sup>. In addition many groups have reported that CHI3L1 is expressed on many different types of human solid cancers<sup>[3,10,18,19]</sup>. Surprisingly, CHI3L1 seems to be a useful “prognosticator”, or indicator of prognosis, and may also be a potential “tumor marker” in screening and monitoring of cancer patients<sup>[20-23]</sup>. In this review article, we will discuss the potentially important biological functions of mammalian chitinases, in particular CHI3L1, during the development of chronic inflammation and the subsequent inflammation-mediated oncogenic processes in epithelial cells.

## INFLAMMATORY DISORDERS MEDIATED BY MAMMALIAN CHITINASES

Chitin, a polymer of GlcNAc, is produced copiously by a wide variety of organisms such as crustaceans (e.g. shrimp and crab), insects, fungi, amphibians, parasitic nematodes, and other marine organisms<sup>[24,25]</sup>. However, chitin is completely absent in mammals including humans and mice<sup>[26]</sup>. Therefore, it was believed for a long time that mammals were not capable of producing chitinolytic endoglucosaminases in the body, but Hollak *et al*<sup>[27]</sup> first discovered CHIT1, a functional and structural homolog to the chitinases of other species, in serum samples of Gaucher disease patients. Gaucher disease is a genetic disorder causing a lack of the lysosomal enzyme glucocerebrosidase and is characterized by accumulation of macrophage-like Gaucher cells which have glycosphingolipids in cytosolic compartments<sup>[28]</sup>. The serum level of CHIT1 is upregulated approximately 1000-fold in patients with Gaucher disease as compared to normal individuals and the enzyme shows glycohydrolase activity against chitin and other chitin-like substances (e.g. *p*-nitrophenyl chitooligosaccharides)<sup>[29]</sup>. Subsequent studies revealed that the increased CHIT1 activity in Gaucher disease patients is associated with aberrant macrophage activation<sup>[28]</sup>, and the activity can be used as a marker of disease severity and therapeutic response in Gaucher disease<sup>[30]</sup>. Boot *et al*<sup>[31]</sup> reported that approximately 6% of various ethnic groups have a homozygous mutant allele of 24 bp duplication of the CHIT1 gene, resulting in a

complete lack of CHIT1 enzymatic activity. Increased rates of nematode infection seemed to be associated with the mutation<sup>[32]</sup>. The CHIT1 cDNA was cloned by Boot *et al*<sup>[33]</sup> revealing that this molecule has a strong sequential homology to other chitinases belonging to the family 18 of glycosyl hydrolases. CHIT1 contains a complete chitin-binding domain in the C-terminus region that connects with the catalytic groove by a hinge region<sup>[34]</sup>. The chitin-binding domain of CHIT1 efficiently binds with chitin polymers as shown by structural analysis<sup>[35]</sup>. Under inflammatory conditions, CHIT1 expression of pathogenic macrophages is significantly upregulated in the inflamed tissues<sup>[29]</sup>. In addition to CHIT1, there is another mammalian chitinase, AMCase, which shows high structural homology with CHIT1, has an optimal enzymatic activity at around pH2 and exhibits glycohydrolase enzymatic activity. Full-length cDNA of mouse and human AMCase was first cloned in 2001<sup>[36]</sup>, but an exciting biological role of AMCase was revealed by Zhu *et al*<sup>[14]</sup> only recently: the group noticed a formation of crystals in lung tissues of mice with an asthma-like disease model and identified that the crystals were mammalian chitinase<sup>[37]</sup>. The same group further identified that overproduction of AMCase is highly dependent on a Th2 cytokine IL-13, which further induces production of IP-10 [interferon (IFN)-inducible protein-10] and ITAC (IFN-inducible T cell alpha chemoattractant)<sup>[14]</sup>. The production of AMCase was significantly upregulated in the epithelial cells and tissue macrophages of patients with asthma, but the expression at messenger RNA level was undetectable from patients without lung disease<sup>[14,37]</sup>. Interestingly, anti-AMCase specific antibody as well as pan-chitinase inhibitor allosamidin efficiently ameliorate airway hyperresponsiveness and inflammatory cell infiltrations in the lung of aeroallergen-challenged mice<sup>[14]</sup>, suggesting that AMCase would be an attractive therapeutic target in allergic asthma. A common genetic variant within exon 4 of AMCase from A to G at position 47 (termed A47G) and another variant K17R showed significant association with pediatric asthma. These genetic results support strongly that AMCase is associated with the development of asthma. Recently, Elias's group found that not only AMCase but also CHI3L1 levels in serum as well as lung tissue were significantly elevated in 3 cohorts of patient (at Yale University, University of Paris, and University of Wisconsin) with asthma<sup>[15]</sup>. Expression levels of CHI3L1 in the serum and lungs closely correlated with the severity of asthma, suggesting that the CHI3L1 molecule plays both a primary and a secondary role in asthma patients<sup>[15]</sup>. In addition, another group also identified that a promoter single nucleotide polymorphism (C131G) in CHI3L1 was strongly associated with elevated serum levels of CHI3L1, pulmonary function, asthma, and bronchial hyperresponsiveness<sup>[38]</sup>. From those results, it appears mammalian chitinases are somehow associated with the development of inflammatory conditions in mucosal tissues. The association between the mammalian chitinases and inflammatory disorders is summarized in Table 1.

Table 1 Mammalian chitinases in inflammatory disorders

Location	Disorders	Chitinase type	Ref.
Airway	Bacteremia with <i>S. pneumoniae</i>	CHI3L1	[91,92]
	Bronchial asthma	CHI3L1,	[14,38]
		AMCase, Ym1	
	COPD	CHI3L1	[93]
	Cystic fibrosis	AMCase, CHIT1	[94]
	Rhinosinusitis	AMCase	[95]
Blood	Sarcoidosis	CHIT1	[96,97]
	Bacterial septicemia	CHI3L1	[98]
Brain	Encephalitis	CHI3L1	[99,100]
	Meningitis	CHI3L1	[99]
Disc/Joint	Intervertebral disc degeneration	CHI3L1	[101]
	Juvenile idiopathic arthritis	CHIT1	[102]
	Osteoarthritis, RA	CHI3L1	[103,104]
Eye	Conjunctivitis	AMCase	[105,106]
		CHI3L1, CHIT1	[107,108]
GI tract	Helicobacter gastritis	CHI3L1	[10,109]
	Inflammatory bowel disease	CHI3L1	[10,109]
Heart	Acute myocardial infarction	CHI3L3, CHI3L1	[110,111,112]
	Coronary artery disease	CHIT1	[113]
Liver	Chronic hepatitis C, LC	CHI3L1	[114, 115]
	Fatty liver disease	CHIT1	[116,117]
	Hepatic fibrosis	CHI3L1	[118]
Oral cavity	Periodontitis	Chitinase	[119,120]
Systemic	Gaucher disease	CHIT1	[27]
	Systemic sclerosis	CHI3L1	[121,122]

COPD: Chronic obstructive pulmonary disease; LC: Liver cirrhosis; RA: Rheumatoid arthritis; CHI3L1: Chitinase 3-like-1; AMCase: Acidic mammalian chitinase; CHIT1: Chitotriosidase; Ym1: Chitinase-like lectin.

## ROLE OF CHI3L1 IN THE PATHOGENESIS OF CHRONIC INFLAMMATION

Although CHI3L1 was first identified in 1993<sup>[3]</sup>, its biological function has been largely undetermined. CHI3L1 possesses a functional carbohydrate-binding motif which allows binding with a polymer or oligomer of GlcNAc, but CHI3L1 lacks enzymatic activity entirely. The lack of enzymatic activity in CHI3L1 can be explained by the substitution of leucine for an essential glutamic acid residue within the active site of CHI3L1<sup>[59]</sup>. Therefore, chitinases without enzymatic activity (including CHI3L1) act as chi-lectin<sup>[40]</sup> because of the presence of a preserved carbohydrate-binding motif. Recently, Recklies *et al*<sup>[39]</sup> reported that CHI3L1 promotes the growth of human synovial cells and fibroblasts, raising the possibility that this protein plays a role in the pathological conditions leading to arthritis and tissue fibrosis. Of note, increased circulating levels of CHI3L1 have been reported in the serum of patients with several inflammatory conditions including IBD [Crohn's disease (CD) and ulcerative colitis (UC)]<sup>[41]</sup>, asthma<sup>[15,38]</sup> and liver cirrhosis<sup>[42]</sup>. Serum CHI3L1 is rarely detectable in healthy individuals<sup>[41]</sup>, and therefore CHI3L1 has recently been proposed as a useful marker for indicating inflammatory activity and poor clinical prognosis for IBD<sup>[41]</sup>. A soluble form of CHI3L1 seems to be secreted by a wide variety of mammalian cells *in vitro*, including activated neutrophils, granulocytes, differentiated macrophages and colonic epithelial cells (CECs)<sup>[10,19,43]</sup>. CHI3L1 is strongly expressed by macrophages in the synovial membrane of patients with rheumatoid arthritis (RA) and a polarized IFN $\gamma$ -

mediated proinflammatory Th1-type immune response has been observed in half the patients with RA. In contrast, peripheral mononuclear cells from healthy individuals strongly react against the CHI3L1 antigen and eventually produce a regulatory cytokine IL-10<sup>[44]</sup>. These results strongly suggest that CHI3L1-mediated immune responses in RA patients are somehow shifted from an IL-10 dominated immunoregulatory response to an IFN $\gamma$ -dominated proinflammatory phenotype<sup>[44]</sup>. In addition, serum levels of CHI3L1 are positively correlated with the severity of arthritis<sup>[45]</sup>. Interestingly, peripheral blood T cells from RA patients proliferate in response to RA-associated DR4 (DRB1\*0401) peptide which contains a potential self-reactive motif preserved within human CHI3L1<sup>[46]</sup>. In fact, the specific motif within CHI3L1 is responsible for the development and relapse of joint inflammation seen in Balb/c mice, suggesting that CHI3L1 is able to serve as an auto-antigen for arthritis in mice as well as humans<sup>[46]</sup>. Intranasal as well as oral auto-antigen administration is one of the most effective strategies for inducing immuno-tolerance<sup>[47,48]</sup>. Indeed, several groups have tried to administer CHI3L1 intranasally in animal models of arthritis<sup>[49,50]</sup> as well as RA patients with moderate disease activity<sup>[51]</sup>, and the protein administration effectively suppresses the disease activity by downregulating the Th1-type immune response without showing any adverse effects. Therefore, CHI3L1 seems to be the cross-tolerance inducing protein in chronic arthritis which effectively downregulates the pathogenic immune responses. It is possible that nasal administration of CHI3L1 represents an attractive approach for suppressing the clinical manifestation of chronic types of inflammation as well as autoimmune diseases.

Our group recently identified that CHI3L1 plays a unique role during the development of intestinal inflammation: the molecule is induced in both colonic lamina propria macrophages and CECs during the course of intestinal inflammation in experimental colitis models as well as in patients with IBD<sup>[10]</sup>. Gentamicin protection assays using intracellular bacteria, including *Salmonella typhimurium* and adherent invasive *Escherichia coli* show that CHI3L1 is required for the enhancement of adhesion and invasion of these bacteria on/into CECs and acts as a pathogenic mediator in acute colitis. It has been suggested that a genetic defect against intracellular bacterial infection is strongly associated with the development of CD, and an increased prevalence of intracellular bacteria in the ileal biopsies and surgical specimens of CD patients has been reported previously<sup>[52,53]</sup>. We also identified that the CHI3L1 molecule particularly enhances the adhesion of chitin-binding protein-expressing bacteria to CECs through the conserved amino-acid residues<sup>[11]</sup>. Therefore, overexpression of CHI3L1 may be strongly associated with the intracellular bacterial adhesion and invasion on/into CECs in CD patients, who presumably have mutations in the susceptibility genes of CD including NOD2 (CARD 15), IL-23R, ATG16L1 and XBP-1<sup>[54]</sup>. In contrast, in an aseptic condition such as bronchial asthma, epithelium-expressing CHI3L1 seems to play a regulatory role by rescuing Th2-type immune responses<sup>[16]</sup>. Further

study will be required to fully prove the exact role of epithelium-expressing CHI3L1 in inflammatory conditions.

## EXPRESSION OF CHI3L1 IN VARIOUS SOLID TUMORS

The biological function of CHI3L1 is still unclear, but it has been strongly hypothesized that CHI3L1 plays a pivotal role as a growth stimulating factor for solid tumors or has a suppressive/protective effect in the apoptotic processes of cancer cells<sup>[18]</sup> and inflammatory cells<sup>[16]</sup>. Based on an amino acid database search at the National Center for Biotechnology Information, CHI3L1 is expressed in a wide variety of human solid tumors as summarized in Table 2. In addition, elevated levels of CHI3L1 in serum and/or plasma have been detected in patients with different types of solid tumors (Table 2). Therefore, it is reasonable to predict that the serum level of CHI3L1 can be a reliable marker of progression of certain kinds of tumors and of a “bad prognosis” in patients with certain types of malignant tumors<sup>[18]</sup>.

Many clinical laboratories have reported that CHI3L1 could be used as a novel tumor marker for ovarian cancer<sup>[55,56]</sup>, small cell lung cancer<sup>[22]</sup>, metastatic breast cancer<sup>[57]</sup>, and metastatic prostate cancer<sup>[23]</sup>. In addition, several groups have reported that CHI3L1 is one of the most significant prognosis markers for cervical adenocarcinoma<sup>[58]</sup>, recurrent breast cancer<sup>[4]</sup> and metastatic breast cancer<sup>[21]</sup>, as well as advanced stages of breast cancer<sup>[59]</sup>. Interestingly, the CHI3L1 serum level could be a useful and sensitive biomarker for recurrence in locally advanced breast carcinoma<sup>[59]</sup>, ovarian carcinoma<sup>[60]</sup>, endometrial cancer<sup>[61]</sup>, squamous cell carcinoma of the head and neck<sup>[62]</sup>, metastatic prostate cancer or melanoma<sup>[63,64]</sup>, Hodgkin's lymphoma<sup>[65]</sup> and colon cancer<sup>[20]</sup>. Based on extensive research by Johansen and colleagues, CHI3L1 may be used as a novel and sensitive predictor of any cancer<sup>[66,67]</sup>. They categorized patients into 5 distinct levels according to the amount of plasma CHI3L1 detected by ELISA, and found that participants with the highest level of plasma CHI3L1 had a median survival rate of only 1 year after the cancer diagnosis<sup>[66]</sup>. In contrast, the patients with the lowest level of plasma CHI3L1 had a survival rate of more than 4 years. Although the variation of CHI3L1 serum levels in healthy subjects in this study was relatively small, subsequent measurements would be required to determine cancer risk since the serum level of CHI3L1 could also be elevated in patients with other inflammatory diseases or autoimmune disorders<sup>[68]</sup>. From the results, it has been highly predicted that serum CHI3L1 levels seem to be a potential and promising biomarker for malignant tumors.

The expression of CHI3L1 is relatively restricted to a limited number of cell types: it is totally absent in monocytes<sup>[69]</sup> and marginally expressed in monocyte-derived dendritic cells<sup>[70]</sup>, but is strongly induced during the late stages of human macrophage differentiation<sup>[43]</sup>. Rehli *et al*<sup>[43]</sup> clearly demonstrated that promoter

Table 2 Expression of CHI3L1 in solid tumors

Solid tumors	Location	Ref.
Glioma, Oligodendroglioma, glioblastoma	Brain	[74,123-132]
Squamous cell carcinoma of the head and neck	Head and neck	[18,22,62]
Lung cancer (small cell carcinoma)	Lung	[133,134]
Breast cancer	Breast	[4,8,21,55,57,62,67,68,75,132-141]
Colorectal cancer	Colon	[20,132]
Kidney tumor	Kidney	[132,142,143]
Hepatocellular carcinoma	Liver	[21,66]
Ovarian tumor, endometrial cancer	Ovary	[55-61,133,138,139]
Primary prostate cancer	Prostate	[23,63,144]
Metastatic prostate cancer		
Papilloma thyroid carcinoma, thyroid tumor	Thyroid	[136,145]
Extracellular myxoid-chondrosarcoma	Bone	[146]
Multiple myeloma	Bone marrow	[147,148]
Hodgkin's lymphoma	Lymph node	[65]
Malignant melanoma	Melanocyte	[18,64]
Myxoid liposarcoma	Fat cells	[146]

elements (in particular, the proximal -377 base pairs of the CHI3L1 promoter region) control the expression of CHI3L1 in the macrophage used. CHI3L1 is also expressed in neutrophils<sup>[71]</sup>, chondrocytes<sup>[3]</sup>, fibroblast-like synoviocytes<sup>[72]</sup>, vascular smooth muscle cells<sup>[5]</sup>, vascular endothelial cells<sup>[73]</sup>, ductal epithelial cells<sup>[67]</sup>, hepatic stellate cells<sup>[18]</sup>, and colonic epithelial cells<sup>[10]</sup>. In physiological concentrations CHI3L1 tends to promote proliferation of these cell types. CHI3L1 is a transmembrane protein whose extracellular domain can undergo cleavage<sup>[39]</sup>. The cleaved components bind to putative receptor(s) on the cell surface or soluble receptor(s), but these receptors have not been identified yet<sup>[18,40]</sup>. Some reports suggest that CHI3L1 can play an important role in tumor invasion. Nigro *et al*<sup>[74]</sup> showed that immortalized human astrocytes stably transfected with CHI3L1 increased invasion across a chemotactic gradient *in vitro*. Roslind *et al*<sup>[75]</sup> demonstrated that metastatic tumor cells in blood vessels, lymph nodes and skin displayed the same pattern of CHI3L1 as primary breast cancer cells by immunohistochemical analysis: normal epithelial cells display the widespread strong dot-like staining in the whole cytoplasm, while the dot-like staining is localized in the restricted area of cytoplasm in malignant tumor cells. Indeed, CHI3L1 is strongly expressed in the invasive front of lobular carcinoma which is adjacent to normal epithelial cells. These studies suggest that CHI3L1 may assist the migration of malignant cells and support metastasis of cancer cells by promoting the malignant transformation of adjacent normal epithelial cells.

## A POSSIBLE BIOLOGICAL ROLE OF CHI3L1 IN ONCOGENIC PROGRAMMING PROCESS

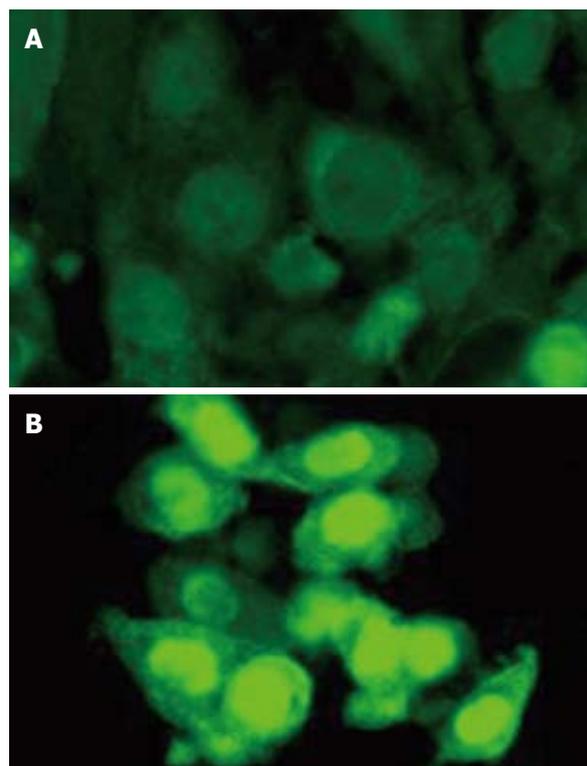
It has been predicted that CHI3L1 can have a growth

**Table 3** Inflammation-associated carcinogenic change

Inflammatory disorder	Carcinogenic formation	Ref.
Human papillomavirus infection	Cervical carcinoma	[149-152]
Crohn's disease, ulcerative colitis	Colorectal carcinoma	[153-159]
Chronic cholecystitis	Gall bladder carcinoma	[160-162]
Hepatitis B, C infection	Hepatocellular carcinoma	[163-167]
Asbestosis, asthma, <i>C. pneumoniae</i> infection, chronic obstructive lung disease, middle lobe syndrome, silicosis	Lung carcinoma	[168-174]
Pelvic inflammatory disease	Ovarian carcinoma	[175-177]
Chronic pancreatitis	Pancreatic carcinoma	[178-183]
<i>H pylori</i> infected gastritis	Gastric carcinoma, lymphoma	[184-186]
Chronic cystitis, schistosomiasis	Bladder carcinoma	[187-190]
Primary sclerosing cholangitis	Cholangio carcinoma, colorectal carcinoma, liver carcinoma, pancreatic carcinoma	[191]

stimulating effect since a family of CHI3L molecules in the fruit fly *Drosophila melanogaster* regulates the growth of imaginal disc cells<sup>[76]</sup>. Two of the major biological functions of CHI3L1 are a growth stimulating effect on connective tissue cells<sup>[39,72]</sup> and a potent migration enhancing effect for endothelial cells<sup>[73]</sup>. CHI3L1 also stimulates angiogenesis and reorganization of vascular endothelial cells<sup>[73]</sup>. Insulin-like growth factor-1 is a well-characterized growth factor in connective tissue cells, and works synergistically with CHI3L1 to enhance the response of human synovial cells isolated from patients with osteoarthritis<sup>[39]</sup>. In addition, CHI3L1 strongly promotes the activation of 2 major signaling pathways associated with mitogenesis and cell survival: MAPK (mitogen-activated protein kinase) pathways and PI-3K (phosphoinositide 3-kinase)-mediated pathways in fibroblast cells. The putative cell surface receptor and/or adaptive molecule for CHI3L1 are still unidentified, but the purified human CHI3L1 molecule efficiently leads to phosphorylation of MAPK p42/p44 in human synovial cells, fibroblasts, articular chondrocytes<sup>[39]</sup> and human colonic epithelial cells (Mizoguchi E, unpublished observation) in a dose-dependent manner. It has been suggested that guanine nucleotide-binding protein (G-protein)-regulated MAPK networks are involved in the action of most non-nuclear oncogenes and subsequent carcinogenesis and tumor progression<sup>[77]</sup>. The networks are involved in the activation of MAPK p42/p44 which may enhance the carcinogenic change of epithelial cells during upregulated CHI3L1 expression under inflammatory conditions.

It is believed that IBD is a risk factor of cancer development based on the severity of the disease course. As previously reported, serum CHI3L1 concentration is elevated in patients with IBD<sup>[41]</sup> and primary colorectal cancer<sup>[20]</sup>. People with CD have a 5.6-fold increased risk of developing colon cancer<sup>[78]</sup>; therefore screening for colon cancer by colonoscopy is strongly recommended for patients who have had CD for several years<sup>[79]</sup>. Inflammation was recently recognized as an important



**Figure 1** Activation of  $\beta$ -catenin in colonic epithelial cells by purified Chitinase 3-like-1 (CHI3L1). SW480 human colon cancer cells were cultured without (A) or with purified human CHI3L1 protein (50 ng/mL) for 24 h (B), and cells were stained with mouse anti-human  $\beta$ -catenin monoclonal antibody followed by FITC-horse anti-mouse IgG staining. Purified CHI3L1 significantly stimulates the nuclear translocation of  $\beta$ -catenin in SW480 cells. Original magnification, objective 40  $\times$ .

factor in the pathogenesis of malignant tumors<sup>[80]</sup> and we summarize some examples of inflammation-mediated carcinogenesis in Table 3. Of interest, most of the diseases listed in Table 3 express CHI3L1 during the course of inflammation and the subsequent tumorigenesis. CHI3L1 protects cancer cells from undergoing apoptosis and also has an effect on extracellular tissue remodeling by binding specifically with collagen types I, II, and III<sup>[81]</sup>. Therefore, CHI3L1 is strongly associated with cell survival and cell migration during the drastic tissue remodeling processes by interacting with extracellular matrix components<sup>[39,40]</sup>.

The canonical (Wnt/ $\beta$ -catenin) pathway is known to play a crucial role in UC-related tumor progression<sup>[82]</sup>. Recently, our group identified that the SW480 human colon cancer cell line shows significantly upregulated expression and trans-nuclear translocation of  $\beta$ -catenin after extensive stimulation with low dose (50 ng/mL) purified CHI3L1 protein (Qidel, San Diego, CA) (Figure 1). This result strongly suggests that CHI3L1 may have a direct but not a secondary role for inflammation-associated tumorigenesis by continuously activating the Wnt/ $\beta$ -catenin canonical signaling pathway in CECs. As previously demonstrated, CHI3L1 expression is enhanced by proinflammatory cytokine interleukin-6<sup>[10,83]</sup>, which also has a critical tumor-promoting effect during early colitis-associated cancer tumorigenesis<sup>[84]</sup>. It has been proven that

activation of gp130/STAT3 transcription factor regulates cell cycle progression as well as survival of enterocytes during chronic colitis-associated tumor promotion<sup>[85-88]</sup>. Therefore, the blocking of interleukin-6 mediated CHI3L1 expression by anti-CHI3L1 specific antibody or pan-family 18 chitinase inhibitors including allosamidin and methylxanthine derivatives (e.g. theophylline, caffeine, and pentoxifylline)<sup>[14,89,90]</sup> would be a useful strategy in preventing both chronic mucosal inflammation and the subsequent inflammation-associated carcinogenic change in epithelial cells. In summary, CHI3L1 could be a useful and attractive target for potential anti-cancer therapies, in particular against highly invasive and metastatic solid tumors.

## CONCLUSION

Mammalian chitinases, including CHI3L1 and AMCCase, actively participate in the pathogenesis of acute and chronic inflammation and presumably the following inflammation-associated tumorigenesis. Further understanding of the biological and physiological functions of mammalian chitinases would be very important to develop novel anti-inflammatory as well as anti-cancer therapies for several inflammatory disorders and inflammation-associated cancers in the near future.

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## Experimental evidence of obesity as a risk factor for severe acute pancreatitis

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### Abstract

The incidence of acute pancreatitis, an inflammation of the pancreas, is increasing worldwide. Pancreatic injury is mild in 80%-90% of patients who recover without complications. The remaining patients may develop a severe disease with local complications such as acinar cell necrosis, abscess and remote organ injury including lung injury. The early prediction of the severity of the disease is an important goal for physicians in management of patients with acute pancreatitis in order to optimize the therapy and to prevent organ dysfunction and local complications. For that purpose, multiple clinical scale scores have been applied to patients with acute pancreatitis. Recently, a new problem has emerged: the increased severity of the disease in obese patients. However, the mechanisms by which obesity increases the severity of acute pancreatitis are unclear. Several hypotheses have been suggested: (1) obese patients have an increased inflammation within the pancreas; (2) obese patients have an increased accumulation of fat within and around the pancreas where necrosis is often located; (3) increase in both peri- and intra-pancreatic fat and inflammatory cells explain the high incidence of pancreatic inflammation and necrosis in obese patients; (4) hepatic dysfunction associated with obesity might enhance the systemic inflammatory response by altering the detoxification of inflammatory mediators; and (5) ventilation/perfusion

mismatch leading to hypoxia associated with a low pancreatic flow might reduce the pancreatic oxygenation and further enhance pancreatic injury. Recent experimental investigations also show an increased mortality and morbidity in obese rodents with acute pancreatitis and the implication of the adipokines leptin and adiponectin. Such models are important to investigate whether the inflammatory response of the disease is enhanced by obesity. It is exciting to speculate that manipulation of the adipokine milieu has the potential to influence the severity of acute pancreatitis.

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**Key words:** Acute pancreatitis; Obesity; Adiponectin; Leptin

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### INTRODUCTION

The incidence of acute pancreatitis is increasing worldwide<sup>[1]</sup>. Most episodes of acute pancreatitis are mild and self-limiting, requiring only a short hospitalization<sup>[2]</sup>. However, 10% of the patients (with a significant proportion of obese patients) develop a severe disease with local and extra-pancreatic complications characterized by early development and persistence of hypovolemia and multiple organ dysfunction<sup>[3,4]</sup>. Following the initial pancreatic edema, necrosis is the most severe local complication. The patchy areas of nonviable parenchyma are initially sterile but may be infected by bacteria originated mostly from the gut whose permeability increases during the disease. The most important extra-pancreatic complication is lung injury with a high incidence in severe

pancreatitis, ranging from 15% to 55 %<sup>[5]</sup>. The severity of pulmonary complications varies greatly from mild hypoxemia without clinical or radiological abnormalities to severe acute respiratory distress syndrome. Two peaks of pulmonary complications were observed during the early phase of severe acute pancreatitis, the first peak being described upon admission with new radiological abnormalities by day 5<sup>[6]</sup>. Hepatic injury is mild in acute pancreatitis but may participate in the propagation of inflammation from pancreas to other organs, mostly lungs<sup>[7,8]</sup>.

### **INCREASED SEVERITY OF ACUTE PANCREATITIS IN OBESE PATIENTS**

Several clinical investigations showed that obesity increases the severity of the disease by favoring local complications within the pancreas and injuries in remote organs as well as by increasing the mortality rate<sup>[9-13]</sup>. Obesity increases the incidence of early shock, renal and pulmonary failure<sup>[14]</sup> and extends the hospital stay<sup>[15]</sup>. However, other studies have questioned such findings<sup>[11,16,17]</sup>.

The mechanisms by which obesity increases the severity of acute pancreatitis is unclear, but one hypothesis might be that obese patients have an increased inflammatory response within the pancreas<sup>[11,18]</sup>. In the study by Sempere *et al*<sup>[12]</sup>, among 85 consecutive patients with acute pancreatitis, 74% had a mild disease while the remaining patients were severely ill. Serum concentrations of interleukin-1 $\alpha$  (IL-1 $\alpha$ ), IL-1 receptor antagonist (IL-1-ra), IL-6, IL-8, IL-10 and IL-12p70 were significantly increased in patients with acute pancreatitis as compared with volunteers, and the concentrations were significantly higher in obese patients. One explanation is that obesity per se induces a chronic inflammatory state<sup>[11,19]</sup>. A second hypothesis is that obese patients have an increased accumulation of fat within and around the pancreas where necrosis is often located. The risk of pancreatic infection and inflammation would be proportional to the increased amount of peri-pancreatic fat. Accordingly, patients with intra-pancreatic fat are more prone to develop local complications following pancreatic surgery<sup>[11,20]</sup>. Interestingly, cytokine expression in fat tissue is higher in obese than in lean subjects<sup>[21]</sup>. In obese patients, the cytokine expression is also higher in visceral than in subcutaneous fat, cytokines being produced mainly by macrophages located in the stromal-vascular fraction of fat tissues<sup>[21]</sup>. Thus, increase in both peri- and intra-pancreatic fat and presence of inflammatory cells in adipose tissues might explain the high incidence of pancreatic inflammation and necrosis in obese patients. Weight loss improves the inflammatory profile of fat tissue with an increased expression of anti-inflammatory factors such as IL-10 and IL-1-ra<sup>[21]</sup>. Similarly, in inflammatory bowel diseases, visceral fat is also a source of inflammatory signals<sup>[22,23]</sup>. A third hypothesis is that pancreatic microcirculation is lower in obese than in non-obese patients, which increases the risk of

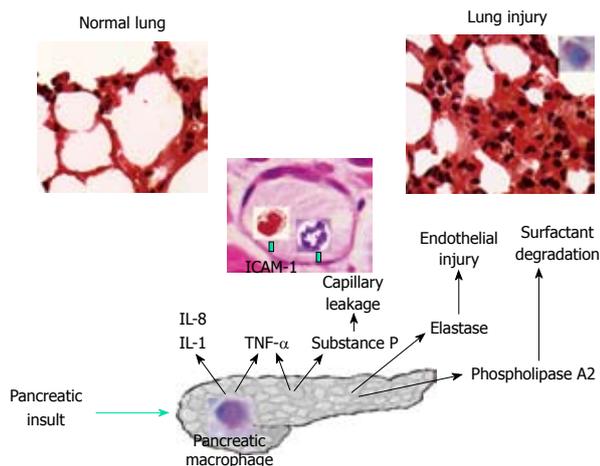
ischemic injury and subsequent local infections. Moreover, obese patients might be immunodeficient, a condition that increases the risk of local infections<sup>[24]</sup>. Finally, because obesity restricts the movement of the chest wall and diaphragm, inspiratory capacity of obese patients is reduced. Ventilation/perfusion mismatch may lead to hypoxemia that, in conjunction with low blood flow, further decreases tissue oxygenation to the pancreas.

### **BRIEF OVERVIEW OF THE PATHOPHYSIOLOGY OF ACUTE PANCREATITIS**

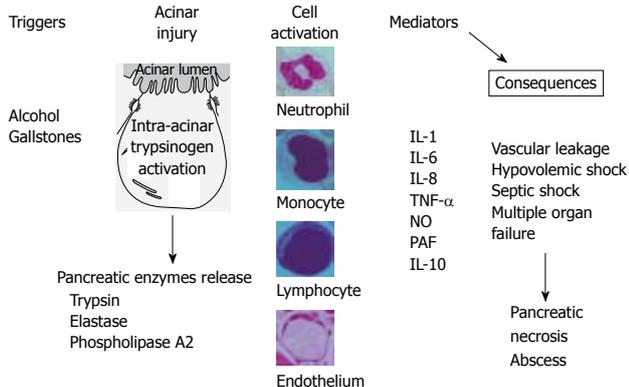
Although controversial, most observers believe that acute pancreatitis is caused by the dysregulated activation of trypsin within pancreatic acinar cells. Enzyme activation within the pancreas leads to autodigestion of the gland followed by local inflammation. The main factors that trigger the acute disease are pancreatic hyperstimulation (mainly observed in experimental models), gallstones and alcohol abuse in humans. Acute pancreatitis occurs when intracellular protective mechanisms designed to prevent trypsinogen activation or reduce trypsin activity are decreased or overwhelmed. Following the activation of trypsinogen into active trypsin within acinar cells, numerous enzymes such as elastase and phospholipase A2, as well as complement and kinin systems, are activated<sup>[25]</sup> (Figures 1 and 2). Additionally, inflammation is initiated with local production of mediators such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-1 and IL-8 from neutrophils, macrophages and lymphocytes<sup>[26,27]</sup>. In addition to these events, activation of endothelial cells permits the transendothelial migration of leukocytes that release other harmful mediators<sup>[28]</sup>. Thus, regardless of the initial trigger of the disease, the severity of pancreatic damage is related to the injury of acinar cells and to the activation of inflammatory and endothelial cells. Local complications such as acinar cell necrosis may develop and injury in remote organs (lungs) results from the release of numerous mediators from the pancreas or extrapancreatic organs (Figure 1).

### **EXPERIMENTAL PANCREATITIS, OBESITY, ADIPONECTINS AND INFLAMMATION**

Fat tissues are likely to contribute to the increased inflammation in obese rodents. Thus, adipokines secreted by adipocytes are potent regulators of the inflammatory response, leptin being considered as a pro-inflammatory adipokine, while adiponectin functions as an anti-inflammatory mediator (Table 1). Adipose tissues also produce cytokines that participate in the inflammatory response of obesity and organ dysfunction. Thus, pancreatic necrosis following intrapancreatic duct injection of taurocholic acid (TA) was similar in obese *fa/fa* rats and lean



**Figure 1** Overview of the major events occurring in experimental acute pancreatitis and pancreatitis-associated lung injury. Inflammatory cells adhere to the endothelial walls via the expression of the intercellular molecules (ICAM-1) and translate the local inflammation to remote organs.



**Figure 2** Mediators involved in the pathogenesis of acute pancreatitis. After the acinar cell injury occurs, inflammatory cells will synthesize and release proinflammatory cytokines and chemokines that induce systemic effects (vascular leakage, etc.). All these consequences will finally dictate the occurrence of pancreatic necrosis and abscess.

*fa/+* rats, but the survival was lower in obese rats<sup>[29]</sup>. The pancreatic expression of TNF- $\alpha$  and IL-6 was higher in obese than in lean rats while IL-10 expression was lower<sup>[30]</sup>. TNF- $\alpha$  expression was also higher in liver and lungs from obese rats. Moreover, obese rats with acute pancreatitis had steatohepatitis while livers in lean rats with acute pancreatitis were normal. Thus, dysregulation between inflammatory and anti-inflammatory mediators in obese rats is an important issue to explain the increased severity of the disease. These investigators also determined whether high-fat feeding vs. normal diet may influence the severity of a “two-hit” injury (TA-induced pancreatitis followed by intraperitoneal injection of endotoxin 3 h later)<sup>[31]</sup>. Endotoxin administration lowers the survival rate of lean rats by increasing peripancreatic fat necrosis and systemic inflammatory response while chronic high fat diet does not aggravate pancreatic injury induced by TA plus endotoxin<sup>[31]</sup>.

Obese *fa/fa* rats have a mutation with dysfunction of the leptin receptor and develops obesity by week 5<sup>[32]</sup>. Littermates *fa/+* rats are used as controls. By week 14,

**Table 1** Severity of acute pancreatitis and adipokines

Adipocytokine	Effects	Final effect
Leptin	↑ TNF- $\alpha$ ↑ Chemotaxis ↑ Neutrophil activation ↑ IL-6 ↑ T-cell proliferation	Pro-inflammatory
Adiponectin	↓ Endothelial cell adhesion ↓ TNF- $\alpha$ ↓ IL-6 ↓ Phagocytosis ↑ IL-10 ↑ IL-1RA	Anti-inflammatory

Adiponectin may be considered as an anti-inflammatory compound whereas leptin acts as a proinflammatory mediator. Both adipokines may influence the severity of pancreatitis.

body weight doubles in *fa/fa* rats. Restricted feeding does not modify the occurrence of obesity. The obesity is associated with an increase in the number and size of adipocytes. These rats have hyperlipidemia and hypercholesterolemia. The pancreas weight is similar in *fa/fa* and *fa/+* rats but, according to the increased body weight of *fa/fa* rats, the ratio between pancreas and body weight is lower in *fa/fa* rats<sup>[33]</sup>. Of note, amylase content is lower in *fa/fa* than *fa/+* rats and treatment with ciglitazone that increases the insulin sensitivity partially prevents this low pancreatic amylase content<sup>[34]</sup>. Administration of cholecystokinin (CCK) is known to decrease food intake but the obese rats have a higher threshold than lean rats for this effect<sup>[35]</sup>. Moreover, the exocrine response to cerulein and carbamylcholine is decreased in acini isolated from *fa/fa* rats while the response to secretin or vasoactive intestinal peptide is identical<sup>[36,37]</sup>. When CCK is incubated in isolated acini, less amylase is secreted in *fa/fa* rats than in lean rats<sup>[37]</sup>. In *fa/fa* and *fa/+* rats, the expression of CCK<sub>A</sub> and CCK<sub>B</sub> receptors is unknown. To overcome the absence of effects on the receptor, circulating leptin expression is increased. Leptin mRNA is detected in adipose tissue but not in pancreas, lungs, or liver<sup>[38]</sup>. The expression of leptin decreases gradually from epididymal to retroperitoneal, subcutaneous, and interscapular brown adipose tissue. In isolated adipocytes, leptin mRNA expression is also significantly higher in *fa/fa* than in control rats. Finally, TNF- $\alpha$  protein expression in adipose (perirenal and epididymal) tissues is similar in both strains<sup>[33]</sup>.

The severity of acute pancreatitis has been investigated in other experimental models of obesity, such as *ob/ob* and *db/db* mice. Both mice are congenitally obese but manifest this phenotype via different mechanisms. *Ob/ob* mice have a spontaneous mutation of the *ob* (leptin) gene and produce no leptin while *db/db* mice have a spontaneous mutation of the leptin receptor and have increased circulating concentrations of leptin. *Ob/ob* mice may reach three times the normal weight of wild-type mice. Obesity is characterized by an increase in the number and size of adipocytes. Although hyperphagia contributes to obesity, excess of weight is also

observed with a restrictive diet sufficient for lean mice. Interestingly, *ob/ob* mice have impaired pulmonary bacterial clearance of *Streptococcus pneumoniae* and increased pulmonary concentrations of cytokines<sup>[39]</sup>. *Ob/ob* mice are less hyperglycemic than *db/db* mice. Moreover, in pancreas from *ob/ob* mice, content of triglycerides, free fatty acids, cholesterol and total fat is higher than in lean mice<sup>[40]</sup>. Serum concentrations of IL-1 and TNF- $\alpha$  are also increased.

In a model of acute pancreatitis induced by two injections of IL-12 plus IL-18, all *ob/ob* mice died within 48 h while all wild-type mice survived<sup>[41]</sup>. To differentiate the contribution of obesity or leptin deficiency to the severity of the disease, a group of *ob/ob* mice had leptin replacement therapy (obesity with normal leptin). Interestingly, this group had severe acute pancreatitis, suggesting that obesity *per se* and not leptin deficiency was responsible for the severe acute pancreatitis. Moreover, the authors generated "slim" *ob/ob* mice that had a less severe acute pancreatitis, reinforcing the finding that obesity rather than leptin deficiency was responsible for the severity of acute pancreatitis. In another study, three experimental groups received injections of cerulein: C57BL/6J (lean), *ob/ob* (obese mice with leptin deficiency), and *db/db* (obese mice with leptin receptor deficiency and increased circulating leptin) mice<sup>[42]</sup>. Both *ob/ob* and *db/db* mice developed a significantly more severe disease than wild-type lean mice associated with an increase in pancreatic inflammatory cytokines. Finally, in patients matched for body mass index, the circulating leptin did not correlate with the severity of the disease<sup>[43]</sup>. These studies confirmed the feeling that leptin production does not relate to the severity of acute pancreatitis.

However, the role of leptin in acute pancreatitis remains puzzling (Table 1). In pancreatitis induced by cerulein injections in lean rats, serum leptin concentrations increased by 12 h and remained high for 36 h<sup>[44]</sup>. When a more severe disease was induced (arginine model), leptin concentrations were high at 12 and 24 h but were similar at 48 h in both experimental models. Thus, in lean rodents, the serum concentrations of leptin increase in acute pancreatitis but no relationship is found between severity of the disease and circulating leptin concentrations. To further investigate the role of leptin in the severity of the disease, rats with acute pancreatitis were treated with leptin (10  $\mu\text{g}/\text{kg}$  ip) after the last cerulein injection<sup>[45]</sup>. Leptin treatment increased the survival rate over 48 h and this beneficial effect was associated with a reduced serum expression of TNF- $\alpha$ , IL-1, MIP, sICAM and lung nitric oxide. Pancreatic and pulmonary CD40 expression was significantly reduced by leptin as was pancreatic and pulmonary injuries at histological examinations<sup>[45]</sup>.

The anti-inflammatory adipokine adiponectin has also been investigated in experimental pancreatitis. Adiponectin is decreased in obesity and inversely mirrors the severity of experimental pancreatitis<sup>[46]</sup>. Adiponectin acts through the receptors AdipoR1 and AdipoR2. Both receptors are expressed in rodent pancreas but AdipoR1

expression is significantly decreased in the pancreas of *ob/ob* and *db/db* mice as compared with wild-type lean mice<sup>[46]</sup>. To investigate the role of adiponectin in the severity of acute pancreatitis, adiponectin knockout (APN-KO) and wild type mice were injected with a low dose of cerulein two weeks after normal or high-fat-diet<sup>[47]</sup>. Whereas APN-KO mice fed a high-fat-diet treated with cerulein developed pancreatic damage and inflammation, wild-type mice did not. Finally, adenovirus-mediated over-expression of adiponectin attenuates the severity of acute pancreatitis in APN-KO mice<sup>[47]</sup>. All these data clearly demonstrate that adiponectin plays a protective role in the cerulein model of acute pancreatitis.

## INTERACTION OF CHOLECYTOKININ, DIGESTIVE PROENZYMES AND LEPTIN

Confusion on the protective or deleterious role of leptin in acute experimental pancreatitis may also rise from the fact that a crosstalk between CCK and leptin pathways is observed in acinar cells. CCK is produced in endocrine cells present in the mucosa of the small intestine following the ingestion of proteins and fat. CCK stimulates the contraction of gallbladder and relaxes the sphincter of Oddi (facilitating bile secretion into the intestine) and stimulates pancreatic secretion by acinar cells either by a direct effect or through acetylcholine released by the vagus nerve that possesses receptors for CCK. CCK is also a proliferative hormone for the pancreas and by delaying gastric emptying induces satiety. Leptin, produced and secreted from white adipocytes, regulates food intake and energy consumption<sup>[48]</sup>. Intravenous administration of leptin diminishes the postprandial pancreatic secretions<sup>[49]</sup>. Administration of leptin does not affect the volume of bile and pancreatic juice while the protein and trypsin output is reduced<sup>[49]</sup>. The effect of leptin becomes stronger when protein and trypsin secretions are stimulated by CCK. In contrast, leptin does not affect basal and CCK-8-stimulated amylase release in pancreatic acini, suggesting that leptin does not act directly on pancreatic acinar cells but inhibits the secretion of pancreatic enzymes through CCK-vagal-dependent mechanism<sup>[49]</sup>. In contrast to the intravenous administration, intraduodenal leptin administration to fasted rats increases pancreatic protein and amylase secretions, this effect being related to the stimulation of CCK release through activation of duodeno-pancreatic reflexes<sup>[50]</sup>.

Otsuka Long-Evans Tokushima Fatty (OLETF) rats are spontaneously diabetic rats with polyuria, polydipsia, hyperglycemia, mild obesity and diabetes<sup>[51]</sup>. These rats do not express the CCK-A receptor mRNA in pancreas. This lack of CCK-A receptors results in a reduced ability to produce nutrient-induced satiety signals which leads to increase in meal size, overall hyperphagia and obesity. Administration of increasing doses of CCK8 induced a biphasic dose-response curve of pancreatic juice and protein secretion in control Long-Evans Tokushima Otsuka (LETO) rats whereas the OLETF rats did not respond to CCK-8<sup>[52]</sup>. Cerulein injections induce acute

pancreatitis in LETO rats but did not increase serum amylase or lipase activities in OLETF rats.

Finally, the rat pancreatic acinar tumor (AR42J) cell lines do express the leptin receptor<sup>[53]</sup>. The binding of leptin is specific to the leptin receptor and does not cross-react with CCK pathway. Leptin does not modify basal amylase release but inhibits amylase release stimulated by CCK. Leptin alone has no effect on intracellular Ca<sup>2+</sup> mobilization but pre-treatment with leptin enhances the Ca<sup>2+</sup> response to CCK. Thus, AR42J cells express a functional leptin receptor that modulates the action of CCK on Ca<sup>2+</sup> mobilization and amylase release<sup>[53]</sup>. Relationship between enzyme release from acinar cells and signals of satiety such as leptin in lean and obese rodents are complex and further investigations are needed.

## CONCLUSION

The prevalence of obesity has increased worldwide. Despite numerous clinical investigations, the precise mechanisms involved in the pathogenesis of acute pancreatitis remain elusive, and currently no specific medical therapy is available beyond general support. Investigating the mechanisms, by which acute pancreatitis develops from novel angles such as obesity, offers potentially new observations that may ultimately lead to the development of useful treatment. It is exciting to speculate that manipulation of the adipokine milieu has the potential to influence the severity of acute pancreatitis. Thus, investigations along these lines are warranted.

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ORIGINAL ARTICLE

## Validation of Fujinon intelligent chromoendoscopy with high definition endoscopes in colonoscopy

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pattern compared to white light. The mucosal surface was best assessed using filter 4. However, the views obtained were not rated significantly better than those observed with white light. The "gold standard", indigo carmine (IC) dye, was found to be superior to both white light and filter 4. Filter 6 (RGB wavelengths of 580, 520, and 460 nm, respectively) allowed for exploration of the IC-stained mucosa. When assessing mucosal polyps, both FICE with magnification, and magnification following dye spraying were superior to the same techniques without magnification and to white light imaging. In the presence of suboptimal bowel preparation, observation with the FICE mode was possible, and endoscopists considered it to be superior to observation with white light.

**CONCLUSION:** FICE-filter 4 with magnification improves the image quality of the colonic vascular patterns obtained with WLE.

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**Key words:** Colonoscopy; Computed virtual chromoendoscopy; Fujinon intelligent chromoendoscopy; Magnifying colonoscopy; Polyp diagnosis

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### Abstract

**AIM:** To validate high definition endoscopes with Fujinon intelligent chromoendoscopy (FICE) in colonoscopy.

**METHODS:** The image quality of normal white light endoscopy (WLE), that of the 10 available FICE filters and that of a gold standard (0.2% indigo carmine dye) were compared.

**RESULTS:** FICE-filter 4 [red, green, and blue (RGB) wavelengths of 520, 500, and 405 nm, respectively] provided the best images for evaluating the vascular

### INTRODUCTION

Chromoendoscopy has been proven to be a valuable tool for the study of colorectal neoplasia. Indigo carmine (IC) can be used with conventional endoscopy<sup>[1]</sup> or with magnifying colonoscopy<sup>[2-5]</sup>.

Endoscopy manufacturers have developed artificial image enhancements, including "virtual chromoendoscopy", as a quicker and less messy alternative to dye spraying.

The Olympus narrow band imaging (NBI) system employs a narrow band light to increase the contrast. This has been shown to enhance the views, and it provides similar diagnostic accuracy to chromoendoscopy<sup>[4-6]</sup>. Computed virtual chromoendoscopy (Fujinon intelligent chromoendoscopy, FICE) was subsequently developed<sup>[7]</sup>; with this technique, the wavelength does not change. Instead, the endoscopy processor reconstructs the image and displays what the mucosa would look like if illuminated using a certain wavelength. FICE has 10 pre-programmed settings, some of which have been tested in the upper gastrointestinal tract<sup>[7]</sup> and in Barrett's mucosa<sup>[8]</sup>. In a third preliminary study, we used one of the FICE settings to compare detection of colonic polyps with white light endoscopy (WLE)<sup>[9]</sup>. However, this study did not compare the views obtained using different FICE settings and did not comment on the capabilities in the presence of suboptimal bowel cleansing or after the application of indigo carmine dye. Our study aimed to: (1) systematically compare the 10 pre-set FICE settings with normal white light and indigo carmine dye in terms of their ability to enhance views of normal mucosa and polyps, (2) assess FICE in the presence of suboptimal bowel preparation, (3) determine whether poor bowel preparation markedly degrades the capabilities of "virtual chromoendoscopy" systems<sup>[10]</sup>.

## MATERIALS AND METHODS

High definition magnification colonoscopes (EC-590ZW/M), and the Fujinon EPX-4400 medical video endoscope system were used in the study.

### *The quality of mucosal and vascular pattern views*

In study one, 560 areas of normal colonic mucosa in 10 patients were observed in real time by two experienced endoscopists (Parra-Blanco A and González N), who assessed the quality of mucosal and vascular pattern views. The views were obtained with white light, 0.5% indigo carmine dye, and the different FICE settings (Table 1) both with and without magnification ( $\times 100$ ). The quality of the mucosal views was scored according to the following scale: 0 (poor), 1 (fair), 2 (good), and 3 (excellent). Each endoscopist was blinded to the other endoscopist's assessments.

### *Diagnostic accuracy*

In study two, 114 pictures were taken of 19 mucosal polyps (14 tubular adenomas, 4 hyperplastic polyps, and 1 normal mucosa). The pictures were obtained with white light, 0.5% indigo carmine (IC) dye, and the highest rated FICE settings according to study one (FICE setting 4), both with and without magnification ( $\times 100$ ). Polyp size and shape were estimated by comparison with closed biopsy forceps (Radial Jaw 3, Boston Scientific Corporation). The mean size of the polyps was 2.8 mm (range 1-5 mm). Figure 1 illustrates the sequence of images corresponding to one of the cases.

A panel of 5 fully trained endoscopists was asked to classify each lesion into one of the following categories: benign (for types I and II crypt patterns), neoplastic (types III and IV crypts), and invasive (type V pattern)<sup>[11]</sup>. For the

**Table 1** Spectral specification of each FICE filter estimation set with assignments to the color channels

	Blue (B) channel	Green (G) channel	Red (R) channel
0	500	445	415
1	500	470	420
2	550	500	470
3	540	490	420
4	520	500	405
5	500	480	420
6	580	520	460
7	520	450	400
8	540	415	415
9	550	500	400

FICE: Fujinon intelligent chromoendoscopy.

assessment of the capillary pattern, Sano's classification was used<sup>[10]</sup>. The panel members were asked to grade the degree of certainty in the prediction according to the following scale: (1) very uncertain, (2) uncertain, (3) reasonably certain, (4) absolutely certain.

### *The feasibility of FICE colonoscopy in the presence of suboptimal bowel preparation*

In study three, the feasibility of FICE colonoscopy in the presence of suboptimal bowel preparation was evaluated. Images from 17 colonic areas with small or moderate amounts of liquid or solid stools were recorded, both with the conventional view and with FICE setting 4. The panel of endoscopists rated: (a) the image quality of FICE setting 4 as superior, equal, or inferior to that of WLE; (b) the ability of FICE-4 to trace the fecal contents as compared to WLE (superior, equal, or inferior).

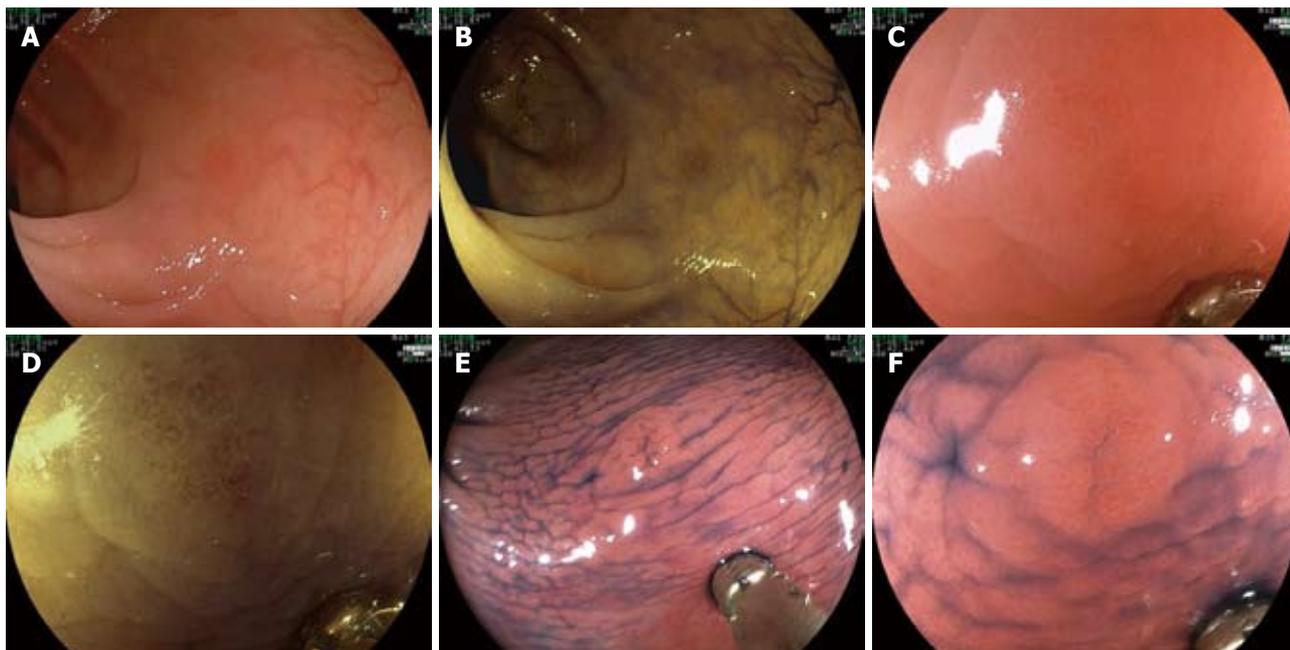
### *The ability of FICE to trace the polyp margin and recognize the irregularities of the polyp surface*

In study four, the ability to trace the polyp margin and recognize the irregularities of the polyp surface was compared between FICE setting 4, conventional endoscopy, and IC. Ninety images corresponding to 15 polyps were obtained and presented to the panel. The panel rated the ability of FICE to trace the margin and surface as superior, equal, or inferior to that of the other techniques.

### *The feasibility of FICE colonoscopy in IC-stained mucosa*

In study five, the feasibility of FICE colonoscopy in IC-stained mucosa was tested. In a preliminary phase, both endoscopists participating in study one had determined that colonoscopy with FICE setting 6 was the most adequate to observe the IC-stained mucosa, with a global image quality that was at least as good as with WLE. Then, 48 images corresponding to 12 polyps were taken, including views with IC and with IC plus FICE setting 6. The members of the panel were asked to rate the ability of FICE-6 plus IC to trace the margin and surface of the polyps as superior, equal, or inferior to the performance of IC.

The study was approved by the Research Ethics Committee of Hospital Universitario de Canarias, and a signed informed consent document was obtained from all participating patients.



**Figure 1** Presentation of a sequence of images for the different diagnostic modalities corresponding to a case included in study two (histopathological diagnosis: tubular adenoma with mild dysplasia). A: Circumscribed erythema with loss of vascular pattern, which can be difficult to detect; B: Observation with Fujinon intelligent chromoendoscopy (FICE) 4. Note that the innominate grooves can be seen in the normal surrounding mucosa; C: Observation under 0.5% indigo carmine (IC), which reveals a 0-II c depressed lesion 3 mm in size; D: High magnification view, showing vascular pattern type Sano II; E: Same magnification image observed under FICE 4, which provides enhanced vascular contrast; F: Under 0.5% IC plus magnification, the depression and the vascular patterns are evident.

### Statistical analysis

No sample sizes were calculated, as this was a pilot study, and no data were available when the study was performed regarding colonoscopy with FICE.

Categorical variables are expressed with frequencies and percentages. Proportions were compared using the  $\chi^2$  test or Fisher's exact test whenever required. Means were compared with the unpaired Student's *t* test, and means and standard deviations (SD) are reported.

For the same endoscopist, comparisons between techniques on quality of the images were carried out using the Wilcoxon test.

Calculated  $P < 0.05$  values were considered to indicate statistical significance.

## RESULTS

### Normal mucosa and vascular pattern

The assessment of FICE settings is shown in Figure 2. In evaluating the normal mucosa, only filter 4 was scored by both assessors as superior to conventional WLE (Table 2). The gold standard for mucosal observation, indigo carmine, was regarded as far superior to any FICE setting according to all assessors. In fact, the normal colonic innominate grooves could only be seen with the aid of dye spraying.

In evaluating the vascular pattern, FICE setting 4 (corresponding to RGB wavelengths 520, 500, and 405 nm, respectively) and FICE setting 2 were found to provide the best image quality. When mucosal and vascular pattern ratings were taken together, FICE setting 4 was considered the best by both assessors.

### Endoscopic diagnosis

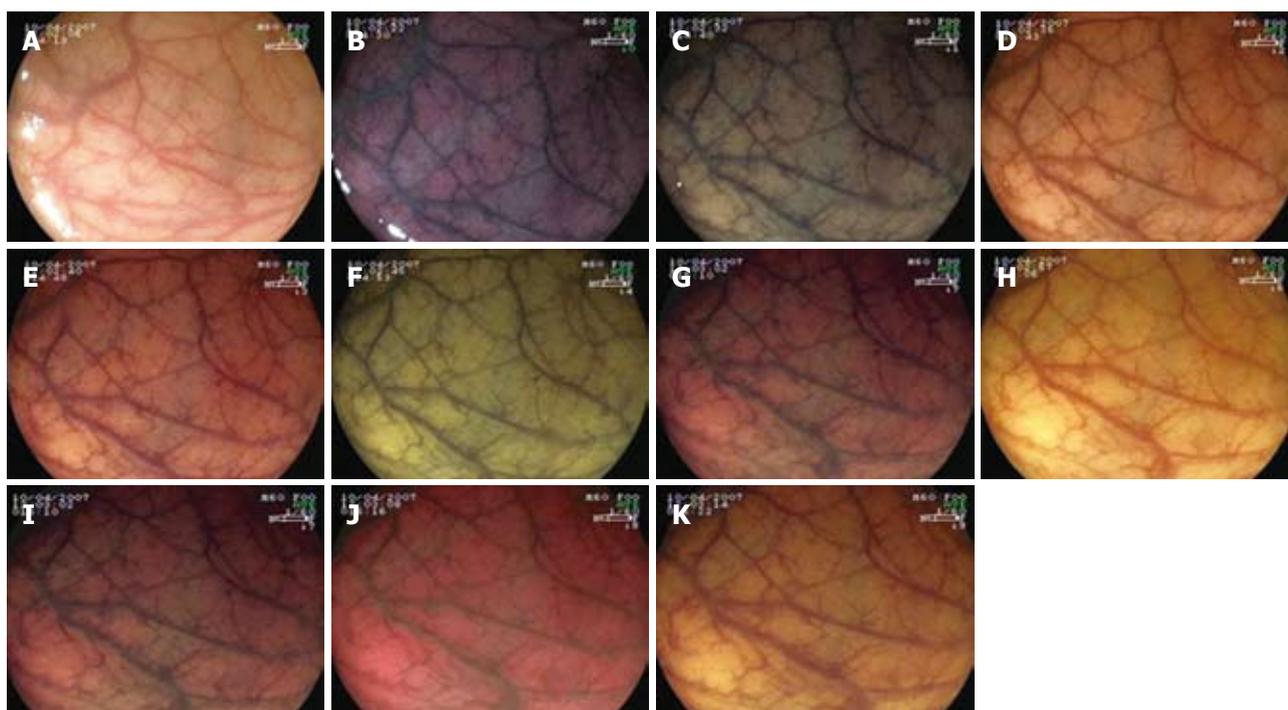
Study two (Figure 1): The histological predictions for the five participants are indicated in Table 3. In general, the diagnostic accuracy was related to the level of experience of the endoscopists in the panel. IC plus magnification, FICE setting 4 plus magnification, and IC without magnification were the most accurate techniques. The following differences among the techniques were statistically significant (Table 3): Conventional *vs* IC-Magnification:  $P = 0.002$ ; Conventional-Magnification *vs* FICE-Magnification:  $P = 0.02$ ; Conventional *vs* IC:  $P = 0.02$ ; Conventional *vs* IC-M:  $P = 0.001$ ; FICE *vs* FICE-Magnification:  $P = 0.048$ ; FICE *vs* IC:  $P = 0.048$ ; FICE *vs* IC-Magnification:  $P = 0.004$ .

The following differences among endoscopists were statistically significant (Table 3): Endoscopist 2 *vs* 4:  $P = 0.003$ ; Endoscopist 2 *vs* 5:  $P = 0.006$ ; Endoscopist 3 *vs* 4:  $P < 0.001$ ; Endoscopist 3 *vs* 5:  $P = 0.002$ .

The endoscopists' level of confidence in the endoscopic diagnosis was increased with these techniques compared to making the diagnosis with white light only (Figure 3).

### FICE and mucosa, polyps

In study three, FICE was particularly useful in evaluating the mucosa in the presence of suboptimal bowel preparation, and the panel found that in that situation, the view was generally better with FICE setting 4 than with WLE (Table 4). Small solid feces showed up as a vivid yellow color and transparent liquid as a darker yellow color; its distinction from colonic mucosa could be clearly seen (Figure 4).



**Figure 2** Images corresponding to the first study examining the evaluation of the vascular pattern without magnification. A: Plain endoscopy; B: Filter 0; C: Filter 1; D: Filter 2; E: Filter 3; F: Filter 4; G: Filter 5; H: Filter 6; I: Filter 7; J: Filter 8; K: Filter 9.

**Table 2** Individual scores for mucosal and vascular patterns with conventional imaging, indigo carmine, and FICE with each of the 10 filters

Type of endoscopy	Endoscopist 1				Endoscopist 2			
	Mucosal pattern	Vascular pattern	Sum score	<i>n</i>	Mucosal pattern	Vascular pattern	Sum score	<i>n</i>
Without FICE								
Conv	12	35	44	38	21	40	61	38
Indigo	59	-	59	20	59	-	59	20
FICE								
0	6	20	26	38	8	23	31	38
1	8	32	40	38	15	33	48	38
2	13	42	55	38	20	50	70	38
3	14	43	57	38	17	48	65	38
4	15	45	60	38	25	49	74	38
5	11	32	43	38	19	38	57	38
6	11	38	49	37	19	52	71	37
7	10	28	38	38	14	34	48	38
8	11	30	41	38	15	30	45	38
9	14	44	58	38	18	48	66	38

**Table 3** Accuracy of prediction of histopathological diagnosis among the five endoscopists with the different techniques applied *n* (%)

Endoscopists	1 ( <i>n</i> = 19)	2 ( <i>n</i> = 19)	3 ( <i>n</i> = 19)	4 ( <i>n</i> = 19)	5 ( <i>n</i> = 19)	Global ( <i>n</i> = 95)
Conv	9 (47.4)	13 (68.4)	12 (63.1)	10 (52.6)	10 (52.6)	54 (56.8)
Conv-M	12 (63.1)	12 (63.1)	14 (73.7)	6 (31.6)	9 (47.4)	53 (55.8)
FICE	10 (52.6)	11 (57.9)	12 (63.1)	13 (68.4)	10 (52.6)	56 (58.9)
FICE-M	15 (78.9)	14 (73.7)	15 (78.9)	11 (57.9)	12 (63.1)	67 (70.5)
Indigo	15 (78.9)	15 (78.9)	15 (78.9)	10 (52.6)	12 (63.1)	67 (70.5)
Indigo-M	17 (89.4)	16 (84.2)	15 (78.9)	13 (68.4)	12 (63.1)	73 (76.8)
Mean percentage	77.9	85.3	87.3	66.3	68.4	

Conv: Conventional; Conv-M: Conventional + magnification; FICE-M: FICE + magnification; Indigo-M: Indigo + magnification.

In study four, FICE setting 4 was found to be better than conventional endoscopy for tracing the margin of the polyps and observing the surface irregularities, and IC was found to

be better than FICE setting 4 (Tables 5 and 6, Figure 5).

In study five, when the polyps were observed under IC plus FICE setting 6, the view was not considered inferior

**Table 4** Evaluation of image quality of FICE-4 as compared to conventional WLE in the presence of remaining liquid or solid fecal content

Endoscopists	Quality				Fecal content			
	FICE better (%)	FICE equal (%)	FICE worse (%)	P <sup>1</sup>	FICE better (%)	FICE equal (%)	FICE worse (%)	P <sup>1</sup>
1	87.5	12.5	0		87.5	12.5	0	
2	93.8	6.2	0		100	0	0	
3	28.6	71.4	0		57.1	42.9	0	
4	87.5	12.5	0		100	0	0	
5	87.5	12.5	0	< 0.001	93.8	6.2	0	
Global mean	77.0			< 0.001	87.7			< 0.001

<sup>1</sup>P values are comparing the observed proportion of better with respect to 50%; WLE: White light endoscopy.

**Table 5** Comparison of the observation quality of the margins and surfaces of the polyps with FICE vs conventional WLE

Endoscopists	Margin				Surface			
	FICE better (%)	FICE equal (%)	FICE worse (%)	P <sup>1</sup>	FICE better (%)	FICE equal (%)	FICE worse (%)	P <sup>1</sup>
1	73.3	26.7	0		73.3	26.7	0	
2	75.0	25.0	0		43.8	56.3	0	
3	13.3	80.0	6.7		68.8	25.0	6.2	
4	68.8	25.0	6.2		31.3	68.8	0	
5	81.3	18.8	0		87.5	12.5	0	
Global mean	62.3			0.02	60.9			0.013

<sup>1</sup>P values are comparing the observed proportion of better with respect to 50%.

to that of IC alone (Table 7, Figure 6). Therefore, it can be concluded that colonoscopy with FICE setting 6 in the IC-stained mucosa is feasible.

## DISCUSSION

Virtual chromoendoscopy has been developed by endoscope manufacturers to reduce the need for dye spraying. FICE comes with 10 pre-installed settings, which have not previously been evaluated and compared to normal dye spraying techniques and WLE.

In our study, setting 4 was found to be best when assessing mucosal and vascular architecture. Teixeira *et al*<sup>[12]</sup> have reported on the effectiveness of FICE with magnification endoscopes in the differential diagnosis of neoplastic and non-neoplastic lesions. The FICE setting employed was R 500 nm, G 520 nm, and B 405 nm, as we felt that it provided the best imaging of the capillary vessels, after having compared multiple filter settings. However, those comparisons were not probably performed in a structured fashion, as no specific data about the comparisons is available in the study.

We described the settings as provided by the manufacturer. Because it is possible to personalize the filter settings and rename them, care should be taken not to misinterpret the filters used in different studies. Paradoxically, we found that the FICE settings that most closely correlated with the Olympus NBI mode provided the worst mucosal views. NBI and FICE are indeed different technologies, and the results obtained with one of them cannot be extrapolated to the other.

FICE was generally better at assessing vascular architecture than the mucosal surface. Moreover, indigo carmine dye spraying was significantly superior to any

FICE setting. In particular, the innominate grooves, important features when recognizing flat lesions, were perfectly outlined by indigo carmine dye.

Magnification appears to be as effective with FICE as with IC, whereas IC would be superior to FICE in the absence of magnification. Because this is a pilot study, these results should be confirmed in larger studies. The endoscopists participating in image interpretation were far more familiar with IC than with FICE. A learning curve in diagnostic interpretation of FICE may exist. Another possible explanation for the superiority of IC over FICE in our study could be that the former seems to provide a better mucosal contrast, and in the absence of magnification, this might lead to significant differences. In the only study available regarding FICE for the diagnosis of colonic polyps, Pohl *et al*<sup>[9]</sup> also found that IC and FICE were superior to WLE both with and without magnification. However, no difference was found when IC and FICE were compared. Because the mean size of polyps in their study was 7 mm, and lesions up to 20 mm in size were included, their results cannot be directly compared to those from our study, as our polyps were significantly smaller. Although they performed a sub-analysis on the subgroup of lesions  $\leq$  5 mm in size, no information was provided about possible differences between IC and FICE. Moreover, images were evaluated for diagnostic performance by one single endoscopist, whereas five endoscopists were involved in our study.

The diagnostic accuracy with the different techniques in our study was lower than in some other series<sup>[2,5,13-16]</sup>. Possible explanations for such differences are that some series included larger polyps, which are probably easier to diagnose<sup>[5,13-15]</sup>, or that diagnosis was estimated with a live video-endoscopy image and not with stored

**Table 6 Comparison of the observation quality of the margins and surfaces of the polyps with FICE vs IC**

Endoscopists	Margin			P <sup>1</sup>	Surface			P <sup>1</sup>
	FICE better (%)	FICE equal (%)	FICE worse (%)		FICE better (%)	FICE equal (%)	FICE worse (%)	
1	0	20.0	80.0		6.7	13.3	80.0	
2	0	50	50.0		0	25.0	75.0	
3	0	31.3	68.8		0	25.0	75.0	
4	50.0	25.0	25.0		0	68.8	31.3	
5	0	50.0	50.0		0	18.8	81.3	
Global mean		35.3		< 0.001		30.2		< 0.001

<sup>1</sup>P values are comparing the observed proportion of equal with respect to 50%. IC: Indigo carmine.

**Table 7 Comparison of the observation quality of the polyps with FICE-6 + IC vs IC**

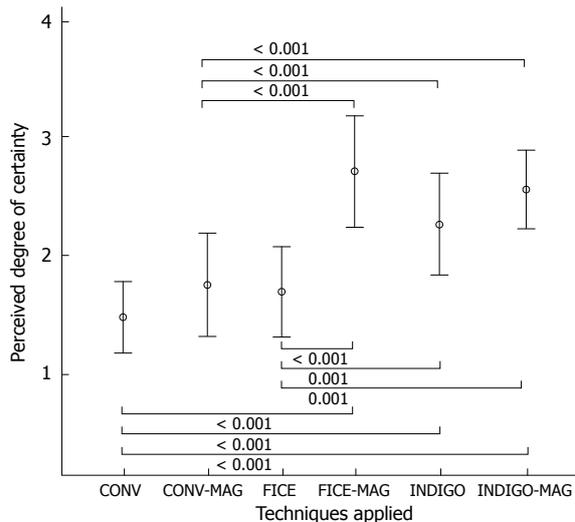
Endoscopists	Quality			P <sup>1</sup>
	FICE-6 + IC better (%)	FICE-6 + IC equal (%)	FICE-6 + IC worse (%)	
1	53.8	46.2	0	
2	61.5	30.8	7.7	
3	30.8	69.2	0	
4	30.8	69.2	0	
5	69.2	30.8	0	
Global mean	49.2			0.37

<sup>1</sup>P values are comparing the observed proportion of better with respect to 50%.

pictures<sup>[2,13]</sup>, which could enable a more exact diagnosis. Some series used absorptive stains, which can be more accurate<sup>[13,14]</sup>. Moreover, endoscopists with varying degrees of experience in magnifying colonoscopy participated in our study, and there were significant differences in diagnostic accuracy among them. In a recent study, East *et al*<sup>[16]</sup> reported a diagnostic accuracy of 69%-72% for magnification with methylene blue and 72%-84% for magnification plus NBI, which is similar to our findings. Further larger studies should compare FICE with IC without magnification in order to ascertain possible differences between the techniques.

The perceived degree of certainty in the diagnosis given with the different techniques applied was also evaluated. We believe that this issue deserves consideration when test performance is evaluated, as it could have an influence on the therapeutic strategy chosen by the endoscopist. We found that IC (with and without magnification) and FICE plus magnification - the techniques with a higher diagnostic accuracy - were associated with a greater perception of accurate diagnosis, and in fact they were more accurate.

Virtual chromoendoscopy techniques are expected to increase mucosal contrast. In fact, they should probably do so if they are to replace endoscopic stains. In our study, FICE was superior in highlighting the contour of lesions compared with WLE. However, IC remains the gold standard as it was found to be superior to FICE. Machida *et al*<sup>[4]</sup> came to a similar conclusion when comparing NBI with dye staining. One very important issue that will have to be explored in future studies is whether FICE or NBI can aid in the recognition of depressed lesions (Paris type 0-IIc). These lesions are

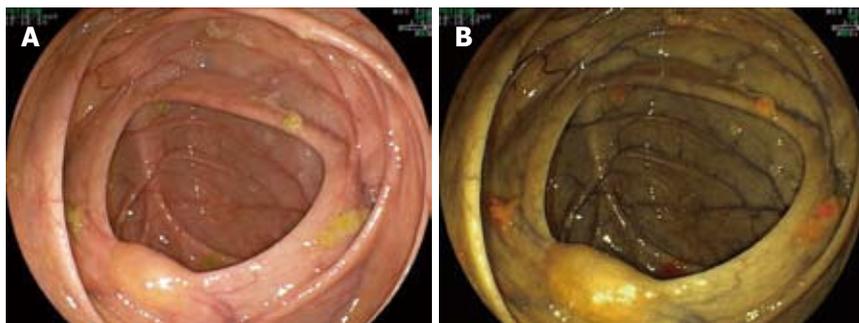


**Figure 3 Degree of certainty in the endoscopic diagnosis for all participating endoscopists with the six diagnostic modalities employed, when endoscopic images were sequentially shown for each case (mean ± SD). INDIGO-MAG: Indigo carmine plus magnification; FICE-MAG: FICE plus magnification; CONV-MAG: Conventional imaging plus magnification; CONV: Conventional.**

frequently small but frequently harbour high grade dysplasia or early invasive cancer<sup>[11,17]</sup>.

Adequate bowel cleansing is a prerequisite for the application of chromoendoscopy, as the mixture of stains and remaining fecal content worsens endoscopic observation<sup>[18]</sup>. It appears important to determine whether FICE can be applied when there is some fecal content remaining, because in most colonoscopies, at least some liquid material persists. when the NBI technology is used, suboptimal bowel preparation appears to negatively affect the endoscopic view. According to our initial impression and the endoscopists' subjective opinion measured in the study, FICE setting 4 was better than WLE for adequately observing the colonic mucosa when bowel content was present and for localizing and individualizing small fecal particles. Liquid and especially small fecal particles showed up as a vivid yellow color. This property of FICE might be useful to differentiate more accurately between small polyps and feces, allowing for targeted washing when small particles are detected.

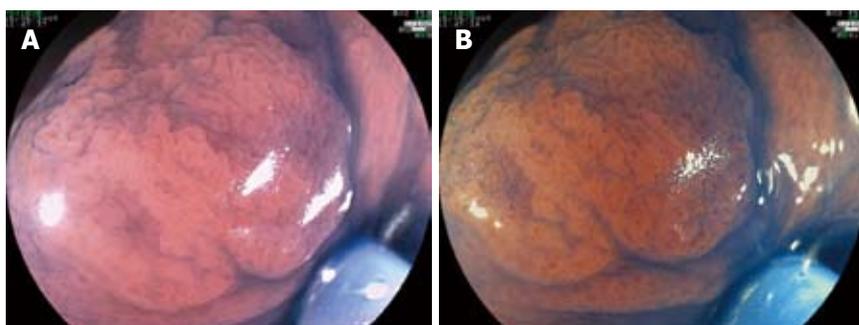
Finally, it is important to know the effect of IC on FICE observation. It is possible that both techniques could be applied in combination for certain indications, as FICE allows for better observation of the vascular



**Figure 4** Observation of the colonic mucosa in the presence of remaining stool particles. A: Plain endoscopy; B: FICE 4.



**Figure 5** Sequence of images shown for the evaluation of mucosal contrast, in a 0-II a flat elevated lesion, 3 mm in maximum diameter (histopathological diagnosis: tubular adenoma with mild dysplasia). A: Plain endoscopy plus magnification; B: FICE 4 plus magnification; C: 0.5% IC plus magnification.



**Figure 6** A flat elevated tubular adenoma, shown for the evaluation of FICE 6 plus IC. A: 0.5% IC plus magnification; B: 0.5% IC plus magnification plus FICE 6.

pattern and IC more clearly highlights the lesion edge and surface shape. FICE 6 did not interfere with endoscopic observation. The effect of IC on NBI observation has not been described, but in our experience, the endoscopic view is worsened, resulting in a greenish color.

It is noteworthy that in all cases, FICE was applied with magnifying high definition endoscopes (1 300 000 pixels). When we tried conventional endoscopes (resolution 410 000 pixels), we felt that the image quality was greatly worsened when any FICE filter was applied. Therefore, the results of our study apply only to high resolution endoscopes.

Our study has several limitations: first, the determination of the image quality provided by the different filters is unavoidably a subjective issue. However, we used a scale, a method similar to that used in previous studies<sup>[4]</sup>, and each endoscopist was blinded to the assessments of the other endoscopists. Moreover, although filter 4 had the highest scores, other filters also seemed to provide clear endoscopic images, but they were not evaluated in the current study. The effect of these filters in the colon could represent a field of future research. A second limitation is that our pilot study on polyps only included small

lesions, and none of the lesions was invasive; therefore, the usefulness of FICE for the prediction of invasiveness was not assessed. Finally, one investigator selected the endoscopic images and also participated in the prediction of histopathological diagnosis. However, he was unaware of the diagnosis, and in fact, he did not review any of the histopathological reports for any endoscopic procedure during the study period. When this investigator's assessments were excluded, there were no changes in the statistical results for the diagnostic accuracy of the techniques.

In conclusion, FICE setting 4 seems to be the most suitable for the observation of the colonic mucosa with high definition endoscopes. Its performance in the diagnosis of small polyps appears similar to IC when magnification is applied; however, in our pilot study, IC was superior to FICE in the absence of magnification. FICE seems to depict the polyp shape better than WLE but less clearly than IC. The presence of small amounts of liquid or solid feces or IC does not appear to interfere with endoscopic observation when FICE is applied. This is an initial description, and future studies should clarify the role of FICE in colonoscopic diagnosis.

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## COMMENTS

### Background

Fujinon intelligent chromoendoscopy (FICE) provides virtual chromoendoscopy, without the need for endoscopic stains. However, knowledge about the most adequate FICE settings in colonoscopy, and how it compares with conventional chromoendoscopy is still limited.

### Research frontiers

Many issues regarding colonoscopic imaging with FICE have to be explored, including comparison with other virtual chromoendoscopy technologies, traditional chromoendoscopy with stains, and even the influence of the quality of colonic cleansing.

### Innovations and breakthroughs

In this study a structured methodology was applied in order to determine which one among the multiple FICE-filters available provides a better observation of the vascular and mucosal patterns. After the most adequate filter was selected, it was applied for the examination of colonic polyps. In previous studies such a methodology to choose the best FICE-filter was not applied or described in detail. Additionally, in this study certain features of FICE imaging are explored, such as the possibility of applying it to observe indigo carmine-stained mucosa, or whether the existence of fecal contents has an influence on FICE imaging.

### Applications

Our results suggest that FICE is adequate for the observation of the vascular pattern, that indigo carmine is better for examining the fine mucosal surface and the margin and shape of the lesion, and that endoscopic stains (like indigo carmine) can be applied in combination with FICE-6. As opposed to narrow band imaging, indigo carmine or colonic fecal contents do not seem to interfere with FICE imaging. In fact, FICE-4 could clarify whether small mucosal irregularities are really mucosal lesions or adherent particles, and this could have an application in screening colonoscopy.

### Terminology

Virtual chromoendoscopy: The contrast of mucosal and vascular patterns is enhanced without the need of any endoscopic stain, by just pressing a button in the endoscope. FICE: Virtual chromoendoscopy is obtained by means of spectral estimation technology, i.e. computerized image reconstruction by the endoscopic processor. FICE filters or settings: there are 10 pre-programmed filters which are switched on the keyboard, each of which has different settings for estimated R, G, and B wavelengths.

### Peer review

This is a prospective study to validate high definition endoscopes with FICE in colonoscopy. This paper is quite interesting.

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BRIEF ARTICLE

## Diabetes mellitus as a risk factor for gastrointestinal cancer among American veterans

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### Abstract

**AIM:** To assess the risk of biliary and pancreatic cancers in a large cohort of patients with type 2 diabetes mellitus (DM).

**METHODS:** Eligibility for this study included patients with type 2 DM (ICD-9 code 250.0) who were discharged from Department of Veteran Affairs hospitals between 1990 and 2000. Non-matched control patients without DM were selected from the same patient treatment files during the same period. Demographic information included age, sex and race. Secondary diagnoses included known risk factors based on their ICD-9 codes. By multivariate logistic regression, the occurrence of biliary and pancreatic cancer was compared between case subjects with DM and controls without DM.

**RESULTS:** A total of 1172496 case and control subjects were analyzed. The mean age for study and control subjects was  $65.8 \pm 11.3$  and  $64.8 \pm 12.6$  years, respectively. The frequency of pancreatic cancer in subjects with DM was increased (0.9%) in comparison to control subjects (0.3%) with an OR of 3.22 (95% CI: 3.03-3.42). The incidence of gallbladder and

extrahepatic biliary cancers was increased by twofold in diabetic patients when compared to controls. The OR and 95% CI were 2.20 (1.56-3.00) and 2.10 (1.61-2.53), respectively.

**CONCLUSION:** Our study demonstrated that patients with DM have a threefold increased risk for developing pancreatic cancer and a twofold risk for developing biliary cancer.

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**Key words:** Diabetes mellitus; Pancreatic neoplasms; Adenocarcinoma; Gallbladder neoplasms

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Jamal MM, Yoon EJ, Vega KJ, Hashemzadeh M, Chang KJ. Diabetes mellitus as a risk factor for gastrointestinal cancer among American veterans. *World J Gastroenterol* 2009; 15(42): 5274-5278 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5274.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5274>

### INTRODUCTION

The prevalence of type 2 diabetes mellitus (DM) is rapidly growing globally and has become a major public health problem that is approaching epidemic proportions worldwide<sup>[1,2]</sup>. Even though cardiovascular complications are a major cause of morbidity and mortality in patients with diabetes, this disease has also been associated with several cancers, most notably of the liver, endometrium, kidney, and pancreas<sup>[3-5]</sup>.

To the best of our knowledge, the relationship of DM with gallbladder and extrahepatic biliary cancers has not been reported clearly in the literature. There has also been previous discussion over the role of diabetes in the development of pancreatic cancer; and there are recent studies that have suggested an elevated risk of pancreatic cancer in patients with DM<sup>[6-8]</sup>.

Although clinical conditions associated with high levels of insulin, such as acromegaly, are related to an increased risk of colon cancer, esophageal and gastric cancer, the relationship between type 2 DM and these gastrointestinal cancers has not been well established in large studies<sup>[9,10]</sup>.

It is of great significance to investigate the relationship between type 2 DM and gastrointestinal malignancies from an epidemiological standpoint, to determine preventive measures and implement screening strategies. Distinct from most previous studies that have involved either a limited sample size or a specified cancer site, we conducted a comprehensive assessment of the risk of gastrointestinal malignancies in a large cohort of patients with DM.

## MATERIALS AND METHODS

### Data collection and data sources

The Austin Automation Center has maintained the Patient Treatment File (PTF) since July 1969. The PTF documents inpatient treatment from all Veterans Health Administration (VHA) hospitals, extended care discharges and non-VHA hospital discharges at the Veterans Administration (VA) expense. The PTF contains demographic characteristics of patients and discharge diagnoses. Since 1984, a primary diagnosis and up to nine secondary diagnoses have been recorded according to ICD9-CM<sup>[11]</sup>.

### Identification of cases and controls

Cases were defined as patients who were diagnosed with type 2 DM. Cases with ICD9-CM code 250.0 were identified from the PTF from 1990 to 2000<sup>[11]</sup>. The date of a patient's first appearance in the PTF with type 2 DM was considered the date of diagnosis. Non-matched control patients without DM were selected from the same PTF during the same time period. Controls were gathered with a 3:1 ratio in proportion to cases. A method of random selection without replacement was used to ensure that no individual control subject was selected more than once.

### Calculation of comorbidity index

A comorbidity index was calculated for cases and controls. An adaptation of the Charlson Comorbidity Index as applied to administrative databases was employed<sup>[12,13]</sup>.

### Collected information and extraction of secondary diagnoses

Demographic information consisting of age, sex and ethnicity was obtained from the computerized records for cases and controls at the time of selection. Individual social security numbers were used to search the inpatient files from 1990 to 2000 for the following malignancies: gallbladder cancer (156.0), biliary cancers (156.1, 156.8 and 156.9), and pancreatic cancer (157.0, 157.1, 157.2, 157.3, 157.8 and 157.9)<sup>[11]</sup>. To ensure an appropriate temporal relationship between diabetes and these selected malignancies, cases were excluded if diabetes was not diagnosed at least 3 years prior to the diagnosis of the selected malignancy.

We searched the inpatient files for secondary diagnoses of potential risk factors for biliary and pancreatic cancer which included the following: cholelithiasis (574.0, 574.01, 574.10, 564.11, 574.20, 574.21, 574.60, 574.61, 474.70, 574.71, 574.80, 574.81, 574.90 and 574.91); choledocholithiasis (574.30, 574.31, 574.40, 574.41, 574.50 and 574.51); cholecystitis (575.0, 575.10, 575.11 and 575.12); other gallbladder diseases (575.2, 575.3, 575.4, 575.5, 575.6, 575.8, 575.9, 576.2, 576.3, 576.4, 576.8 and 576.9); sclerosing cholangitis (576.1), pancreatitis (577.0, 577.1 and 577.2); other pancreatic diseases (251.8, 251.0, 577.8, 577.9, 579.4 and 251.9); smoking or history of smoking (305.1, 989.84, E869.4 and V15.82); obesity (278.00 and 278.01); and hypercholesterolemia (272.0)<sup>[11]</sup>.

### Statistical analysis

Statistical analysis was performed using SAS/STAT Software (SAS Institute, Cary, NC, USA)<sup>[14]</sup>.  $P < 0.05$  was interpreted as being indicative of statistical significance. Continuous variables were analyzed by unpaired  $t$  tests. Binary variables were analyzed using the  $\chi^2$  and Fisher's exact tests. Quantitative variables were expressed as means  $\pm$  SD. In the multivariable analysis, a logistic regression model was used to assess the occurrence of gallbladder, biliary and pancreatic cancers using age, ethnicity, and potential risk factors as predictor variables, while controlling for differences in comorbid conditions. OR and 95% CI were used to indicate the strength of influence.

## RESULTS

We evaluated 278 761 patients with DM and 836 283 control patients hospitalized between 1990 and 2000. Case and control groups were well matched according to their demographic information. The mean age for case and control subjects was  $65.8 \pm 11.3$  and  $64.8 \pm 12.6$  years, respectively ( $P = \text{NS}$ ). There was a preponderance of male subjects in case and control groups (97.8% and 97.4%, respectively). Also, in the DM group, 66.3% were Caucasian compared to 68.3% in the control group (Table 1). As expected, there was a greater proportion of obesity among patients with DM compared to controls. Also, smokers were seen in 5.8% of the DM group compared to 6.8% in the control group.

Table 1 shows the distribution of pancreatic, gallbladder, and extrahepatic biliary cancers in case and control groups. The frequency of gallbladder cancer was 0.03% among patients with diabetes and there were no patients detected with gallbladder cancer in the control group. Extrahepatic biliary cancer was also five times more common in patients with diabetes when compared to control patients, 0.1% *vs* 0.02%, respectively. Pancreatic cancer in subjects with diabetes (0.9%) was three times more common when compared to control subjects (0.3%).

The occurrence of other biliary and pancreatic disorders is also depicted in Table 1 in both groups, as some of these disorders are potential risk factors for biliary or pancreatic cancer. Cholelithiasis was observed more commonly in DM patients (4.5%) in comparison to controls (2.8%). In addition, cholecystitis, sclerosing cholangitis and other

**Table 1** Demographics and predictive factors in diabetes mellitus (DM) patients and controls (%)

Variable	Diabetes (n = 278 761)	Controls (n = 836 283)	P value
Age ± SD (yr)	65.8 ± 11.3	64.8 ± 12.7	NS
Male sex	97.8	97.4	< 0.001
Caucasian	65.3	68.3	< 0.001
Obesity	15.7	7.5	< 0.001
Smoking	5.8	6.8	< 0.001
Gallbladder cancer	0.03	0	< 0.001
Extrahepatic biliary cancer	0.1	0.02	< 0.001
Pancreatic cancer	0.9	0.3	< 0.001
Pancreatitis	5.1	2.7	< 0.001
Cholelithiasis	4.5	2.8	< 0.001
Choledocholithiasis	0.8	0.5	< 0.001
Cholecystitis	0.9	0.5	< 0.001
Sclerosing cholangitis	0.4	0.2	< 0.001
Other biliary diseases	1.2	0.6	< 0.001
Other pancreatic disease	1.7	0.3	< 0.001

**Table 3** Pancreatic diseases associated with DM: univariate and multivariate analysis

Variables	Unadjusted OR (95% CI)	P value	Adjusted OR (95% CI)	P value
Age > 50 yr	1.51 (1.49-1.54)	< 0.001	1.51 (1.53-1.57)	< 0.001
Male sex	1.18 (1.14-1.22)	< 0.001	1.09 (1.06-1.22)	< 0.001
Caucasian	0.86 (0.85-0.87)	NS	0.91 (0.90-0.92)	NS
Smoking	1.16 (1.14-1.19)	< 0.001	0.99 (0.95-1.02)	NS
Obesity	2.30 (1.70-3.10)	< 0.001	2.68 (2.62-2.75)	< 0.001
Pancreatic cancer	2.48 (2.26-2.72)	< 0.001	3.22 (3.03-3.42)	< 0.001
Cholelithiasis	1.66 (1.62-1.70)	< 0.001	1.94 (1.89-1.98)	< 0.001
Choledocholithiasis	1.52 (1.44-1.62)	< 0.001	1.82 (1.72-1.93)	< 0.001
Pancreatitis	1.98 (1.94-2.03)	< 0.001	2.30 (2.25-2.36)	< 0.001
Other pancreatic disorders	5.16 (4.88-5.45)	< 0.001	6.15 (5.82-6.50)	< 0.001

biliary diseases occurred more commonly in diabetic patients. Furthermore, other pancreatic diseases were significantly higher in patients with diabetes when compared to controls (1.7% vs 0.3%, respectively).

Table 2 illustrates our first model of multivariate logistic regression, which analyzed gallbladder and extrahepatic biliary cancer in relation to DM. The presence of gallstone disease, smoking, and obesity were controlled for in both groups before the ORs were calculated. We found that gallbladder and extrahepatic biliary cancer was significantly and independently associated with DM with an OR and 95% CI of 2.20 (1.56-3.00) and 2.10 (1.61-2.53), respectively. Included in Table 2 are the associations between diabetes and other biliary diseases, which were accounted for to eliminate potential confounding factors. The presence of DM was found to be associated with cholelithiasis and other biliary disorders. (OR, 1.81; 95% CI, 1.77-1.85 and OR, 2.10; 95% CI, 1.86-2.36, respectively).

Table 3 demonstrates our second model of multivariate analysis, which examined the association between diabetes and the incidence of pancreatic cancer. The results were obtained after controlling for recognized risk factors, such as smoking, obesity, pancreatitis, and other pancreatic disorders. After all case and control patients

**Table 2** Gallbladder diseases associated with DM: univariate and multivariate analysis

Variables	Unadjusted OR (95% CI)	P value	Adjusted OR (95% CI)	P value
Age > 50 yr	1.51 (1.49-1.54)	< 0.001	1.56 (1.54-1.58)	< 0.001
Gender M	1.18 (1.15-1.22)	< 0.001	0.80 (0.75-0.85)	NS
Race W	0.86 (0.85-0.87)	NS	0.75 (0.69-0.79)	NS
Smoking	1.16 (1.14-1.19)	< 0.001	0.86 (0.84-0.88)	NS
Obesity	2.30 (1.70-3.10)	< 0.001	2.52 (2.47-2.58)	< 0.001
Gallbladder cancer	2.01 (1.47-2.76)	< 0.001	2.20 (1.56-3.00)	< 0.001
Extrahepatic biliary cancer	1.81 (1.44-2.27)	< 0.001	2.10 (1.61-2.53)	< 0.001
Cholelithiasis	1.66 (1.62-1.70)	< 0.001	1.81 (1.77-1.85)	< 0.001
Choledocholithiasis	1.52 (1.44-1.62)	< 0.001	1.47 (1.43-1.50)	< 0.001
Cholecystitis	1.97 (1.87-2.08)	< 0.001	2.25 (2.02-2.52)	< 0.001
Sclerosing cholangitis	1.93 (1.78-2.10)	< 0.001	2.37 (1.76-3.12)	< 0.001
Other biliary diseases	1.99 (1.90-2.09)	< 0.001	2.10 (1.86-2.36)	< 0.001

were considered, a significant relationship between diabetes and pancreatic cancer was established (OR, 3.22; 95% CI, 3.03-3.42). Diabetes was also associated with pancreatitis and other pancreatic disorders with an OR of 2.30 (95% CI, 2.25-2.36) and 6.15 (95% CI, 5.82-6.50).

## DISCUSSION

Type 2 DM is one of the most common and challenging problems faced today because of the many complications that result from this disease. Approximately 140 million people worldwide currently have diabetes, and this number is projected to reach up to 300 million by the year 2025<sup>[15]</sup>. The massive prevalence of this disease may now be unmasking additional, yet to be discovered, complications such as pancreatic and biliary cancer. A recent study has revealed a relationship between increasing fasting serum glucose and the incidence of gastrointestinal malignancies in Korean men and women<sup>[16]</sup>.

Pancreatic cancer is the fifth leading cause of cancer-related mortality in the United States<sup>[17,18]</sup>. The incidence of pancreatic cancer is higher among men and African Americans<sup>[18,19]</sup>. Previous studies have documented risk factors for the development of pancreatic cancer<sup>[6-8]</sup>. The most well-established risk factors for pancreatic cancer are smoking, genetic predisposition, chronic pancreatitis, and DM. A recent study has shown an increase in the incidence of pancreatic cancer among overweight patients, and that moderate exercise is associated with a lower incidence of pancreatic cancer<sup>[20]</sup>.

The linkage between pancreatic cancer and diabetes is well recognized. However, it has been debatable whether diabetes is a risk factor for, or a consequence of, pancreatic cancer. Earlier studies could not address this question adequately because the presence of type 2 diabetes has not been documented before the onset of disease. A more recent meta-analysis has shown that a history of diabetes for  $\geq 5$  years increases the incidence of pancreatic cancer by twofold<sup>[21]</sup>. In a hospital based case-control study, Bonelli *et al*<sup>[22]</sup> have demonstrated that the risk of pancreatic cancer was increased by 6.2-fold in patients with diabetes, which necessitated insulin therapy

for > 5 years. Our study supported these findings by showing that the occurrence of pancreatic cancer was increased by threefold in DM patients when compared to controls.

Primary carcinoma of the gallbladder is the fifth most common malignancy of the gastrointestinal tract in the United States. Approximately 5000-6000 adults are diagnosed annually, and most will die within 1 year of diagnosis<sup>[23,24]</sup>. Although the etiology of primary gallbladder carcinoma is not well understood, several factors have been postulated to place patients at a greater risk. These risk factors include gallstone disease, obesity, female sex, tobacco use, and an anomalous pancreaticobiliary ductal union<sup>[24,25]</sup>. Perhaps no risk factor has been attributed to gallbladder cancer more than gallstone disease, but the relationship between diabetes and gallbladder cancer has not been established conclusively. However, there has been one previous population-based study that showed an excess risk for all biliary cancers in patients with diabetes<sup>[5]</sup>.

This is one of the first studies to demonstrate a clear association of diabetes with gallbladder and extrahepatic biliary cancer. In our study, patients with diabetes had a twofold increased risk of having gallbladder or extrahepatic biliary cancer after controlling for known risk factors.

Our understanding of DM has changed dramatically over the past few years. Initially thought of as a disease secondary to a lack of insulin production, type 2 DM is now recognized as an ailment of insulin resistance and resultant hyperinsulinemia. First-line therapy now specifically targets insulin-mediated glucose utilization in the liver and peripheral tissue<sup>[26,27]</sup>. The over-production of both insulin and insulin-like growth factors (IGFs) has been postulated to increase carcinogenesis and may have played an essential role in the development and progression of pancreatic and biliary cancer in our patients with diabetes<sup>[28]</sup>. It would be of great interest to see whether there is a trend towards a greater risk for pancreatic and gallbladder cancer among diabetics with an increasing degree of hyperinsulinemia.

Evidence from animal and human studies has suggested that abnormal glucose metabolism plays an important role in pancreatic carcinogenesis<sup>[29,30]</sup>. Recent evidence supports the suggestion that insulin and IGF-1 act through a tyrosine kinase growth factor cascade in enhancing tumor cell proliferation.

Another contributor to this problem might be the rising prevalence in all age groups of the metabolic syndrome, specifically insulin resistance, in the United States<sup>[31]</sup>. The metabolic syndrome is associated with a chronic inflammatory state with accompanying cytokine abnormalities, which could also contribute to tumor progression. Furthermore, diabetic patients are at a higher risk for developing gallstones, which have also been recognized as a risk factor for the development of gallbladder and other biliary cancers<sup>[32]</sup>.

The strength of our study was that it encompassed a large patient population, dramatically more than any other study that has examined this association. This

approach allowed for a large sample of diabetics in the VA population, thereby increasing the overall power of the study.

The weakness of our study was that the population we studied was restricted to the VA population and therefore was composed mainly of men. Obtaining a larger female sample could have been helpful in allowing us to observe any differences in the associations according to sex. Other limitations to our study were the potential for misclassification bias (errors in cancer diagnosis ICD9-CM code classification) and cancers diagnosed post mortem. However, given the large size of our patient population, the significance of misclassification and post mortem cancer diagnosis were thought to be inconsequential toward the overall study results. Also, because of our current dearth of knowledge regarding the development of biliary and pancreatic malignancies, our results might have been affected by unknown confounding factors. However, our study did address the known risk factors for these malignancies and controlled for these so that they would not affect our overall results.

In summary, we found that type 2 DM was associated with an increased risk of gallbladder, biliary and pancreatic cancer, independent of other known risk factors such as cholelithiasis, pancreatitis, obesity and smoking. This information should further heighten our awareness of the many complications associated with insulin resistance and modify the intensity of our approach to these patients. This could warrant a keener eye by the primary care physician for any abnormalities in his or her diabetic patients, which indicate the possibility of cardiovascular, renal and ophthalmic complications, as well as the rare but foreseeable possibility of a fatal pancreaticobiliary malignancy.

## COMMENTS

### **Background**

The prevalence of type 2 diabetes mellitus (DM) is growing globally and has become a major public health problem that is approaching epidemic proportions worldwide. This disease has been associated with several cancers, most notably of the liver, endometrium, kidney and pancreas. To the best of our knowledge, the relationship of DM with gallbladder and extrahepatic biliary cancers has not been reported clearly in the literature.

### **Research frontiers**

It is of great significance to investigate the relationship between type 2 DM and gastrointestinal malignancies from an epidemiological standpoint, to determine preventive measures and implement screening strategies.

### **Innovations and breakthroughs**

Distinct from most previous studies that have involved a limited sample size or a specified cancer site, we conducted a comprehensive assessment of the risk of gallbladder, biliary and pancreatic malignancies in a large cohort of patients with DM.

### **Applications**

Type 2 DM poses an increased risk of gallbladder, biliary and pancreatic cancer, independent of other known risk factors such as cholelithiasis, pancreatitis, obesity and smoking. This information heightens awareness of the many complications associated with insulin resistance and should modify the intensity of our approach to these patients. This warrants a keener eye by primary care physicians for any abnormalities in their diabetic patients, which indicate the possibility of cardiovascular, renal and ophthalmic complications, as well as the rare but foreseeable possibility of a fatal pancreaticobiliary malignancy.

### **Terminology**

Type 2 DM is associated with an increase in the occurrence of gallbladder,

biliary and pancreatic cancer among American veterans, independent of other known risk factors.

### Peer review

The study deals with an interesting epidemiological analysis of the question if DM is a risk factor for several gastrointestinal cancers. It reports associations between type 2 DM and incidence of certain cancers.

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## Comorbidity negatively influences prognosis in patients with extrahepatic cholangiocarcinoma

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analysis, tumor resection, CCI score, and serum CA 19-9 levels correlated significantly with outcome. In the multivariate analysis, only resection (HR 0.10; 95% CI, 0.02-0.51,  $P = 0.005$ ) and a CCI score  $\geq 2$  (HR 3.36; 95% CI, 1.0-10.9,  $P = 0.045$ ) were found to independently predict survival.

**CONCLUSION:** Tumor resection and comorbidity emerged as significant prognostic variables in extrahepatic cholangiocarcinoma. Comorbidity evaluation instruments should be applied in the clinical management of such patients.

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**Key words:** Charlson index; Cholangiocarcinoma; Comorbidity; Prognosis; Survival

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### Abstract

**AIM:** To study the outcome and prognostic factors in a series of patients with extrahepatic cholangiocarcinoma and determine the impact of comorbidity on survival.

**METHODS:** A retrospective analysis of 68 patients with extrahepatic cholangiocarcinoma (perihilar,  $n = 37$ ; distal,  $n = 31$ ) seen at a single tertiary-care institution during the period 1999-2003 was performed. Data on presentation, management, and outcome were assessed by chart review. Pathologic confirmation was obtained in 37 cases (54.4%). Comorbidity was evaluated by using the Charlson comorbidity index (CCI).

**RESULTS:** Mean age at diagnosis was  $73.4 \pm 11.5$  years. Jaundice was the most common symptom presented (86.8%). Median CCI score was 1 (range, 0 to 4). Nineteen patients (27.9%) underwent tumor resection. Palliative biliary drainage was performed in 39 patients (57.4%), and 6 patients (8.8%) received only best supportive care. Tumor-free margin status (R0) was achieved in 15 cases (78.9% of resection group). Baseline serum carbohydrate antigen 19-9 (CA 19-9) level was revealed to be an independent predictor of surgical treatment ( $P = 0.026$ ). Overall median survival was  $3.1 \pm 0.9$  mo, with 1- and 2-year survival rates of 21% and 7%, respectively. In the univariate

### INTRODUCTION

Cholangiocarcinoma is a relatively uncommon malignant tumor arising in the intrahepatic or extrahepatic biliary ducts. It accounts for about 3% of all gastrointestinal cancers globally and constitutes the second most common primary hepatic malignant disease, with approximately 5000 new cases being diagnosed annually in the United States<sup>[1]</sup>. Although the entire biliary tree is potentially at risk, the perihilar region is the most frequently involved site, accounting for about 60% of all tumors<sup>[1]</sup>. However, it has been suggested that the incidence of intrahepatic forms of the disease is currently increasing in the US and the United Kingdom<sup>[2]</sup>. Cholangiocarcinoma has been characterized as a slow-growing and late metastasizing tumor, tending to spread longitudinally along the bile ducts with neural, perineural, and subepithelial extensions.

Because of the late presentation of symptoms, tumors are usually diagnosed in their later stages, and thus most therapeutic approaches are not curative<sup>[1]</sup>. The prognosis for patients with unresectable disease is dismal, with the reported median survival time less than 1 year from diagnosis<sup>[3]</sup>.

In developed countries, the peak age at diagnosis of cholangiocarcinoma has shifted from the sixth decade of life during the 1970s, toward the seventh decade and older nowadays. According to Patel, the median age at the time of death in individuals with intrahepatic cholangiocarcinoma - based on US vital-statistics data referring to the period between 1973 and 1997 - was 71 years for males and 74 years for females<sup>[2]</sup>. Recent works from various European institutions have reported a median age at diagnosis of 64 to 75 years<sup>[4-7]</sup>. Due to the increased ageing of the Western population, there is an emerging need to develop a means to characterize the "functional age" of older patients in order to optimize therapeutic strategies for cancer and design new multi-disciplinary approaches. Multiple studies have demonstrated that the amount of comorbidity (defined as the presence of diseases or disorders which exist before cancer diagnosis and are not treatment-related adverse effects) significantly impacts on various prognostic outcomes in oncologic patients, such as functional status<sup>[8]</sup>, healthcare resources use<sup>[9]</sup>, therapeutic decision-making<sup>[10]</sup>, and overall survival<sup>[11]</sup>. The Charlson comorbidity index (CCI) was originally developed by Mary Charlson and colleagues in 1987 from the study of 1-year all-cause mortality in a cohort of more than 500 patients admitted to a medical unit of a teaching hospital<sup>[12]</sup>. This index has been validated in predicting mortality risk associated with a wide range of medical conditions, and constitutes one of the most commonly used comorbidity indices to date. Correlations between the severity of comorbid conditions, assessed by means of the CCI, and diverse outcomes have been observed in patients with colorectal<sup>[13]</sup>, head and neck<sup>[14]</sup>, non-small cell lung<sup>[15]</sup>, bladder<sup>[10]</sup>, clear cell renal<sup>[16]</sup>, and ovarian cancer<sup>[17]</sup>. Some reports indicate that the impact of comorbidity on survival may vary among populations with different cancers. To the best of our knowledge, no previous studies have focused on the role of age and comorbidity, quantified using the CCI, on treatment decisions and clinical outcomes for patients with biliary tract malignancies. Thus, we conducted a single-center analysis of consecutive Spanish patients with extrahepatic cholangiocarcinoma to investigate the impact of these variables on the choice of therapeutic approach and patients' overall survival.

## MATERIALS AND METHODS

The tumor registry of the University Hospital "12 de Octubre" compiles data on all new cancer cases in Health Area 11 of the Community of Madrid (Central Spain), with 593931 inhabitants in 2007. We carried out a retrospective analysis of all the patients with extrahepatic cholangiocarcinoma (code C24.0 according to the 2nd edition of the International Classification of

Diseases for Oncology) consecutively diagnosed at our institution between January 1, 1999 and December 31, 2003. Full clinical documentation was available for 68 subjects; they were then included in the study. Diagnosis of cholangiocarcinoma was based on clinical, imaging, cytologic or histopathologic findings. All patients underwent ultrasound examination of the liver and gallbladder as the first diagnostic imaging approach. Further procedures included triple phase helical computed tomography in 60 patients (88.2%), cholangio-magnetic resonance imaging in 14 patients (20.6%), and cholangiography by means of either the percutaneous transhepatic (PTC) or endoscopic retrograde approach (ERCP) in 39 (57.4%) and 32 (47.1%) patients, respectively. Pathologic confirmation was established in 37 cases (54.4%), a proportion in accordance with previously published series<sup>[4,6,18,19]</sup>, and was based on either histologic or cytologic samples (24 and 13 cases, respectively).

Demographic data, predisposing factors, clinical manifestations at admission, laboratory and imaging findings, pathology reports, therapeutic approaches, and all-cause mortality were assessed by review of medical records. Additional variables such as length of hospital stay or presence of perioperative complications were specifically recorded in patients who underwent surgical resection as the first-line treatment. Extrahepatic cholangiocarcinomas were classified as perihilar (those involving or requiring resection of the hepatic duct bifurcation) or distal types (those involving the distal extrahepatic, or intrapancreatic portion of the bile duct and potentially amenable to pancreatoduodenectomy). The American Joint Committee on Cancer (AJCC) 2003 criteria were used for TNM (Tumor, Node, Metastases) staging of the tumor<sup>[20]</sup>. Perihilar tumors were classified according to the modified Bismuth-Corlette classification<sup>[21]</sup>. CCI scores were calculated by the method previously reported by Charlson *et al*<sup>[12]</sup>, in which each specific comorbid condition is weighted and scored (Table 1). These scores were determined by one of the authors (Fernández-Ruiz M) who was blinded to survival status.

The primary endpoint of the study was overall survival, defined as the interval (in months) between tumor diagnosis and death or completion of the follow-up period (December 31, 2004). Patients lost during this period were recorded as of the last known contact in our institution. Quantitative data are shown as mean  $\pm$  SD, or median  $\pm$  range or 95% confidence interval (95% CI), as appropriate. Qualitative variables are expressed as absolute and relative frequencies. Nominal variables were compared by the  $\chi^2$  test or Fisher's exact test. Two-tailed Student's *t* test (or *U* Mann-Whitney test when the assumption of normality did not hold) were applied for continuous variables. We used logistic regression analysis in order to identify factors predictive of tumor resectability. Survival curves were estimated by the Kaplan-Meier product-limit method, and differences between groups were compared with the log-rank test (univariate analysis). Multivariate analysis was based on the stepwise forward Cox proportional hazards model, using survival as the dependent variable and those factors demonstrating statistical significance

**Table 1 The Charlson comorbidity index (CCI)<sup>1,2</sup>**

Weight <sup>1</sup>	Comorbid condition
1	Myocardial infarction Congestive heart failure Peripheral vascular disease Cerebrovascular disease (except hemiplegia) Dementia Chronic obstructive pulmonary disease Connective tissue disease Peptic ulcer disease Mild liver disease
2	Diabetes (without complications) Hemiplegia Moderate or severe renal disease Diabetes with end-organ damage (retinopathy, neuropathy, etc) Any second solid tumor (nonmetastatic), leukemia or lymphoma
3	Moderate or severe liver disease
6	Metastatic solid tumor AIDS

<sup>1</sup>Optionally, the age index leads to adding 1 point for each decade over 40 years.

in the univariate analysis as covariates. To assess the role of CCI as a predictor of mortality, survival analysis was carried out with the cohort divided into 2 groups based on its median value (CCI score equal or lower than 1, or greater than 1). We also dichotomized other continuous variables by using their mean or median values, except for total bilirubin (cut-off value at 10 mg/dL) and hemoglobin (cut-off value at 12 g/dL). Differences were considered significant at *P* < 0.05. All statistical analysis was performed using the software package SPSS, version 12.0 (SPSS Inc., Chicago, IL, USA).

## RESULTS

### Patients' characteristics

A total of 68 consecutive patients diagnosed with extrahepatic cholangiocarcinoma during the study period were analyzed. Their baseline characteristics stratified by primary tumor location are summarized in Table 2. There were 34 males and 34 females, with a mean age at diagnosis of 73.4 ± 11.5 years (range, 42 to 96 years). Forty-seven patients were older than 70 (69.1%) years. Regarding the risk factors for the development of cholangiocarcinoma, only 1 patient from the cohort had a previous diagnosis of primary sclerosing cholangitis (PSC). A history of underlying chronic liver disease was recognized in 5 cases (7.4%): hepatitis B and C infection (2 patients each), and chronic alcoholism (1 patient). Five patients (7.4%) had previously undergone cholecystectomy. No cases of Caroli's disease, choledochal cyst, hepatolithiasis, or exposure to chemical agents were found. A family history of malignancy was reported in 5 patients (7.4%). Major clinical symptoms at admission were jaundice (86.8%), abdominal pain (36.7%), and weight loss (27.9%). Age and sex distribution, predisposing factors, clinical manifestations, and duration of symptoms were similar between patients with perihilar and distal lesions. The serum lactate dehydrogenase level in patients with distal cholangiocarcinoma (169 ± 54 IU/L) was lower than that in patients with perihilar tumors (269 ±

**Table 2 Demographic, clinical, and laboratory data of patients at baseline (mean ± SD) *n* (%)**

Variable	Perihilar ( <i>n</i> = 37)	Distal ( <i>n</i> = 31)	Total ( <i>n</i> = 68)
Age (yr)	74.2 ± 10.5	72.4 ± 12.6	73.4 ± 11.5
Sex (M/F)	18/19	16/15	34/34
Smoking	13 (35.1)	8 (25.8)	21 (30.9)
Predisposing factor	5 (13.5)	1 (3.2)	6 (8.8)
Family history of malignancy	1 (2.7)	4 (12.9)	5 (7.4)
Clinical manifestations			
Jaundice	33 (89.2)	26 (83.9)	59 (86.8)
Abdominal pain	16 (43.2)	9 (29.0)	25 (36.7)
Weight loss	11 (29.7)	8 (25.8)	19 (27.9)
Fever	0 (0)	3 (9.7)	3 (4.4)
Casual diagnosis	0 (0)	1 (3.2)	1 (1.5)
Symptoms duration (mo) [median (range)]	0.3 (0.01-12)	0.5 (0.05-3.5)	0.5 (0.01-12)
AST (IU/L)	146 ± 104	129 ± 121	138 ± 111
ALT (IU/L)	211 ± 166	172 ± 139	193 ± 144
γ-GT (IU/L)	629 ± 377	811 ± 660	712 ± 529
LDH (IU/L) <sup>a</sup>	269 ± 180	169 ± 54	223 ± 174
Albumin (g/dL)	3.3 ± 0.4	3.2 ± 0.7	3.2 ± 0.6
Bilirubin (mg/dL)	15.6 ± 9.1	12.6 ± 9.5	14.2 ± 9.3
Hemoglobin (g/dL)	13.4 ± 1.4	12.7 ± 2.3	13.1 ± 1.9
Platelets (× 1000/μL)	257 ± 99	294 ± 103	274 ± 102
Creatinine (mg/dL)	0.8 ± 0.4	0.8 ± 0.3	0.8 ± 0.3
CA 19-9 (IU/L)	989	87.6	269
[median (range)] <sup>b</sup>	(2.9-65920)	(9-11 641)	(2.9-65920)

<sup>a</sup>Data available for 29 patients; <sup>b</sup>*P* = 0.005; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CA 19-9: Carbohydrate antigen 19-9; γ-GT: γ-glutamyltranspeptidase; LDH: Lactate dehydrogenase.

180 IU/L, *P* = 0.005), while there was a nearly significant difference in serum carbohydrate antigen 19-9 (CA 19-9) levels at diagnosis (87.6 IU/L *vs* 989 IU/L, respectively, *P* = 0.057). Other hematologic and liver function tests were similar in both groups.

The median CCI score was 1 (range, 0 to 4). Thirty-one patients (45.6%) had no comorbidities (CCI score of 0), 18 (26.5%) had a modest comorbidity level (CCI score of 1), and 19 (27.9%) had a high comorbidity level (CCI score ≥ 2). The most common comorbid conditions encountered were hypertension (44%), diabetes mellitus (17.6%), chronic obstructive pulmonary disease (16%), coronary heart disease (11.7%), and cerebrovascular disease (8.8%). A history of previous malignancy was identified in 4 patients (5.8%).

### Treatment approaches

After initial assessment, 23 patients (33.8%) were considered to have potentially resectable disease and underwent laparotomy with curative intent. At exploration, 4 patients had findings (locally advanced tumor) that precluded resection. Surgical therapy in the remaining 19 patients (27.9%) consisted of partial duodenopancreatectomy with radical lymphadenectomy in 13 cases, excision of the extrahepatic biliary tree in 4 cases, and extrahepatic duct resection associated with left hemihepatectomy in 2 cases. Median hospital stay was 30 d (range, 15 to 66 d). Major postoperative complications occurred in 14 patients (60.8% of the surgical group) and included sepsis (4 cases), surgical wound infection (3 cases), digestive tract bleeding

Table 3 Analysis of predictive variables for resectable tumor

Variable	Univariate			Multivariate		
	OR	95% CI	P	OR	95% CI	P
Age $\geq$ 73 yr	0.24	0.0-0.7	0.012	-		
Distal location	5.27	1.6-17.1	0.004	-		
CEA $\geq$ 5 IU/L	0.09	0.0-0.6	0.013	-		
CA 19-9 $\geq$ 270 IU/L	0.05	0.0-0.5	0.003	0.07	0.0-0.7	0.026

CEA: Carcinoembryonic antigen; OR: Odds ratio.

(3 cases), and death within 30 d after procedure (3 cases). Primary tumor location, age, and baseline serum levels of carcinoembryonic antigen (CEA) and CA 19-9 were identified as predictive variables for resectable disease, whereas CCI scores did not differ significantly between patients who had surgery and those who did not (Table 3). In logistic regression multivariate analysis, only serum CA 19-9 levels  $\geq$  270 IU/L predicted unresectability (OR, 0.07; 95% CI, 0.0-0.7;  $P = 0.026$ ).

Palliative biliary drainage was performed in 39 subjects (57.4%) with non-resectable tumors. Thirteen patients (19.1%) underwent endoscopic biliary stenting by ERCP, whereas percutaneous approach by PTC was required in 26 patients (38.2%), associated with stent placement in 14 cases. Chemo- or brachy-radiotherapy were employed as adjuvant treatment in 3 and 7 subjects, respectively. Finally, 6 patients (8.8%) received only best supportive care.

### Macroscopic and microscopic appearance

TNM staging distribution by primary location is presented in Table 4. The majority of tumors were pathologically classified as T3 (36.7%) and metastatic spread was identified in 13.2% of patients. According to the Bismuth-Corlette classification of perihilar cholangiocarcinomas, 6 out of the 37 patients with such tumors were diagnosed as stage I, 8 as stage II, 5 as stage IIIa, 7 as stage IIIb, and 7 as stage IV. Four patients remained unclassified. Of the 19 patients who underwent resection, 15 (78.9%) had negative histologic margins (R0 resection), whereas in 3 cases (15.8%) the margins were microscopically involved with the tumor (R1 resection).

### Survival analysis

Median follow-up time for the entire cohort was 2.7 mo (range, 0.07 to 49.4 mo). Five patients (7.4%) were lost to follow-up. At the end of observation, 60 of 68 patients (88.2%) had died, with an overall median survival of 3.1 mo (95% CI, 1.4-4.8). Survival rates at 1, 2, and 3 years were 21%, 7%, and 2% respectively. Tumor progression (27.9%), infection (13.2%), liver failure (8.8%), and bleeding (4.4%) were the most frequently recorded causes of death. The clinical, tumor-related, and treatment-related variables evaluated by univariate and multivariate analysis to determine their impact on outcome are presented in Table 5. For the univariate log-rank analysis, surgical resection, lower comorbidity index (CCI score  $<$  2), and lower serum CA 19-9 levels ( $<$  270 IU/L) correlated significantly with better survival. Patients

Table 4 AJCC-TNM staging distribution

	Perihilar (n = 37)	Distal (n = 31)	Total (n = 68)
Tumor status			
T1	3	3	6
T2	8	5	13
T3	16	9	25
T4	3	6	9
Unknown	7	8	15
Lymph node status			
N0	27	19	46
N1	10	10	20
Unknown	1	1	2
Metastases status			
M0	31	28	59
M1	6	3	9

AJCC: American Joint Committee on Cancer; TNM: Tumor, Node, Metastases.

who underwent resection had a longer median survival than those who did not undergo such treatment ( $8.7 \pm 5.7$  mo *vs*  $2.3 \pm 0.4$  mo,  $P = 0.015$ ) (Figure 1A). Regarding the presence and number of comorbid conditions, the median survival in patients with a CCI score of 0 or 1 was longer than that in patients with a higher score ( $4.7 \pm 0.8$  mo *vs*  $1.4 \pm 0.5$  mo,  $P = 0.017$ ) (Figure 1B). This difference remained significant in the subgroup of patients who did not undergo surgical resection ( $3.6 \pm 1.0$  mo *vs*  $1.1 \pm 0.5$  mo,  $P = 0.001$ ). Within the resection group, median survival in patients with a CCI score of 0 or 1 ( $17.7 \pm 7.9$  mo) was also longer than that in patients with a score  $\geq 2$  ( $8.3 \pm 7.5$  mo), although the difference did not achieve statistical significance ( $P = 0.25$ ). On Cox multivariate analysis, the performance of surgical resection [Hazard Ratio (HR) 0.10; 95% CI, 0.02-0.51,  $P = 0.005$ ] and the number of comorbid conditions (HR 3.36; 95% CI, 1.0-10.9,  $P = 0.045$ ) emerged as independent predictors of survival (Table 5).

## DISCUSSION

Our study is a retrospective analysis of the clinical and evolutive characteristics of a consecutive series of Spanish patients diagnosed with extrahepatic cholangiocarcinoma. The results of the study are in addition to the limited works published to date in our country regarding this condition<sup>[22-24]</sup>. We have demonstrated that the presence and number of comorbid conditions, as assessed by the CCI, act as independent factors of unfavorable prognosis. Patients with higher associated comorbidity (CCI score  $\geq 2$ ) had significantly shorter median survival than those with a lower burden of comorbidity (CCI score  $<$  2). This difference was upheld specifically in patients not subject to surgery. To the best of our knowledge, our work is the very first study to demonstrate the impact of comorbidity - as assessed by the CCI criteria - on the survival of patients with malignancies of the biliary tract, and is in accordance with previous studies focused on other solid-organ malignancies<sup>[10,13-17]</sup>.

Extrahepatic cholangiocarcinoma is a rare condition in the Western world. In a study performed in Spain between

Table 5 Univariate and multivariate analysis for prognostic survival variables						
Variable (n)	Median survival (mo)	95% CI	P (univariate)	P (multivariate)	HR	95% CI
Age at diagnosis						
< 73 yr (30)	4.7	0.7-8.7	0.054	-		
≥ 73 yr (38)	2.2	0.7-3.8				
History of weight loss						
No (49)	3.6	0.9-6.2	0.085	-		
Yes (19)	2.1	0.5-3.6				
CCI score						
0-1 (49)	4.7	3.1-6.3	0.017	0.045	3.36	1.0-10.9
≥ 2 (19)	1.4	0.4-2.3				
Total bilirubin						
< 10 mg/dL (23)	3.6	0.7-6.6	0.583	-		
≥ 10 mg/dL (45)	2.3	0.0-4.6				
Hemoglobin						
≥ 12 g/dL (49)	3.6	1.4-5.7	0.097	-		
< 12 g/dL (19)	2.2	0.1-4.4				
CA 19-9 <sup>1</sup>						
< 270 IU/L (15)	8.4	0.0-18.3	0.012	0.089	-	-
≥ 270 IU/L (14)	2.7	0.2-5.2				
Primary location						
Perihilar (37)	3.1	1.1-5.2	0.994	-		
Distal (31)	2.5	0.0-6.3				
Lymph node status <sup>2</sup>						
No (46)	2.3	0.0-5.0	0.286	-		
Yes (20)	3.6	1.5-5.8				
Metastases						
No (59)	3.6	1.3-6.1	0.068	-		
Yes (9)	0.7	0.3-1.1				
Tumor resection						
No (49)	2.3	1.4-3.2	0.015	0.005	0.10	0.02-0.51
Yes (19)	8.7	0.0-19.8				
Adjuvant therapy <sup>3</sup>						
No (41)	2.1	1.0-3.1	0.129	-		
Yes (8)	5.1	2.5-3.2				

<sup>1</sup>Data available for 29 patients; <sup>2</sup>Data available for 66 patients; <sup>3</sup>Denotes chemotherapy or brachy-radiotherapy in patients not receiving surgical resection; HR: Hazard ratio; CCI: Charlson comorbidity index.

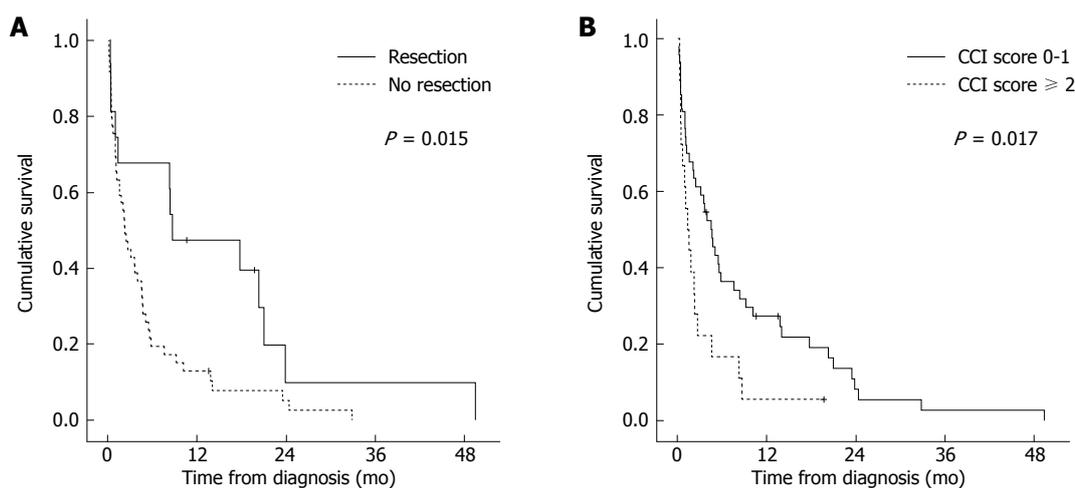


Figure 1 Kaplan-Meier survival curves stratified by treatment approach (log-rank test) (A) and Charlson comorbidity index (CCI) score (log-rank test) (B).

1994 and 1996, Mena *et al.*<sup>[22]</sup> estimated its incidence as 3.23 new cases per 100 000 inhabitants per year. A more recent work, based on data from the nationwide Danish Cancer Registry, revealed a reduced progression of incidence from 1978 (1.05 cases per 100 000 inhabitants per year) up to 2002 (0.74 cases per 100 000 inhabitants per year)<sup>[25]</sup>. In contrast, the incidence and mortality of intrahepatic

forms of the disease seem to have experienced a sustained increase in the last few decades<sup>[1,2]</sup>. The cause of this rise is unknown and does not appear to be explained simply by improvements in diagnosis or changes in coding practice. PSC remains the most common predisposing condition in the development of cholangiocarcinoma in Western countries<sup>[1]</sup>. Cirrhosis of any cause and,

more specifically, hepatitis B and C virus infection, have recently been linked to this type of cancer<sup>[2]</sup>. In our study, only a reduced percentage of patients were associated with some of these risk factors, including 1 case of PSC. These circumstances are common in the literature<sup>[5-7,18]</sup> and appears to suggest the concurrence of other etiopathogenic mechanisms yet to be clarified. Our experience confirms the poor prognosis associated with extrahepatic cholangiocarcinoma, with a median survival of 3.1 mo and a 3-year survival probability of 2%, slightly lower than that described in previous studies with similar clinical and epidemiologic characteristics<sup>[6,7,24]</sup>. The mean age at diagnosis of the patients analyzed in this study (73.4 years) was higher than that reported by other authors (64 years in the study by Figueras *et al*<sup>[23]</sup>, 67 years in the study by Weber *et al*<sup>[5]</sup>) and may have conditioned the reduced rate of resectability obtained in our series (27.9%). Distal tumor location was associated with a higher probability of receiving surgical resection in the univariate analysis, a finding previously reported in the literature<sup>[19,26,27]</sup>. Determination at diagnosis of serum CA 19-9 levels  $\geq$  270 IU/L emerged in the logistic regression multivariate analysis as the only factor independently predictive of unresectability (OR, 0.07; 95% CI, 0.0-0.7). Gerhardt *et al*<sup>[6]</sup> revealed that initial levels of this tumor marker in patients with perihilar cholangiocarcinoma subject to resection were significantly lower in comparison to those affected by unresectable disease. Kau *et al*<sup>[28]</sup> reported similar findings in subjects with periampullar carcinoma. As suggested by these authors, the presence of high serum levels of CA 19-9 probably reflects a greater tumor mass. Consequently, the univariate analysis of survival in our study showed worse prognosis in patients with higher serum levels of CA 19-9 at the time of diagnosis ( $\geq$  270 IU/L), while this difference did not remain significant in the Cox multivariate model.

As early as 1964, Feinstein highlighted the role of comorbidity when explaining the difference between estimated survival according to the TNM system in patients with lung cancer and that observed in clinical practice<sup>[29]</sup>. Since then, various comorbidity scales have been designed; the CCI, the Kaplan-Feinstein Index (KFI), the Cumulative Illness Rating Scale (CIRS), and the Index of Co-Existent Disease (ICED) feature among the most widely used, although no single index has yet emerged as clearly superior to the others<sup>[11]</sup>. The majority of these scales were not designed specifically for subjects with neoplastic disease; as commented, the CCI was developed to analyze 1-year mortality on the basis of data from an internal medicine inpatient department<sup>[12]</sup>. The KFI was created by these authors in 1974 from a cohort of diabetic subjects<sup>[11]</sup>. Only in the last few years have some specific instruments been developed and validated for oncologic patients. The National Institute on Aging (NIA) and National Cancer Institute (NCI) Comorbidity Index<sup>[30]</sup> and the Adult Comorbidity Evaluation-27 (ACE-27)<sup>[31]</sup> are probably the most notable. The latter was developed by Piccirillo *et al*<sup>[31]</sup> and modifications as well as additions of comorbid ailments have been carried out on the KFI, which is currently available online ([\[bidity.wustl.edu\]\(http://cancercomorbidity.wustl.edu\)\). This index has demonstrated its usefulness when analyzing the influence of comorbidity on the prognosis of adult patients with carcinoma of unknown primary site \(CUP\)<sup>\[32\]</sup>, resected colon cancer<sup>\[33\]</sup>, or recently diagnosed head and neck cancer<sup>\[34\]</sup>. However, in spite of the progress made in the last few decades on the design and perfection of new scales, the CCI remains one of the most popular and extensively validated comorbidity instruments. Since its original formulation by Charlson \*et al\*<sup>\[12\]</sup> the CCI has exhibited good prognostic value for predicting cancer patient survival in numerous retrospective studies<sup>\[10,13-17\]</sup>. Reviews of the CCI suggest it has good reliability, excellent correlation with mortality and progression-free survival outcomes, and is easily modified, particularly to account for the effect of age<sup>\[11\]</sup>. Versions of the index adapted to databases \*via\* the International Classification of Diseases \(ICD\)-9 or based on the evaluation of self-reported comorbidities have been also developed. One of its limitations when applied to oncologic patients is characterized by the exclusion of certain comorbidities, such as nonmalignant hematopoietic disorders \(i.e. anemia\) or polyneuropathy<sup>\[10,11\]</sup>.](http://cancercomor-</a></p></div><div data-bbox=)

Not surprisingly, comorbidity has a greater impact on biologically indolent cancers (e.g. prostate or breast), rather than aggressive tumors<sup>[31]</sup>. Chronic conditions, such as those included in the CCI, exert their influence on survival at mid-term and long-term follow-up, losing part of its relevance in the presence of aggressive entities. Although malignancies of the biliary tract are supposed to be relatively slow-growing<sup>[4]</sup>, the late diagnosis at advanced stages of the disease determines its poor prognosis as we reproduced in our series (median survival of 3.1 mo). Consequently, it would be reasonable to suppose that the burden of comorbidity should exert a minor influence on prognosis in patients with extrahepatic cholangiocarcinoma. In support of this hypothesis, tumor progression was the most frequently recorded cause of death in our study (27.9%), ahead of others more directly related to associated comorbidities (e.g. infection or liver failure). Nonetheless, our findings clearly reveal that patients with a higher comorbidity level (CCI score  $\geq$  2) presented significantly lower survival in comparison to the remainder of the cohort, and that this effect was independent of other prognostic variables. The literature contains some equivalent examples in relation to other tumors with aggressive biological behaviour and poor outcome. Seve *et al*<sup>[32]</sup> demonstrated in a cohort of 389 patients with CUP and a median survival of just 12 wk, that the number of comorbid conditions (as assessed by the ACE-27) entailed a worse prognosis, specifically in subjects with impaired functional class. Firat *et al*<sup>[35]</sup> found that comorbidity (evaluated by the CIRS and CCI) was an important prognostic factor in stage III non-small cell lung cancer and concluded that comorbidity should be taken into account even in advanced-stage disease. Similar findings have been reported in ovarian cancer patients with regional spread (FIGO stage II and III) or distant metastases (FIGO stage IV)<sup>[17]</sup>. It has been hypothesized that the type of comorbidities and cancer may interact on a physiologic

level resulting in increased aggressiveness and metastatic potential<sup>[36]</sup>. Another plausible explanation may be related to the nature of therapeutic management. Older and sicker patients are often excluded from prospective clinical trials and are not usually eligible for aggressive cancer therapies, such as duodenopancreatectomy or resection of the biliary tree<sup>[10]</sup>. However, we have not been able to demonstrate the presence of differences in the CCI scores between surgical and nonsurgical groups. The impact of comorbidity on patient survival in our series is also upheld after specific analysis according to the type of treatment, while that difference did not attain statistical significance in subjects who underwent surgical resection (17.7 mo vs 8.3 mo in patients with CCI score < 2 or  $\geq$  2, respectively;  $P = 0.25$ ).

There are a number of limitations in our single-center study to be considered. The retrospective design hinders identification and control of variables with a potential influence on survival, such as performance status or conditions not included in the CCI. We had no control over the quality of the medical records reviewed. The reduced size of the sample analysed, in particular among subjects undergoing surgical resection ( $n = 19$ ), could have hindered demonstration of significant differences in the survival of this subgroup according to their level of comorbidity. Therefore, we cannot generalize the validity of our results on other cohorts of younger patients subject to a higher proportion of surgical procedures. As we previously pointed out, the CCI presents some drawbacks in the evaluation and quantification of comorbidity in individuals with malignant diseases, in comparison with other more recent and specific indices<sup>[30,31]</sup>. Nonetheless, its broad dissemination and simple application guarantee the usefulness of the CCI both in clinical practice and in the context of retrospective studies. As highlighted by Extermann, this index exhibits excellent test-retest reliability, that ranged from 0.86 among a cohort of elderly cancer subjects to 0.92 in surgical patients; its inter-rater reliability is also acceptable, attaining 0.945 in some series<sup>[11]</sup>. A recent study which compared its capacity for prognostic prediction in patients undergoing resection of colorectal carcinoma with that of other recently developed instruments (ACE-27 and NIA/NCI) has concluded the existence of close similarities between them<sup>[33]</sup>.

In conclusion, we have demonstrated that comorbidity exerts an adverse impact on survival in patients diagnosed with extrahepatic cholangiocarcinoma, even after multivariate adjustment for other well-known prognostic factors such as age at diagnosis, primary tumor location, lymph node status, metastatic spread, or nature of the therapeutic approach. With this aim in mind, the CCI constitutes an easy and intuitive instrument in daily clinical practice. In our experience, patients with a CCI score  $\geq$  2 had higher all-cause mortality compared to patients with a CCI score < 2. These data suggest that comorbidity evaluation should be added to the decision-making process in patients with extrahepatic cholangiocarcinoma in order to improve therapy selection and prognostic estimation. However, further prospective studies should be conducted to specifically examine the relationship between comor-

bidity and treatment outcomes such as mid-term efficacy and incidence of postoperative complications.

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## COMMENTS

### Background

Cholangiocarcinoma is a relatively uncommon malignant tumor that is associated with a poor prognosis. Many studies have reported that the presence of comorbidity influences various prognostic outcomes in cancer patients, including treatment decision-making and survival.

### Research frontiers

A correlation between the severity of comorbid conditions, as assessed by the Charlson comorbidity index (CCI), and overall survival has been described in patients with various solid-organ malignancies, such as colorectal, head and neck, bladder, clear cell renal, or ovarian cancer.

### Innovations and breakthroughs

The number of comorbid conditions has an adverse impact on survival in patients diagnosed with extrahepatic cholangiocarcinoma, even after multivariate adjustment for other established prognostic factors such as age, tumor location, or type of treatment. Patients with a CCI score  $\geq$  2 had higher all-cause mortality compared to patients with a CCI score < 2. No previous studies have focused on the role of comorbidity on treatment decisions and clinical outcomes for patients with this disease.

### Applications

The study results suggest that comorbidity evaluation should be routinely added to the decision-making process in subjects with extrahepatic cholangiocarcinoma with the aim of improving their treatment selection criteria and survival estimation.

### Terminology

Comorbidity in cancer patients may be defined as the presence of diseases or disorders which exist before cancer diagnosis and are not treatment-related adverse effects. The CCI, in which each specific comorbid condition is weighted and scored, was originally developed from the study of 1-year all-cause mortality in a cohort of patients admitted to the medical unit of a teaching hospital.

### Peer review

This paper is a retrospective research about the impact of comorbidity on extrahepatic cholangiocarcinoma. We are sure that this kind of research is fairly important in the clinical treatment of biliary tract malignancies. Although the data was accurate and stated in detail, this research included a relatively small size of sample, so it can not get more significant results and weakened the conclusion. Anyway, this paper gave us some new information about the prognostic of biliary tract malignancies.

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## Mucosal bacterial microflora and mucus layer thickness in adolescents with inflammatory bowel disease

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### Abstract

**AIM:** To assess the mucosa-associated bacterial microflora and mucus layer in adolescents with inflammatory bowel disease (IBD).

**METHODS:** Sixty-one adolescents (mean age 15 years, SD  $\pm$  4.13) were included in the study. Intestinal biopsies from inflamed and non-inflamed mucosa of IBD patients and from controls with functional abdominal pain were cultured under aerobic and anaerobic conditions. The number of microbes belonging to the same group was calculated per weight of collected tissue. The mucus thickness in frozen samples was measured under a fluorescent microscope.

**RESULTS:** The ratios of different bacterial groups in inflamed and non-inflamed mucosa of IBD patients and controls were specific for particular diseases. *Streptococcus spp.* were predominant in the inflamed mucosa of Crohn's disease (CD) patients (80% of all bacteria), and *Lactobacillus spp.* were predominant in ulcerative colitis patients (90%). The differences were statistically significant ( $P = 0.01-0.001$ ). Lower number of bifidobacteria was observed in the whole IBD group. A relation was also found between clinical and endoscopic severity and decreased numbers of *Lactobacillus* and, to a lesser extent, of *Streptococcus* in biopsies from CD patients. The mucus layer in the inflamed sites was significantly thinner as compared to controls ( $P = 0.0033$ ) and to non-inflamed areas in IBD patients ( $P = 0.031$ ).

**CONCLUSION:** The significantly thinner mucosa of IBD patients showed a predominance of some aerobes specific for particular diseases, their numbers decreased in relation to higher clinical and endoscopic activity of the disease.

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**Key words:** Adolescents; Crohn's disease; Mucosa-associated bacterial microflora; Mucus layer; Ulcerative colitis

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### INTRODUCTION

The term inflammatory bowel disease (IBD), used to describe the chronic intestinal processes, includes ulcerative colitis (UC) and Crohn's disease (CD). Despite very

extensive studies, the pathogenesis of IBD still remains unclear. Genetic, environmental and immunological factors seem to play a substantial role<sup>[1]</sup>. However, an increasing number of both clinical and laboratory studies support the idea of the leading role of bacterial microflora in the onset and persistence of inflammation<sup>[2]</sup>. It is well known that the anatomic sites with the highest concentration of luminal bacteria are most often involved in the inflammatory process<sup>[3]</sup>. Gjaffer *et al*<sup>[4]</sup> showed in 1991 that intestinal flora of patients with active CD is different from that of quiescent patients, UC subjects and controls with functional abdominal pain. Patients with Crohn's disease presented with elevated numbers of aerobic bacteria, mainly *Escherichia coli*, and some anaerobic bacteria. There was also evidence of decreased numbers of *Bifidobacteria*. The results of research based on animal models support the importance of luminal bacteria in experimental colitis. Transgenic mice kept under germ free conditions did not develop inflammation until bacteria were introduced<sup>[5]</sup>. In recent years, the results of several reviews on gut microflora and IBD have suggested that inflammation may be due to a shift in the balance of healthy flora, which leads to dysregulation of the microflora and triggers or maintains the inflammation<sup>[6,7]</sup>. Moreover, it is also known, that microbes which were in close contact with the epithelium were different from those found in stools, and probably more important in the process of gut inflammation<sup>[8]</sup>.

There is very little information about the specificity of the gut microbiota in adolescents with IBD. Up to now, only one study in the literature by Conte *et al*<sup>[9]</sup> where both culture and PCR amplification methods were used, found higher numbers of mucosa-associated bacteria in biopsies from IBD children in comparison to controls. On the other hand, the occurrence of strict anaerobes including *Bacteroides* was low in specimens from patients with CD, UC and indeterminate colitis.

It has also been demonstrated that mucus which is an integral part of the mucosal barrier may play an important role in hindering penetration of the mucosa by luminal bacteria and their products thus preventing the inflammatory process. A study by Pullan *et al*<sup>[10]</sup> showed that a reduction of mucus layer thickness in adult IBD patients caused increased exposure of the microbes to the gut immune system, which in turn sustained the inflammation.

Both mucus and mucosa-associated bacterial microflora form a specific environment in the gut and its disruption may play a crucial role in the development of IBD, promoting specific bacterial colonization and immunological response.

Assessment of the multiple interactions among bacterial microflora and mucus in the very early stage of IBD, seen in newly diagnosed disease, is essential for a better understanding of disease pathogenesis and may help in suggesting therapeutic options such as the use of specific probiotics or antimicrobial treatment.

The aim of our study was to assess mucosa-associated bacterial microflora and mucus layer thickness in relation

to the inflammatory process in adolescents with IBD in comparison to controls.

## MATERIALS AND METHODS

### Ethics

The trial was approved by the Jagiellonian University Bioethical Committee and informed consent was obtained from all patients' legal guardians and/or patients over 16 years of age enrolled in the study.

### Patients

Sixty-one adolescents (25 boys and 36 girls, age:  $15 \pm 4.13$  years) were enrolled in the study from January 2004 to October 2006. There were 12 patients with UC (5 boys and 7 girls, age:  $14.74 \pm 2.9$  years), 22 with CD (9 boys and 13 girls, age:  $16.22 \pm 3.8$  years) and 3 with indeterminate colitis (IC) (1 boy and 2 girls, age:  $15.01 \pm 7.9$  years) in the study group and 24 control subjects (10 boys, 14 girls, age:  $14.13 \pm 4.4$  years) who underwent colonoscopy because of chronic abdominal pain, which was finally diagnosed as functional abdominal pain, without any organic abnormalities. The mean duration of symptoms in the UC group was  $96.5 \pm 75.1$  d,  $153 \pm 146.7$  d in the CD group and  $40.5 \pm 27.6$  d in the IC group. IC patients were not considered for further analysis as a separate group due to the small number of subjects. Patients with UC were younger at disease onset ( $11.64 \pm 4.44$  years) than those with CD ( $13.67 \pm 3.28$  years). Nutritional status of the patients assessed according to the Cole index was comparable in both groups (UC group - 84.57, CD - 82.89). Abdominal pain as the main complaint was more frequent in the group with CD (85.7%) than UC (81.1%), whereas bloody diarrhea and sideropenic anemia were more frequent in UC (81.2% and 36.4%, respectively) than CD patients (40.9% and 19.1%, respectively). Only 9% of UC patients presented with fever at disease onset, as compared to 27.2% of CD patients.

The diagnosis of CD or UC was based on endoscopic, histopathological, immunological and radiological criteria. Disease activity was assessed according to the Pediatric Crohn's Disease Activity Index (PCDAI) for CD patients and the modified Trulove-Witts score for UC patients<sup>[11]</sup>. Depending on the score value the patients were classified into three subgroups of disease activity: mild, moderate and severe. The majority of patients in both groups were classified as having mild (UC - 4 and CD - 8) to moderate (UC - 8 and CD - 12) stages of clinical activity. Only 2 patients with CD (and none with UC) were classified as having severe clinical activity. For the evaluation of endoscopic changes we used Roth's score<sup>[12]</sup>. Seven UC patients showed severe endoscopic activity compared to 6 patients with CD. A minority of patients in the UC group showed mild (2 children) to moderate (3 patients) endoscopic activity, as compared to the CD group (7 and 6 patients, respectively). Histology was assessed blindly by an independent histopathologist. All patients with IBD were in the active phase of the disease.

The use of antibiotics 30 d prior to enrolment,

infectious diarrhea, malabsorption, immunodeficiencies and intestinal enteropathies were the exclusion criteria.

### Sampling of mucosa

All subjects underwent the same type of preparation prior to colonoscopy, with oral sodium phosphate at a dose of 0.6-0.8 mL/kg (up to 45 mL) and bowel cleansing, consisting of 4 saline enemas. During colonoscopy, patients received intravenous sedation or general anesthesia, as required. Biopsy samples from IBD patients were obtained from both, the inflamed and the non-inflamed colonic mucosa. Four biopsy specimens were taken from both sites: two for culture, and one each for standard histopathological assessment and mucus thickness measurement. In the control group, the biopsy samples were taken from a normal sigmoid colon for the same assessments. The biopsy samples were transferred directly into Schaedler Anaerobic Broth (SAB) medium (Difco, BD, Franklin Lakes, USA) with 10% of glycerol. The samples were immediately snap frozen on dry ice and kept at -80°C or on dry ice until analysis. All procedures were performed as fast as possible, using sterile instruments and ensuring the integrity of the intestinal tissue. The codes of the biopsy samples were blinded before performing microbiological analysis.

### Bacteriology

The frozen samples were thawed, weighed, homogenized in 1 mL of SAB and quantitatively analyzed for the main bacterial constituents by cultures on differential media in aerobic and anaerobic conditions<sup>[9]</sup>. All these manipulations were done aseptically in an anaerobic chamber (MACS - MG 500 Work Station, DW Scientific, Shipley, UK) in N<sub>2</sub> (85%) + H<sub>2</sub> (10%) + CO<sub>2</sub> (5%) atmosphere. Homogenized samples were serially diluted with SAB and 100 µL aliquots plated on the following media: McConkey Agar (Oxoid, Basingstoke, UK) for *Enterobacteriaceae*, Columbia Blood Agar (Difco) with 5% sheep blood for streptococci, Enterococcosel Agar (BBL, BD, Franklin Lakes, USA) for enterococci, MRS agar (Oxoid) for lactobacilli and other lactic acid bacteria (LAB), BL agar for bifidobacteria<sup>[13]</sup>, and Wilkins-Chalgren Agar Base with supplements for *Bacteroides*.

The dilutions were then spread over the plate surface using a glass rod and the plates were then incubated aerobically at 37°C for 24 h, except for the cultures for anaerobic bacteria, which were kept in the anaerobic chamber for up to 4 d depending on the type of medium. The morphology of the grown colonies was analyzed under magnifying glass and several colony picks of each morphological type were subcultured on appropriate aerobic and anaerobic media and Gram-stained. After further incubation and culture purity checks, phenotypic identification was performed using commercial identification systems (API 20E, API20A, APIStaph, APIStrept, bioMerieux, Marcy l'Etoile, France; BBL Crystal ID System, BD, Franklin Lakes, USA).

### Measurement of the mucus layer thickness

The frozen biopsy samples, collected as described

above, were cut into 5 µm sections, thawed and fixed to microscopic slides and stained with fluorescein labeled *Maackia amurensis* lectin MAA lectin (EY Labs, San Mateo, USA) which binds to 2-3 linked sialic acid<sup>[14]</sup>. Briefly, the lectin solution (20 µg/mL in 0.05% sodium azide solution in a buffer supplied by the producer) was applied in a 50 µL volume to each slide and incubated under cover in a dark moist chamber at room temperature for 0.5 h. Then the unbound lectin was washed out with the buffer and the slides were counterstained with DAPI (Sigma, St. Louis, USA), washed again and dried. The mucus thickness was measured under a fluorescent microscope (Olympus BX51) with 100 × magnification.

### Statistical analysis

Comparisons were made using the Student's *t* test for variables with a normal distribution and  $\chi^2$  test. For comparison of bacterial populations and ratios of particular bacterial groups the likelihood ratio was used. The Wilcoxon test and Wilcoxon signed-rank test for matching pairs were used to compare mucus thickness. These statistical methods were chosen because data distribution was significantly different from normal distribution. All analyses were conducted using SAS 9.1 package and SAS Enterprise Guide 3.0 (SAS Institute, USA). Data are expressed as mean ± SD.

## RESULTS

All biopsy specimens from IBD patients and controls showed the presence of all groups of bacteria in the cultures although of different populations. The total number of mucosa-associated bacteria was higher in IBD patients compared to the controls with functional abdominal pain, however, the differences were statistically significant only in the UC group. Because of high diversities among the isolated bacteria and their numbers, the ratios of bacterial groups present were calculated as pooled numbers of bacteria weighted by the number of patients in the groups (Table 1).

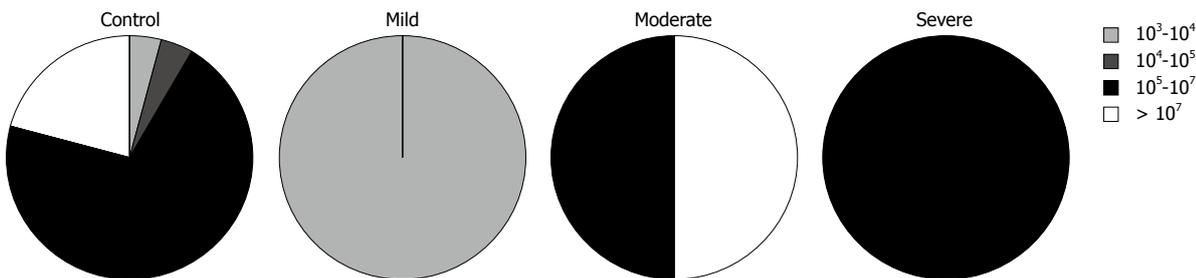
The ratios of different bacterial groups cultured from biopsies obtained both from inflamed and non-inflamed sites in IBD patients were different in samples taken from children with specific diseases. The differences were statistically significant ( $\chi^2$  test,  $P < 0.0001$ ). *Streptococci* were the predominant group of bacteria in inflamed mucosa of CD patients. About 80% of all microbiota cultured from the tissue belonged to these genera. On the other hand, patients with UC showed predominance of *Lactobacillus* (90%) in inflamed sites (Table 1). Both CD and UC patients showed a significant reduction in the ratios for anaerobes such as *Bifidobacterium* in samples taken from inflamed compared to non-inflamed mucosa. Considering the ratios of bacteria found in non-inflamed mucosa of IBD patients as compared with those found in controls, it appeared that bifidobacteria were the predominant group in all these sites (Table 1).

We also looked at the relationship between the total numbers of bacteria and disease activity, measured using PCDAI for CD patients and the modified Trulove-Witts

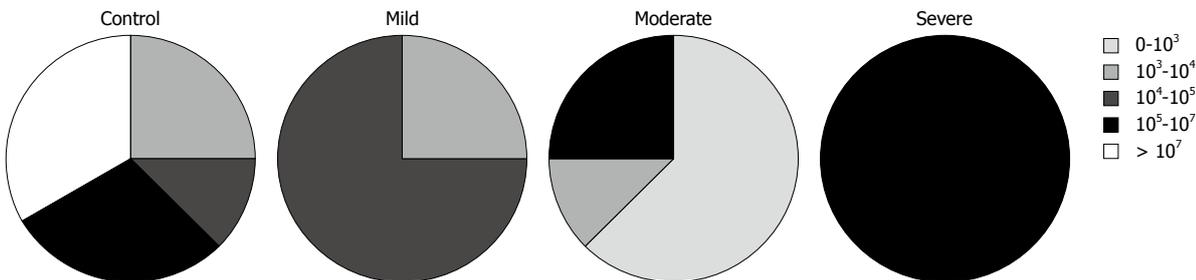
**Table 1** Median number of isolated bacteria per gram in different groups from inflamed and non inflamed tissue taken from children with CD, UC and from the control group

Bacterial groups (taxons)	Children with UC (n = 12)		Children with CD (n = 22)		Children in control group (n = 24)
	Inflamed site	Non-inflamed	Inflamed site	Non-inflamed	
<i>Enterobacteriaceae</i>	$4.92 \times 10^5$	$5.55 \times 10^6$	$2.17 \times 10^7$	$9.71 \times 10^6$	$1.94 \times 10^7$
<i>Enterococcus</i>	$1.80 \times 10^6$	$1.27 \times 10^6$	$1.36 \times 10^6$	$1.43 \times 10^6$	$6.82 \times 10^5$
<i>Streptococcus</i>	$6.83 \times 10^5$	$1.07 \times 10^6$	$1.29 \times 10^{6b}$	$1.33 \times 10^6$	$5.39 \times 10^6$
<i>Lactobacillus</i>	$9.28 \times 10^{7a}$	$7.30 \times 10^6$	$3.37 \times 10^6$	$7.76 \times 10^6$	$1.01 \times 10^7$
<i>Bifidobacterium</i>	$5.56 \times 10^{6a}$	$7.14 \times 10^7$	$8.62 \times 10^{6a}$	$3.15 \times 10^7$	$1.99 \times 10^8$
<i>Bacteroides</i>	$1.63 \times 10^6$	$5.13 \times 10^2$	$1.24 \times 10^5$	$2.31 \times 10^5$	$1.63 \times 10^6$
Total number of cultured bacteria	$5.83 \times 10^{8a}$	$4.79 \times 10^8$	$3.93 \times 10^8$	$2.30 \times 10^8$	$1.77 \times 10^8$

<sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 differences between inflamed site and the control group; CD: Crohn’s disease; UC: Ulcerative colitis.



**Figure 1** Relationships between endoscopic activity and total numbers of bacteria cultured from biopsies of UC patients and the control group. Likelihood ratio = 9.535, *P* = 0.0490. UC: Ulcerative colitis.



**Figure 2** Relationships between disease activity and numbers of *Lactobacillus* group of bacteria cultured from biopsies of CD patients and the control group. Likelihood ratio = 13.209, *P* = 0.0398. CD: Crohn’s disease.

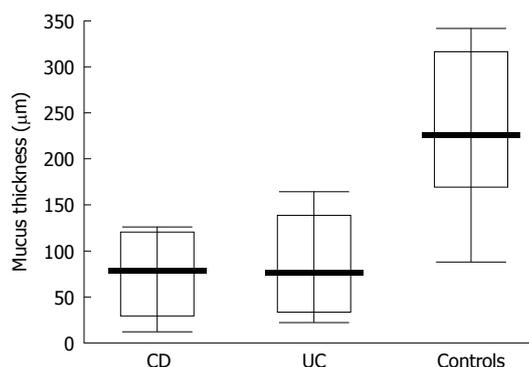
score for UC in addition to the Roth’s endoscopic scoring index. To express these relationships we adopted the five interval scale of bacterial populations from Swidsinski *et al*<sup>[5]</sup>: 0-10<sup>3</sup> cfu/g, 10<sup>3</sup>-10<sup>4</sup> cfu/g, 10<sup>4</sup>-10<sup>5</sup> cfu/g, 10<sup>5</sup>-10<sup>7</sup> cfu/g, and > 10<sup>7</sup> cfu/g.

The total numbers of cultured bacteria found in biopsies from inflamed mucosa of UC patients was increased with severity of the disease estimated by endoscopic activity (Figure 1). This relationship was statistically significant (likelihood ratio = 9.535, *P* = 0.0490). In relation to specific groups of bacteria, this analysis showed that the number of lactobacilli, which was the predominant group in the samples from inflamed mucosa of patients with UC, was lower in samples taken from patients with severe endoscopic activity. This was, however, not statistically significant (likelihood ratio = 6.959, *P* = 0.1381).

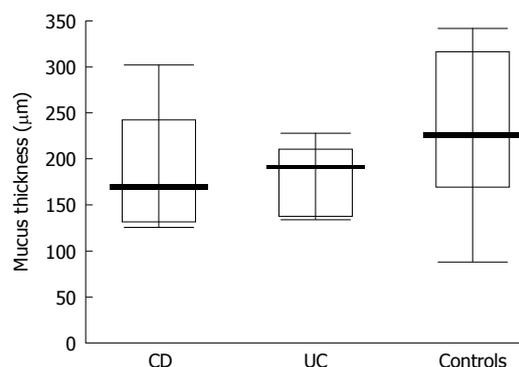
There was no relationship between the total numbers of bacteria in samples from inflamed sites and severity of the disease in CD patients. In relation to various groups of bacteria, the analysis showed that numbers

of lactobacilli were lower in samples with higher clinical activity (likelihood ratio = 13.209, *P* = 0.0328, Figure 2). The same tendency, although less significant, was observed for numbers of streptococci in samples from CD patients (likelihood ratio = 13.889, *P* = 0.0847).

When studying the thickness of the mucus layer in biopsies taken from inflamed sites of the gut in adolescents with IBD, it appeared thinner when compared to the controls (CD-74 ± 40 μm; UC-83.35 ± 49.93 μm; controls- 218 ± 81.07 μm) and these differences were statistically significant (*P* = 0.0047 and *P* = 0.0093, respectively) (Figure 3). Also, the matching pair test analysis showed a statistically significant thinner mucus layer in inflamed as compared to non-inflamed sites in the same IBD patients (*Z* = 10.50, *P* = 0.031). Focusing on specific IBD groups and comparing inflamed to non-inflamed sites, the difference was significant in CD (*P* = 0.0109), but not in UC patients (*P* = 0.0662). On the other hand, there was no difference between the CD and control group (*P* = 0.7003) and the UC and control



**Figure 3** The average thickness of the mucus layer in biopsy samples obtained from inflamed areas in CD ( $H = 8.0$ ,  $P = 0.0047$ ) and UC ( $H = 6.76$ ,  $P = 0.0093$ ) patients and controls. The horizontal line represents median value with 25% and 75% quartile.



**Figure 4** The average thickness of the mucus layer in biopsy samples obtained from non-inflamed areas in CD ( $H = 0.1481$ ,  $P = 0.7003$ ) and UC ( $H = 0.987$ ,  $P = 0.3137$ ) patients and controls. The horizontal line represents median value with 25% and 75% quartile.

group ( $P = 0.3137$ ) (Figure 4) when comparing the thickness of the mucus layer in biopsies from non-inflamed areas in IBD patients to those of the control group.

When trying to assess the histopathological activity and goblet cell depletion according to the scoring index proposed by Kudo *et al.*<sup>[16]</sup>, we found no statistically significant differences between thinner and thicker mucus layer sites both in the UC and CD groups. However, histopathological activity and goblet cell depletion was more severe in the UC group (77% and 61.5%, respectively) than in the CD group (59.1% and 32%, respectively).

Considering the numbers of bacteria contained in the biopsy samples, their ratios and the mucus thickness no relationship was found between age, gender and disease status of the patients.

## DISCUSSION

It is well known that bacterial microflora in the human gastrointestinal tract play an important role in maintaining human health and may also contribute to the pathogenesis of IBD<sup>[17]</sup>. The data from animal models of IBD suggest that intestinal inflammation is dependent on the presence of intestinal microflora, although no specific pathogen has been identified and confirmed to be responsible for this process. Some studies performed on adult patients showed a strong correlation between disease activity and increased numbers of bacteria attached to the mucosa<sup>[18,19]</sup>. Although in our study we did not observe a significant increase in microflora contained in the biopsy samples in IBD adolescents in relation to controls, we did find a higher number of bacteria in biopsies taken from UC patients, particularly in those with the most advanced stage of changes as estimated at endoscopy. Increasing disease activity was inversely related to the numbers of lactobacilli in samples from CD (but not from UC patients) and to a minor degree to *Streptococcus*.

In various studies carried out on adult IBD patients, it is possible to find conflicting results relating to the numbers of specific groups or genera of bacteria with respect to inflammation. Some authors reported increased numbers of aerobic Gram-negative rods (*E. coli*), while others found increased numbers of non-spore forming

anaerobes (*Proteobacteria* and *Bacteroides*) and decreased numbers of spore-forming anaerobes like *Clostridium*<sup>[20-24]</sup>. There are also papers in which the authors found no differences between the bacterial flora in inflamed and non-inflamed tissue in the same patient<sup>[25,26]</sup>. On the contrary, Sepehri *et al.*<sup>[27]</sup> using a PCR method showed significant differences in bacterial flora between the inflamed and non-inflamed mucosa, and an increase in microbial diversity in controls and the non-inflamed tissue from adult IBD patients.

We found differences in microflora composition between endoscopically inflamed and non-inflamed mucosal sites in newly diagnosed IBD adolescents which were specific for each form of the disease. In the controls and in the endoscopically non-inflamed mucosa of IBD children there were high numbers of bifidobacteria compared with decreased populations of these bacteria in inflamed sites of IBD patients. Thus, our observations are in concordance with the earlier findings of Seksik *et al.*<sup>[28]</sup> who also showed decreased numbers of *Bifidobacterium* and *Enterobacteriaceae* in adult patients with active and inactive CD. Our CD and UC patients also had decreased numbers of *Bifidobacterium* in inflamed mucosal sites compared with controls. It should also be noted that the numbers of lactobacilli, known for their protective effect in IBD, were lower in samples from CD adolescents with severe inflammation in our study<sup>[29]</sup>. Seksik *et al.*<sup>[28]</sup> showed that the numbers of *Lactobacillus* strains were low in active and inactive CD adult patients.

Conte *et al.*<sup>[9]</sup> recently reported the first, to our knowledge, study on gut-associated bacterial microflora in children with IBD. They found higher numbers of aerobic and facultative anaerobic bacteria in biopsy specimens from IBD children in comparison to controls, but lower numbers of *Bacteroides* in CD patients.

It should be noted that more than 50% of intestinal bacteria are not cultivable and this may represent a limitation of the study. Furthermore, molecular analytical methods have utility in discriminating between bowel microbiota of altered compositions and could be used in future studies in children. These techniques enable characterization as well as quantification of the microbiota; composition of the microbiota may be identified with

clone libraries, fingerprinting techniques may be used to analyze microbial community structure and dot blot hybridization or fluorescent *in situ* hybridization (FISH) may be used to analyze a multitude of given taxa<sup>[30-32]</sup>. In our previous pilot study (results not included here) we used FISH to analyze colonic microflora in children with IBD, however, this approach was limited by low sensitivity of the method.

Considering the conflicting results on microflora in IBD presented in the literature one should remember that the patient populations as well as stages of the illness in various studies were different. Other factors that could strongly affect the results and cause obvious discrepancies were: different sites from which the biopsies were taken, the cleaning procedures and microbiological methods used to identify the microflora. Moreover, it is possible that gut bacterial flora in children is generally less stable than in adults. All these factors could be responsible for the observed variations.

We have carried out our investigation using samples of tissue and feces which were stored at -80°C during transport from the ward to the laboratory. According to Achá *et al*<sup>[33]</sup> and Dan *et al*<sup>[34]</sup>, freezing does not influence viability of fecal samples.

Colonic mucus is an adherent, water insoluble gel that has several functions, including protection of the epithelium from mechanical trauma, toxins, allergens and from microbial invasion<sup>[35]</sup>. Thus, the mucus layer plays a protective role in the intestinal mucosa<sup>[36,37]</sup>. The gut mucus lubricates the passage of food and protects the epithelial cells from direct contact with bacterial flora. The mucus consists of glycoproteins called mucins, which are encoded by many *MUC* genes - to date more than 20 types of *MUC* genes have been described<sup>[38]</sup>. Thickness of the mucus layer is related to the dynamic equilibrium between mucus secretion and its subsequent removal into the lumen. In UC, the absence of an adherent layer in some areas may be a result of inadequate secretion or excessive removal. The findings of various studies on mucus synthesis in colitis have suggested that the epithelial layer remains intact in the presence of mild to moderate inflammation. In the presence of severe inflammation or inactivity, however, it tends to be lower than normal<sup>[10]</sup>.

In our study we have shown that the thickness of the mucus layer lining the intestinal lumen of adolescents with IBD is about three times thinner in both CD and UC patients compared with the control group. A similar relationship was observed in biopsies taken from inflamed and non-inflamed mucosa in the same IBD patients and these differences were statistically significant. In addition, we have demonstrated that mucus layer thickness measured in tissue samples taken from normal sites in patients with IBD was only slightly thinner than that in the control group and the difference was not statistically significant.

At present, there is no data in the literature on the mucus layer in children and adolescents with CD. There is also no clear explanation of observed differences, however, the results of the latest studies are in accordance

with our findings. The course of CD and localization of inflammatory lesions in childhood may be quite different from than in adults. The majority of CD adolescents (63.6%) in our study had colon involvement on endoscopic assessment, similar to UC which may have influenced the observed mucus layer depletion in this group of patients. Moreover, Gersemann *et al*<sup>[39]</sup> using a real-time PCR method in adult IBD patients, showed that goblet cells were diminished in the intestinal biopsies of both UC and CD patients, however, in the CD group enhanced differentiation was found.

In addition, using immunostaining Ardesjö *et al*<sup>[40]</sup> reported the presence of immunoreactive agents in the serum of IBD patients, which reacted with goblet cells in the intestine.

A reduction in mucus layer thickness leads to bacteria from the intestinal lumen having closer contact with the intestinal epithelium, also causing a different selection of microorganisms, which may induce an inflammatory process in the bowel<sup>[10]</sup>. In adult patients suffering from IBD, the numbers of bacterial cells adhering to the intestinal mucus layer are much higher as compared to controls without IBD, and this dependence is proportional to the severity of the clinical course<sup>[19,21]</sup>. Whether this is a result or cause of bowel inflammation still remains unclear, however, the protective property of the mucosa is significantly weakened by this process.

In summary, our results showed that non-inflamed mucosa in both IBD patients and controls was covered with a thick mucus layer with the attached microflora showing a predominance of *Bifidobacterium*. In contrast, in inflamed sites there was a reduction in mucus layer thickness with the prevalence of specific bacterial groups which were different for CD and UC: *Streptococcus* in CD and *Lactobacillus* in UC. However, numbers of these bacteria decreased proportionally with the intensity of inflammation. Thus, our results also support the idea of a disruption of the balance between the different protective and harmful intestinal bacteria in the gastrointestinal tract. These results also provide evidence which suggest that the therapeutic use of probiotics, especially those containing *Bifidobacterium* and *Lactobacillus* may have some positive effects in patients with IBD.

## COMMENTS

### Background

The pathogenesis of inflammatory bowel disease (IBD) still remains unclear. Genetic, environmental and immunological factors seem to play a substantial role. However, an increasing number of both clinical and laboratory studies support the idea of the leading role of bacterial microflora in the onset and persistence of inflammation. It is well known, that the anatomic sites with the highest concentrations of luminal bacteria are most often involved in the inflammatory process. The intestinal flora of patients with active Crohn's disease (CD) is different from that of quiescent subjects, ulcerative colitis (UC) patients and healthy controls. Transgenic mice kept under germ-free conditions did not develop inflammation until bacteria were introduced. In recent years several studies on gut microflora have suggested that inflammation may be due to a shift in the balance of healthy flora, which leads to dysbiosis and triggers or maintains inflammation.

### Research frontiers

The multiple interactions among bacterial microflora, mucus and host immunity

in the children's mucosa play a crucial role in IBD pathogenesis. We suggest that the local mucus layer in the intestine, especially in childhood, determines bacterial colonization and metabolism. The aim of our study was to assess mucosa-associated bacterial microflora and mucus layer thickness in relation to the inflammatory process in adolescents with newly diagnosed IBD in comparison to controls.

### Innovations and breakthroughs

Up to now, this is the second study focused on the mucosa-associated bacterial microflora in children with IBD, used both culture and a PCR amplification method, and found significant differences between CD and UC children. The authors went a step further and assessed both the mucosa-associated bacterial microflora and intestinal mucosa.

### Applications

Assessment of the multiple interactions among bacterial microflora and mucus in the very early stage of IBD, seen in newly diagnosed disease, is essential for a better understanding of disease pathogenesis and may help in suggesting therapeutic options such as the use of specific probiotics or antimicrobial treatment.

### Terminology

Mucosa-associated bacterial microflora consists of bacteria that are located very close to the mucus layer. This layer forms a specific environment in the gut and its disruption may play a crucial role in the development of IBD, promoting specific bacterial colonization and immunological response. The colonic mucus layer is an adherent, water insoluble gel that has several functions, including protection of the epithelium from mechanical trauma, toxins, allergens and from microbial invasion. The mucus consists of glycoproteins called mucins, which are encoded by many *MUC* genes - to date more than 20 types of *MUC* genes have been described. Thickness of the mucus layer is related to the dynamic equilibrium between mucus secretion and its subsequent removal into the lumen.

### Peer review

The authors demonstrated changes of mucosa-associated microbiota in adolescents with IBD, using classical culture techniques. They also showed depletion of the mucus layer in IBD patients. This paper is well documented.

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## Fructose-sorbitol ingestion provokes gastrointestinal symptoms in patients with eating disorders

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### Abstract

**AIM:** To evaluate gastrointestinal (GI) symptoms and breath hydrogen responses to oral fructose-sorbitol (F-S) and glucose challenges in eating disorder (ED) patients.

**METHODS:** GI symptoms and hydrogen breath concentration were monitored in 26 female ED inpatients for 3 h, following ingestion of 50 g glucose on one day, and 25 g fructose/5 g sorbitol on the next day, after an overnight fast on each occasion. Responses to F-S were compared to those of 20 asymptomatic healthy females.

**RESULTS:** F-S provoked GI symptoms in 15 ED patients and one healthy control ( $P < 0.05$  ED vs control). Only one ED patient displayed symptom provocation to glucose ( $P < 0.01$  vs F-S response). A greater symptom response was observed in ED patients with a body mass index (BMI)  $\leq 17.5$  kg/m<sup>2</sup> compared to those with a BMI  $> 17.5$  kg/m<sup>2</sup> ( $P < 0.01$ ). There were no differences in psychological scores, prevalence of functional GI disorders or breath hydrogen responses between patients with and without an F-S response.

**CONCLUSION:** F-S, but not glucose, provokes GI symptoms in ED patients, predominantly those with low BMI. These findings are important in the dietary management of ED patients.

### INTRODUCTION

A high prevalence of gastrointestinal (GI) symptoms, fulfilling criteria for functional GI disorders (FGID), is present in patients with eating disorders (ED), with more than half of a large sample of ED inpatients meeting the symptom criteria for irritable bowel syndrome (IBS)<sup>[1]</sup>. In addition to the distress caused by these chronic or recurrent GI symptoms, such symptoms may potentially interfere with the nutritional rehabilitation of ED patients.

Ingestion of fructose-sorbitol (F-S) is an established means of GI symptom provocation in IBS patients<sup>[2,3]</sup>. It remains controversial whether such symptom provocation is related to hydrogen production in the colon as a result of incomplete small bowel absorption. Because both fruit and sorbitol-containing "diet" products are frequently consumed by ED patients<sup>[4]</sup>, we hypothesized that ingestion of both these substances together may be an important factor in the genesis of the GI symptoms in ED patients. Furthermore, we hypothesized that certain characteristics associated with ED, such as body weight, and behavioral and psychological features, could influence the responses to F-S symptom provocation.

The specific aims of this study were therefore: (1) to determine the prevalence of F-S symptom provocation in female ED patients when compared to healthy female subjects; (2) to determine the specificity of any positive symptom response to F-S by evaluating whether, in ED patients, symptom provocation is greater after F-S

ingestion than after glucose ingestion, a substance not recognized to provoke GI symptoms; (3) to examine the relationships between body mass index (BMI), menstrual status, behavioral and psychological characteristics, type of ED and the F-S symptom responses; (4) to determine if symptom provocation is related to the presence of symptoms compatible with IBS, or to the number of FGIDs present in an ED patient; and (5) to determine whether F-S symptom provocation in ED patients, if present, is related to incomplete small bowel absorption of F-S detected by breath hydrogen testing.

## MATERIALS AND METHODS

### Subjects

Twenty-six consecutive female ED inpatients ( $23 \pm 7$  years, BMI  $18.6 \pm 3.6$  kg/m<sup>2</sup>) from the Eating Disorder Unit at the Northside Clinic, Sydney, Australia, participated in the study. Inclusion criteria were eating disorder diagnosis<sup>[5]</sup> confirmed by a specialist psychiatrist and a specialist psychologist, minimum age 16 years, and no known organic GI or other systemic disease. The study was conducted 2 wk after admission to hospital. ED assessment included current BMI, menstrual status, and ED behaviors (self-induced vomiting, laxative abuse, binge eating). Ten patients had a diagnosis of anorexia nervosa, 5 bulimia nervosa, and 11 eating disorder not otherwise specified (EDNOS: 6 purging type and 5 restricting type). Twenty asymptomatic, normal weight, healthy women ( $31 \pm 9$  years) who underwent F-S provocation breath testing in our laboratory using the same protocol as the ED patients formed a control group. Informed consent was obtained from all subjects, and the study was approved by the Ethics Committee of the Northside Clinic.

### GI symptom, psychological and behavioral assessment

The following self-report questionnaires were administered to the ED patients: the Rome II modular questionnaire<sup>[6]</sup>, the Beck Depression Inventory (BDI)<sup>[7]</sup>, the Eating Attitudes Test (EAT)<sup>[8]</sup>, the Eysenck Personality Questionnaire - neuroticism (EPQ)<sup>[9]</sup>, the Spielberger State-Trait Anxiety Inventory (STAI)<sup>[10]</sup>, the Brief Symptom Inventory - somatization (BSI)<sup>[11]</sup>, and the Quality of Life Eating Disorder Scale (QOL ED)<sup>[12]</sup> of the computerized Eating and Exercise Examination<sup>[13]</sup>.

### Experimental protocol

On the evening prior to the test day, subjects ate a carbohydrate-free dinner, and then fasted from midnight until the end of testing the following day. Immediately prior to testing the subjects brushed their teeth, and subjects were not allowed to eat, drink, or smoke cigarettes during the testing<sup>[3]</sup>.

In the ED patients, substrate testing took place on 2 consecutive days. On the first day patients underwent a challenge with a glucose solution and on the second day with an F-S solution. Three baseline breath hydrogen samples were obtained on each day. Patients then ingested 50 g glucose dissolved in 250 mL water (2200 mOsm/L), or an F-S mixture of 25 g fructose and 5 g sorbitol

dissolved in 250 mL water (1680 mOsm/L)<sup>[3,14]</sup>. Breath samples were obtained at 10 min intervals for a 3 h period, and the hydrogen concentration (ppm) of each sample was determined<sup>[3]</sup> on a portable instrument (Gastrolyser II, Bedford Pty Ltd., UK). The instrument was calibrated with research grade hydrogen, as per the manufacturer's recommendations. Patients were blind to the order of substrate challenge, and were not aware of the hydrogen concentrations obtained.

### Symptom assessment during provocative testing

A standard proforma assessment of GI symptoms was completed before, and at 1, 2, and 3 h following ingestion of the test solutions<sup>[3]</sup>. The symptoms rated were: abdominal pain, abdominal discomfort, abdominal bloating, abdominal distension, belching, nausea, loose or increased frequency of bowel motions, sensation of fullness, borborygmi, and flatulence. These symptoms were each rated on a score of 0 to 3, as follows: absent (score 0); mild (score 1); moderate (score 2); or severe (score 3).

### Statistical analysis

An hourly symptom score was determined by summing the individual symptom scores (corrected for baseline) for each symptom at hour 1, hour 2 and hour 3. A total symptom score was obtained by summing the 3-hourly symptom scores. A symptom response was defined as a total symptom score of 5 or greater; this measure was used to relate symptom provocation to BMI, behavioral characteristics and psychological measures, type of ED and presence of FGIDs. A BMI  $\leq 17.5$  kg/m<sup>2</sup> was used to determine 2 categories of ED patients<sup>[5]</sup>. The total number of FGIDs was determined for each ED patient. A breath hydrogen level  $\geq 20$  ppm above baseline was used as a cut-off value to categorize each subject as either a "malabsorber", or an "absorber" (i.e. no breath hydrogen response)<sup>[3]</sup>. Mouth-to-cecum transit time was defined as the time between ingestion of the solution and an increase in breath hydrogen of 10 ppm in 3 consecutive samples<sup>[15]</sup>. Peak hydrogen level was taken as the maximum hydrogen value recorded during the 3-h test period. To test for differences between groups, the Chi square with Fisher's Exact test for low cell numbers, ANOVA, and the Student's *t*-test were used where appropriate. Unless otherwise noted, results are presented as frequencies or mean  $\pm$  SD, and *P* values  $< 0.05$  are considered significant.

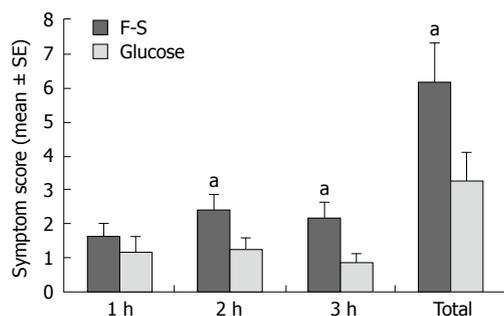
## RESULTS

### F-S symptom provocation in ED patients and controls

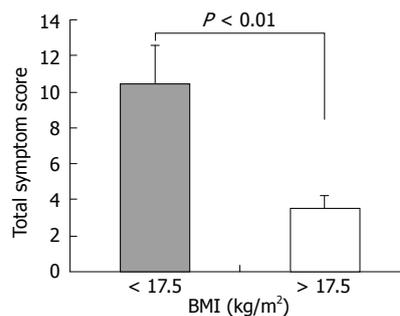
Fifteen (58%) ED patients compared to one (5%) control subject reported one or more individual symptoms at 3 h after F-S provocation (*P*  $< 0.05$ ).

### F-S vs glucose symptom provocation in ED patients

Hourly and total symptom scores following F-S and glucose ingestion are shown in Figure 1. There was a significantly greater symptom score following F-S than



**Figure 1** Hourly and total symptom scores in eating disorder patients following ingestion of 25 g fructose-5 g sorbitol (F-S) and 50 g glucose (<sup>a</sup>*P* < 0.05 vs glucose).



**Figure 2** Total symptom scores (mean ± SE) in eating disorder patients following ingestion of 25 g fructose-5 g sorbitol, grouped according to body mass index (BMI).

**Table 1** Type of eating disorder and ED behaviors in patients according to symptom response to fructose-sorbitol

	No symptom response ( <i>n</i> = 12)	Symptom response ( <i>n</i> = 14)
Type of eating disorder	f (%)	f (%)
Anorexia nervosa	2 (17)	8 (57)
Bulimia nervosa	4 (33)	1 (7)
EDNOS	6 (50)	5 (36)
Eating disorder behaviors		
Self-induced vomiting	7 (58)	6 (43)
Laxative abuse	1 (8)	1 (7)
Binge eating	7 (58)	2 (14) <sup>1</sup>

ED: Eating disorder; EDNOS: Eating disorder not otherwise specified; f: Frequency; <sup>1</sup> $\chi^2 > 5.54$ , *df* = 1, *P* < 0.05 (Fisher's exact test).

there was following glucose at both 2 and 3 h. Fourteen (55%) patients exhibited a symptom response to F-S, while only one (4%) patient exhibited a symptom response to glucose (*P* < 0.01).

**F-S symptom provocation and ED characteristics**

Total symptom score for patients with a BMI ≤ 17.5 kg/m<sup>2</sup> was significantly higher than that of patients with a BMI >17.5 (Figure 2). Excluding 7 patients who were taking oral contraception, 7 (64%) of the 11 patients with a symptom response were suffering from oligo- or secondary amenorrhoea, compared with 2 (25%) of the 8 patients with no symptom response; this difference did not reach statistical significance.

There was no significant difference in type of ED diagnosis in patients with and without an F-S symptom response (Table 1), although 80% of anorexia nervosa patients showed a symptom response. There was a significant difference in binge eating behavior between patients with and without an F-S symptom response (Table 1). There were no differences in psychological or QOL ED scores between ED patients with and without an F-S symptom response (Table 2).

**F-S symptom provocation and FGID**

Sixteen patients fulfilled the criteria for IBS, while 8 patients had 3 or more FGID diagnoses. There was no significant difference in the prevalence of IBS or in the proportion of patients with 3 or more FGIDs, in ED

**Table 2** Psychological characteristics and QOL ED in ED patients according to symptom response to fructose-sorbitol (mean ± SD)

	No symptom response ( <i>n</i> = 12)	Symptom response ( <i>n</i> = 14)
Eating attitudes test	62 ± 26	67 ± 19
BSI somatization	14.0 ± 8.1	13.6 ± 7.1
BDI depression	30.4 ± 12.6	30.8 ± 7.0
STAI trait anxiety	63.6 ± 9.7	61.0 ± 7.2
STAI state anxiety	60.1 ± 9.4	63.3 ± 10.2
EPQ neuroticism	19.3 ± 2.5	20.6 ± 2.5
QOL ED global	17.0 ± 3.2	14.7 ± 2.4
QOL ED psychological	3.3 ± 1.1	3.2 ± 1.3

BSI: Brief Symptom Inventory; BDI: Beck Depression Inventory; STAI: Spielberger State-Trait Anxiety Inventory; EPQ: Eysenck Personality Questionnaire; QOL ED: Quality of Life Eating Disorder Scale.

patients with (73%, 36% respectively) and without (80%, 40%) an F-S symptom response.

**F-S breath hydrogen response in ED patients and controls**

Thirteen (50%) ED patients and 14 (70%) control subjects malabsorbed F-S; this difference was not significant. There were no significant differences in the peak hydrogen levels between ED “malabsorbers” and control subject “malabsorbers” (71 ± 31 ppm *vs* 48 ± 30 ppm). In ED patients, there were no significant differences in total symptom scores between F-S “malabsorbers” (8 ± 7) and “absorbers” (5 ± 3). When the ED “malabsorbers” were compared with the ED “absorbers” there were no significant differences in the prevalence of IBS (85% *vs* 63%), or the number of ED patients with ≥ 3 FGIDs (46% *vs* 25%). The mouth-to-cecum transit time for ED “malabsorbers” was significantly longer (106 ± 35 min) than for control subject “malabsorbers” (54 ± 25 min, *P* < 0.001).

**DISCUSSION**

This study is the first to examine the GI symptom responses to F-S ingestion in patients with eating disorders. The key findings are that F-S provoked symptoms in more than half of female ED patients.

This is a significantly greater proportion than that found in healthy individuals. Moreover, the response was specific for F-S ingestion. Additionally, there was a greater symptom response in patients at lower BMI values; consistent with this finding, symptom provocation was more common in anorexia nervosa patients. Symptom provocation was not related to the patients' psychological characteristics, to the presence of chronic digestive tract symptoms such as IBS, or to the presence of a positive breath hydrogen response to F-S.

These findings are clinically relevant to the day-to-day management of ED patients. Eating disorder patients preferentially select low energy foods, including fruit and diet drinks<sup>[4]</sup>. Fructose, a monosaccharide, is found naturally in fruit, including apples, grapes and stone fruit<sup>[16]</sup>. Sorbitol, a sugar alcohol, is present in diet drinks, chewing gum, artificial sweeteners and in some fruits such as apples. These 2 substances are therefore likely to be commonly ingested by ED patients, representing a potential source of GI distress that would impact negatively on their nutritional management. In this context, F-S provocative testing could prove valuable in identifying those patients with symptom sensitivity to these substances. Such testing may be of particular importance in very low weight ED patients. Breath testing in association with the F-S challenge, however, does not appear to provide additional clinically useful information. Thus an F-S challenge without breath testing would be sufficient. This approach would also obviate the need for dietary restriction prior to the challenge, a restriction that is contraindicated in the treatment of ED patients in whom "normal" eating behavior is being established.

We chose a dose of F-S (25 g fructose and 5 g sorbitol) that had been previously evaluated in IBS patients<sup>[2,3]</sup>, and shown to produce a greater symptom response than a dose of 20 g fructose and 5 g sorbitol<sup>[9]</sup>. A recent study has supported a dose of 25 g fructose as the optimal challenge for testing for fructose malabsorption<sup>[17]</sup>. The dose of glucose was chosen based on that recommended for the evaluation of small bowel bacterial overgrowth<sup>[18]</sup>.

The mechanisms involved in the symptom response to F-S are not clear. The response was not related to the presence of F-S malabsorption or to the psychological characteristics of the patients. An osmotic effect is unlikely, given that the glucose challenge - of similar osmolality to the F-S solution - did not provoke symptoms. The mechanism, however, does appear to be a genuine physiologic phenomenon. A factor associated with low body weight, and presumably a significant negative energy balance, appears to be involved. A role of negative energy balance is also indicated by the observed increased menstrual disturbance among the symptom responders. Underlying disordered gut physiology in the ED patients, as evidenced by a prolongation of mouth-cecum transit time, is another factor of potential relevance. Despite this, there was no evidence for the presence of small bowel bacterial overgrowth in our patients, based on the glucose breath hydrogen testing and using accepted definitions<sup>[14,18]</sup>.

In conclusion, given the novel findings observed in

this study, further research should be undertaken to enable the formulation of more precise guidelines regarding F-S sensitivity and the dietary management of ED patients. Future studies should address the role of different F-S doses in relation to body weight and the extent to which various foods containing F-S provoke symptoms.

## ACKNOWLEDGMENTS

The assistance of the staff and patients of the Eating Disorders Unit, the Northside Clinic, Sydney, Australia is gratefully acknowledged.

## COMMENTS

### Background

Eating disorder (ED) patients display a high prevalence of gastrointestinal symptoms and functional gastrointestinal disorders such as irritable bowel syndrome (IBS). These symptoms may interfere with their nutritional management. Ingestion of fructose-sorbitol (F-S) is an established means of gastrointestinal symptom provocation in irritable bowel syndrome patients. Surprisingly, although ED patients are known to consume "diet" products containing fructose and sorbitol, their gastrointestinal symptom responses to F-S provocation have not been studied.

### Research frontiers

It remains controversial whether ingestion of F-S (25 g fructose and 5 g sorbitol) provokes symptoms in IBS by hydrogen production in the colon as a result of incomplete small bowel absorption. Because both fruit and sorbitol-containing "diet" products are frequently consumed by ED patients, ingestion of both these substances together may be an important factor in the genesis of gastrointestinal symptoms in ED patients. Furthermore, certain characteristics associated with ED, such as body weight, and behavioral and psychological features, could influence the responses to F-S symptom provocation.

### Innovations and breakthroughs

The key findings of this study are that F-S provoked gastrointestinal symptoms in more than half of the female ED patients, a significantly greater proportion than that found in healthy individuals; the response was specific for F-S ingestion; and there was a greater symptom response in patients at lower BMI values. Consistent with this last finding, symptom provocation was more common in anorexia nervosa patients. Hence negative energy balance appears to play a role in F-S sensitivity in these patients.

### Applications

As fructose and sorbitol are likely to be commonly ingested by ED patients, representing a potential source of gastrointestinal distress that would impact negatively on their nutritional management, F-S provocative testing could prove valuable in identifying those patients with symptom sensitivity to these substances. Further studies could address the role of different F-S doses in relation to body weight and the extent to which various foods containing F-S provoke symptoms.

### Terminology

Fructose: A monosaccharide, found naturally in fruit, including apples, grapes and stone fruit; Sorbitol: A sugar alcohol, present in diet drinks, chewing gum, artificial sweeteners and in some fruits such as apples.

### Peer review

This study documents the provocation of symptoms in patients with eating disorders who have been challenged with a fructose-sorbitol dose.

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BRIEF ARTICLE

## Small bowel MRI enteroclysis or follow through: Which is optimal?

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aged in the supine position. MRE and MRFT are equivalent for distal SB distension and artefact effects. Proximal SB distension is frequently less optimal in MRFT than in MRE. MRE is, therefore, the preferred MR examination method of the SB.

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**Key words:** Magnetic resonance enteroclysis; Magnetic resonance enterography; Magnetic resonance follow through; Artefact

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### Abstract

**AIM:** To determine if a nasojejunal tube (NJT) is required for optimal examination of enteroclysis and if patients can be examined only in the supine position.

**METHODS:** Data were collected from all patients undergoing small bowel (SB) magnetic resonance imaging (MRI) examination over a 32-mo period. Patients either underwent a magnetic resonance (MR) follow-through (MRFT) or a MR enteroclysis (MRE) in the supine position. The quality of proximal and distal SB distension as well as the presence of motion artefact and image quality were assessed by 2 radiologists.

**RESULTS:** One hundred and fourteen MR studies were undertaken (MRFT-49, MRE-65) in 108 patients in the supine position only. Image artefact was more frequent in MRE than in MRFT (29.2% vs 18.4%), but was not statistically significant ( $P = 0.30$ ). Adequate distension of the distal SB was obtained in 97.8% of MRFT examinations and in 95.4% of MRE examinations, respectively. Proximal SB distension was, however, less frequently optimal in MRFT than in MRE ( $P = 0.0036$ ), particularly in patients over the age of 50 years ( $P = 0.0099$ ). Image quality was good in all examinations.

**CONCLUSION:** All patients could be successfully im-

### INTRODUCTION

Investigation of small bowel (SB) pathology can be very difficult. Assessment of the terminal ileum (TI) by endoscopy is not optimal and conventional barium radiology (SB follow-through or SB enteroclysis) has a sensitivity of 23%-80% for the detection of lesions typical of Crohn's disease (CD)<sup>[1-3]</sup>. Wireless capsule endoscopy may allow for excellent visualisation of the SB mucosa and any abnormalities, but its specificity is lower than other methods<sup>[3]</sup> and it often does not clearly localise the area of the small intestine where a lesion is identified. There is also a small but definite capsule retention rate that contraindicates its use in SB strictures<sup>[4]</sup>. Computed tomography enteroclysis (CTE) has a good sensitivity of 71%-95% and an impressive specificity of 90%-98% and is superior to conventional enteroclysis<sup>[5,6]</sup>. A single abdominal CT, however, may increase a patient's lifetime risk of malignancy<sup>[7]</sup>, which is even greater in the younger population<sup>[8]</sup>. The sensitivity and specificity of SB magnetic resonance imaging (MRI) examinations are also higher in the evaluation of CD than CTE<sup>[9]</sup>. Although MRI cannot provide the consistently good mucosal detail as conventional enteroclysis, it, however, correlates with pathologic findings and does not use ionising radiation<sup>[10-12]</sup>.

Bowel contrast is required for SB distension and

adequate distension is required for optimal information on mucosal abnormalities, bowel distensibility and passage of the contrast. The difference between magnetic resonance (MR) SB enteroclysis (MRE) and MR SB follow-through (MRFT) is the administration of bowel contrast *via* a nasojejunal tube (NJT) for MRE, which is ingested orally for MRFT. It has been proposed that SB distension is best obtained with the use of a NJT, but patients often describe that placement of a NJT is very unpleasant. A NJT also reduces a potential benefit of MRI as it requires fluoroscopic radiation exposure to insert the tube<sup>[13]</sup>. Recent studies also suggest that MRFT is as sensitive as MRE in the diagnosis of ileal CD<sup>[14]</sup>. Although MRFT and MRI enteroclysis are excellently correlated with disease findings<sup>[15]</sup>, proximal SB distension may not be as optimal as that of MRFT<sup>[12,14,15]</sup>, suggesting that a NJT may still be required.

CD is one of the major indications for investigation of the SB, as approximately 70% of such patients will have inflammatory involvement of the TI. CD is a chronic, relapsing condition with an increasing prevalence<sup>[16]</sup> and may frequently present with intestinal fibrosis, stenosis and obstruction. Fibrosis and stricture formation are the most common indications for intestinal surgical resection<sup>[17]</sup>. Patients undergoing SB radiological investigations are usually examined in the prone position, as it has been suggested that this may result in better SB distension. This, however, is an unpopular technique especially in patients with stomas<sup>[18]</sup>, and it has been reported that the prone and supine positions are equivalent in terms of lesion detection and bowel wall feature visualisation<sup>[19]</sup>.

If optimal SB distension can be obtained without insertion of a NJT, then fluoroscopic radiation could be avoided, which is of particular importance because the predilection of IBD to affect younger patients who frequently require multiple investigations over their lifetime. The aims of this study were thus to assess the difference in the quality of imaging between MRE and MRFT in proximal and distal SB distension and the presence of artefact impairing diagnostic assessment, and to confirm whether imaging of patients in the supine position with a surface array coil can provide abdominal compression and good SB distension in quality studies.

## MATERIALS AND METHODS

All subjects were patients of a 450-bed tertiary institution located in Perth of Western Australia, which is also the site for the "Centre for Inflammatory Bowel Diseases", a specialist unit for the management of inflammatory bowel diseases. Data were collected from all patients undergoing MRFT or MRE over a 32-mo period.

### MRI preparation

Patients were randomly investigated by either MRFT or MRE. If a patient refused a NJT, or placement of the NJT failed due to technical reasons, a MRFT was undertaken. All patients drank only clear fluids for 6 h prior to their MRI and were nil by mouth for 2 h. The bowel contrast agent used was a polyethylene glycol-

water (PEG) solution (glycoprep-C, Pharmatel Fresenius Kabi, Australia). Patients who were to undergo a MRFT attempted to drink 1000 mL or more of the bowel contrast agent over 20 min. For a MRE, a NJT (Bilbao-Dotter, Cook, Australia) was placed under fluoroscope. PEG was injected manually through the NJT (from 60 mL/min to 120-150 mL/min). A total of 800-2000 mL was usually required to distend the SB to the TI, which varied depending on previous bowel resections, the presence of stenosing disease, and patient tolerance.

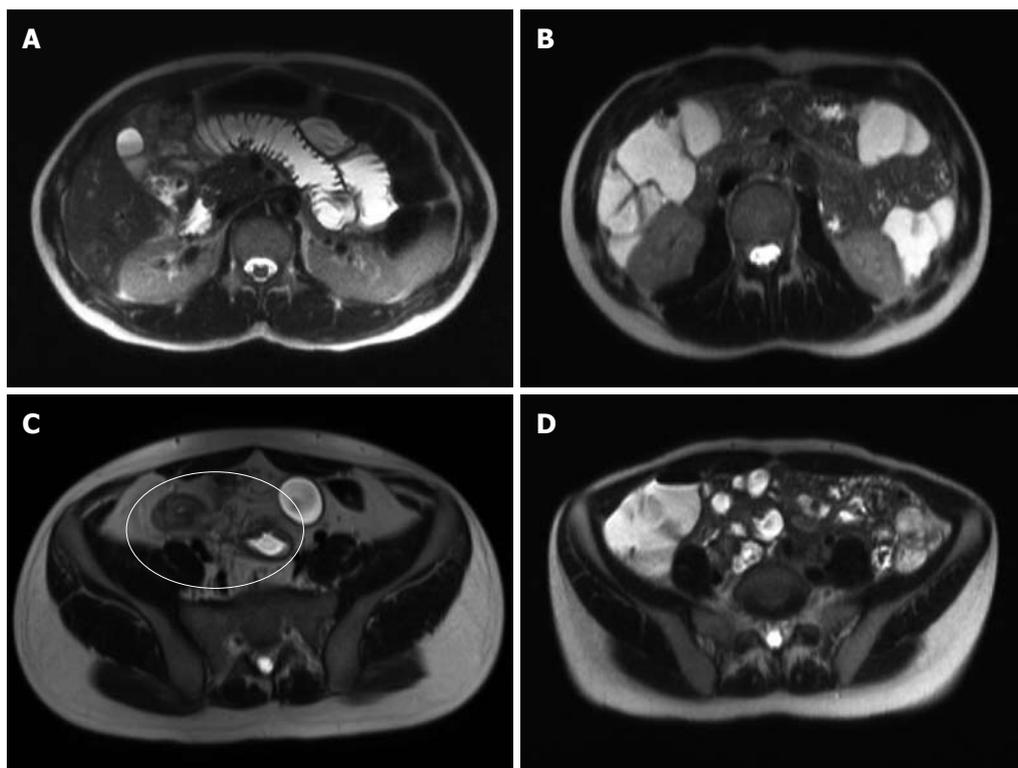
### MRI technique

Patients were imaged in the supine position using a 1.5T MRI system (Avanto SQ, Siemens Medical Solutions, Erlangen, Germany) with a surface array coil providing compression. In patients having undergone an ileostomy, a sponge was placed between the surface coil and stoma with the stoma bag empty.

A scout image was acquired to ensure adequate coverage. SB filling was dynamically assessed using a coronal 150 mm-thick single slab T2-weighted (HASTE) fat saturated sequence (TR 4500 ms, TE 749 ms, flip angle 180°, bandwidth 150 Hz/Px, FOV 350 mm, averages 1, concatenation 1, distance factor 50%, GRAPPA acceleration factor 2), which was acquired repeatedly without breath-holding to monitor retrograde stomach filling and SB distension. These images, combined into a cine loop, were used to assess stenotic lesions. If there was a doubt as to TI contrast filling, a single breath-hold coronal T2-weighted sequence (HASTE) (TR 2000 ms, TE 118 ms, flip angle 180°, bandwidth 195 Hz/Px, FOV 350 mm, averages 1, concatenations 2, no parallel imaging) with a 5 mm-thick slice was obtained with a 50% gap.

To reduce bowel peristalsis and prolong SB distension, 10 mg intravenous hyoscine butylbromide (Buscopan, Boehringer Ingleheim, Australia) was given if there were no contraindications. Once there was adequate SB filling, a coronal pre-contrast T1-weighted 3D gradient echo (VIBE) (TR 9.38 ms, TE 4.46 ms, flip angle 20°, bandwidth 630 Hz/Px, FOV 400 mm, averages 1, concatenation 1, phase over-sampling 25%, distance factor 20%, GRAPPA acceleration factor 2) with a 2.5 mm-thick slice was obtained. Gadodiamide (Omniscan, Amersham, Australia) was intravenously injected (0.2 mL/kg) with post contrast imaging commencing at 60 s. Post-contrast VIBE sequences were obtained in the coronal (imaging factors the same as pre-contrast VIBE) and axial (TR 3.37 ms, TE 1.22 ms, flip angle 12°, bandwidth 490 Hz/Px, FOV 320 mm, averages 1, concatenation 1, phase oversampling 0%, distance factor 20%, GRAPPA acceleration factor 2, a 2 mm-thick slice). The axial plane required 2-3 overlapping sections covering the upper and lower abdomen.

Further imaging was obtained in 2-3 blocks of the upper and lower abdomen, including a coronal steady-state free precession sequence (true FISP) (TR 3.65 ms, TE 1.83 ms, flip angle 64°, bandwidth 501 Hz/Px, FOV 380mm, averages 1) with a 6 mm-thick slice and 30% gap obtained with and without fat saturation, an axial steady-state free precession sequence (true FISP) (TR 3.69 ms, TE 1.83 ms, flip angle 64°, bandwidth 501 Hz/Px, FOV



**Figure 1** Images demonstrating good and poor proximal and distal small bowel (SB) distension. A: Axial T2 weighted HASTE image of the upper abdomen demonstrating good proximal SB distension; B: Coronal T2 weighted HASTE image demonstrating poor proximal SB distension; C: Axial T2 weighted HASTE image of the lower abdomen demonstrating good distal SB distension, abnormal bowel thickening and fistulous tracts between the abnormal bowel loops (circled); D: Axial T2 weighted HASTE image of the lower abdomen demonstrating poor distal SB distension.

350 mm, averages 1) with a 6 mm-thick slice and 30% gap obtained without fat saturation in 2-3 blocks of the upper and lower abdomen, a coronal T2-weighted half Fourier single shot turbo spin echo (HASTE) sequence (imaging factors as above) with a 5 mm-thick slice and 30% gap, and an axial T2-weighted half Fourier single shot turbo spin echo (HASTE) sequence (TR 1000 ms, TE 85 ms, flip angle 150°, bandwidth 391 Hz/Px, FOV 350 mm, averages 1, concatenation 1, GRAPPA acceleration factor 2) with a 6 mm-thick slice and 30% gap. If a site of pathology was identified at an overlap point on the axial images, then a further set of targeted axial true FISP and HASTE images were obtained.

For MRFT, an initial single breath-hold coronal T2-weighted sequence (HASTE) (imaging factors as above) with a 6 mm-thick slice and 30% gap was obtained. If there was adequate filling of the TI, buscopan was injected and routine imaging was obtained as above. With suboptimal filling of the TI, but sufficient bowel contrast proximally, reassessment was performed at 5-min intervals for 15 min. If there was still inadequate filling of SB loops, the patient was removed from the MRI unit and drank a further 500 mL of contrast prior to recommencing imaging.

#### **MRI assessment**

Each MRI was evaluated by consensus of two radiologists (CW and PS) with experience in both gastrointestinal and MR imaging. Image analysis was performed using a standardised worksheet. The quality of proximal and distal SB distension and the presence of artefact were assessed. Good distension

was defined as luminal fluid present within the bowel lumen allowing clear visualisation of both endoluminal surfaces. Poor distension was lack of the above (Figure 1). Artefact, generally related to poor breath-holding, was present if it rendered the images diagnostically impaired on both sequence planes through a region (Figure 2).

#### **Ethics**

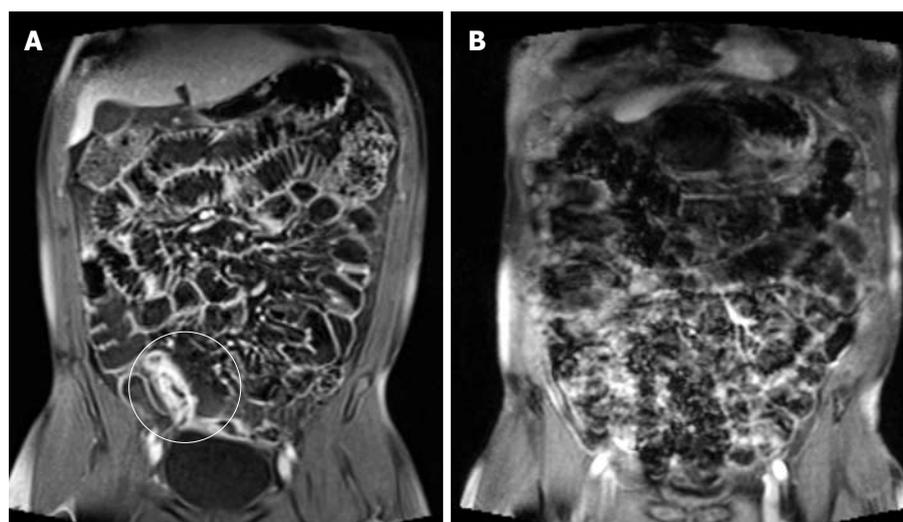
The Fremantle Hospital Human Research Ethics Committee deemed that patient consent was not required for this study as both MRFT and MRI are considered to be standard examination methods.

#### **Statistical analysis**

Logistic regression was undertaken by a statistician (KM) and considered significant at the 0.05 level. The significant effects and odds ratios were presented, along with 95% confidence intervals. Logistic regression was used to model separately the three responses: bowel distension proximal (good *vs* poor), bowel distension distal (good *vs* poor), and artefact (good *vs* poor), with potential predictors of treatment (tube or oral), sex, and age group subdivided into low age group under the age of 30 years, medium age group at the age of 30-50 years, and high age group over the age of 50 years.

## **RESULTS**

Data were collected from 114 MR examinations of SB performed in the supine position on 108 patients



**Figure 2** Images demonstrating the effect of motion artefact. A: Coronal post gadolinium VIBE demonstrating good distension of SB loops with negligible artefact and distal SB wall thickening with strong contrast enhancement consistent with active inflammatory CD (circled); B: Coronal post gadolinium VIBE demonstrating significant motion artefact precluding assessment of contrast enhancement and motion insensitive sequences (HASTE and TruFISP) which are, however, of diagnostic quality (images not shown).

**Table 1** Indication for 114 consecutive MRFT or MRE examinations performed on 108 patients

	Patients	MRFT	MRE
Gender: Male	41.2% (47/114)	32.65% (16/49)	47.7% (31/65)
Age (yr): mean (range) $\pm$ SE	40.7 (14-78) $\pm$ 16.5	39.1 (14-74) $\pm$ 15.4	43.6 (17-78) $\pm$ 17.6
Indication			
Known or suspected CD	86.0% (98/114)	93.8% (46/49)	80.0% (52/65)
Iron deficiency anaemia	7.0% (8/114)	0%	12.3% (8/65)
Resistant coeliac disease	3.5% (4/114)	6.1% (3/49)	1.5% (1/65)
Other	3.5% (4/114)	0%	6.2% (4/65)

MRFT: MR follow-through; MRE: MR enteroclysis; CD: Crohn's disease.

**Table 2** Small bowel (SB) distension and artefact in 114 consecutive MRFT or MRE examinations performed on 108 patients

	All Patients	MRFT	MRE
Good proximal bowel distension	76.3% (87/114)	65.3% (32/49)	84.6% (55/65)
Good distal bowel distension	93.9% (107/114)	97.8% (45/49)	95.4% (62/65)
No artefact	75.4% (86/114)	81.6% (40/49)	70.8% (46/65)
Good proximal and distal bowel distension	75.4% (86/114)	63.3% (31/49)	84.6% (55/65)

(41.7% males, 45/108) over a 32-mo period. A total of 49 MRFT and 65 MRE were assessed. No difference was found in male sex (32.7% *vs* 47.7%,  $P = 0.079$ ) or in age (39.1  $\pm$  15.4 years *vs* 43.6  $\pm$  17.6 years) between the MRE or MRFT patient groups. The primary indication for SB MRI imaging was assessment of CD, either for level of inflammatory activity or for investigation of obstructive symptoms (68.4%, 78/114). The remaining MRI imaging was for suspected CD involving the small intestine (17.5%, 20/114). Of the 114 examinations, 98 (86.0%) were thus for CD or suspected CD. Of the remaining 16 investigations, 8 were for unexplained iron deficiency anaemia, 4 for resistance coeliac disease, 1 for a suspected SB mass seen on wireless capsule endoscopy, 1 for unexplained abdominal pain, and 2 for investigation of SB obstruction, respectively (Table 1).

#### MRFT vs MRE

Proximal and distal SB distension and artefact were

assessed (Table 2). Proximal SB distension was more likely to be suboptimal (Figure 1) in MRFT than in MRE (65.3% *vs* 84.6%). No statistical difference was detected in good distal SB distension (97.8% *vs* 95.4%,  $P = 0.77$ ) between MRFT and MRE or between proximal SB distension and patient age or sex. Although the image artefact, which rendered the study diagnostically impaired (Figure 2), was present more frequently in MRE than in MRFT (29.2% *vs* 18.4%), it was not statistically significant ( $P = 0.30$ ). Good proximal and distal SB distension was observed in 84.6% (55/65) of the patients undergoing a MRE and in 63.3% (31/49) of the patients undergoing a MRFT, respectively.

Logistic regression findings were correlated with patient age (under 30 years, 30-50 years and over 50 years) and sex. While proximal SB distension was overall more likely to be suboptimal in MRFT than in MRE (OR = 4.365, 95% CI = 1.62-11.77,  $P = 0.0036$ ), age also had an impact on the presence of good proximal SB

**Table 3** Assessment of proximal SB distension in relation to MRI investigation, patient age and sex

	Proximal SB distension			P value
	Good proximal SB distension	Odds ratio	95% CI	
MRE	84.6% (55/65)	4.365	1.619-11.772	0.004
MRFT	65.3% (32/49)	Ref		
30-50 yr	86.7% (39/45)	4.882	1.463-16.294	0.010
< 30 yr	71.0% (22/31)	1.561	0.495-4.917	0.447
> 50 yr	68.4% (26/38)	Ref		
Female	77.6% (52/67)	1.646	0.616-4.395	0.320
Male	74.5% (35/47)	Ref		

distension. Patients aged 30-50 years were more likely to have good proximal SB distension than those aged over 50 years (OR = 4.882, 95% CI = 1.463-16.294,  $P = 0.009$ ). No statistically significant difference, however, was found between the patients aged less than 30 years, 30-50 years and over 50 years (Table 3). No statistically significant effect was observed in sex and no significant difference was detected in the diagnostic accuracy of the studies between the various age groups.

All patients were examined in the supine position alone. Nine examinations were undertaken in patients having undergone ileostomy (7.9% of examinations), 4 and 5 of these patients underwent a MRFT and a MRE, respectively. Good proximal and distal bowel distension was achieved in 77.8% (7/9) and in 100% of the patients (9/9), respectively, and image artefact was observed in only 1 patient (11.1%). These findings were very comparable to those in the overall group and no statistically significant difference was found in these parameters between the patients whether they underwent and did not undergo ileostomy.

## DISCUSSION

In our institution, CD or suspected CD is by far the most common indication for SB MR imaging, accounting for more than 85% of cases, which is primarily due to the frequency of involvement of the TI in CD and its transmural nature that may result in obstructive symptoms secondary to intestinal inflammation and/or fibrosis. Radiological investigations have undergone an evolution with both CTE and MRE demonstrating an impressive specificity and sensitivity in the assessment of SB CD. CTE, however, delivers ionising radiation, while MR provides a good soft tissue contrast without radiation, which may potentially differentiate between intestinal inflammation and fibrosis and may thus be superior to CT scanning<sup>[12,20,21]</sup>. For long-term safety issues regarding cumulative radiation exposure and potentially better diagnostic capabilities, MR examination of the SB appears preferable to CT.

Examination of the SB requires a bowel contrast to achieve adequate SB distension, which may either be taken orally or *via* a NJT. SB follow-through (SBFT) is dependent on a number of factors, such as the presence or absence of disease, the ability of patients to ingest a sufficient volume of oral contrast over a short period

of time, inter-individual variation in bowel transit time, and the type of contrast agent used<sup>[22]</sup>. Most radiological facilities routinely place a NJT despite a strong patient preference against it due to its discomfort. Our MRI service has paediatric radiology experience in performing MRFT studies with no placement of a NJT, using PEG according to the published techniques<sup>[23,24]</sup>. Adaptation of this technique for adults was used in this study, but proved difficult due to the significant time variability required for adequate SB filling with tightly scheduled MRI appointments. Since the MR technique used in our facility also requires real-time monitoring, overlapping images can be obtained when needed, thereby enhancing the quality of MR studies.

Our experience is similar to the published studies where the total investigation time (18-27 min, average 22.4 min) was achieved with NJT placement<sup>[11]</sup>, but it could vary as many as 15-240 min (average 65 min) when performed as a follow-through study<sup>[25]</sup>. It should be noted that the imaging time within a MRI unit is approximately the same for both MRFT and MRE. The extra time frequently required for a MRFT is due to the movement of patients in and out of the MRI unit more than once in order to determine SB filling and the need for further oral contrast, which, however, must be weighed against the extra time and radiologist skills required for NJT placement, the need for fluoroscopic radiation, and the strong patient preference against a NJT. Other methods, described to streamline the MRFT technique, however, still require 20-96.6 min of the study time and recurrent periods in and out of the magnet<sup>[22]</sup>, although it is potentially possible to reduce the imaging time to 15-20 min following oral ingestion of PEG over 30-45 min<sup>[26]</sup>.

Our findings regarding the image quality of MRFT and MRE are consistent with previous reports that oral contrast is as sensitive as NJT for distal ileal CD<sup>[27]</sup>. Our results, however, indicate that MRFT is inferior to MRE for proximal SB distension, particularly in the older age group (over 50 years), which may primarily be due to the fact that many of these patients have difficulty in consuming a sufficient volume of oral contrast over a short period of time. We did not record or assess the volume of bowel contrast used. It has been suggested that 900 mL of either an osmotic or a nonosmotic solution is sufficient to obtain duodenal distension and 1350 mL is sufficient to distend the distal jejunum and ileum *via* mouth<sup>[28]</sup>. We strongly encouraged our patients to drink at least 1000 mL of the oral contrast agent. There is often, however, a limit to how much a patient with obstructive symptoms can ingest orally and we have to accept that the finally ingested volume is the best possible for the individual. A recent paper has addressed this specific concern and identified that the SB could be reliably analyzed in healthy volunteers with a volume of 450 mL, and not unexpectedly, less reliably with a volume of 300 mL and 150 mL, respectively<sup>[29]</sup>.

The unpopular technique of imaging in the prone position, could be avoided in our cohort of patients, especially in patients with stomas<sup>[18]</sup>, by putting the patient in the supine position and using a large surface coil, and

sponge over the stoma if present, to apply abdominal pressure. This technique delivered similar SB distension in the patients having undergone ileostomy as in those who did not undergo ileostomy. Our findings in 15.4% of supine MRE studies having either poor proximal or distal SB distension are consistent with SBFT/enterography showing poor SB distension in 13.8% of patients examined in the prone position, and 16.1% of patients examined in the supine position<sup>[19]</sup>. In our study, and as has been observed by other investigators<sup>[26]</sup>, the image quality was still considered good despite the poor SB distension, and was not considered to have reduced the diagnostic accuracy of the examination. Our findings, and studies comparing SB distension examined in the prone and supine positions<sup>[19]</sup>, suggest that the two methods are equivalent in lesion detection and bowel wall feature visualisation.

The findings presented here suggest that both MRE and MRFT are comparable investigations with regard to distal SB bowel distension, image artefact and quality. We also observed no difference in the quality of studies performed in the limited number of patients having undergone ileostomy. Proximal SB distension, however, is frequently less optimal in MRFT than in MRE, but the examination of patients in the supine position is a viable option. Due to reduction in study duration and improved proximal SB distension, the authors prefer to primarily perform all studies in adult patients in the supine position with a NJT. In patients who are unable to tolerate a NJT, or the study is requested to examine the distal SB, a MRFT with oral contrast is an acceptable alternative, particularly in patients under the age of 50 years.

## COMMENTS

### Background

If optimal small bowel (SB) distension can be obtained without insertion of a nasojejunal tube (NJT), then fluoroscopic radiation could be avoided. This study was to assess magnetic resonance (MR) SB enteroclysis (MRE) and MR SB follow-through (MRFT) for proximal and distal SB distension, the presence of movement artefact, and whether imaging in the supine position provides quality studies with good SB distension.

### Research frontiers

Patients often describe that placement of a NJT is very unpleasant. A NJT also requires fluoroscopic radiation exposure to insert the tube. Recent studies suggest that MRFT is as sensitive as MRE in the diagnosis of ileal disease. This study assessed the optimal method of SB examination by MR and if patients could be examined in the supine position only.

### Innovations and breakthroughs

The findings demonstrate that the image quality of MRFT and MRE is equivalent. The results, however, indicate that MRFT is inferior to MRE for proximal SB distension, particularly in patients over the age of 50 years. The unpopular technique of imaging in the prone position, especially in patients with stomas, can be avoided by putting the patient in the supine position and using a large surface coil and sponge over a stoma if present to apply abdominal pressure.

### Applications

The findings demonstrate that MRE and MRFT are comparable investigations with regard to distal SB bowel distension, image artefact and quality, and examination of patients in the supine position is a viable option. Proximal SB distension, however, is less optimal in MRFT. Due to reduction in study duration and improved proximal SB distension, the authors prefer to perform all studies in the supine position with a NJT. In patients who are unable to tolerate a NJT, or the study is requested to examine the distal SB, a MRFT with oral contrast is an acceptable alternative, particularly in patients under the age of 50 years.

## Terminology

Magnetic resonance imaging (MRI) is a cross sectional imaging technique that does not utilize radiation and provides excellent tissue differentiation. MRE has the administration of bowel contrast via a NJT and patients undergoing a MRFT drink the bowel contrast. Good bowel distension is defined as luminal fluid present within the bowel lumen allowing clear visualisation of both endoluminal surfaces. Artefact, generally related to poor breath-holding, is present if it renders the images diagnostically impaired on both sequence planes through a region.

## Peer review

The paper is a large study assessing the quality of MRIs with the bowel contrast administered through a tube (ME enteroclysis) and contrast injected orally (MR follow-through) and whether or not MRI examination of patients in the supine position is adequate. The authors show that tube administration is the best and that patients in the supine position are OK.

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## Effects on coagulation factor production following primary hepatomitogen-induced direct hyperplasia

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### Abstract

**AIM:** To investigate the molecular mechanisms involved in coagulation factor expression and/or function during direct hyperplasia (DH)-mediated liver regeneration.

**METHODS:** Direct hyperplasia-mediated liver regeneration was induced in female C57BL/6 mice by administering 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP), a representative hepatomitogen. Mice were weighed and sacrificed at various time points [Day 0 (D0: prior to injection), 3 h, D1, D2, D3, and D10] after TCPOBOP administration to obtain liver and blood samples. Using the RNA samples extracted from the liver, a comprehensive analysis was performed on the hepatic gene expression profiling of coagulation-related factors by real-time RT-PCR (fibrinogen, prothrombin, factors V, VII, VIII, IX, X, XI, XII, XIII $\beta$ , plasminogen, antithrombin, protein C, protein S, ADAMTS13, and VWF). The

corresponding plasma levels of coagulation factors (fibrinogen, prothrombin, factors V, VII, VIII, IX, X, XI, XII, XIII, and VWF) were also analyzed and compared with their mRNA levels.

**RESULTS:** Gavage administration of TCPOBOP (3 mg/kg body weight) resulted in a marked and gradual increase in the weight of the mouse livers relative to the total body weight to 220% by D10 relative to the D0 (control) ratios. At the peak of liver regeneration (D1 and D2), the gene expression levels for most of the coagulation-related factors (fibrinogen, prothrombin, factors V, VII, VIII, IX, XI, XII, XIII $\beta$ , plasminogen, antithrombin, protein C, ADAMTS13, VWF) were found to be down-regulated in a time-dependent manner, and gradually recovered by D10 to the basal levels. Only mRNA levels of factor X and protein S failed to show any decrease during the regenerative phase. As for the plasma levels, 5 clotting factors (prothrombin, factors VIII, IX, XI, and XII) demonstrated a significant decrease ( $P < 0.05$ ) during the regeneration phase compared with D0. Among these 5 factors, factor IX and factor XI showed the most dramatic decline in their activities by about 50% at D2 compared to the basal levels, and these reductions in plasma activity for both factors were consistent with our RT-PCR findings. In contrast, the plasma activities of the other coagulation factors (fibrinogen, factors V, VII, XIII, and VWF) were not significantly reduced, despite the reduction in the liver mRNA levels. Unlike the other factors, FX showed a temporal increase in its plasma activity, with significant increases ( $P < 0.05$ ) detected at D1.

**CONCLUSION:** Investigating the coagulation cascade protein profiles during liver regeneration by DH may help to better understand the basic biology of the liver under normal and pathological conditions.

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**Key words:** Coagulation factor; 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene; Direct hyperplasia; Liver regeneration

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## INTRODUCTION

The normal adult liver is largely quiescent and only a small percentage of the cells are undergoing cell division at any one time. However, the liver is one of the few organs in the mammalian system that can undergo rapid proliferation in response to various stimuli through a process known as liver regeneration. Liver regeneration is a well-orchestrated process in which hepatocytes and non-parenchymal cells rapidly proliferate *via* induction by two distinct pathways: (1) compensatory regeneration; and (2) direct hyperplasia (DH)<sup>[1,2]</sup>. In the former pathway, liver regeneration is triggered by a loss of functional liver mass, which can be observed in situations where the liver becomes necrotic due to chemical administration or portions of the liver lobes are physically removed by partial hepatectomy<sup>[3-5]</sup>. In the latter DH pathway, the liver regeneration can be initiated by a direct administration of hepatocyte mitogens, such as 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP), thyroid hormone T<sub>3</sub>, phenobarbital or bile acids<sup>[3,4,6-8]</sup>.

The process of liver regeneration is known to involve a complex interaction of cytokine-mediated responses as well as pro-proliferative gene expression during the initiation, propagation, and termination phase<sup>[1,2]</sup>. Liver regeneration appears to adversely affect the production of other liver-specific proteins not directly involved in the regenerative process, including albumin and ornithine transcarbamylase. In terms of hemostasis, the production and secretion of the coagulation factors, anti-coagulant factors, and fibrinolytic factors are highly dependent on the hepatocytes in the liver<sup>[9-12]</sup>. There is evidence that some of the liver-specific proteins have temporal suppression in their production during compensatory liver regeneration<sup>[5,13,14]</sup>. Although the kinetics for the coagulation and/or fibrinolytic factors during compensatory liver regeneration have been investigated in the past<sup>[15,16]</sup>, comprehensive analyses assessing the gene expression profiles of the coagulation factors and their related proteins in combination with their plasma activities during DH-mediated liver regeneration have not yet been documented.

Recently, several reports have elucidated that fibrinolytic factors such as plasminogen and urokinase-type plasminogen activator are largely involved in promoting cancer cell invasion and liver regeneration<sup>[17,18]</sup>. It could thus be speculated that coagulation factors, which have an opposite function to fibrinolytic factors, might participate in modulating the liver regeneration process. In this context, elucidation of the coagulation factor profiles during the liver regeneration process may provide an important clue in clarifying the mechanism of liver

regeneration and in understanding the biological process of the blood coagulation system. To date, coagulation factor profiles during the TCPOBOP-induced liver regeneration process have yet to be documented, to the best of our knowledge.

In the present study, we induced liver regeneration in C57BL/6 mice by administering TCPOBOP; the most commonly used experimental procedure to induce DH-mediated liver regeneration. During this liver regeneration process, we investigated the gene expression profiles of coagulation factors and fibrinolytic factors as well as plasma activities of these factors at different time points following TCPOBOP administration.

## MATERIALS AND METHODS

### Animals

A total of 48 female C57BL/6 mice (10-12 wk old; The Jackson Laboratory, Bar Harbor, ME) were housed in an environmentally controlled room with alternating 12-h dark/light cycles (8:00 am lights on/8:00 pm lights off). The mice had *ad libitum* access to food and water. Female mice were exclusively used in this study, since there was a previous report documenting a gender-dependent effect by the liver in the response to TCPOBOP<sup>[19]</sup>. Experimental protocols were developed in accordance with the guidelines outlined by the local animal committee at Nara Medical University.

### Direct hyperplasia (DH)-mediated induction of liver proliferation

DH to the liver was induced by a single intragastric injection of a primary hepatomitogen, TCPOBOP (kindly provided by Dr. BA Diwan) as previously described<sup>[3,8,20]</sup>. Briefly, TCPOBOP was dissolved in dimethyl sulfoxide (DMSO)/corn oil solution and administered by gavage at a dosage of 3 mg/kg body weight.

### Liver harvesting and plasma sampling

Mice were weighed and sacrificed at various time points [Day 0 (D0: prior to injection), 3 h, D1, D2, D3, and D10] after the injection of TCPOBOP ( $n = 8$  mice/time point). Before sacrifice, blood samples were obtained from the retro-orbital plexus and anti-coagulated with 0.1 vol of 3.8% sodium citrate. The blood solution was centrifuged at 4°C, and plasma was separated for storage at -80°C until analysis. After sacrifice, the livers were harvested and weighed, and the liver lobes were snap-frozen in liquid nitrogen and kept at -80°C to preserve the integrity of the RNA until extraction.

### RNA isolation and quality controls

Total RNA was extracted from each tissue sample using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. DNase I was used to digest and remove genomic DNA contamination. Aliquots of the total RNA samples were diluted in TE buffer and the concentration of each sample was measured at a wavelength of 260 nm ( $A_{260}$ ) using the

Table 1 Description of analyzed gene primers used in the real-time RT-PCR assay

Symbol	Gene name	Assay ID	Amplicon length (bp)
Housekeeping genes			
<i>PPIA</i>	Peptidylprolyl isomerase A	Mm02342430_g1	148
<i>RPL4</i>	Ribosomal protein L4	Mm00834993_g1	129
Target genes			
Fbg	Fibrinogen, beta chain	Mm00805336_ml	154
F2	Coagulation factor II (prothrombin)	Mm00438843_ml	68
F5	Coagulation factor V	Mm00484202_ml	61
F7	Coagulation factor VII	Mm00487329_ml	78
F8	Coagulation factor VIII	Mm00433174_ml	110
F9	Coagulation factor IX	Mm01308427_ml	74
F10	Coagulation factor X	Mm00484177_ml	81
F11	Coagulation factor XI	Mm00511167_ml	115
F12	Coagulation factor XII	Mm00491349_ml	64
F13b	Coagulation factor XIII $\beta$ subunit	Mm00491938_ml	62
AT	Antithrombin	Mm00446573_ml	94
PC	Protein C	Mm00435966_ml	52
PS	Protein S ( $\alpha$ )	Mm01343426_ml	62
Plg	Plasminogen	Mm00447087_ml	83
VWF	Von Willebrand factor	Mm00550376_ml	63
ADAMTS13	A disintegrin-like and metalloproteinase with thrombospondin type 1 motif, 13	Mm01218030_g1	77

UV-1600 spectrophotometer (Shimadzu, Kyoto, Japan). Gene expression analyses were performed only when the  $A_{260}/A_{280}$  ratios ranged between 1.9 and 2.1, and the integrity of each RNA sample was confirmed by agarose gel electrophoresis.

#### Reverse transcription (RT) coupled to quantitative real-time PCR

Total RNA (1  $\mu$ g) was reverse-transcribed using oligod(T)<sub>16</sub> primers as described by the manufacturer (Omniscript RT Kit, QIAGEN). First-strand cDNA samples were subjected to quantitative PCR amplification using the StepOne Real-time PCR System (Applied Biosystems Japan Ltd., Tokyo, Japan). Each of the cDNA samples was examined to assess gene expression levels for housekeeping and coagulation factor genes. To normalize the gene expression of the coagulation cascade genes between the various time points following the activation of liver proliferation, the geometric mean of the two genes, peptidylprolyl isomerase A (*PPIA*) and ribosomal protein L4 (*RPL4*), were utilized as previously identified by our lab<sup>[20,21]</sup>. The coagulation factors analyzed in our study included fibrinogen (Fbg), prothrombin (PT), factor V (FV), factor VII (FVII), factor FVIII (FVIII), factor IX (FIX), factor X (FX), factor XI (FXI), factor XII (FXII), factor XIII  $\beta$  subunit (FXIII  $\beta$ ), and von Willebrand factor (VWF). In addition, gene expressions of plasminogen (Plg), antithrombin (AT), protein C (PC), protein S (PS), and a disintegrin-like and metalloproteinase with thrombospondin type 1 motif 13 (ADAMTS13) were also analyzed. TaqMan probes and primers for the target genes were purchased from Applied Biosystems (TaqMan Gene Expression Assay, Table 1). All the PCR analyses were performed using the following cycling conditions: 10 min at 95°C (initial melt), followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The specificity of the primers was verified by 2% agarose gel electrophoresis of the

amplicons derived from naïve mouse liver cDNAs. For quantification of gene expression, the cDNAs derived from total RNA extracted from pooled normal mouse livers were serially-diluted, and used to generate the reference standard curves.

#### Coagulation assays

Plasma activity of coagulation factors was measured by one-stage clotting assay using human plasma that was deficient for each specific coagulation factor (Sysmex, Kobe, Japan). The activities of FVIII, FIX, FXI, and FXII were measured based on the activated partial thromboplastin time (aPTT) and those of PT, FV, FVII, and FX were measured based on the prothrombin time (PT) using the KC10A Coagulometer (Amelung, Lemgo, Germany). Plasma mouse VWF antigen levels were assayed by ELISA using a primary antibody against human VWF (Dako, Glostrup, Denmark), and a secondary goat anti-human VWF-HRP antibody (Dako). Fibrinogen levels were measured by the Clauss method using bovine thrombin, and FXIII levels were measured by the Berichrom FXIII chromogenic assay (Dade Behring, Marburg, Germany). For all the assessments, pooled plasma collected from 50 normal C57Bl/6 mice was used as a standard.

#### Statistical analysis

Significant differences between two groups were analyzed by two-tailed Wilcoxon *t*-test using Excel (Microsoft) with ystat2006 software (Igakutosyosyuppan, Tokyo, Japan).  $P < 0.05$  was considered significant.

## RESULTS

#### Induction of the DH mode of liver regeneration using hepatomitogen TCPOBOP

As shown in Table 2, the mouse livers treated with

**Table 2** Increase in liver weight during the DH-mediated regenerative phase following TCPOBOP induction

	Body weight (g)	Liver weight (g)	LW/BW × 100
D0	22.44 ± 0.95	1.06 ± 0.06	4.73 ± 0.14
3 h	21.05 ± 0.88	1.10 ± 0.07	5.22 ± 0.26
D1	28.28 ± 2.08	1.63 ± 0.15	5.70 ± 0.16
D2	26.71 ± 1.49	2.05 ± 0.10	7.70 ± 0.12
D3	27.55 ± 2.29	2.38 ± 0.19	8.62 ± 0.09
D10	29.81 ± 0.91	3.15 ± 0.11	10.42 ± 0.16

LW/BW: Liver weight to body weight; TCPOBOP: 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene. Quantitative changes in LW/BW ratio during the DH-regeneration phase at specific time points (D0, 3 h, D1, D2, D3, D10) following intragastric TCPOBOP injection (3 mg/kg body weight) ( $n = 8$  mice/time point). Livers were harvested and weighed to calculate the relative liver weight (as a percentage of body weight). All values were represented as the mean ± SE.

TCPOBOP were found to display an increase in organ size over defined time points as determined by the quantitative changes in the liver weight to body weight (LW/BW) ratio expressed as a percentage. Naïve LW/BW ratio (D0) was measured at  $4.73\% \pm 0.14\%$ , and there was a significant ( $P < 0.05$ ) increase in the ratio to  $5.22\% \pm 0.26\%$  as early as 3 h post-TCPOBOP administration. Over the 10 d period following the TCPOBOP injection, the ratio gradually increased ( $5.70\% \pm 0.16\%$ ,  $7.70\% \pm 0.19\%$ ,  $8.62\% \pm 0.09\%$ , and  $10.42\% \pm 0.16\%$ , at D1, D2, D3, and D10, respectively). At D10, the LW/BW ratio increased to 220% of the naïve LW/BW ratio at D0. We also observed that the body weight increased during the 10 d period, but its magnitude of increase was far less than that of liver weight. Furthermore, administering only the solvent (DMSO/corn oil) to the mice did not affect the LW/BW ratio significantly, confirming that the regenerative responses were caused by TCPOBOP (data not shown).

#### Gene expression levels of coagulation factors and other factors in the liver

Initial experiments were performed to assess the mRNA expression profile of 10 different coagulation factors (Fbg, PT, FV, FVII, FVIII, FIX, FX, FXI, FXII, and FXIII $\beta$ ) following TCPOBOP injection at distinct time points ( $n = 8$  livers/time point) using real-time RT-PCR. All of the data were normalized to the geometric mean of two housekeeping genes, *PPLA* and *RPLA*, and then calculated as a comparative ratio to the quiescent (control) liver at D0. Compared with values at D0, mRNA expression levels of all the analyzed coagulation factors, except for FX, were significantly ( $P < 0.05$ ) but temporally down-regulated during the most active phase of DH-mediated liver regeneration as shown in Figure 1. FX mRNA levels did not decrease during the DH-liver regenerative phase, and ultimately, the levels were significantly increased at D10 compared with D0. For most of the coagulation factor mRNAs that were down-regulated, the mRNA levels gradually recovered by D10 to the levels observed at D0, but there were several factors, particularly FXI, that remained significantly

lower ( $P < 0.05$ ) until D10.

In addition to coagulation factors, we assessed the mRNA expression levels for other factors involved in the regulation of the coagulation cascade, including Plg, AT, PC, PS, VWF, and ADAMTS13. As shown in Figure 2, the mRNA levels of Plg, AT, PC, and ADAMTS13 were down-regulated at D1 and D2 after the TCPOBOP injection, and slowly recovered by D10 to the levels detected in the quiescent state (D0). PS mRNA tended to be increased and, at times, significantly ( $P < 0.05$ ) elevated, throughout the DH-mediated liver regenerative phase.

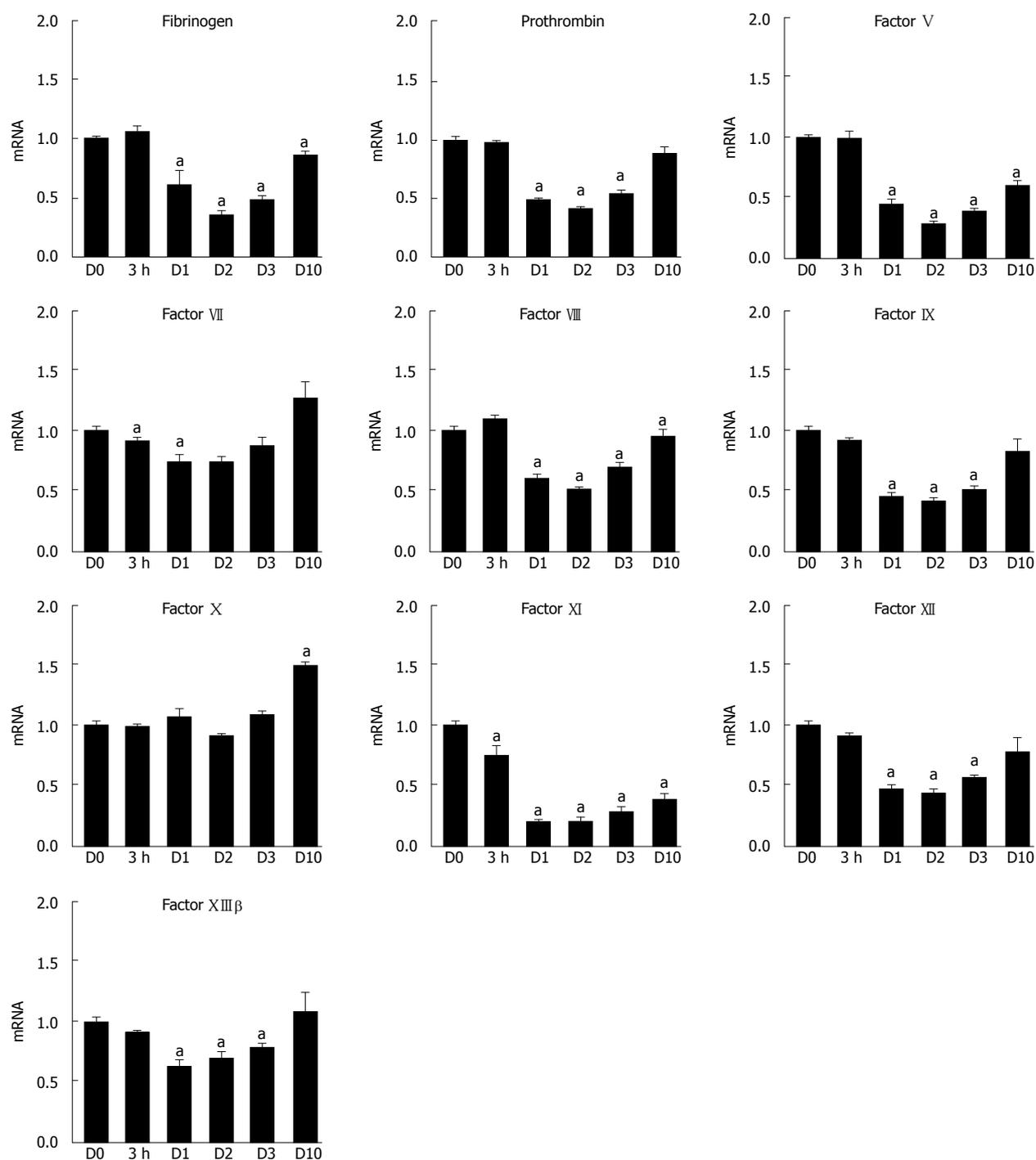
#### Plasma levels of coagulation factors

We then determined the changes in the plasma activity of the coagulation factors during the DH-mediated liver regenerative phase. As shown in Figure 3, we detected a significant decrease ( $P < 0.05$ ) in the plasma activities for 5 of the clotting factors (PT, FVIII, FIX, FXI, and FXII) during the regeneration phase compared with D0. Among these 5 factors, FIX and FXI showed the most dramatic decline in plasma activity by about 50% at D2 compared to the D0 levels. These reductions in plasma activity for FIX and FXI were consistent with our RT-PCR findings (Figure 1) following TCPOBOP injection. In contrast, the plasma activities of the other coagulation factors, Fbg, FV, FVII, and FXIII, as well as VWF, were not significantly reduced despite the reduction in the liver mRNA levels. Unlike the other coagulation factors, FX showed a temporal increase in its plasma activity, with significant increases ( $P < 0.05$ ) detected at D1.

## DISCUSSION

The present study demonstrated a comprehensive gene expression profile in the liver with corresponding plasma activities of coagulation factors and fibrinolytic factors during the liver regeneration phase induced by TCPOBOP, which is a hepatomitogenic compound. At the peak regenerative phase (i.e. D2 after TCPOBOP injection), the mRNA expression levels for most of the analyzed genes were temporally down-regulated followed by a compensatory recovery back towards the normal levels upon the completion of the regenerative activity (i.e. D10 after TCPOBOP injection). In contrast to the mRNA expression profile, the plasma activities for the coagulation factors showed varied responses. More specifically, PT, FVIII, FIX, FXI, and FXII demonstrated a temporal decrease in activity, FX showed a temporal increase in activity, and Fbg, FV, FVII, FXIII, and VWF did not markedly change throughout the regenerative period.

Liver regeneration is known to be an intrinsic function that can be induced through two distinct pathways: (1) compensatory regeneration and (2) direct hyperplasia (DH). In compensatory regeneration, tumor necrosis factor, interleukin-6, nuclear factor- $\kappa$ B, STAT3, and AP-1 are associated with the initiation of hepatocyte proliferation, but the process of DH-mediated liver regeneration appears to be independent of these cytokines<sup>[7,22]</sup>. At the level of gene expression, there are

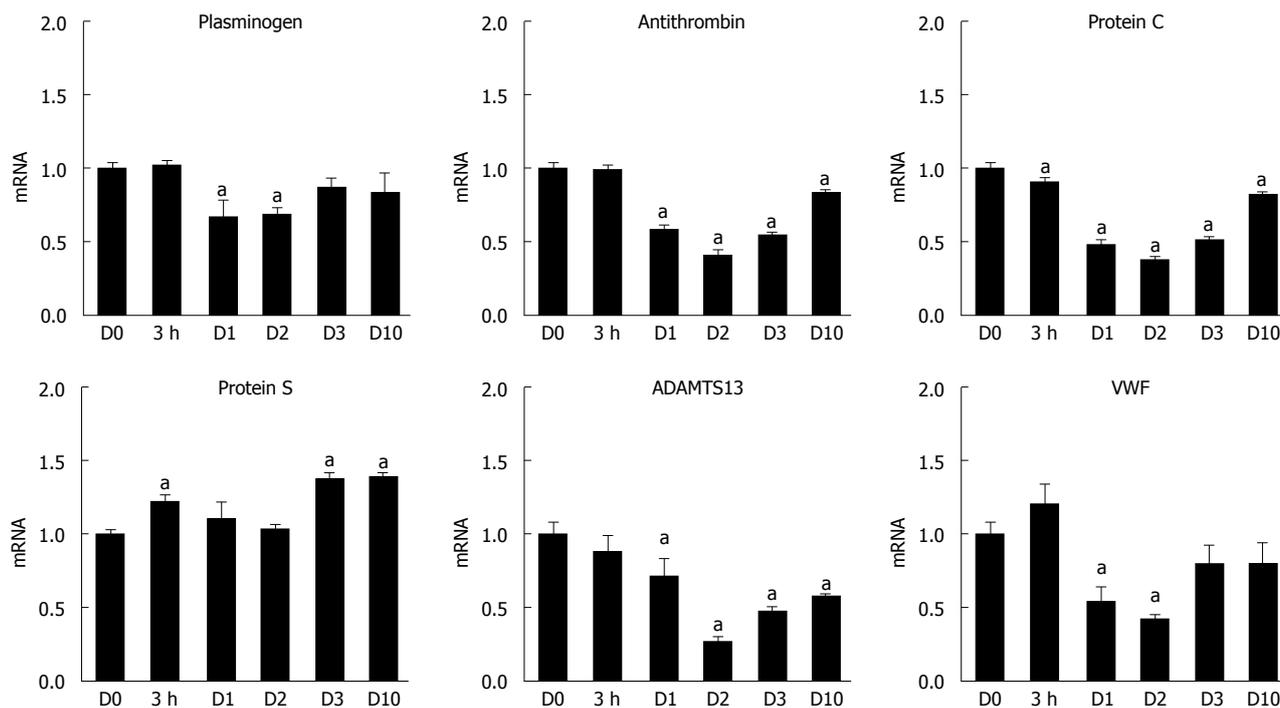


**Figure 1** Gene expression profiling of coagulation factors in the liver during the DH-mediated regenerative phase following TCPOBOP induction. The mRNA expression levels of 10 coagulation factors (fibrinogen, prothrombin, factors V, VII, VIII, IX, X, XI, XII, and XIII $\beta$ ) were assessed from mouse livers at D0, 3 h, D1, D2, D3, and D10 by real-time RT-PCR ( $n = 8$ /time point). The mRNA levels of each gene were normalized to the geometric mean of *PPIA* and *RPL4* mRNA levels. The values were expressed as a comparative ratio to the day 0 samples, and represented as the mean  $\pm$  SE. <sup>a</sup> $P < 0.05$  vs D0.

distinct genetic pathways that are activated depending on the mode of liver regeneration. For example, there is a rapid induction of cyclin D1 as early as 8 h after the induction of the mode of DH, whereas cyclin D1 does not appear to be induced at any time point during compensatory regeneration<sup>[23]</sup>.

In this paper, we focused on the DH mode of liver regeneration, which is a unique process that mediates hepatocellular proliferation due to inoculation with primary hepatocyte mitogens<sup>[6,8]</sup>. Following the

administration of the mitogens, hepatocytes and other liver cell types demonstrate a rapid activation of DNA synthesis in which the peak of the cell division occurs between D1 to D2 leading to a subsequent increase in liver size and weight. Among the currently available hepatocyte mitogens, TCPOBOP, a synthetic ligand to the constitutive androstane receptor (CAR), has been shown to produce robust proliferation and growth in mouse livers<sup>[6,8]</sup>. The rate of regenerative activity at the peak phase of DH-mediated proliferation

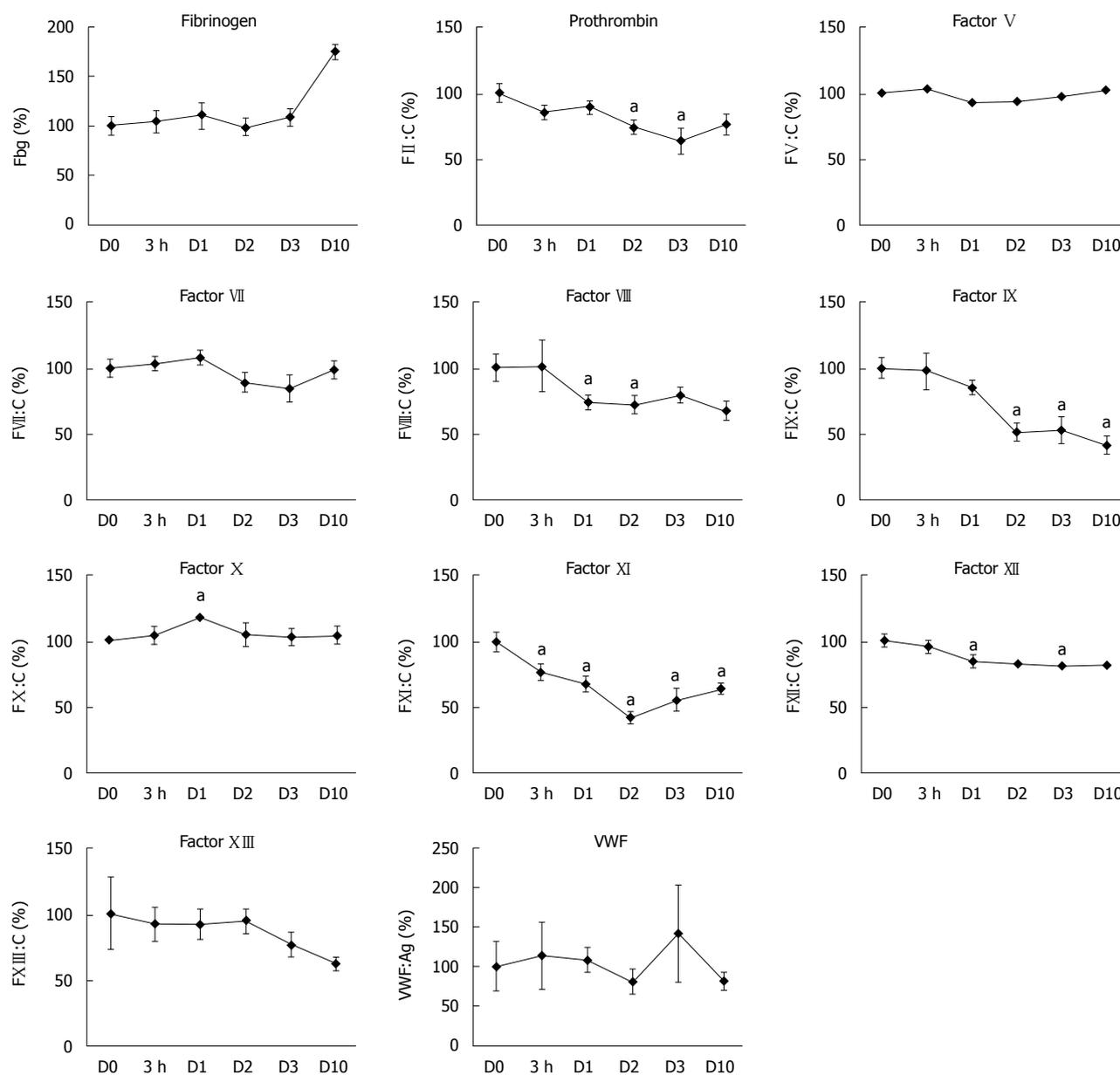


**Figure 2** Gene expression profiling of coagulation-related factors in the liver during the DH-mediated regenerative phase following TCPOBOP induction. The mRNA expression levels of 6 coagulation-related factors [plasminogen, antithrombin, protein C, protein S, a disintegrin-like and metalloproteinase with thrombospondin type 1 motif 13 (ADAMTS13), and von Willebrand factor (VWF)] were assessed in mouse livers at D0, 3 h, D1, D2, D3, and D10 by real-time RT-PCR ( $n = 8/\text{time point}$ ). The mRNA levels of each gene were normalized to the geometric mean of *PPIA* and *RPL4* mRNA levels. The values were expressed as a comparative ratio to the D0 samples, and represented as the mean  $\pm$  SE. <sup>a</sup> $P < 0.05$  vs D0.

following TCPOBOP administration is more robust compared to the regenerative effect attributed to a partial hepatectomy-induced compensatory regeneration as observed by the BrdU labeling of proliferating hepatocytes<sup>[8]</sup>. Consequently, an active proliferation of hepatocytes at the same levels of previous reports was induced appropriately by the administration of TCPOBOP in the present study (Table 2)<sup>[8,20]</sup>.

In the face of a proliferative response in the liver, liver function can be adversely affected. It has been reported that gene expression levels of liver-specific proteins are temporally suppressed, which suggests that the regenerating liver sacrifices the production of liver-specific proteins, mainly plasma proteins, in order to promote the protein production required for cell division and growth<sup>[24,25]</sup>. These findings are supported by our previous report that gene expression of essential structural proteins for the cell, including  $\beta$ -actin, were significantly increased during liver regeneration<sup>[5,20]</sup>. In contrast to compensatory liver regeneration, there remains a paucity of information regarding the molecular changes in gene expression and production of liver-specific proteins, including coagulation factors, during liver regeneration through the DH mode. Since the majority of the coagulation factors and anti-coagulation factors are produced by hepatocytes, analysis of the liver to profile the gene expression of these factors would provide a comprehensive determination of the effect of proliferation on gene expression. In our analyses, we demonstrated that livers in the active phase of liver regeneration (D1 and D2) significantly down-regulated

the gene expression of almost all of the coagulation and fibrinolytic factors analyzed (Figures 1 and 3). The reduction in the coagulation cascade gene expression is consistent with previous studies in our lab whereby TCPOBOP-induced hepatocyte proliferation led to a marked down-regulation of other liver-specific genes, including albumin and ornithine transcarbamylase<sup>[8,20]</sup>. Interestingly, the decrease in gene expression for the coagulation cascade proteins did not necessarily correlate with the plasma protein activity for many of the analyzed genes, such as Fbg, FV, FVII, FX and FXIII. For these proteins, the plasma protein activity appeared to be maintained or elevated during the liver proliferation and growth phase even though the mRNA levels of these proteins were significantly reduced. The lack of reduced plasma activity could be related to a number of factors, including the threshold of mRNA expression needed for efficient translation of the coagulation factor, an extended half-life of the protein, enhanced translation and subsequent secretion of the proteins into the circulation, or the mass of liver needed to produce the coagulation proteins. Unlike the other coagulation factors, FIX activity remained significantly reduced throughout the liver regenerative phase even though the FIX mRNA profile returned towards normal levels in the face of increased liver mass. In order for FIX to become available in a biologically active and secretable form, it has been described that several post-translational modification steps are required within the hepatocytes. It may be speculated that the disparity between the low plasma FIX activity and revived liver



**Figure 3** Determination of plasma levels and activity for coagulation factors during the DH-mediated regenerative phase following TCPOBOP induction. Plasma activity levels of 10 coagulation factors (fibrinogen, prothrombin, factor V, VII, VIII, IX, X, XI, XII, and XIII) and plasma von Willebrand factor (VWF) antigen levels were assessed in mice during the DH-mediated regenerative phase. Activity levels of prothrombin, factor V, VII, VIII, IX, X, XI, and XII were measured by one-stage clotting assay, fibrinogen activities were measured by the Clauss method, factor XIII activities were measured by chromogenic assay, and VWF antigen levels were measured by specific ELISA. The plasma samples were obtained at D0, 3 h, D1, D2, D3, and D10 after TCPOBOP injection ( $n = 8$ /time point). The data were described as percentage of pooled normal plasma, and represented as the mean  $\pm$  SE. <sup>a</sup> $P < 0.05$  vs D0.

mRNA levels is the result of insufficient recovery of such post-translational modification systems in the regenerated hepatocytes. Since FIX is a critical protein for mediating blood coagulation<sup>[11,12]</sup>, further investigation needs to be addressed for the elucidation of the mechanism producing the biologically active FIX during the regeneration phase.

The effect of proliferative stimuli on coagulation factors not produced in the hepatocytes is not well-established. VWF is known to be an important glycoprotein for platelet adhesion to wound sites as well as a carrier protein for FVIII, and to be largely produced in endothelial cells throughout the body, including the liver<sup>[26]</sup>. ADAMTS13, which is produced exclusively by hepatic stellate cells, is a

VWF-cleaving enzyme essential for preventing excessive thrombotic events in the body<sup>[27]</sup>. These proteins were found to have their mRNA expression significantly down-regulated during the active phase of DH-mediated regeneration (Figures 1 and 3). Proliferation of non-parenchymal cells in the liver has been shown to occur following DH-mediated stimuli, but the peak level of proliferation was found to occur 1-2 d following the peak normally observed in hepatocytes<sup>[8]</sup>. One possible explanation for the down-regulation of VWF and ADAMTS13 at the early regeneration phase could be associated with the early initiation, but lagging pace, of cell-cycle progression in the non-parenchymal cells.

In conclusion, this is the first detailed study to

investigate the liver gene expression profiles with corresponding plasma protein activity levels for a panel of coagulation factors and related coagulation cascade proteins during liver regeneration mediated by direct hyperplasia. Temporal down-regulation of gene expression was found to be associated with this mode of liver regeneration, while plasma activity levels showed mixed changes in activity depending on the factor being examined. These results suggest that there may be a common feature between two mechanisms involved in liver regeneration whereby the liver in the proliferative phase sacrifices the production of liver-specific proteins that are not essential for hepatocyte division and growth until the liver has completed or nearly completed the regenerative event. However, it is clear that the genetic profiles of these proteins cannot be simply answered by determining the expression and protein activity, but that other mechanisms are involved requiring further investigation as to how liver-specific proteins not involved in cell division and growth are regulated during DH-mediated liver regeneration.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Liver regeneration is a well-orchestrated process in which hepatocytes and non-parenchymal cells undergo rapid proliferation *via* induction by two distinct pathways: (1) compensatory regeneration; and (2) direct hyperplasia (DH). In the former pathway, liver regeneration is triggered by a loss of functional liver mass, which can be observed in situations where the liver becomes necrotic due to chemical administration or portions of the liver lobes are physically removed by partial hepatectomy. In the latter DH pathway, the liver regeneration can be initiated by direct administration of hepatocyte mitogens, such as 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP), thyroid hormone T<sub>3</sub>, phenobarbital or bile acids.

### Research frontiers

There is evidence that some of the liver-specific proteins have temporal suppression in their production during compensatory liver regeneration. Although the kinetics for the coagulation and/or fibrinolytic factors during compensatory liver regeneration have been investigated, comprehensive analyses assessing the gene expression profiles of the coagulation factors and their related proteins in combination with their plasma activities during the DH-mediated liver regeneration have not yet been documented.

### Innovations and breakthroughs

In the present study, the authors induced liver regeneration in C57BL/6 mice by administering TCPOBOP; the most commonly used experimental procedure to induce DH-mediated liver regeneration. During this liver regeneration process, they investigated the gene expression profiles of coagulation factors and fibrinolytic factors as well as plasma activities of these factors at different time points following TCPOBOP administration.

### Applications

The systematic and thorough investigation of the molecular changes in the coagulation cascade during liver regeneration by DH will help to better understand the basic biology of the liver under normal and pathological conditions.

### Terminology

TCPOBOP is one of the currently available hepatocyte mitogens, and functions as a synthetic ligand to the constitutive androstane receptor, which has been shown to produce robust proliferation and growth in mouse livers.

## Peer review

In this study, the authors investigated the changes in expression and activity of several factors of the coagulation cascade during liver regeneration. The study is well presented and the figures are convincing.

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BRIEF ARTICLE

## Sickle cell cholangiopathy: An endoscopic retrograde cholangiopancreatography evaluation

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biliary sludge and bile duct stones in SCD, endoscopic sphincterotomy might prove helpful in these patients.

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**Key words:** Sickle cell disease; Hepatobiliary; Cholestatic jaundice; Sickle cell hepatopathy; Sickle cell cholangiopathy; Endoscopic retrograde cholangiopancreatography

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### Abstract

**AIM:** To evaluate the role of endoscopic retrograde cholangiopancreatography (ERCP) in patients with sickle cell disease (SCD).

**METHODS:** Two hundred and twenty four SCD patients with cholestatic jaundice (CJ) had ERCP. The indications for ERCP were based on clinical and biochemical evidence of CJ and ultrasound findings.

**RESULTS:** Two hundred and forty ERCPs were performed. The indications for ERCP were: CJ only in 79, CJ and dilated bile ducts without stones in 103, and CJ and bile duct stones in 42. For those with CJ only, ERCP was normal in 42 (53.2%), and 13 (16.5%) had dilated bile ducts without an obstructive cause. In the remaining 22, there were bile duct stones with or without dilation. For those with CJ, dilated bile ducts and no stones, ERCP was normal in 17 (16.5%), and 28 (27.2%) had dilated bile ducts without an obstructive cause. In the remaining 58, there were bile duct stones with or without dilation. For those with CJ and bile duct stones, ERCP was normal in two (4.8%), and 14 (33.3%) had dilated bile ducts without an obstructive cause. In the remaining 26, there were bile duct stones with or without dilatation.

**CONCLUSION:** Considering the high frequency of

### INTRODUCTION

Sickle cell disease (SCD) is common in the Eastern Province of Saudi Arabia and it can affect any part of the body<sup>[1-3]</sup>. One of the main organs to be affected by SCD is the hepatobiliary system. However, this is variable in character and severity, and can be presented as a variety of symptoms, including cholelithiasis, choledocholithiasis, and cholestatic jaundice (CJ). CJ can be caused by several diseases and one of the common intrahepatic causes is sickling of red blood cells (RBC), which is also called hepatic crisis or hepatic sequestration (sickle cell hepatopathy)<sup>[4,5]</sup>. This can mimic extrahepatic bile ducts obstruction, which causes diagnostic and therapeutic dilemmas. There is also a high frequency of cholelithiasis and choledocholithiasis in patients with SCD<sup>[3,6-8]</sup>. It is of paramount importance to differentiate between these causes and one of the diagnostic and therapeutic modalities in the evaluation of patients with CJ is endoscopic retrograde cholangiopancreatography. Thus, we evaluated the role of endoscopic retrograde cholangiopancreatography (ERCP) in patients with SCD, with an emphasis on SCD cholangiopathy.

### MATERIALS AND METHODS

Over a period of 15 years (1993-2008), 224 patients with

**Table 1 ERCP findings in patients with cholestatic jaundice only (Total No. of patients = 79)**

Finding	n (%)
Normal	42 (53.2)
Dilated CBD without stones	11 (13.9)
Dilated CBD with stones	10 (12.7)
Dilated bile ducts with stones	6 (7.6)
Dilated bile ducts without stones	1 (1.3)
Dilated CHD without stones	1 (1.3)
Normal CBD with stones	1 (1.3)
Edematous inflamed papilla	4 (5.1)

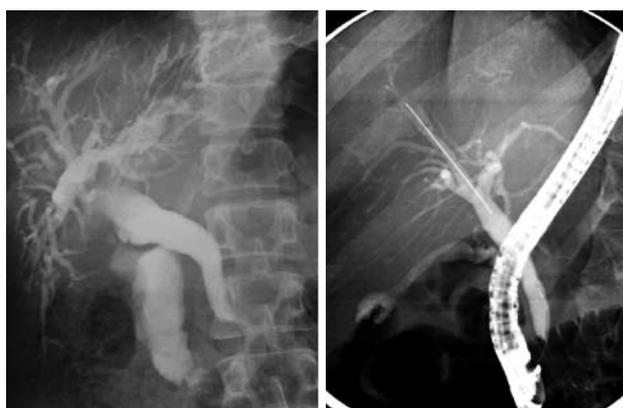
**Table 2 ERCP findings in patients with cholestatic jaundice and dilated bile ducts on ultrasound (Total No. of patients = 103)**

Finding	n (%)
Normal	17 (16.5)
Dilated CBD without stones	17 (16.5)
Dilated CBD with stones	30 (29.13)
Dilated bile ducts without stones	11 (10.7)
Dilated bile ducts with stones	17 (16.5)
Normal CBD with a stone	1 (0.98)
Choledochoduodenal fistula	2 (1.94)
Edematous inflamed papilla	8 (7.8)

ERCP: Endoscopic retrograde cholangiopancreatography; CBD: Common bile duct; CHD: Common hepatic duct.

**Table 3 ERCP findings in patients with cholestatic jaundice and bile duct stones (Total No. of patients = 42)**

Finding	n (%)
Normal	2 (4.8)
Dilated CBD without stones	7 (16.7)
Dilated CBD with stones	14 (33.3)
Dilated bile ducts without stones	7 (16.7)
Dilated bile ducts with stones	7 (16.7)
Normal CBD with a stone	1 (2.4)
Edematous inflamed papilla	4 (9.5)



**Figure 1 Endoscopic retrograde cholangiopancreatography (ERCP) showing dilated CBD and common hepatic duct without an obstruction.**

SCD underwent 240 ERCP procedures. Their medical records were reviewed and the following information was obtained: age, sex, clinical presentation, investigations, indication for ERCP, abdominal ultrasound results, ERCP findings, therapeutic procedures at the time of ERCP, complications and outcome. All had an abdominal ultrasound and the indications for ERCP were based on clinical and biochemical evidence of CJ and ultrasound findings. Based on abdominal ultrasound findings, the patients were divided into three groups as follows: (1) those with normal ultrasound, (2) Those with dilated bile ducts but no stones, and (3) Those with bile duct stones. All ERCPs were performed in the radiology department using an Olympus TJF 240 or JF 260 side-viewing duodenoscope. This was done under general anesthesia with nasotracheal intubation for children less than 10 years of age, and under sedation using meperidine (1 mg/kg) and diazepam (0.1-0.2 mg/kg) for those above 10 years of age. The ampulla of Vater was cannulated with tapered or regular catheters and the biliary ducts were deliberately visualized under fluoroscopy using Hexabrix (320 mg diluted to 50%). Appropriate radiographs were obtained and, where indicated, sphincterotomy was performed using a 5F sphincterotome (Olympus) and bile duct stones, if found, were extracted with a basket, balloon extractor, or mechanical lithotripter.

**RESULTS**

Two hundred and twenty four patients with SCD underwent 240 ERCP procedures. There were 144 males and 80 females. Their mean age was 22.4 years (5-70 years). Their

mean hemoglobin S (HbS) was 76.8% (64.7%-92.3%) and their mean hemoglobin F (HbF) was 20.4% (5.1%-34.0%). Their mean total bilirubin was 224 g/L (55-395 g/L). Their mean direct bilirubin was 134 g/L (40-263 g/L). Their mean alkaline phosphatase was 486 IU/mL (81-1189 IU/mL) (Normal: 50-136 IU/mL). Their mean alanine transaminase (ALT) was 234.3 IU/mL (50-761 IU/mL) (Normal: 30-56 IU/mL) and their mean aspartate transaminase (AST) was 206.3 IU/mL (63-317 IU/mL) (Normal: 15-37 IU/mL). The indications for ERCP were: CJ only in 97, CJ and dilated bile ducts on ultrasound in 103, and CJ and bile duct stones on ultrasound in 42. The ERCP findings in each of these three groups are shown in Tables 1-3. In those with CJ only, there was a group of 13 patients (16.5%) with dilated bile ducts without an obstructive cause. In those with CJ and dilated bile ducts on ultrasound, there was a group of 28 patients (27.2%) with dilated bile ducts without an obstructive cause. In those with CJ and bile duct stones on ultrasound, there was a group of 14 patients (33.3%) with dilated bile ducts without an obstructive cause. In total 55 patients (24.6%) had dilated bile ducts without an obstructive cause or previous history of biliary duct stones (Figures 1 and 2).

The therapeutic procedures performed during ERCP are shown in Table 4. This included endoscopic sphincterotomy only in 42 out of the 55 patients (76.4%) who had dilated bile ducts without an obstructive cause. The remaining 13 patients were treated early in the study and no sphincterotomies were done. There was no mortality. Four patients developed minor bleeding from



**Figure 2** An ERCP showing markedly dilated bile ducts without an obstructive cause. Note the nasobiliary tube for drainage in the first image.

the sphincterotomy site. This was controlled with local adrenaline injection. Eight patients (3.3%) developed transient mild pancreatitis.

## DISCUSSION

Sickle cell disease is one of the common hemoglobinopathies in the Eastern Province of Saudi Arabia, where the frequency of Sickle cell trait can reach as high as 25% in some areas<sup>[1-3]</sup>. One of the common manifestations of SCD is jaundice, which can be caused by a variety of hepatobiliary diseases including CJ<sup>[3-5]</sup>. There are certain causes of CJ that are SCD related. One of these causes is intrahepatic sickling of RBC<sup>[4,5,9]</sup>. This is also called hepatic crisis or hepatic sequestration (sickle cell hepatopathy)<sup>[4,5]</sup>. This can lead to cholestasis and a clinical picture that can resemble extrahepatic bile duct obstruction which causes diagnostic and therapeutic dilemmas. Sickle cell intrahepatic cholestasis on the other hand is a more serious condition, characterized by acute onset of hepatomegaly, hyperbilirubinemia, coagulopathy, and acute liver failure<sup>[4,5]</sup>. Early identification of these is important as the process of sickling can be reversed by hydration and simple, or in severe cases, exchange blood transfusion. There is also a high frequency of cholelithiasis and choledocholithiasis in patients with SCD<sup>[3,6-8]</sup>. The frequency of cholelithiasis in patients with SCD is variable, ranging from 4% to 55%, and this increases with age<sup>[2,3,6-8]</sup>. In the general population with cholelithiasis, the incidence of common bile duct (CBD) stones has been reported to be 10%-15%, whereas in those with SCD it ranges from 18%-30%<sup>[10,11]</sup>. Due to this high incidence, routine intraoperative cholangiography has been advocated<sup>[10]</sup>. With the recent advances in laparoscopic cholecystectomy (LC), exclusion of CBD stones prior to LC is of great importance. ERCP has been shown to be valuable, both for the diagnosis and management of CBD stones, in patients with SCD who are undergoing or have undergone LC<sup>[12-14]</sup>. ERCP is also of great importance in evaluating SCD patients with CJ, whether this is due to intrahepatic or extrahepatic causes. Ultrasound is a simple, non invasive imaging technique, and although gallstones and intrahepatic and

**Table 4** Therapeutic procedures during ERCP

Procedure	n (%)
Endoscopic sphincterotomy only	42 (18.8)
Endoscopic sphincterotomy and stone extraction	79 (35.3)
Insertion of biliary stent	8 (3.6)
Endoscopic sphincterotomy, mechanical lithotripsy, and stone extraction	4 (1.8)
Insertion of a nasobiliary tube	4 (1.8)

extrahepatic bile duct dilatation are readily detected by ultrasound, common bile duct stones might be missed. ERCP, on the other hand, is more invasive but is the procedure of choice in suspected cases of extrahepatic bile duct obstruction. It provides direct visualization of the biliary tree and demonstrates the site and nature of the obstruction in more than 90% of patients. ERCP also provides therapeutic interventions, including endoscopic sphincterotomy and stone extraction, dilatation of strictures, and placement of stents and biliary drainage catheters<sup>[15-19]</sup>. This was the case in our series, where we found ERCP valuable both as a diagnostic and therapeutic procedure. The majority of bile duct stones (95.4%) in our series were removed via ERCP. ERCP however was normal and unnecessary in a significant number of our patients (27%) with SCD and CJ. This was specially so in those who presented with CJ only (53.2%). These patients most likely had CJ secondary to intrahepatic sickling of RBC. Hepatic crisis and hepatic sequestration resemble each other clinically, and the only differentiating point between the two is a sudden drop in hematocrit, as well as a sudden increase in liver size in those with hepatic sequestration<sup>[4,5]</sup>. Sickle cell intrahepatic cholestasis, on the other hand, is a more serious and often fatal complication of SCD where there is widespread intrahepatic sickling within the liver sinusoids leading to their blockage, vascular stasis with hepatic ischemia, and the striking feature is the highly elevated plasma bilirubin level<sup>[4,5,12,13]</sup>. It is possible that our patients with CJ and normal ERCP represent a benign variant of intrahepatic cholestasis or a form of what is called benign hyperbilirubinemia<sup>[5]</sup>. All our patients had hyperbilirubinemia, but their plasma AST, ALT and alkaline phosphatase levels were only moderately elevated. To support this, these patients subsequently recovered with conservative treatment including observation, hydration and, where indicated, blood transfusion. To overcome this and decrease the number of patients with normal ERCP, patients with CJ only should be evaluated further prior to ERCP, including endoscopic ultrasound (EUS) and magnetic resonance cholangiopancreatography (MRCP). However, these investigations are not readily available, and in these situations a period of observation and conservative management is to be advocated.

We found an interesting group of patients with SCD and CJ who had dilatation of the bile ducts without an obstructive cause (24.6%). There are several causes for bile duct dilatation, such as CBD stones, tumor of the head of pancreas or Ampulla of Vater,



**Figure 3** ERCP showing biliary sludge in a patient with SCD. An arrow indicates the site of biliary sludge.

and tumors or masses at the porta hepatis. None of our patients had an obstructive cause for the bile duct dilatation. The exact etiology of this dilatation is not known. However, we think this is a form of cholangiopathy that is a consequence of sickling in the end arteries of the biliary arterial tree leading to hypoxia and dilatation<sup>[16,17]</sup>. The bile ducts are supplied via the hepatic arteries and ischemic bile duct injury might occur when these vessels are injured or occluded. This will ultimately result in ischemic stricture of the bile ducts. This however, depends on the extent and velocity of the occlusive process. In patients with SCD, we feel that the occlusion, which is usually not complete, is of the peribiliary vascular plexus and is a result of sickling within these vascular channels. This will ultimately lead to hypoxia of the bile ducts leading to their dilatation rather than ischemia and stricture formation. This is SCD cholangiopathy and the extent of this is also variable, as was demonstrated in our series. We found patients with bile duct dilatation limited to the common bile duct, but there were also those who had dilatation involving both extra and intrahepatic bile ducts (Figures 1 and 2). Documenting this is of great importance, as these patients need to be followed up regularly for the possibility of developing bile duct stones. Cunningham, in a study of the common hepatic duct diameter in SCD patients, found only two patients with clinically silent enlargement based on ultrasonic evaluation of 95 patients<sup>[20]</sup>. Their patients, in contrast to our patients, were young with a mean age group of 16.8 years. Taking in consideration the high frequency of biliary sludge and the possibility of bile duct stones formation in these patients, 42 (76.4%) of our patients with dilated bile ducts without an obstructive cause had endoscopic sphincterotomy, as this could obviate the future development of bile duct stones (Figure 3). Thus, the value of endoscopic sphincterotomy in this group of patients needs to be evaluated further, as endoscopic sphincterotomy is not without complications.

## COMMENTS

### Background

Cholestatic jaundice (CJ) is common in patients with sickle cell disease (SCD) and can be caused by both intra and extra hepatic pathology. However, there are certain causes of CJ that are specific to patients with SCD. One of the common intrahepatic causes is sickling of red blood cells (RBC), which is also called hepatic crisis or hepatic sequestration (sickle cell hepatopathy). This can mimic extrahepatic bile ducts obstruction which causes diagnostic and therapeutic dilemmas. Endoscopic retrograde cholangiopancreatography (ERCP) is one of the diagnostic and therapeutic measures used in patients with CJ. The value of ERCP for patients with SCD needs to be evaluated.

### Research frontiers

CJ is common in patients with SCD and it is of therapeutic importance to define the cause as soon as possible. Ultrasound is a valuable, non invasive, investigation but it has limitations. ERCP, on the other hand, is invasive but more valuable, both as a diagnostic and therapeutic procedure. It provides direct visualization of the biliary tree and demonstrates the site and nature of obstruction. This is of importance in excluding extrahepatic causes of CJ in patients with SCD, and once this is excluded emphasis is concentrated on intrahepatic causes.

### Applications

Taking in consideration the high frequency of biliary sludge, and the possibility of bile duct stones formation in these patients, endoscopic sphincterotomy might obviate the future development of bile duct stones.

### Terminology

Sickle cell disease is a form of hemoglobinopathy that results from a single amino acid substitution of valine for glutamic acid in the 6th position among the 146 amino acids of the hemoglobin-beta chain.

### Peer review

This is an interesting study evaluating sickle cell cholangiopathy by ERCP.

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## Celiac disease in patients with presumed irritable bowel syndrome: A case-finding study

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### Abstract

**AIM:** To estimate the prevalence of celiac disease (CD) in adult patients with presumed irritable bowel syndrome (IBS).

**METHODS:** Between March 2005 and December 2008, 742 consecutive patients (293 male, median age 43 years, range 18-69 years) fulfilling the Rome II criteria for IBS were prospectively enrolled in the study. IBS was diagnosed *via* self-completed Rome II modular questionnaires. Anti-tissue transglutaminase (anti-tTG) serology was checked to initially recognize possible CD cases. Patients with a positive test were offered endoscopic duodenal biopsy to confirm the diagnosis of CD.

**RESULTS:** Thirty two patients (15 male, median age 41 years, range 19-59 years) were found to have organic diseases other than CD. Twenty four patients tested positive for anti-tTG antibodies, and duodenal biopsies confirmed the diagnosis in all of them. Thus, in this patient population with presumed IBS, 3.23% actually had CD.

**CONCLUSION:** CD is common in patients with presumed IBS. Routine screening for CD in patients with symptoms of IBS is recommended.

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**Key words:** Irritable bowel syndrome; Celiac disease; Anti-tissue transglutaminase; Case-finding; Screening

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### INTRODUCTION

Irritable bowel syndrome (IBS) is a highly prevalent disorder. It is found in 10% to 20% of individuals using standard diagnostic tools such as the Rome II criteria<sup>[1]</sup>.

Diagnostic approaches to suspected IBS rely on eliciting symptoms that satisfy specific criteria and performing limited tests to exclude organic diseases that produce similar symptoms<sup>[2]</sup>.

IBS can sometimes be difficult to distinguish clinically from adult-onset celiac disease (CD)<sup>[3-8]</sup>. A broad spectrum of symptoms and signs may be associated with untreated CD. In fact, many patients - especially those presenting in adulthood - have minimal or atypical symptoms<sup>[5,7-10]</sup>.

The recent development of highly sensitive and specific serologic assays for CD has led to the increased realization that the disease is more common than it was believed<sup>[11-15]</sup>. This justifies the concern that some IBS-labeled patients may in fact have CD.

Reports of prevalence of CD in IBS patients from the Middle East are scanty.

We aimed to estimate the prevalence of CD in patients masquerading as IBS, and also to describe their clinical features.

### MATERIALS AND METHODS

#### Study population

Ethical approval of the study was obtained from the

Table 1 The modified Marsh classification of celiac disease

Type	Intraepithelial lymphocytes per 100 enterocytes	Crypts	Villi
0	< 40	Normal	Normal
1	> 40	Normal	Normal
2	> 40	Increased	Normal
3a	> 40	Increased	Mild atrophy
3b	> 40	Increased	Marked atrophy
3c	> 40	Increased	Absent

Table 2 The distribution of patients according to gender and IBS type *n* (%)

IBS type	Gender		Total
	Male	Female	
C-IBS	140 (47.8)	217 (48.3)	357 (48.1)
D-IBS	84 (28.7)	122 (27.2)	206 (27.8)
C/D-IBS	69 (23.5)	110 (24.5)	179 (24.1)
Total	293	449	742

IBS: Irritable bowel syndrome; C-IBS: Constipation-predominant IBS; D-IBS: Diarrhea-predominant IBS; C/D-IBS: Alternating constipation-diarrhea IBS.

Institutional Review Board at King Abdullah University Hospital. The potential implication of a positive result for CD was explained to all participants, and their written consent was obtained.

The Rome II criteria for IBS were applied to 891 consecutive patients upon their first visit to our outpatient gastroenterology clinic in the period between March 2005 and December 2008. The inclusion criteria were: age greater than 18 years, fulfilling the Rome II criteria for IBS; condition not previously investigated; absence of lactose intolerance or giardiasis. The exclusion criteria were: history of gastrointestinal alarm symptoms or signs; unwillingness to be submitted to esophagogastroduodenoscopy and/or colonoscopy. Only 764 individuals were eligible to participate in the study, and 22 (2.9%) of these did not agree to sign a written consent and thus were excluded from the study.

### Laboratory testing

Testing for anti-tissue transglutaminase (anti-tTG) serology was performed using the ORG 540A Anti-Tissue-Transglutaminase IgA (ORGENTEC Diagnostika GmbH®).

Quantitative IgA anti-tTG test was determined using the ELISA method. The sensitivity and specificity of this test in our laboratory was previously estimated at 98% and 96%, respectively (unpublished data). Patients with a positive test were submitted to duodenal biopsy to confirm the possibility of CD.

Other investigations included complete blood count, serum chemistry panel, erythrocyte sedimentation rate, thyroid function tests, occult blood stool testing, and stool analysis for ova and parasites. Additionally, patients with diarrhea were put on a 3-wk lactose-free diet to exclude lactose intolerance.

Table 3 The prevalence of celiac disease in different types of IBS patients

	No. of study patients	No. of celiac cases	Prevalence of CD (95% CI)
C-IBS	357	6	1.68 (0.35, 3.01) <sup>a</sup>
D-IBS	206	14	6.80 (3.36, 10.23) <sup>a</sup>
C/D-IBS	179	4	2.23 (0.07, 4.40)
Total	742	24	3.23 (1.96, 4.50)

<sup>a</sup>Significantly different (*P*-value = 0.0026); CI: Confidence interval.

### Colonoscopy

All patients older than 45 years or with a family history of colorectal cancer, and those with a positive occult stool blood test were submitted to colonoscopy to rule out structural disease. Furthermore, in patients with diarrhea random colonic biopsies were taken to rule out microscopic colitis.

### Intestinal biopsy

Using a standard biopsy forceps, six specimens were taken from the second and third portion of the duodenum. All biopsies were reviewed independently by two histopathologists, and changes of CD were reported using the modified Marsh criteria (Table 1).

### Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS, version 15). Continuous data were described using mean, median, standard deviation, and range wherever appropriate. Categorical variables were described using proportions. The 95% confidence interval (CI) was used to calculate the interval estimate of the prevalence of CD. Differences in prevalence rates according to different types of IBS were tested using  $\chi^2$  test; a *P*-value of less than 0.05 was considered statistically significant.

## RESULTS

This study included a total of 742 patients (293 males and 449 females). Their distribution according to gender and IBS type is shown in Table 2. Thirty two patients (15 males and 17 females) with a median age of 41 years (range 19-59 years) were found to have organic diseases other than CD [14 hypothyroidism, three microscopic colitis (two collagenous and one lymphocytic colitis), six lactose intolerance, three ulcerative colitis, and six Crohn's disease]. Twenty four patients [14 diarrhea-predominant IBS (D-IBS), six constipation-predominant IBS (C-IBS), and four alternating constipation-diarrhea IBS (C/D-IBS)] tested positive for anti-tTG. The prevalence of CD in different types of IBS is summarized in Table 3. The prevalence of CD in patients with D-IBS (6.80%, 95% CI: 3.36, 10.23) was significantly higher than that in patients with C-IBS (1.68%, 95% CI: 0.35, 3.01). Duodenal biopsies confirmed the diagnosis in all 24 patients. The modified Marsh criteria (Table 1) were used for the grading of severity of histopathological

**Table 4** The demographic and clinical features of celiac disease patients

Variable	n (%)
Gender	
Female	13 (54.2)
Male	11 (45.8)
Age (yr)	
18-25	8 (33.3)
26-35	8 (33.3)
36-61	8 (33.3)
mean (SD)	33.5 (11.5)
Body mass index, mean (SD)	26.6 (3.5)
Duration of symptoms (mo), mean (SD)	26.8 (18.1)
IBS type	
C-IBS	6 (25.0)
D-IBS	14 (58.3)
C/D-IBS	4 (16.7)
Malabsorptive features	
Hypoalbuminemia	2 (8.3)
Hypocalcemia	2 (8.3)
Iron deficiency	4 (16.7)
None	16 (66.7)

changes. Among our CD patients, two had Marsh type 1, seven had Marsh type 3a, 10 had Marsh type 3b, and five had Marsh type 3c. Thus, in this patient population with presumed IBS, 3.23% (95% CI: 1.96-4.50) actually had CD. The age of patients with CD (13 females and 11 males) ranged from 18 to 61 years with a mean (SD) of 33.5 (11.5). About 58% of these patients belonged to the D-IBS type, while less than 17% had the C/D-IBS type.

The demographic and clinical features of CD patients are summarized in Table 4. Interestingly, only eight of the CD patients had signs of intestinal malabsorption on further laboratory testing, with iron deficiency being the most common abnormality. The duration of IBS symptoms before the diagnosis of CD ranged between 6 and 72 mo (average 26.8 mo). The body mass index (BMI) of our CD patients was surprisingly higher than the expected, with an average of 26.6. All patients diagnosed with CD were started on a gluten-free diet, with subsequent improvement of their IBS-like symptoms in periods ranging from 2 to 6 wk.

## DISCUSSION

A high prevalence of CD in patients with presumed IBS was found in the present study. This implies that even with strict application of the Rome II criteria, IBS patients may have undetected CD. The majority of our CD patients had severe histopathological changes in duodenal specimens according to the modified Marsh criteria<sup>[16]</sup>. The advanced histopathological changes most probably reflect long-standing, untreated disease in our patient population.

The prevalence of CD in several recent population studies from North America ranged from 0.5% to 1%<sup>[17-19]</sup>. Studies in Europe have shown that up to 1% of the adult population may have CD<sup>[11]</sup>. In contrast to its high prevalence in Western countries, CD is considered rare in

non-Western populations. However, recent studies from the Middle East, Africa and India showed prevalence as high as 7.6% in selected groups of patients<sup>[20-22]</sup>. To the best of our knowledge, studies on the prevalence of CD in adult Jordanians have never been carried out. A prevalence study of CD in thousands of blood donors in Jordan is being conducted by the authors of the present study. Based on an interim analysis of the data obtained thus far, a prevalence of one in 200 could be projected. Therefore, IBS patients would be 6.5 times more likely to have CD than the general population.

The diagnosing of CD is often delayed, perhaps owing to a failure to recognize the protean manifestations of this disease, especially in the adult population. Patients often have few or no gastrointestinal symptoms and can even be obese<sup>[5,8,10]</sup>. In fact, in the present study none of the patients had typical symptoms of CD, such as steatorrhea or weight loss, and the majority of them had an average BMI in the overweight range.

Previously regarded as a mainly childhood problem it is now recognized that CD affects mostly adults, with about one quarter of patients being diagnosed at over 60 years of age. In a study by Green *et al.*<sup>[8]</sup>, data obtained on 1138 people with biopsy-proven CD showed that the majority of individuals were diagnosed in their 4th to 6th decades. Our study showed that adult CD can manifest at any age. However, it appears that the Jordanian adult patient population tends to present at a relatively younger age, possibly because of differences in gene penetrance as well as a larger wheat consumption by Jordanians (135 kg/head year); data from the Department of Agriculture, Jordan).

Survey data in the United States indicate that the median time to diagnosis in CD patients is 12 mo, and that over 20% of patients have symptoms for 10 years before CD is suspected and diagnostic testing performed<sup>[5]</sup>. However, the true denominator of undiagnosed CD is not well defined, and evolving data from both Europe and the United States indicate that many adult CD patients probably remain undetected<sup>[6,11,23]</sup>. In our CD patient population the time to diagnosis ranged between 8 and 72 mo. The delay in diagnosis could be ascribed to the atypical manifestations of the disease, but we believe that limited access to tertiary health care centers in our country could be another contributing factor to the long lag time before diagnosis.

CD can present with a wide spectrum of insidious symptoms. These can mimic symptoms of IBS. Several studies have suggested that the incidence of CD in patients with presumed IBS is higher than that of the normal population. In their case-control study of 300 subjects fulfilling the Rome II criteria for IBS, Sanders and colleagues found that the patients were 7 times more likely to have biopsy-proven CD than matched controls<sup>[4]</sup>. Sixty six patients with IBS tested positive for the antibodies, and 4.6% had active CD as compared with 0.66% of the non-IBS matched controls. The authors concluded that patients who meet the Rome II criteria for IBS should be investigated routinely for CD. More recently, the same investigators reported a primary

care-based cross-sectional study in which 1200 patients were recruited and evaluated serologically for CD<sup>[24]</sup>. The prevalence of CD in this population sample was 1%, while in the 123 participants with IBS the prevalence of CD was 3.3%. Once again, the authors recommended that a low threshold for serological screening of patients with IBS symptoms would be an optimal strategy.

In a recent primary care-based, multicenter study from North America, 976 subjects with symptoms or conditions known to be associated with CD, including IBS, were serologically tested for CD<sup>[25]</sup>. A diagnosis of CD was established in 22 patients, and thus the prevalence of CD in the serologically screened sample was 2.25%. The most frequent reasons for CD screening in these 22 cases were bloating (12/22), thyroid disease (11/22), IBS (7/22), unexplained chronic diarrhea (6/22), chronic fatigue (5/22), and constipation (4/22). The authors concluded that an active case-finding strategy in the primary care setting is an effective way to improve the diagnostic rate of CD. Of interest, the group of patients with IBS or symptoms of IBS - such as bloating, diarrhea, and constipation - constituted the largest proportion of screening-detected patients in their study.

Conversely, other investigators suggested that the prevalence of CD is not increased in patients with IBS symptoms. Locke III and colleagues recently published the results of a case-control study<sup>[26]</sup>. Using a self-completed questionnaire, the researchers evaluated 150 adult subjects, of whom 72 reported having symptoms of IBS and dyspepsia, and 78 controls who reported no gastrointestinal symptoms. The total number of individuals with CD in each group was surprisingly high: two out of 50 with IBS (4%), two out of 24 with dyspepsia (6%), and two of the 78 controls (2.6%). The researchers concluded that CD alone cannot explain the presence of IBS or dyspepsia in the subjects. The results of their study are interesting, but the sample size is probably not large enough to reach statistical significance.

Other studies suggested that the prevalence of CD in adult patients with IBS is not higher than that in the general population. Hin and colleagues conducted a case-finding study of CD in a primary care setting<sup>[27]</sup>. None of their 132 patients with IBS symptoms had positive results for CD, suggesting that CD rarely masquerades as IBS. Yet, we believe that this study is limited because of the small sample size.

It is arguable that, similar to IBS patients, other patients with various functional gastrointestinal disorders (i.e. functional dyspepsia) could have a higher prevalence of CD. In a recent study from Italy<sup>[28]</sup>, the authors reported biopsy-proven CD in 2% of their 726 patients with presumed functional dyspepsia, and suggested that routine duodenal biopsy should be considered in all dyspeptic patients undergoing diagnostic esophagogastroduodenoscopy. The results of this Italian study are interesting, but we believe that further prevalence studies of CD in the functional dyspepsia population are needed to corroborate these results.

There are some limitations in the present study. First, IgA test was not checked for all patients, which could

underestimate the prevalence of positive serology for CD because of the strict dependence of the anti-tTG test on the level of IgA. Second, because of limited resources, we used the lactose-free diet test to rule out lactose intolerance instead of the more accurate hydrogen breath test. Because of the lower sensitivity and specificity of the lactose-free diet test, some patients with lactose intolerance could be misdiagnosed as IBS or vice versa.

In conclusion, the prevalence of CD in patients with presumed IBS is high. Therefore, serological testing should be considered for all individuals with symptoms of IBS. However, larger, multicenter studies are needed to settle the debate on the utility - or futility - of screening IBS patients for CD.

## COMMENTS

### Background

Celiac disease (CD) may present with a broad spectrum of subtle or atypical symptoms. Some patients with irritable bowel syndrome (IBS) may have minimally symptomatic CD. The value of screening IBS patients for CD remains a matter of debate.

### Research frontiers

There were several early published studies showing minimal, atypical clinical presentations of CD. The widespread availability of highly sensitive and specific serologic tests for CD has allowed for large population-based serologic surveys that have shown this disorder to be very common, especially in at-risk groups such as patients with symptoms of IBS.

### Innovations and breakthroughs

The present study underscores the importance of screening IBS patients for CD. The high prevalence of CD found in our large study population is in accordance with the findings of other investigators. However, scattered reports from different regions of the world assert that the prevalence of CD is not increased in patients believed to have IBS. Based on the results of our study, we believe that screening IBS patients for CD should be routinely performed.

### Applications

Because the manifestations of CD respond to a gluten-free diet, testing for CD in patients with presumed IBS may prevent prolonged morbidity and unnecessary health care costs. The results of the present study may contribute to raising the awareness of the protean manifestations of CD, as well as settling the ongoing debate on the utility of screening IBS patients for CD. Larger, case-control studies of undetected CD in patients believed to have IBS are required to establish vigorous clinical guidelines for both primary care physicians and gastroenterologists.

### Terminology

CD is an immune-mediated enteropathy triggered by the ingestion of gluten-containing grains in genetically susceptible individuals. A case-finding study is a term used to describe a method which involves screening a smaller group of people for specific disorders based on specific baseline clinical characteristics.

### Peer review

The peer reviewers of this article believe that it is a valuable contribution to the pertinent literature. The study draws attention to the protean manifestations of CD and the need to be aware of this common disease in particular in conditions such as IBS.

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BRIEF ARTICLE

## Usefulness of a scoring system in the interpretation of histology in neonatal cholestasis

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**CONCLUSION:** A 7-feature, 15-point histological scoring system had good diagnostic accuracy in the interpretation of liver histology in neonatal cholestasis.

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**Key words:** Histology scoring system; Liver biopsy; Neonatal cholestasis

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### Abstract

**AIM:** To ascertain the usefulness of a histological scoring system devised to assist in the interpretation of liver histology in neonatal cholestasis (NC).

**METHODS:** Liver biopsy specimens obtained from infants with NC referred to a tertiary pediatric unit in Malaysia were prospectively studied. The first author, blinded to the final diagnosis, devised the histological diagnosis based on a 7-feature (portal ductal proliferation, bile plugs in portal ductules, porto-portal bridging, lymphocytic infiltration in portal region, multinucleated hepatocytes, neutrophilic infiltration, hepatocellular swelling), 15-point (0 to 15) scoring system. The author classified the histological diagnosis as either biliary atresia (BA) or neonatal hepatitis (NH, all other diagnoses), and subsequently compared the author's diagnosis with the final diagnosis.

**RESULTS:** Eighty-four biopsy specimens obtained from 78 patients were reviewed. Without the scoring system, BA was correctly diagnosed by the author histologically in 30 cases, labelled as NH in 3. For other diagnoses, BA was excluded correctly in 33 cases and mislabeled as BA in 2 cases. The overall sensitivity for BA was 91%, specificity 86% and accuracy 88%. With the scoring system, a score of  $\geq 7$  had the best diagnostic utility to differentiate BA from other intrahepatic cholestasis histologically (sensitivity 88%, specificity 94%, accuracy 92%). Four patients with a score  $< 7$  had BA, and 3 patients with a score  $\geq 7$  had NH.

### INTRODUCTION

A priority in the investigation of infants with neonatal cholestasis (NC) is to distinguish non-obstructive from obstructive causes to facilitate timely surgery for infants with biliary atresia (BA)<sup>[1,2]</sup>. Liver biopsy is one of the most important diagnostic steps in the evaluation of NC and may be performed safely even in the smallest infants with local anaesthesia and sedation<sup>[3,4]</sup>. Percutaneous liver biopsy has been found to be particularly helpful in infants suspected of having BA in developing countries<sup>[5]</sup>.

Bile ductular proliferation, bile plugging, multinucleated giant cells, focal necrosis of liver parenchyma, extramedullary haemopoiesis and inflammatory cell infiltrate in the portal area are all well-recognised histological features of BA<sup>[3]</sup>. Bile ductular proliferation is considered a highly specific and sensitive feature, while the others are less specific and sensitive<sup>[6,7]</sup>. The histological features of neonatal hepatitis, on the other hand, are more heterogeneous<sup>[8,9]</sup>, with giant-cell hepatitis a prominent feature, but others such as diffuse hepatocyte degeneration, minimal degree of bile ductular proliferation, and portal infiltration of lymphocytes, eosinophils and neutrophils less specific<sup>[8,9]</sup>. Other authors noted that the only consistent hepatic histological feature of neonatal hepatitis was extramedullary haemopoiesis<sup>[10]</sup>. Recently, a mathematical approach have been devised to identify the most useful histological feature differentiating BA from

other causes of neonatal cholestasis<sup>[11]</sup>. However, it has not received wide attention due to its complex nature<sup>[11]</sup>.

We devised an objective, 7-feature, 15-point histological scoring system for the interpretation of liver histology to differentiate BA from other causes of NC. Its diagnostic usefulness was tested prospectively in a cohort of infants with NC referred to a tertiary pediatric unit in Malaysia.

## MATERIALS AND METHODS

This prospective, descriptive study was conducted in the Department of Paediatrics, University of Malaya Medical Centre (UMMC), Kuala Lumpur and was approved by the institutional medical ethics committee.

### *The process*

The first author (Lee WS), blinded to the final clinical diagnosis, conducted the entire histological interpretation process alone and reviewed the histology of liver biopsy specimens obtained from patients with NC. Histological diagnosis based on the biopsy materials available were made, initially without and subsequently with the histological scoring system. The histological diagnosis was then compared with the final clinical diagnosis to ascertain the diagnostic usefulness of the histological scoring system.

### *The patients*

All patients with NC referred to the Department of Paediatrics, UMMC from May 2002 to June 2005 were enrolled. NC was defined as the onset of clinically apparent jaundice within the first four months of life, with the conjugated bilirubin greater than 17  $\mu\text{mol/L}$  if the total bilirubin was less than 85  $\mu\text{mol/L}$ , or the conjugated bilirubin more than 20% of the total bilirubin if the total bilirubin was more than 85  $\mu\text{mol/L}$ <sup>[12]</sup>. All cases of BA were confirmed with operative cholangiogram, or demonstration of atretic gallbladder and/or extrahepatic biliary tree intra-operatively<sup>[13,14]</sup>. Diagnosis of other causes of NC were made according to standard and acceptable clinical practices and have been described previously<sup>[1,15]</sup>. The histology review process was conducted in December 2007, more than two years after the final patient in the study cohort was recruited to allow a firm clinical diagnosis to be made in all patients.

### *The biopsy materials*

All patients with NC who had a liver biopsy were identified. The liver histology slides were traced and were screened for suitability of interpretation by the first author. Any material with faded stain was noted. The original paraffin-embedded tissue block was re-sectioned and re-stained with H&E stain. The biopsy materials were screened for adequacy in size and the number of portal tracts present. All the portal tracts were observed for the number, size and shape of portal bile ductules. The average number of bile ductules present in all the visible portal tracts was noted.

## *Histology of neonatal cholestasis*

A review of the literature on the histological interpretation of neonatal cholestasis was performed<sup>[2,9-11,16-19]</sup>. The histological features indicative of BA are bile ductular proliferation, porto-portal bridging, and the presence of bile plugs in the portal ductules<sup>[3,10-12,16]</sup>. Histological features more indicative of neonatal hepatitis are portal lymphocytic infiltration, neutrophilic infiltration, multinucleated giant cells, and hepatocellular swelling<sup>[2,7,16]</sup>.

### *The histological scoring system*

For the purpose of histological interpretation, the histological diagnosis was designated as either BA or non-BA. A 15-point scoring system based on histology of liver biopsy, consisting of seven histological criteria regarded as sufficiently specific to differentiate BA from non-BA, was devised (Table 1). Generally a higher score was indicative of BA, while a lower score was less favourable for BA. From the distribution of the score tabulated against the final diagnosis, a cut-off value with highest sensitivity, specificity and diagnostic accuracy differentiating BA from non-BA was determined.

Bile ductular proliferation was considered to be present if the average number of ductules in the portal tract was more than five. The following criteria were used to grade the degree of bile ductular proliferation: no proliferation - average number of bile ductules per portal tract < 5; mild - average number of bile ductules per portal tract between 5 to 9; moderate - average number of bile ductules per portal tract  $\geq 10$ ; marked proliferation - elongated, attenuated, angulated bile ductules in addition to proliferation (average number of bile ductules per portal tract  $\geq 10$ ).

### *Blinded histological interpretation*

The author personally interpreted the histology slides blindly without knowledge of the clinical data or the final clinical diagnosis. The route of biopsy was noted by observing the size and shape of the biopsy materials. Generally biopsy materials obtained *via* the percutaneous route were long and slender, while materials obtained *via* laparotomy were wedge-shaped. The adequacy of the size of the materials and the number of portal tracts available for interpretation was noted.

A blinded histological diagnosis was made for each histological specimen and was labelled as BA, non-BA (including other causes of cholestasis) or a paucity of bile ductules. For the purpose of statistical analysis, biopsies obtained *via* different routes (percutaneous or operative) from the same patient were considered as separate cases.

For the purpose of ascertaining the diagnostic accuracy of the newly devised histological scoring system, a two-step interpretation was performed, firstly without the scoring system and them with the scoring system.

### *Un-blinding and comparing with final clinical diagnosis*

The author (Lee WS) was un-blinded and the initial histological diagnosis was compared with the final

**Table 1** Seven-feature, 15-point histological scoring system devised for the interpretation of liver biopsy materials in neonatal cholestasis<sup>1</sup>

Parameter	Histological characterization	Histological grade
Portal ductal proliferation	None	0
	Mild	1
	Moderate	2
Bile plug in portal ductules	Marked	3
	Absent	0
Porto-portal bridging	Present	2
	None	0
Lymphocytic infiltrate in portal region	< 50% of portal tracts	1
	> 50% of portal tracts	2
Multinucleated hepatocytes	None	2
	Only around central vein	1
Neutrophils in the infiltrate	Diffuse	0
	Absent or mild	1
Hepatocellular swelling	Moderate or marked	0
	None	2
	Mild/focal	1
	Periportal/diffuse	0

<sup>1</sup>The higher the score, the more likely to be biliary atresia.

diagnosis. The sensitivity, specificity and diagnostic accuracy for various diagnostic methods were calculated using the following formulas where TP = true positive, TN = true negative, FP = false positive, and FN = false negative. Sensitivity = TP/(TP + FN); Specificity = TN/(TN + FP); Positive predictive value = TP/(TP + FP); Negative predictive value = TN/(TN + FN); Diagnostic accuracy = (TN + TP)/(TN + TP + FN + FP).

## RESULTS

During the study period, 84 liver biopsy specimens for histology were obtained from 78 patients who were referred for diagnostic workup of NC. Six patients had both percutaneous and surgical liver biopsies (Table 2). One biopsy specimen (percutaneous) was fragmented and was insufficient for histological interpretation. Thus the remaining 83 specimens were analysed. The final diagnosis of these 83 specimens from 78 patients with NC is shown in Table 3.

### Liver biopsy materials

The median age at biopsy for all patients was 63 d (Table 2). Fifty-three (64%) of the biopsy materials were obtained *via* the percutaneous route while 30 (36%) were obtained surgically. A review of the original histology slides showed that 55 original specimens were adequate for interpretation, while re-staining was necessary in specimens from 28 patients. All 30 specimens obtained during exploratory laparotomy contained more than 5 portal tracts for interpretation. Of the 53 biopsy specimens obtained *via* the percutaneous route, 25 (47%) specimens contained less than 5 portal tracts. In two specimens, no portal tracts were noted.

**Table 2** Characteristics of 83 liver biopsy specimens obtained from 78 patients with neonatal cholestasis

	Biliary atresia (n = 33)	Intra-hepatic cholestasis (n = 50)	All (n = 83)
Age at biopsy (d)			
Median (range)	65 (34-371)	62 (26-180)	63 (26-371)
Route of biopsy			
Percutaneous	16	37	53
Operative	17	13	30
Number of portal tracts			
< 5	4	20	24
≥ 5	29	30	59

**Table 3** Diagnostic accuracy of hepatic histology in patients with neonatal cholestasis-author's personal interpretation

	Author's histological diagnoses			
	Biliary atresia	Neonatal hepatitis	Paucity bile	Final clinical diagnosis ducts
Biliary atresia	33	30	3	
Idiopathic neonatal hepatitis	35	2	33	
Cytomegalovirus hepatitis	6	3	3	
PFIC	4	1	3	
Alagille syndrome	1			1
Parenteral nutrition-associated cholestasis	1		1	
Congenital hypothyroidism	1	1		
Caroli disease	1		1	
Severe asphyxia	1		1	
Total	83	37	45	1

PFIC: Progressive familial intrahepatic cholestasis; Sensitivity: TP/(TP + FN) = 30/(30 + 4) = 91%; Specificity = TN/(TN + FP) = 43/(43 + 7) = 86%; Diagnostic accuracy = (TP + TN)/(TP + TN + FN + FP) = (30 + 43)/83 = 88%.

### Blinded interpretation

The diagnostic accuracy of the author's blinded histological interpretation is shown in Table 3. BA was correctly diagnosed histologically in 30 cases, but was labelled as non-BA in three cases. Non-BA was identified correctly in 33 cases, and labelled as BA in 2 cases. The remaining 15 patients had intrahepatic cholestasis. The author's histological diagnosis was BA in 5 cases and neonatal hepatitis in 9 cases. In the only case with Alagille syndrome, paucity of interlobular bile duct was correctly identified. The overall sensitivity of the author's personal histological interpretation for BA was 91%, sensitivity 86% and diagnostic accuracy 88%.

### Incorrect histological diagnosis

Seven cases of neonatal hepatitis were mislabeled as BA while three cases of BA were mislabeled as non-BA during initial histology without the scoring system (Table 4). Of the 3 cases of BA misdiagnosed as non-BA, liver biopsies in 2 patients were performed early in the course of illness *via* the percutaneous route. The number of portal tracts was inadequate for interpretation in both cases.

The biopsy specimen of the first patient (NKC, performed at 34 d) contained less than 5 portal tracts (Figure 1A) with no portal bile duct proliferation. There was hepatocytic degeneration and swelling, and occasional

**Table 4** Clinical and histological characteristics of 3 patients with biliary atresia diagnosed histologically as neonatal hepatitis by the author

Patient	Bilirubin total/ conj. ( $\mu\text{mol/L}$ )	$\gamma\text{GT}$ (IU/L)	RHBS	Liver biopsy		Reasons for exploratory laparotomy
				Days	Route	
NKC	154/120	179	Non-excretory	34	Percutaneous	Inadequate number of portal tracts. No portal bile duct and pale stools proliferation. Hepatocytic degeneration and swelling, occasional giant cells formation
SQ	189/153	442	Non-excretory	45	Percutaneous	Biopsy material fragmented. Giant cells transformation and pale stools. Intra-hepatocytic cholestasis and bile plug formation
LQY	124/109	144	Non-excretory	78	Operative	Mild ductular proliferation and lymphocytic infiltration of portal tracts

conj.: Conjugated;  $\gamma\text{GT}$ :  $\gamma$  glutamyl transferase; RHBS: Radionuclide hepatobiliary scintigraphic study.

**Table 5** Histological features of biliary atresia and other intrahepatic cholestasis (*n*)

Histological features	Biliary atresia	Intrahepatic cholestasis <sup>1</sup>
Ductular proliferation		
None	1	31
Mild	2	11
Moderate	3	4
Severe	27	2
Bile plug in bile ductules		
Absent	10	42
Present	21	7
Porto-portal bridging		
None	1	33
< 50% of portal tracts	8	6
> 50% of portal tracts	22	6
Lymphocytic infiltration		
Moderate to severe	7	23
Mild	16	24
Absent or mild	10	3
Neutrophilic infiltration		
Moderate to severe	3	15
Absent to mild	30	35
Giant cell transformation of hepatocytes		
Diffuse	1	16
Only around central vein	13	23
None	19	11
Hepatocytes swelling		
Hepatocytes swelling	4	27
Mild/focal	13	22
None	16	1

<sup>1</sup>Including cytomegalovirus hepatitis (*n* = 6), progressive familial intrahepatic cholestasis (*n* = 4), Alagille syndrome (*n* = 1), TPN-associated cholestasis (*n* = 1), hypothyroidism (*n* = 1), Caroli disease (*n* = 1), severe perinatal asphyxia (*n* = 1), idiopathic neonatal hepatitis (*n* = 35).

giant cell transformation. The patient had persistent pale stools. A radionuclide hepatobiliary scintigraphic study was non-excretory. The child had an exploratory laparotomy at 42 d of age, which confirmed BA. A second liver biopsy obtained surgically showed features of BA with bile ductular proliferation, canalicular bile plugs and intracellular cholestasis (Figure 1B).

The biopsy specimen of the second patient (SQ, at 45 d) was fragmented. The liver parenchyma showed giant cell transformation. There was cholestasis within the hepatocytes and bile plug formation was seen within the canaliculi. The patient has persistent pale stools and

had a laparotomy which confirmed BA.

The biopsy specimen of the third patient (LQY), obtained at laparotomy at 78 d of age, showed features of neonatal hepatitis (Figure 1C).

### Histological features indicative of biliary atresia

Seven histological features were assessed individually to ascertain their usefulness in differentiating BA from non-BA (Table 5). Three features were found to be present in more than 50% of cases of BA: moderate to severe bile ductular proliferation (91%), presence of bile plugs in bile ductules (70%) and porto-portal bridging involving more than 50% of portal tracts (71%; Tables 6 and 7).

The presence of moderate or severe bile ductular proliferation had the highest sensitivity (91%), specificity (88%) and positive predictive value (83%) for BA (Table 6). In addition to proliferation, the bile ductules were often elongated and tortuous (Figure 1A). The remaining four features, i.e. lymphocytic and neutrophilic infiltrations, giant cell transformation and hepatocytic swelling were uncommon in BA (between 3% and 21%).

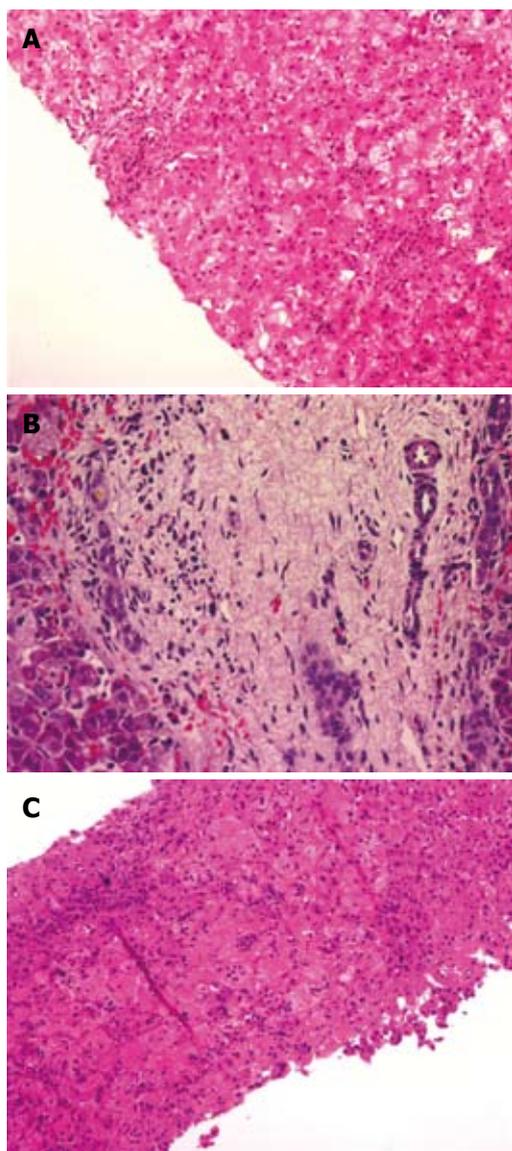
### Histological features of intrahepatic cholestasis

Generally, the features of intra-hepatic cholestasis were less sensitive and specific. The only histological feature noted to be present in more than 50% of patients with intrahepatic cholestasis was periportal or diffuse hepatocellular swelling (54%, Table 7). The remaining three features were noted to be present in 25% to 50% of intrahepatic cholestasis. Features indicative of BA (moderate to severe bile ductular proliferation, bile plugs in bile ductules, and porto-portal bridging involving more than 50% of portal tracts, were not common (between 13% and 14%).

Although not sufficiently sensitive (32%), diffuse giant cell transformations of hepatocytes were highly specific (97%) and has a positive predictive value of 94% for intrahepatic cholestasis. The other criterion, periportal or diffuse hepatocyte swelling was also specific (88%) and had a positive predictive value of 87%. The sensitivity was 54%.

### Histological scoring system (Table 8)

With a score of  $\geq 6$  to differentiate between BA and non-BA histologically, the diagnostic utility of the scoring



**Figure 1** Photomicrograph of liver biopsy (hematoxylin and eosin, original magnification  $\times 200$ ). A: A patient with biliary atresia obtained at 34 d of age (Patient NKC). A portal tract is shown here with no obvious bile ductular proliferation. There was hepatocytic degeneration and swelling. The original blinded histological diagnosis by the author was neonatal hepatitis; B: NKC obtained at 45 d of age. A portal tract is shown here with marked bile ductular proliferation, and bile plugs in bile ductules, typical of biliary atresia; C: A patient with biliary atresia (LQY) obtained at 78 d of life. There are multiple multinucleated giant cells, hepatocellular swelling and moderate lymphocytic infiltration. The original blinded histological diagnosis by the author was neonatal hepatitis.

system was: Sensitivity =  $29/(29 + 4) = 88\%$ ; Specificity =  $44/(44 + 7) = 86\%$ ; Positive predictive value =  $29/(29 + 3) = 91\%$ ; Diagnostic accuracy =  $(29 + 44)/83 = 88\%$ . With a score of  $\geq 7$ , the diagnostic utility of the scoring system was: Sensitivity =  $29/(29 + 4) = 88\%$ ; Specificity =  $47/(47 + 3) = 94\%$ ; Positive predictive value =  $29/(29 + 3) = 91\%$ ; Diagnostic accuracy =  $(29 + 47)/83 = 92\%$ ; Finally, using a score of  $\geq 8$ , Sensitivity =  $25/(25 + 8) = 76\%$ ; Specificity =  $47/(47 + 3) = 94\%$ ; Positive predictive value =  $25/(25 + 3) = 89\%$ ; Diagnostic accuracy =  $(25 + 47)/83 = 87\%$ . A score of  $\geq 7$  had the best diagnostic utility to differentiate between BA and other intrahepatic cholestasis histologically. With this score, four patients

with a score  $< 7$  had a final diagnosis of BA while three patients with a score  $\geq 7$  had neonatal hepatitis.

#### Comparison with pathologists' histological diagnosis

The results of the present study were compared with histological diagnoses made by the reporting pathologists (Table 9). The diagnostic accuracy of the present study (with scoring system, at 92%) was better than the diagnostic accuracy of 81% made by the reporting pathologists.

## DISCUSSION

The Cholestasis Guideline Committee of NASPGN recommends that a liver biopsy should be performed in most infants with undiagnosed cholestasis and should be interpreted by a pathologist with expertise in paediatric liver disease<sup>[12]</sup>. A percutaneous liver biopsy is recommended before performing a surgical procedure to diagnose BA. If the biopsy is performed early in the course of the disease (before 6 wk of age), biopsy may have to be repeated if the results are equivocal<sup>[12]</sup>.

In the present study, using conventional H&E staining in 83 liver biopsies obtained from 78 patients with NC, the overall diagnostic accuracy was 88% (Table 4). BA was correctly diagnosed histologically in 30 of 33 cases while non-BA was correctly diagnosed in 33 of 35 cases. This degree of diagnostic accuracy is in keeping with observations made by other authors<sup>[3,11,18]</sup>, but is lower than the degree of diagnostic accuracy reported by Lai *et al*<sup>[4]</sup>. Moyer *et al*<sup>[12]</sup> reported that with percutaneous liver biopsy, the sensitivity for BA was 89%-99% and specificity was 82.5%-98%.

In the present study, there were 10 histological misdiagnoses with H&E staining without the histological scoring system. Three cases of BA were missed. Of these, the biopsies in 2 cases were obtained early in life (34 and 45 d of age). In all three cases there was no prominent proliferation of bile ductules. In the remaining 7 cases, there was an overdiagnosis of BA in specimens obtained from patients who did not have BA.

With a histological scoring system devised to differentiate BA from other causes of NC using 7 histological criteria, the overall diagnostic accuracy was 92%, which was better than that without the scoring system (88%).

Histological interpretation of hepatic histology in patients with NC requires considerable skills which many general pathologists do not normally possess<sup>[11]</sup>. The advantage of a histological scoring system, in addition to its higher diagnostic accuracy, is that it provides a more objective and systematic way of assessing liver biopsy materials histologically. This may be the most important reason for the very high diagnostic accuracy achieved with this scoring system. In addition, since more than one criterion was used, it is less influenced by the adequacy of the biopsy materials. The seven histological criteria used in the scoring system during the present study were chosen based on the findings of other authors which showed the greatest discriminatory power between BA and other intrahepatic cholestatic disorders<sup>[9,11,16,17]</sup>.

Another advantage of the scoring system used in

**Table 6 Sensitivity, specificity, positive and negative predictive values of various histological features in differentiating biliary atresia from non-BA**

Histological features	BA	Non-BA	Sensitivity for BA (%)	Specificity for BA (%)	Positive PV for BA & negative PV for non-BA (%)	Negative PV for BA & positive PV for non-BA (%)
Bile ductular proliferation						
Moderate or severe	30	6	30/33 (91)	30/33 (91)	30/36 (83)	42/45 (93)
None or mild	3	42				
Bile plug in bile ductules						
Present	21	7	21/31 (68)	42/49 (86)	21/28 (75)	42/52 (81)
Absent	10	42				
Porto-portal bridging						
> 50% of portal tracts	22	6	22/31 (71)	39/45 (87)	22/28 (79)	39/48 (81)
None/< 50% of portal tracts	9	39				

PV: Predictive value; BA: Biliary atresia.

**Table 7 Sensitivity, specificity, positive and negative predictive values of various histological features in differentiating non-biliary atresia from biliary atresia**

Histological features	Non-BA	BA	Sensitivity for BA (%)	Specificity for BA (%)	Positive PV for non-BA & negative PV for BA (%)	Negative PV for non-BA & positive PV for BA (%)
Lymphocytic infiltration						
Moderate to severe	23	7	23/50 (46)	26/33 (79)	23/30 (77)	26/53 (49)
Absent or mild	27	26				
Neutrophilic infiltration						
Moderate to severe	15	3	15/50 (30)	30/33 (91)	15/18 (83)	30/65 (46)
Absent to mild	35	30				
Giant cell transformation of hepatocytes						
Diffuse	16	1	16/50 (32)	32/33 (97)	16/17 (94)	32/66 (48)
None or around central vein	34	32				
Hepatocytes swelling						
Periportal or diffuse	27	4	27/50 (54)	29/33 (88)	27/31 (87)	29/52 (56)
None or mild/focal	23	29				

**Table 8 Total histological score according to underlying diagnosis**

Total score	Biliary atresia	Non-biliary atresia
0	0	3
1	0	6
2	1	6
3	0	11
4	2	10
5	0	8
6	1	0
7	0	3
8	4	0
9	3	1
10	4	1
11	3	0
12	4	0
13	7	1
14	4	0
< 6	4	44
≥ 6	29	7
< 7	4	47
≥ 7	29	3
< 8	8	47
≥ 8	25	3
Total	33	50

**Table 9 A summary of the results and a comparison with the histological diagnosis made by reporting pathologists**

Parameters	Sensitivity	Specificity	Diagnostic accuracy
Author's own interpretation-without scoring system	91	86	88
Author's own interpretation-with scoring system	88	94	92
Pathologists' diagnosis	82	80	81

statistical analyses using a logistic regression method. The accuracy, sensitivity, and specificity were 90.5%, 100% and 75.9%, respectively.

In the present study, the presence of moderate to severe bile ductular proliferation was the most consistent histological feature noted in BA, and was present in 30 of the 33 specimens analysed<sup>[11,16,17]</sup>. The other two features, i.e. bile plug in bile ductules and porto-portal bridging were not as consistent.

On the other hand, no single histological feature was consistently present in intrahepatic cholestasis. All four features analysed, i.e. lymphocytic and neutrophilic infiltration, giant cell transformation, and hepatocellular swelling, were present in 30% to 54% of cases of intrahepatic cholestasis. This is not surprising as intrahepatic cholestasis is a heterogeneous condition. Parenteral nutrition-associated cholestasis may cause

the present study was its non-complicated nature. Other authors have also attempted to devise a histological scoring system<sup>[10,11]</sup>. The scoring system reported by Zerbin *et al*<sup>[11]</sup> involved a series of initial complex

steatosis, bile ductular proliferation and, less commonly bridging fibrosis<sup>[20]</sup>. CMV hepatitis may cause intense hepatitis, giant cell transformation, intracytoplasmic inclusion bodies, bile stasis, inflammation, fibrosis and bile ductular proliferation<sup>[21,22]</sup>. On the other hand, conditions such as PFIC and Alagille syndrome caused a paucity of interlobular bile ducts<sup>[23]</sup>.

There are other advantages in using liver biopsies in the investigation of neonatal cholestasis besides its ability of achieving a high degree of diagnostic accuracy. Firstly, there is a shorter time delay of 1 to 3 d, compared to 3 to 5 d for radionuclide hepatobiliary scintigraphy<sup>[12]</sup>. In addition, percutaneous liver biopsy can be performed safely even in young infants<sup>[12]</sup>.

However, some difficulties remain in the interpretation of hepatic histology in neonatal cholestasis. One of these difficulties is inadequate biopsy materials obtained, especially *via* the percutaneous route. In the present study, while all the biopsy specimens obtained surgically contained an adequate number of portal tracts, slightly less than half (46%) of the specimens obtained *via* the percutaneous route contained less than 5 portal tracts for adequate interpretation. In 2 specimens, no portal tracts were observed. Thus, histological features indicative of BA which depend on an adequate number of portal tracts, such as bile ductular proliferation, bile plugs in bile ductules and porto-portal bridging, were not observed for adequate interpretation.

The present study shows that contrary to the claims made by some authors<sup>[24,25]</sup>, liver biopsy is a sensitive and specific method in the investigation of neonatal cholestasis. This study shows that with proper and adequate training in hepatic histology interpretation, the interpretation of liver histology in neonatal cholestasis can be achieved with sufficient sensitivity, specificity and accuracy.

In conclusion, histological interpretation of liver biopsies based on a 7-criteria scoring system had the highest sensitivity, specificity and diagnostic accuracy to differentiate BA from other intrahepatic cholestasis.

## COMMENTS

### Background

One of the most important priorities in the investigations of infants with neonatal cholestasis is to make an accurate diagnosis of obstructive causes such as biliary atresia to allow timely surgery, as early surgery for biliary atresia is one of the most important prognostic factors of successful outcome. However, there is considerable overlap in the clinical, biochemical and histological features of biliary atresia and other causes of neonatal cholestasis.

### Research frontiers

Making an accurate histological diagnosis for neonatal cholestasis has always been considered a challenge for many pathologists, particularly in the early neonatal period. Many previously reported attempts to histologically differentiate biliary atresia from other causes of neonatal cholestasis using a scoring system have not been widely accepted, mainly due to their complexities.

### Innovations and breakthroughs

In this study, the authors examined the diagnostic usefulness of a 7-feature, 15-point histological scoring system to differentiate biliary atresia from other causes of neonatal cholestasis histologically. It was found that when applied to biopsy specimens obtained from infants with neonatal cholestasis and when interpreted without a scoring system, this new histological scoring system offered good diagnostic accuracy. The diagnostic accuracy was better than the histological diagnosis made by reporting pathologists.

## Applications

By devising a new, objective histological scoring system with high sensitivity, specificity and diagnostic accuracy to differentiate biliary atresia from other non-obstructive causes, practising pathologists can make timely histological diagnoses in infants with neonatal cholestasis to avoid unnecessary surgery in those without biliary atresia, and early surgical correction in those with biliary atresia.

## Terminology

Neonatal cholestasis refers to the response of immature liver cells in the presence of various types of external insults. It is usually seen in newborn infants with somewhat immature liver function, and is characterized physiologically as a measurable decrease of bile flow, pathologically as the histological presence of bile pigment in hepatocytes and bile ducts, and clinically as the accumulation in blood and extrahepatic tissues of substances normally excreted in the bile. Although the insults to the liver may come in many different forms, there is considerable overlap in the clinical, biochemical and histological manifestations of neonatal cholestasis because the response of immature hepatocytes to external injuries is somewhat limited.

## Peer review

The article is a good attempt at making the diagnosis of neonatal hepatitis objectively. The authors have clarified the criteria for selection of the histological features differentiating biliary atresia from other causes of neonatal cholestasis. The basis of allotment of scoring points over each feature has also been clarified. The authors also noted that as compared to the diagnostic accuracy of the reporting histologists (81%), the diagnostic accuracy of the present scoring system (92%) was superior.

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BRIEF ARTICLE

## Synergistic effect of fatty liver and smoking on metabolic syndrome

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### Abstract

**AIM:** To investigate the association of fatty liver and smoking on metabolic syndrome and its components.

**METHODS:** This cross-sectional study enrolled participants who attended annual health screening at Shin Kong Wu Ho-Su Memorial Hospital from January to December 2005. A total of 3455 (1981 men and 1474 women) subjects were included in final analyses. Fatty liver was diagnosed using abdominal ultrasonography by trained gastroenterologists. The modified National Cholesterol Education Program Adult Treatment Panel III was used to define metabolic syndrome. The associations between smoking, fatty liver and metabolic syndrome were analyzed using multiple logistic regression.

**RESULTS:** Subjects with fatty liver, and who smoked tobacco, had the highest odds ratios (ORs) for high waist circumference [OR, 4.5 (95% CI: 3.3-6.1),  $P < 0.05$ ], hypertriglyceridemia [OR, 8.1 (95% CI: 6.0-10.9),  $P < 0.05$ ], low serum high-density lipoprotein cholesterol (HDL-C) [OR, 8.3 (95% CI: 6.1-11.3),  $P < 0.05$ ], and metabolic syndrome [OR, 9.5 (95% CI: 6.7-13.4),  $P < 0.05$ ] compared to subjects without fatty liver who did not smoke tobacco. We also found that the ORs for hypertriglyceridemia, low serum HDL-C, and metabolic syndrome for subjects with fatty liver who smoked tobacco had greater than the sum of the ORs for subjects with fatty liver who did not smoke

plus those who did not have fatty liver and who did smoke.

**CONCLUSION:** Fatty liver and smoking had a synergistic effect on metabolic syndrome and its components, especially for hypertriglyceridemia and low serum HDL-C.

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**Key words:** Smoking; Fatty liver; Synergistic effect; Metabolic syndrome

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### INTRODUCTION

Fatty liver is an increasingly recognized condition with growing prevalence in the modern world and is strongly associated with insulin resistance. The association between fatty liver and metabolic syndrome was broadly discussed in previous studies<sup>[1-9]</sup>. Fatty liver is often associated with central obesity, type 2 diabetes mellitus, dyslipidemia, and hypertension. Two studies based on Asian populations showed that Japanese subjects<sup>[1]</sup> with fatty liver had 5- to 9-fold risk of developing metabolic syndrome and Chinese subjects<sup>[6]</sup> had a 5.2-fold risk of developing metabolic syndrome. Lizardi-Cervera *et al*<sup>[3]</sup> found that the constellation of metabolic disturbances observed in subjects with fatty liver increased the risk of cardiovascular disease compared to those without fatty liver (OR, 4.7 *vs* 2.8). Fatty liver and metabolic syndrome are becoming important public health issues worldwide.

Smoking is a major risk factor for cardiovascular disease<sup>[10]</sup>. Previous studies have shown that smoking reduces insulin sensitivity or induces insulin resistance<sup>[10-15]</sup>

and enhances cardiovascular risk factors such as elevated plasma triglycerides (TG)<sup>[16-19]</sup>, decreased high-density lipoprotein cholesterol (HDL-C)<sup>[16,20]</sup> and hyperglycemia<sup>[11]</sup>. Furthermore, several studies show that smoking is associated with metabolic abnormalities and increases the risk of metabolic syndrome<sup>[21-24]</sup>. Nakanishi *et al.*<sup>[21]</sup> reported that subjects who habitually smoked tobacco had a 1.07- to 1.66-fold risk of developing metabolic syndrome compared to subjects who did not smoke, and the quantity of tobacco smoked had a dose-dependent relationship with the severity of metabolic syndrome.

Habitual smoking is a modifiable risk factor for metabolic syndrome and cardiovascular disease<sup>[10,19]</sup>. The most effective way for smokers to decrease the risk of metabolic syndrome and cardiovascular disease is to stop smoking<sup>[25]</sup>. However, Nakanishi *et al.*<sup>[21]</sup> found that smoking cessation was also associated with a 1.3-fold risk of metabolic syndrome due to subsequent body weight gain. Wada *et al.*<sup>[15]</sup> proposed that the effect of smoking on metabolic syndrome would remain over 20 years after smoking cessation.

The effects of fatty liver and habitually smoking tobacco on metabolic syndrome have not been previously addressed. The purpose of the present study was to investigate the possible interactive effects of fatty liver and smoking on metabolic syndrome and its components.

## MATERIALS AND METHODS

This retrospective cross-sectional study enrolled participants who voluntarily attended annual health screenings at the health center of the Shin Kong Wu Ho-Su Memorial Hospital from January to December 2005. Demographic data, medical histories, family histories regarding risk factors for metabolic syndrome, smoking habits, exercise habits, and alcohol consumption habits were collected through a self-administrated questionnaire. This study was approved by the Shin Kong Wu Ho-Su Memorial Hospital Institutional Review Board, and written informed consent was obtained from all participants. Hepatitis C ( $n = 109$ ), alcohol consumption more than three times per week ( $n = 77$ ), and subjects whose abdominal ultrasonographic findings revealed cirrhosis or other chronic liver parenchymal disease ( $n = 88$ ) were excluded from this study. Thus, a total of 3455 (1981 men and 1474 women) subjects were included in the final analyses.

At the time of examination, sitting blood pressure (mmHg) was measured by an automated oscillometric BP recorder Dinamap DPC 100X-EN (GE Medical Systems, Milwaukee, Wisconsin, USA) applied to the patient's left arm following a sufficiently long period of rest. Height and body weight were determined with a foot-to-foot bioelectric impedance analyzer (BF-220, Tanita Corp., Tokyo, Japan); these measurements were made to the nearest 0.1 cm and 0.1 kg, respectively, with subjects wearing light clothing. Body mass index (BMI, kg/m<sup>2</sup>) was calculated as body weight divided by the square of the individual's height. Waist circumference (WC, cm)

was measured midway between the lowest border of the ribs and the iliac crest in the horizontal plane, and to the nearest 0.5 cm, by trained nurses using an anthropometric non-stretchable tape after normal expiration.

Fasting blood samples were collected from all subjects after at least an 8-h fast. Serum levels of total cholesterol (mg/dL), low-density lipoprotein cholesterol (LDL-C, mg/dL), HDL-C (mg/dL), serum triglycerides (TG, mg/dL), and fasting blood glucose (FPG, mg/dL) were measured by an automated Hitachi 7600 clinical analyzer (Hitachi, Ltd., Tokyo, Japan).

Abdominal ultrasonographic examinations were performed by trained gastroenterologists using a Philips EnVisor M2540R (Philips, Andover, Minnesota, USA) without reference to the participants' history of liver disease, biochemical data, or other clinical findings. Using sonography, fatty liver was diagnosed on the basis of abnormally intense, high-level echoes arising from the hepatic parenchyma, liver-kidney difference in echo amplitude, echo penetration into the deep portion of the liver, and clarity of liver blood-vessel structure.

Subjects were classified as current smokers or non-smokers. Alcohol consumption was quantified as the number of drinking times per week, and subjects who drank more than three times per week were excluded before analysis.

### Definition of metabolic syndrome

Metabolic syndrome was defined as three or more risk factors as defined by the National Cholesterol Education Program Adult Treatment Panel III Guideline criteria and modified by the International Diabetes Federation specifically for the Chinese population, including (1) fasting glucose level  $\geq 100$  mg/dL, (2) serum triglyceride level  $\geq 150$  mg/dL, (3) HDL-C level (men  $< 40$  mg/dL, women  $< 50$  mg/dL), (4) systolic blood pressure  $\geq 130$  mmHg or diastolic blood pressure  $\geq 85$  mmHg, and (5) WC (men  $\geq 90$  cm, women  $\geq 80$  cm). The participants were allocated to one of four study groups according to their smoking status and ultrasonographic liver findings. Group 1 was defined as subjects who did not smoke and did not have fatty liver [fatty liver (-)/smoking (-)]. Group 2 was defined as subjects who did not have fatty liver, but currently smoked [fatty liver (-)/smoking (+)]. Group 3 was defined as subjects who showed ultrasonographic evidence of fatty liver but did not smoke [fatty liver (+)/smoking (-)]. Group 4 was defined as subjects who had ultrasonographic evidence of fatty liver and smoked concurrently [fatty liver (+)/smoking (+)].

### Statistical analysis

ANOVA was used for comparison of continuous variables. The  $\chi^2$  test was used for comparison of categorical variables. The association between smoking, fatty liver and metabolic syndrome components were analyzed using multiple logistic regression. We controlled confounding factors such as age, gender, drinking habit, and physical activity. A *P*-value less than 0.05 was defined as statistically significant. All statistical analyses

**Table 1** Demographic data and lifestyle factors among study subjects ( $n = 3455$ ), stratified by fatty liver and smoking habit (mean  $\pm$  SD)  $n$  (%)

	Fatty liver (-)		Fatty liver (+)		P-value
	Non-smoker ( $n = 1702$ )	Smoker ( $n = 365$ )	Non-smoker ( $n = 1057$ )	Smoker ( $n = 331$ )	
Age (yr)	45.9 $\pm$ 11.9	42.6 $\pm$ 10.8	49.9 $\pm$ 11.0	44.9 $\pm$ 9.4	< 0.0001
BMI (kg/m <sup>2</sup> )	22.0 $\pm$ 2.6	22.3 $\pm$ 2.7	25.7 $\pm$ 3.2	25.9 $\pm$ 3.0	< 0.0001
Male	681 (40)	290 (79.5)	695 (65.8)	315 (95.2)	< 0.0001
Drinking <sup>1</sup>	127 (7.5)	99 (27.1)	122 (11.5)	93 (28.1)	< 0.0001
Physical activity <sup>2</sup>	944 (55.5)	242 (66.5)	568 (53.8)	223 (67.4)	< 0.0001

<sup>1</sup>Alcohol consumption  $\geq$  1 time and  $\leq$  3 times per week (subjects who drank more than 3 times per week were excluded before analysis);

<sup>2</sup>Exercise  $\geq$  1 time per week. BMI: Body mass index.

**Table 2** Clinical and biochemical characteristics of study subjects ( $n = 3455$ ) classified by fatty liver and smoking habit (mean  $\pm$  SD)

	Fatty liver (-)		Fatty liver (+)		P-value
	Non-smoker ( $n = 1702$ )	Smoker ( $n = 365$ )	Non-smoker ( $n = 1057$ )	Smoker ( $n = 331$ )	
WC (cm)	79.9 $\pm$ 8.3	80.8 $\pm$ 7.4	88.1 $\pm$ 8.5	88.8 $\pm$ 7.4	< 0.0001
FPG (mg/dL)	89.2 $\pm$ 19.3	89.8 $\pm$ 21.4	101.9 $\pm$ 33.4	99.7 $\pm$ 33.4	< 0.0001
SBP (mmHg)	113.9 $\pm$ 19.8	111.8 $\pm$ 16.8	125.9 $\pm$ 20.1	122.4 $\pm$ 18.8	< 0.0001
DBP (mmHg)	68.6 $\pm$ 11.3	69.7 $\pm$ 10.9	76.5 $\pm$ 11.4	78.1 $\pm$ 10.6	< 0.0001
TG (mg/dL)	97.7 $\pm$ 49.5	130.1 $\pm$ 74.3	170.6 $\pm$ 129.3	225.9 $\pm$ 238.5	< 0.0001
HDL-C (mg/dL)	60.1 $\pm$ 14.9	52.6 $\pm$ 13.8	48.9 $\pm$ 11.4	44.3 $\pm$ 12.6	< 0.0001

WC: Waist circumference; FPG: Fasting plasma glucose; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; TG: Triglyceride; HDL-C: High-density lipoprotein cholesterol.

**Table 3** Prevalence of metabolic risk factors and metabolic syndrome among study subjects ( $n = 3455$ ) classified by fatty liver and smoking habit  $n$  (%)

	Fatty liver (-)		Fatty liver (+)		P-value
	Non-smoker	Smoker	Non-smoker	Smoker	
Central obesity <sup>1</sup>	497 (29.2)	51 (14.0)	501 (47.4)	137 (41.4)	< 0.0001
FPG $\geq$ 100 mg/dL	164 (9.6)	40 (11.0)	317 (30.0)	76 (23.0)	< 0.0001
High blood pressure <sup>2</sup>	356 (20.9)	63 (17.3)	477 (45.1)	119 (36.0)	< 0.0001
TG $\geq$ 150 mg/dL	207 (12.2)	103 (28.2)	478 (45.2)	197 (59.5)	< 0.0001
Low HDL-C <sup>3</sup>	223 (13.1)	71 (19.5)	320 (30.6)	147 (44.4)	< 0.0001
Metabolic syndrome	123 (7.2)	33 (9.0)	364 (34.4)	121 (36.6)	< 0.0001

<sup>1</sup>Waist circumference in men  $\geq$  90 cm or in women  $\geq$  80 cm; <sup>2</sup>Systolic blood pressure  $\geq$  130 mmHg or diastolic blood pressure  $\geq$  85 mmHg; <sup>3</sup>High-density lipoprotein cholesterol in men < 40 mg/dL or in women < 50 mg/dL.

were performed using SAS statistical software, (SAS for Windows, version 8.02; SAS Institute Inc, Cary, NC, USA).

## RESULTS

Table 1 summarized the demographic data and lifestyle factors among the study subjects stratified by fatty liver and smoking habit. The fatty liver group had more men, higher BMI, and more alcohol drinkers compared to subjects without fatty liver. The frequency of regular exercise was similar for subjects with and without fatty liver. Furthermore, subjects who smoked tobacco also drank alcohol with greater frequency and exercised more regularly compared to subjects who did not smoke.

Table 2 summarized the clinical and biochemical characteristics of the study subjects. Participants with fatty liver had a significantly greater WC, FPG, blood

pressure and TG, and lower serum HDL-C compared to participants without fatty liver. Higher TG levels and lower HDL-C levels were found in the smoking group compared to subjects who did not smoke. Participants with fatty liver who smoked had the highest triglyceride levels (225.9 mg/dL) and the lowest HDL-C levels (44.3 mg/dL) among the four study groups.

The prevalence of metabolic syndrome abnormalities was significantly higher in the fatty liver group compared to subjects without fatty liver (Table 3). Subjects with fatty liver also had a higher prevalence of metabolic syndrome compared to those without fatty liver. Subjects who smoked had a higher prevalence of metabolic syndrome and more metabolic syndrome abnormalities, especially for higher TGs and lower HDL-C compared to subjects who did not smoke.

Table 4 showed the associations among fatty liver, smoking, and metabolic syndrome using multiple

**Table 4** ORs for metabolic risk factors and metabolic syndrome among subgroups of subjects, analyzed by multiple logistic regression, adjusted for age, gender, drinking alcohol, and physical activity ( $n = 3455$ )

	Fatty liver (-)				Fatty liver (+)			
	Non-smoker ( $n = 1702$ )		Smoker ( $n = 365$ )		Non-smoker ( $n = 1057$ )		Smoker ( $n = 331$ )	
	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
Central obesity <sup>1</sup>	1 (reference)		0.7	0.5-1.1	3.5 <sup>a</sup>	2.9-4.2	4.5 <sup>a</sup>	3.3-6.1
FPG $\geq 100$ mg/dL	1 (reference)		1.3	0.8-1.9	3.6 <sup>a</sup>	2.9-4.6	2.8 <sup>a</sup>	2.0-4.1
High blood pressure <sup>2</sup>	1 (reference)		0.7	0.5-1.0	2.7 <sup>a</sup>	2.2-3.2	1.7 <sup>a</sup>	1.3-2.3
TG $\geq 150$ mg/dL	1 (reference)		2.4 <sup>a</sup>	1.8-3.2	5.3 <sup>a</sup>	4.4-6.5	8.1 <sup>a</sup>	6.0-10.9
Low HDL-C <sup>3</sup>	1 (reference)		2.0 <sup>a</sup>	1.4-2.7	3.4 <sup>a</sup>	2.7-4.1	8.3 <sup>a</sup>	6.1-11.3
Metabolic syndrome	1 (reference)		1.4	0.9-2.2	6.6 <sup>a</sup>	5.2-8.4	9.5 <sup>a</sup>	6.7-13.4

<sup>a</sup> $P < 0.05$ . <sup>1</sup>Waist circumference in men  $\geq 90$  cm or in women  $\geq 80$  cm; <sup>2</sup>Systolic blood pressure  $\geq 130$  mmHg or diastolic blood pressure  $\geq 85$  mmHg; <sup>3</sup>High-density lipoprotein cholesterol in men  $< 40$  mg/dL or in women  $< 50$  mg/dL. OR: Odds ratio; CI: Confidence interval.

**Table 5** ORs for metabolic risk factors and metabolic syndrome among fatty liver groups, analyzed by multiple logistic regression, adjusted for age, gender, drinking alcohol, and physical activity ( $n = 1388$ )

	Fatty liver (+)			
	Non-smoker ( $n = 1057$ )		Smoker ( $n = 331$ )	
	OR	95% CI	OR	95% CI
Central obesity <sup>1</sup>	1 (reference)		1.2	0.9-1.6
FPG $\geq 100$ mg/dL	1 (reference)		0.7	0.5-1.1
High blood pressure <sup>2</sup>	1 (reference)		0.6	0.5-0.9
TG $\geq 150$ mg/dL	1 (reference)		1.5 <sup>a</sup>	1.2-2.0
Low HDL-C <sup>3</sup>	1 (reference)		2.7 <sup>a</sup>	2.0-3.7
Metabolic syndrome	1 (reference)		1.3 <sup>a</sup>	1.0-1.8

<sup>a</sup> $P < 0.05$ . <sup>1</sup>Waist circumference in men  $\geq 90$  cm or in women  $\geq 80$  cm; <sup>2</sup>Systolic blood pressure  $\geq 130$  mmHg or diastolic blood pressure  $\geq 85$  mmHg; <sup>3</sup>High-density lipoprotein cholesterol in men  $< 40$  mg/dL or in women  $< 50$  mg/dL.

logistic regression. We found that subjects with fatty liver had significantly increased odds ratios (ORs) for developing metabolic syndrome and its components. Subjects who smoked had higher ORs for developing hypertriglyceridemia and low serum HDL-C, whether they had fatty liver or not. In addition, subjects with fatty liver who smoked had the highest ORs of high WC, hypertriglyceridemia, low HDL-C, and metabolic syndrome. We also found the ORs of hypertriglyceridemia, low HDL-C, and metabolic syndrome for subjects in group 4 [fatty liver (+)/smoking (+)] were greater than the sum of the ORs for subjects in group 3 [fatty liver (+)/smoking (-)] plus group 2 [fatty liver (-)/smoking (+)].

Table 5 showed the ORs for metabolic syndrome components among fatty liver groups. In comparison with subjects with fatty liver who did not smoke, subjects with fatty liver who smoked had significantly increased ORs for hypertriglyceridemia [OR, 1.5 (95% CI: 1.2-2.0),  $P < 0.05$ ], low HDL-C [OR, 2.7 (95% CI: 2.0-3.7),  $P < 0.05$ ] and metabolic syndrome [OR, 1.3 (95% CI: 1.0-1.8),  $P < 0.05$ ].

## DISCUSSION

In this study, we found that fatty liver was associated with an increasing OR for metabolic syndrome. The OR for developing metabolic syndrome increased significantly

among subjects with fatty liver compared to those without fatty liver. We also found that smoking was associated with high TG levels and low HDL-C levels. Subjects who smoked had increased ORs for hypertriglyceridemia and low HDL-C, whether they had fatty liver or not. Moreover, fatty liver and smoking seemed to have a synergistic effect on metabolic syndrome. The ORs for hypertriglyceridemia, low HDL-C, and metabolic syndrome for subjects in group 4 [fatty liver (+)/smoking (+)] had greater than the sum of the ORs for subjects in group 3 [fatty liver (+)/smoking (-)] group plus group 2 [fatty liver (-)/smoking (+)]. In fatty liver groups, subjects who smoked had a significantly increased OR for hypertriglyceridemia, low HDL-C and metabolic syndrome compared to subjects who did not smoke.

Metabolic syndrome and fatty liver were well discussed in previous studies. Insulin resistance played a pivotal role in the pathophysiology of both fatty liver and metabolic syndrome<sup>[26,27]</sup>. Furthermore, several studies showed that smoking induced insulin resistance or hyperinsulinemia and led to metabolic syndrome<sup>[1,3,23,28]</sup>. The associations between fatty liver, smoking, and metabolic syndrome were discussed in many studies individually, but the effect of interaction of fatty liver and smoking on metabolic syndrome was not investigated. In our study, subjects with fatty liver who smoked had a significantly increased risk of metabolic syndrome.

The effects of cigarette smoking inducing high plasma TGs and low HDL-C levels were shown in several studies<sup>[14,16-20,23,24,29,30]</sup>. While these studies provided evidence that smoking increased plasma TG and decreased HDL-C levels, most of these studies did not control potential confounding factors. Fatty liver also increased the risk of dyslipidemia such as hypertriglyceridemia and decreased HDL-C<sup>[3]</sup>. In our study, after controlling for confounding factors such as age, gender, drinking habit, and physical activity, the ORs for hypertriglyceridemia in subjects remained high: group 2 [fatty liver (-)/smoking (+)] 2.4 (95% CI: 1.8-3.2), group 3 [fatty liver (+)/smoking (-)] 5.3 (95% CI: 4.4-6.5), and group 4 [fatty liver (+)/smoking (+)] 8.1 (95% CI: 6.0-10.9) compared to subjects without fatty liver who did not smoke. Additionally, the ORs for low HDL-C were 2.0 (95% CI: 1.4-2.7) for group 2 [fatty liver (-)/smoking (+)], 3.4 (95% CI: 2.7-4.1) for group 3 [fatty liver (+)/smoking (-)], and 8.3

(95% CI: 6.1-11.3) for group 4 [fatty liver (+)/smoking (+)], compared to subjects without fatty liver who did not smoke. Thus, our study provided evidence that fatty liver and smoking increased the risk of hypertriglyceridemia and low HDL-C.

Cigarette smoking decreased insulin sensitivity through increasing circulating levels of insulin-antagonistic hormones (i.e. catecholamines, cortisol, and growth hormone) and increasing lipolysis, resulting in high circulating levels of free fatty acid<sup>[21]</sup>. Nicotine, carbon monoxide, and other metabolites from smoking also played important roles in insulin resistance. Furthermore, several mechanisms by which cigarette smoking promoted dyslipidemia were proposed, including decreased lipoprotein lipase activity, increased 3-hydroxy-3-methylglutaryl-CoA reductase activity, increased glucose-6-phosphatase dehydrogenase activity and increased central obesity<sup>[16]</sup>. Thus, both fatty liver and smoking increased plasma TG and decreased HDL-C by increasing insulin resistance and/or decreasing insulin sensitivity.

While there was no significant difference in WC in subjects without fatty liver, regardless of whether or not subjects smoked, the OR for central obesity was greater among smokers than non-smokers for subjects with fatty liver. These findings were consistent with a previous study<sup>[31]</sup>. Mizuno *et al*<sup>[31]</sup> proposed that WC showed no difference between non-obese subjects with or without a smoking habit. Nonetheless, WC was significantly higher in obese subjects who smoked than those who did not. Smoking seemed to accelerate visceral fat accumulation and promote obesity-related disorders.

Insulin resistance secondary to cigarette smoking decreased insulin-mediated glucose uptake and also resulted in hyperglycemia<sup>[11,32]</sup>. In present study, the ORs for hyperglycemia were 1.3 (95% CI: 0.8-1.9) for group 2 [fatty liver (-)/smoking (+)], 3.6 (95% CI: 2.9-4.6) for group 3 [fatty liver (+)/smoking (-)], and 2.8 (95% CI: 2.0-4.1) for group 4 [fatty liver (+)/smoking (+)], compared to subjects without fatty liver who did not smoke. Thus, fatty liver increased the risk of hyperglycemia, but the effect of smoking on blood sugar was not obvious.

The effect of smoking on blood pressure is controversial. Frati *et al*<sup>[10]</sup> showed that the acute effect of cigarette smoking increased blood pressure and heart rate, but this effect was not seen after the first hour. Geslain-Biquez *et al*<sup>[23]</sup> proposed that the frequencies of increasing blood pressures did not differ between smokers and non-smokers. Moreover, Weitzman *et al*<sup>[24]</sup> found that active smoking was associated with decreasing blood pressure. We found lower ORs for high blood pressure in smoking subjects with or without fatty liver. Fatty liver had a positive effect on elevating blood pressure, but smoking seemed to have a negative effect on raising blood pressure in present study.

There are several limitations in this study. First, since abdominal ultrasonography can only detect fatty liver when steatosis affects more than 33% of the liver parenchyma<sup>[33,34]</sup>, subjects in this study might be affected by fatty liver but not be detected. The impacting effects

of fatty liver on metabolic syndrome and its components might be underestimated. Second, the information of alcohol consumption was collected by the drinking times per week. We did not count the amount of alcohol units and the impacting effects of alcohol on metabolic syndrome components might be diluted. Besides, the smoking groups tended to have a higher prevalence of drinking, even though we excluded subjects who drank more than three times per week and using multiple logistic regression to adjust the impacting effects of alcohol on metabolic syndrome components, the effects of alcohol on metabolic syndrome components can not be totally ruled out. Third, the smoking data was collected by binary criteria in this study so the effects of smoking on metabolic syndrome components might be underestimated<sup>[35]</sup>.

In summary, fatty liver and smoking were closely related to insulin resistance<sup>[1-9,11-14,16,21-23,25,32]</sup>, and our study provided evidence that fatty liver and smoking had a synergistic effect on metabolic syndrome and its components, especially for triglyceride and HDL-C levels. We suggested that smoking cessation would have the great benefit of reducing the risk of metabolic syndrome, especially for subjects with fatty liver.

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## COMMENTS

### Background

Metabolic syndrome is a frequent metabolic abnormality affecting approximately 20% of the nondiabetic population worldwide and increasing risks of cardiovascular diseases and mortality. Fatty liver and smoking increase the risk of developing metabolic syndrome through the pathogenetic factor of insulin resistance and were well discussed in previous studies separately. We aimed to investigate possible interactive effects of fatty liver and smoking on metabolic syndrome.

### Research frontiers

It was recognized that fatty liver, smoking and metabolic syndrome associated with insulin resistance would increase the risks of serious complications such as cirrhosis, hepatocellular carcinoma and cardiovascular diseases. We want to identify the interactive effect of fatty liver and smoking on metabolic syndrome and provide evidence for lifestyle modification and appropriate management.

### Innovations and breakthroughs

The authors provided evidence that fatty liver and smoking had a synergistic effect on metabolic syndrome and its components, especially for triglyceride and high-density lipoprotein cholesterol (HDL-C) levels.

### Applications

After identifying the synergistic effect of fatty liver and smoking on metabolic syndrome, the authors suggested that smoking cessation would have the great benefit of reducing the risk of metabolic syndrome, especially for subjects with fatty liver.

### Terminology

Metabolic syndrome: Metabolic syndrome is a cluster of metabolic abnormalities and is associated with waist circumference, serum triglycerides level, serum HDL-C level, blood pressure and fasting blood glucose (FPG) level. Fatty liver: Fatty liver is a clinicopathologic condition characterized by significant lipid deposition in the liver parenchyma.

### Peer review

This retrospective study investigated the possible interactive effect of fatty liver and smoking on metabolic syndrome. This study was based on a large scale population base ( $n = 3455$ ) and the results are interesting. Study results

suggested that smoking cessation would reduce the risk of metabolic syndrome, especially for people with fatty liver.

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BRIEF ARTICLE

## CDH1 promoter polymorphism (-347G→GA) is a possible prognostic factor in sporadic colorectal cancer

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### Abstract

**AIM:** To investigate the role of the -347G→GA polymorphism in the progression of colorectal cancer (CRC).

**METHODS:** We designed a case-control study based on a population of CRC patients in China and normal healthy controls with no history of tumors or inherited diseases. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analyses were used to genotype the variants, and immunohistochemical staining was performed to measure the expression of E-cadherin in different allele cases among the CRC patients and normal controls.

**RESULTS:** The GA-allele (G/GA heterozygous and GA/GA homozygous) did not increase the risk of CRC compared with the G-allele (OR = 1.232, 95% CI = 0.898-1.691). However, the frequencies of the GA-allele were higher in poorly differentiated ( $P = 0.002$ ) and proximal ( $P = 0.019$ ) CRC patients than in normal controls. We also observed that E-cadherin expression was lower in poorly differentiated CRC patients than in well differentiated CRC patients ( $P = 0.001$ ). Furthermore, E-cadherin expression was lower for the GA-allele than for the G-allele (G/G heterozygous) in CRC patients ( $P = 0.018$ ). In contrast, there was no

significant difference in E-cadherin expression for the G-allele and GA-allele in normal controls ( $P = 0.292$ ).

**CONCLUSION:** The -347G→GA promoter polymorphism in E-cadherin gene is associated with specific CRC features, and may be a prognostic factor rather than a susceptibility factor during the progression of CRC.

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**Key words:** Allele; Colorectal cancer; E-cadherin; Polymorphism; Prognosis

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### INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers in Western countries<sup>[1]</sup>, and is becoming more prevalent in Asian countries, especially China<sup>[2]</sup>. The etiopathogenesis of CRC is considered to be multifactorial, and to include high red meat intake<sup>[3]</sup>, high alcohol intake<sup>[4]</sup> and smoking<sup>[5]</sup>. However, no single environmental or lifestyle factor has consistently been associated with the risk of CRC. Recently, more and more views support genetic predisposition as the basis of many diseases, especially cancers<sup>[6]</sup>. However, CRC is divided into hereditary and sporadic cases, which show distinct genetic alterations and exhibit different key events leading to neoplastic growth.

E-cadherin is one of the major constituents of cell-adhesion complexes in epithelial cells<sup>[7,8]</sup>. It is a 97-kDa transmembrane glycoprotein encoded by the E-cadherin gene (*CDH1*) located on chromosome 16q22.1. It plays important roles in the establishment of adherent-type junctions by mediating calcium-dependent cellular interactions, and is thought to be a tumor suppressor

protein<sup>[7]</sup>. Partial or total loss of E-cadherin expression occurs in the majority of human carcinomas<sup>[9]</sup>. Besides its role in physical cell-cell adhesions, E-cadherin is also thought to be involved in intracellular signaling in normal epithelial cells, since downregulation of this molecule in epithelial cells is frequently associated with tumor formation and differentiation<sup>[10]</sup>.

It is not yet understood how the expression of E-cadherin is regulated, and this may occur *via* loss of heterozygosity, gene mutations or methylation of the coding region. Recently, the promoter region of *CDH1* was reported to be highly polymorphic<sup>[11]</sup>. One of the polymorphisms is the -347G→GA (rs5030625) single nucleotide polymorphism (SNP) upstream from the transcriptional start site<sup>[12]</sup>. Just as nucleotide variations in the coding region of a gene can alter protein expression<sup>[12,13]</sup>, the -347G→GA polymorphism within the promoter region may change the transcriptional efficiency of *CDH1*. For example, the GA-allele has a weak transcriptional factor-binding strength and transcriptional activity compared with the G-allele<sup>[12]</sup>, suggesting that the GA-allele may be associated with tumor formation or differentiation.

In the present study, we carried out a hospital-based case-control study to explore the association of the *CDH1* -347G→GA polymorphism with sporadic CRC in China. In addition, we measured the expression of E-cadherin in different allele cases including CRC patients and normal controls by immunohistochemical staining to check the function of the -347G→GA polymorphism *in vitro*.

## MATERIALS AND METHODS

### Subjects

The study included 290 sporadic CRC patients and 335 normal healthy controls (Table 1) enrolled from The Affiliated Drum Tower Hospital of Nanjing University Medical School between 2004 and 2008. Most of the patients had recently received a final diagnosis of CRC and were scheduled for surgery if no clinical metastases were detected, or would receive chemotherapy. A small number of the CRC patients had previously received surgery or chemotherapy. None of the subjects were blood-related. Patients affected by CRC were considered eligible if they had a histological diagnosis and were free from any known diseases with a genetic predisposition. Controls were selected from trauma patients or puerperal women in the same hospital during this time period. None of the controls had a history of malignancy. All the subjects were interviewed by a trained interviewer using a pretested questionnaire to obtain information on their sociodemographic characteristics, dietary habits, smoking and drinking status, and their individual and family history of cancer. Sporadic CRC cases and controls were matched for age, sex, smoking and drinking history.

The study was approved by the Institutional Review Boards of the Drum Tower Hospital. All the participants provided written informed consent at the time of recruitment and agreed to blood collection.

**Table 1** Clinical characteristics of the CRC patients and normal controls

	CRC patients (%)	Normal controls (%)	P
Sex			
Male	188 (64.8)	212 (63.3)	
Female	102 (35.2)	123 (36.7)	0.688 <sup>1</sup>
Age (yr)			
Range	27-85	15-81	
Mean	63.51 ± 13.24	58.62 ± 17.45	0.407 <sup>2</sup>
Smoking history			
Yes	117 (40.3)	122 (36.4)	
No	173 (59.7)	213 (63.6)	0.314 <sup>1</sup>
Drinking history			
Yes	128 (44.1)	135 (40.3)	
No	162 (55.9)	200 (59.7)	0.332 <sup>1</sup>
Stage			
Dukes A	12		
Dukes B	89		
Dukes C	102		
Dukes D	87		
Location			
Ascending, transverse	138		
Descending, sigmoid	61		
Rectum	91		
Total	290	335	

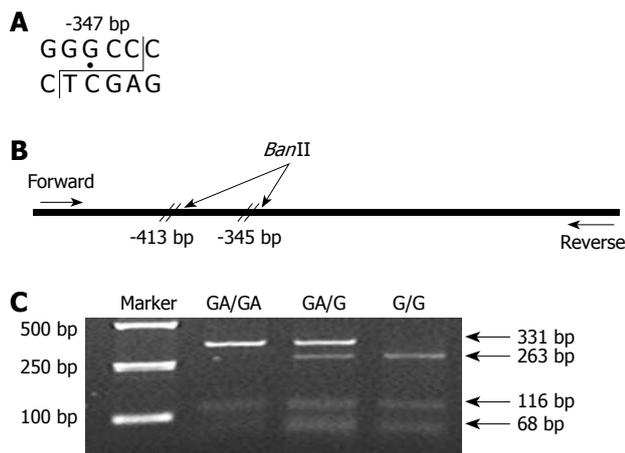
<sup>1</sup>Two-tailed  $\chi^2$ -test; <sup>2</sup>Two-tailed *t*-test; CRC: Colorectal cancer.

### DNA extraction

Peripheral venous blood (5 mL) was drawn from each subject before they received surgery or chemotherapy, and was placed in tubes containing EDTA and stored at -70°C until analysis. Total genomic DNA was extracted using a purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

### Genotyping analysis

The *CDH1* -347G→GA polymorphism was genotyped by the PCR-RFLP method. A 447-bp fragment containing the -347G→GA polymorphism in the *CDH1* promoter was amplified with the following primers: forward, 5'-GCCCCGACTTGTCTCTCTAC-3'; reverse, 5'-GGCCA CAGCCAATCAGCA-3'. PCR amplification was carried out in a volume of 25  $\mu$ L containing 20 ng of genomic DNA, 1  $\mu$ L of primers, 20  $\mu$ mol/L each of the forward and reverse primers, 2.5  $\mu$ L of 10  $\times$  PCR buffer (Mg<sup>2+</sup>-free), 2  $\mu$ L of dNTP, 1.5  $\mu$ L of MgCl<sub>2</sub> and 0.5 U of Taq polymerase (TaKaRa Biotechnology, Dalian, China). The amplification was performed in a programmable thermal cycler (MWG Biotech AG, Ebersberg, Germany) as follows: 1 cycle of 95°C for 2 min; 35 cycles of 94°C for 1 min, 61°C for 1 min and 72°C for 1 min; and a final cycle of 72°C for 10 min. The PCR product was digested with *Ban*II (TaKaRa Biotechnology) at 37°C overnight (Figure 1A and B). After digestion, the products were separated by 3% agarose gel electrophoresis and stained with ethidium bromide (Figure 1C). GA/GA homozygous cases were represented by DNA bands of 332 and 116 bp. G/G homozygous cases were represented by DNA bands of 263, 116 and 68 bp. GA/G heterozygous cases displayed a combination of both alleles (332, 263, 116 and 68 bp).



**Figure 1** Genotyping analysis of the *CDH1* -347G→GA polymorphism by PCR-RFLP analysis. A: Structural diagram of the restriction enzyme analysis for *Ban*II; B: Schematic overview of the E-cadherin gene promoter PCR fragment and the locations of the *Ban*II restriction enzyme sites; C: RFLP analysis of the -347G→GA polymorphism using *Ban*II digestion. GA/GA homozygote: 332 and 116 bp; GA/G heterozygote: 332, 263, 116 and 68 bp; G/G homozygote: 263, 116 and 68 bp.

### Immunohistochemistry

CRC tissue samples were collected from CRC patients who underwent surgery. Normal colon tissue samples were collected from outpatients *via* colonic biopsies during colonoscopy examination.

Immunohistochemistry was carried out using the avidin-biotin-peroxidase complex method. The central region of CRC tissue samples and normal colon tissue samples were cut into 4- $\mu$ m sections, mounted on glass slides, deparaffinized and rehydrated by xylene and graded ethanol solutions. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 20 min at room temperature. For antigen retrieval, the sections were heated in a pressure cooker for 5 min. The sections then were sequentially incubated with primary mouse antibodies against E-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution, 1:150; monoclonal antibody) overnight at 4°C, a biotinylated goat anti-mouse secondary antibody (Santa Cruz Biotechnology) for 30 min, and peroxidase-conjugated streptavidin for 10 min. Samples were colored with diaminobenzidine (Boster, Wuhan, China) and counterstained with hematoxylin. Images were captured under an Olympus-BX50F4 microscope (Olympus Corporation, Tokyo, Japan) and quantitatively analyzed using the Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, USA).

Sections of normal colorectal tissue were used as positive controls for E-cadherin staining. Negative controls were prepared by replacing the primary antibody with nonimmune IgG.

### Statistical analysis

The distributions of the clinical characteristics of the CRC patients and normal controls were analyzed by an unpaired two-tailed *t*-test. This test was also used to compare E-cadherin expression between the G-allele and

GA-allele. The  $\chi^2$ -test was used to test the differences in the allele frequencies between normal controls and CRC patients. The genotype data were further stratified by age, sex, smoking, alcohol intake, tumor location, pathologic grouping and clinical stage of CRC. The odds ratio (OR) and 95% confidence interval (CI) were calculated using an unconditional logistic regression model to evaluate the risk of the *CDH1* -347G→GA polymorphism for CRC. We performed all analyses with the SPSS 15.0 software package (SPSS Inc, Chicago, USA). Values of  $P < 0.05$  were considered statistically significant.

## RESULTS

All the recruited subjects were successfully genotyped. The study included 290 CRC patients and 335 normal controls with available data. The clinical characteristics of the study subjects are summarized in Table 1. No significant differences were noted in the distribution frequencies for sex, smoking and drinking.

As shown in Table 2, the G-allele and GA-allele frequencies were 52.8% and 47.2%, respectively, in the CRC patients, and 57.9% and 42.1%, respectively, in the controls. There was no significant difference between the patients and the controls ( $\chi^2 = 1.671$ ,  $P = 0.198$ ). A logistic regression analysis revealed that the GA-allele did not increase the risk of CRC (OR = 1.232, 95% CI = 0.898-1.691). However, when the CRC patients were stratified by age, sex, smoking, drinking, tumor location, pathologic grouping and clinical stage, the GA-allele frequency was higher in poorly differentiated CRC patients than in normal controls ( $\chi^2$ -test,  $P = 0.002$ ). In addition, the GA-allele frequency was higher in proximal CRC patients than in normal controls ( $\chi^2$ -test,  $P = 0.019$ ).

Immunohistochemical staining for E-cadherin was evaluated in 42 CRC patients (G-allele *vs* GA-allele: 20 *vs* 22) and 37 normal controls (G-allele *vs* GA-allele: 19 *vs* 18) (Figure 2). E-cadherin expression levels with the G-allele and GA-allele were compared between CRC patients and normal controls, as well as between poorly differentiated and well differentiated CRC patients (Figure 3). E-cadherin expression was significantly higher in normal controls, well differentiated CRC patients and the G-allele CRC patients than in CRC patients (*t*-test,  $P < 0.001$ ), poorly differentiated CRC patients (*t*-test,  $P = 0.001$ ) and the GA-allele CRC patients (*t*-test,  $P = 0.018$ ), however, there was no significant difference in E-cadherin expression between the G-allele and GA-allele in normal controls (*t*-test,  $P = 0.292$ ).

## DISCUSSION

Several molecular epidemiological studies have confirmed an association between the *CDH1* -347G→GA polymorphism and the risk of cancers, including gastric, colorectal and esophageal cancers<sup>[14,15]</sup>. The authors of these studies proposed that the *CDH1* -347G→GA polymorphism may be functional, and that the GA-allele could lead to transcriptional downregulation of *CDH1* and low expression of E-cadherin compared with the G-allele,

Table 2 Distribution of the *CDH1* -347G→GA polymorphism between CRC patients and normal controls

	G-allele (%) <sup>1</sup>	GA-allele (%) <sup>2</sup>	OR (95% CI) <sup>3</sup>	$\chi^2$	P
Controls	194 (57.9)	141 (42.1)			
Patients	153 (52.8)	137 (47.2)	1.232 (0.898-1.691)	1.671	0.198
Sex					
Male	98 (52.1)	90 (47.9)	1.264 (0.882-1.809)	1.633	0.233
Female	55 (53.9)	47 (46.1)	1.176 (0.753-1.836)	0.508	0.495
Age (yr)					
> 60	95 (55.2)	77 (44.8)	1.115 (0.770-1.616)	0.333	0.571
≤ 60	58 (49.2)	60 (50.8)	1.423 (0.934-2.169)	2.712	0.107
Smoking					
Yes	57 (48.7)	60 (51.3)	1.448 (0.949-2.210)	2.967	0.105
No	96 (55.5)	77 (44.5)	1.104 (0.762-1.598)	0.273	0.637
Drinking					
Yes	66 (51.6)	62 (48.4)	1.292 (0.859-1.945)	1.516	0.249
No	87 (53.7)	75 (46.3)	1.186 (0.813-1.730)	0.786	0.386
Location					
Proximal <sup>4</sup>	63 (45.7)	75 (54.3)	1.638 (1.099-2.441)	5.919	0.019
Distal <sup>5</sup>	90 (59.2)	62 (40.8)	0.948 (0.642-1.399)	0.073	0.843
Differentiation					
Well	116 (56.3)	90 (43.7)	1.067 (0.752-1.516)	0.133	0.721
Poorly	16 (33.3)	32 (66.7)	2.752 (1.454-5.209)	10.240	0.002
Other	21 (58.3)	15 (41.7)	0.983 (0.489-1.974)	0.002	1.000
Stage					
Dukes A/B	58 (57.4)	43 (42.6)	1.020 (0.650-1.600)	0.007	1.000
Dukes C/D	95 (50.3)	94 (49.7)	1.361 (0.951-1.948)	2.856	0.100

<sup>1</sup>G/G homozygous; <sup>2</sup>GA/GA homozygous; GA/G heterozygous; <sup>3</sup>95% confidence interval; <sup>4</sup>Ascending colon, transverse colon; <sup>5</sup>Descending colon, sigmoid colon, rectum; *CDH1*: E-cadherin gene.

thereby increasing the risk of cancer. However, recent studies have indicated that some functional polymorphisms may play more important roles in the prognosis of cancer than in its formation<sup>[16,17]</sup>. To further investigate the association between the functional *CDH1* -347G→GA polymorphism and sporadic CRC, we conducted the present case-control study in a Chinese population.

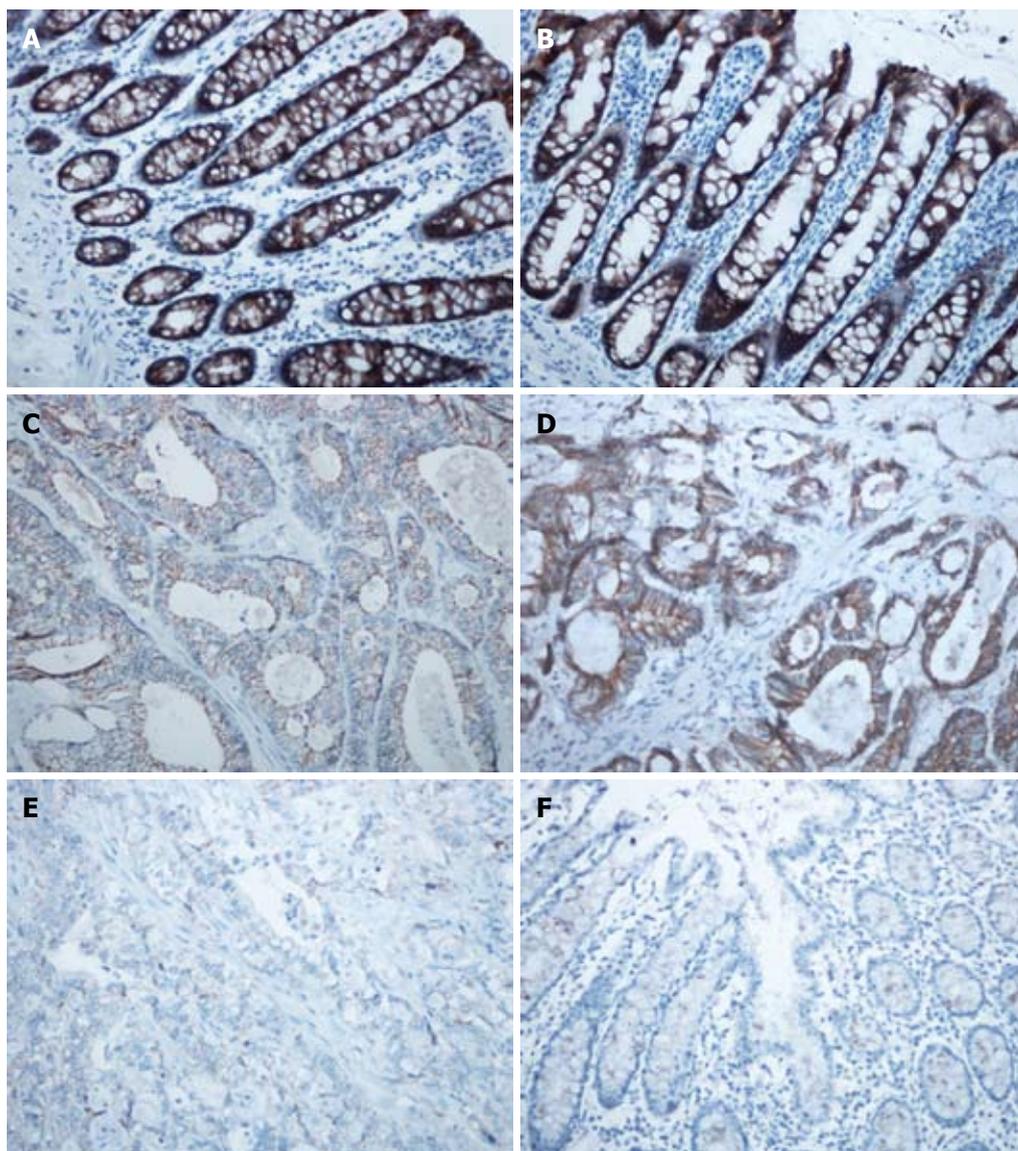
We found that the GA-allele did not increase the risk of CRC compared with the G-allele in this Chinese population. This finding is not consistent with the study by Shin *et al.*<sup>[18]</sup>, who reported that the GA-allele was associated with a significantly increased risk of CRC in Korea. Furthermore, their GA-allele frequency in normal controls (41/147, 27.9%) was obviously lower than the frequency observed in the present study (141/335, 42.1%). These discrepancies may be caused by racial differences. At the same time, we found that there was no significant difference in E-cadherin expression between the G-allele and GA-allele in normal controls, as evaluated by immunohistochemical staining. In other words, the GA-allele did not increase the risk of CRC or influence the expression of E-cadherin in normal controls.

However, the frequency of the GA-allele was significantly higher in our poorly differentiated and proximal CRC patients than in the normal controls. In addition, E-cadherin expression was lower in the poorly differentiated CRC patients than in the well differentiated CRC patients, and the E-cadherin expression was lower for the GA-allele than for the G-allele in CRC patients. Considering the above-described findings, the *CDH1* -347G→GA polymorphism did not appear to influence E-cadherin expression in normal controls. However, it

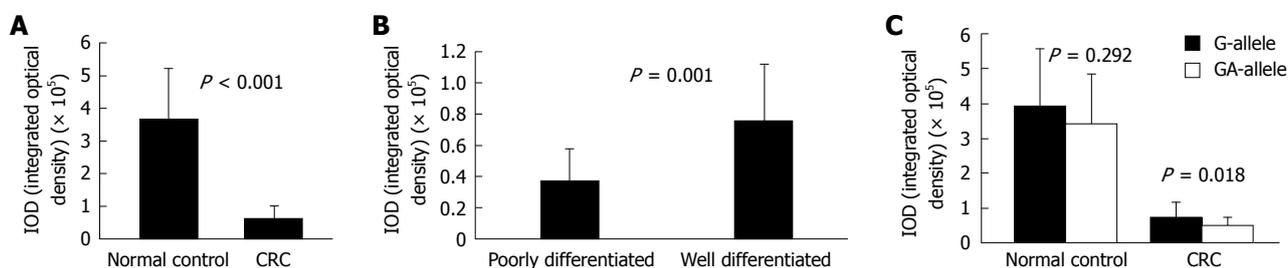
may play a role in the expression of E-cadherin after CRC formation, and may also be associated with the cell differentiation of CRC. These findings are reminiscent of the study of Cano *et al.*<sup>[19]</sup>, who found that the Snail gene family members were expressed in human carcinoma cells, especially poorly differentiated carcinoma cells, and could repress the transcriptional activity of *CDH1* through binding to a specified structure or zone in *CDH1*. We speculate that this specific structure or zone may be associated with certain functional polymorphisms.

Although the idea that E-cadherin is associated with tumors has been accepted by most researchers, the molecular mechanisms for the involvement of E-cadherin in tumor formation remain controversial. For example, it remains unknown whether E-cadherin acts as a susceptibility factor or a prognostic factor. Supporters of the former idea believe that loss of E-cadherin during tumor formation leads to accumulation of  $\beta$ -catenin in the cytoplasm, which could stimulate the  $\beta$ -catenin pathway<sup>[20]</sup>. However, several studies have shown that low expression of E-cadherin can occur after the formation of a tumor in response to alterations in the internal environment, such as hypoxic conditions, growth factor expression, tumor suppressor protein actions etc, all of which can modulate the expression of E-cadherin through various signaling pathways<sup>[21]</sup>. Therefore, it is possible that the *CDH1* -347G→GA polymorphism may change the transcriptional activity of *CDH1* after tumor formation by combining with other factors, and thereby influencing the differentiation of tumor cells.

In conclusion, we found that the *CDH1* -347G→



**Figure 2** Immunohistochemical staining for E-cadherin. A,B: E-cadherin expression in normal colorectal tissue with the G-allele (A) and GA-allele (B) (each,  $\times 200$ ); C,D: E-cadherin expression in well differentiated CRC with the GA-allele (C) and G-allele (D) (each,  $\times 200$ ); E: E-cadherin expression in poorly differentiated CRC with the GA-allele ( $\times 200$ ); F: Negative control ( $\times 200$ ).



**Figure 3** E-cadherin expression in colon tissues of different types. A: E-cadherin expression is significantly higher in normal controls than in CRC patients (*t*-test,  $P < 0.001$ ); B: E-cadherin expression is significantly higher in well differentiated CRC patients than in poorly differentiated CRC patients (*t*-test,  $P = 0.001$ ); C: There is no significant difference in E-cadherin expression between the G-allele and GA-allele in normal controls (*t*-test,  $P = 0.292$ ). However, E-cadherin expression is significantly higher for the G-allele than for the GA-allele in CRC patients (*t*-test,  $P = 0.018$ ).

GA polymorphism was associated with specific CRC features. Furthermore, the GA-allele was a repressive factor for the transcriptional activity of *CDH1*, but may begin to function after the formation of CRC and play

a role in the differentiation of tumor cells. This also implies that the *CDH1* -347G $\rightarrow$ GA polymorphism may be a prognostic factor rather than a susceptibility factor during the progression of CRC.

## COMMENTS

### Background

The progression of colorectal cancer (CRC) is a major cause of cancer death in Western populations, and is becoming more prevalent in Asian countries such as China. Although it is well known that high red meat intake, high alcohol intake and smoking are associated with the risk of CRC, host genetic factors may be one of the critical factors in carcinogenesis.

### Research frontiers

E-cadherin is thought to be involved in intracellular signaling in epithelial cells, since downregulation of this molecule in epithelial cells is frequently associated with tumor formation and differentiation. The -347G→GA polymorphism within the promoter region is considered to be functional, which may change the transcriptional efficiency of E-cadherin gene (*CDH1*).

### Innovations and breakthroughs

Most previous studies concentrated on the association of polymorphisms with the formation of carcinomas. This is probably the first report on the relationship between *CDH1* -347G→GA polymorphism and the prognosis of CRC, and we found that the -347G→GA polymorphism may be a prognostic factor rather than a susceptible factor during the progression of CRC.

### Applications

These findings may help doctors to choose an appropriate treatment for different CRC patients.

### Terminology

E-cadherin: E-cadherin is a 97-kDa transmembrane glycoprotein, which is one of the major constituents of cell-adhesion complexes in epithelial cells. *CDH1*: *CDH1* is the gene which encodes E-cadherin, and is located on chromosome 16q22.1.

### Peer review

It is a well-written and well-designed study, with large observed samples, and with important scientific merit.

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BRIEF ARTICLE

## Radical resection and outcome for malignant tumors of the pancreatic body and tail

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### Abstract

**AIM:** To analyze the factors influencing radical (R0) resection rate and surgical outcome for malignant tumor of the pancreatic body and tail.

**METHODS:** The clinical and operative data and follow-up results of 214 pancreatic body and tail cancer patients were analyzed retrospectively.

**RESULTS:** One hundred and twenty/214 pancreatic body and tail cancer patients underwent surgical treatment; the overall resection rate was 59.2% (71/120), and the R0 resection rate was 40.8% (49/120). Compared with non-R0 treatment, the patients receiving an R0 resection had smaller size tumor ( $P < 0.01$ ), cystadenocarcinoma ( $P < 0.01$ ), less lymph node metastasis ( $P < 0.01$ ), less peri-pancreatic organ involvement ( $P < 0.01$ ) and earlier stage disease ( $P < 0.01$ ). The overall 1-, 3- and 5-year survival rates for pancreatic body and tail cancer patients were 12.7% (25/197), 7.6% (15/197) and 2.5% (5/197), respectively, and ductal adenocarcinoma patients had worse survival rates [15.0% (9/60), 6.7% (4/60) and 1.7% (1/60), respectively] than cystadenocarcinoma patients [53.8% (21/39), 28.2% (11/39) and 10.3% (4/39)] ( $P < 0.01$ ). Moreover, the 1-, 3- and 5-year overall survival rates in patients with R0 resection were 55.3% (26/47), 31.9% (15/47) and 10.6% (5/47), respectively, significantly better than those in patients with palliative resection [9.5% (2/21), 0 and 0] and in

patients with bypass or laparotomy [1.2% (1/81), 0 and 0] ( $P < 0.01$ ).

**CONCLUSION:** Early diagnosis is crucial for increasing the radical resection rate, and radical resection plays an important role in improving survival for pancreatic body and tail cancer patients.

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**Key words:** Pancreatic neoplasm; Body and tail of pancreas; Pancreatectomy; Survival; Cystadenocarcinoma

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### INTRODUCTION

Pancreatic carcinoma is one of the most fatal malignant diseases and ranks fifth in cancer mortality worldwide<sup>[1,2]</sup>. Survival after resection remains disappointing, with 5-year survival rates ranging from 10% to 29%<sup>[3-8]</sup>. Advances in diagnostic and operative techniques and in perioperative care have increased the resectability of pancreatic cancer, and have decreased rates of operative morbidity and mortality. The definition of a resectable tumor has become more clearly defined anatomically based on the availability of high-quality computed tomography (CT) scans<sup>[7-10]</sup>. Such imaging now permits a precise, preoperative, noninvasive assessment of tumor resectability and adds an important level of objectivity to the staging of patients for entry into clinical trials. Importantly, the role of laparotomy is now largely restricted to patients judged "resectable" on preoperative imaging<sup>[9,10]</sup>. For the 80%-90% of patients with pancreatic adenocarcinoma who have unresectable disease, biliary obstruction, when present, can be palliated using minimally invasive endoscopic techniques.

In patients with a malignant neoplasm in the body-

tail of the pancreas, splenectomy has a negative influence on long-term survival after resection<sup>[11,12]</sup>. The incidence of diabetes after spleen-preserving distal pancreatectomy for chronic pancreatitis is less than after *en-bloc* splenectomy<sup>[13-15]</sup>. Spleen salvage eliminates the risk of overwhelming infections. In the past decade, advances in surgical techniques have reduced the operative mortality rate of pancreatic resections to below 5% in high-volume centers, yet morbidity rates have remained essentially unchanged, ranging from 30% to 40%<sup>[11-16]</sup>.

The objective of this study was to analyze factors contributing to radical resection rate and outcome following radical resection for malignant tumors of the pancreatic body and tail.

## MATERIALS AND METHODS

### Patient characteristics

Two hundred and fourteen patients with malignant tumors of the pancreatic body and tail underwent radical pancreatectomy at the First Medical College of Wenzhou Medical College between January 2000 and December 2006, and at the First Affiliated Hospital of Zhejiang University between January 1990 and March 2002 and were eligible for study. The demographic and clinical courses of each patient were collected, including age, sex, and indication for radical pancreatectomy, concomitant splenectomy, symptomatology, diagnostic methods, operative management, pathology report, postoperative morbidity and mortality.

Of the 214 patients, 125 were men and 89 were women, with a mean age of 59.7 years (ranging from 15 to 81 years). Tumors were staged according to American Joint Committee on Cancer staging<sup>[17]</sup>, 11 patients (5.1%) were categorized into stage I, 6 patients (2.8%) into stage II, 16 patients (7.5%) into stage III, 62 patients (29.0%) into stage IVA and 119 patients (55.6%) into stage IVB. The preoperative diagnosis of pancreatic carcinoma was made using abdominal ultrasonography (US), CT, endoscopic retrograde cholangiopancreatography, magnetic resonance cholangiopancreatography and detection of serum tumor markers such as carcinoembryonic antigen, carbohydrate antigen 19-9.

### Surgical technique and definition of R0 resection

The main surgical techniques used for transection and closure of the pancreatic remnant included: (1) Anastomosis: the pancreaticojejunal end-to-end anastomosis was carried out followed Peng's invagination method<sup>[18]</sup>; (2) Closure by suture: the pancreas was transected with a knife, followed by identification of the main pancreatic duct and closure of the duct using single stitches of 2-0 silk suture. The parenchyma was then closed using single stitches of 2-0 silk suture; (3) In some cases, the suture line was reinforced by laying a fibrinogen/thrombin-coated collagen patch (Fibrin Sealant<sup>®</sup>, Guangzhou Bioseal Technology Co. Lt, China) onto the transected end; (4) In our university hospitals, radical resection, especially pancreatoduodenectomy, is performed only by

experienced surgeons who are the professors or directors of general surgery. Recently, most operations for pancreatic tumors have been performed by experienced surgeons in pancreatic disease.

In this study, R0 resection means negative resection margins and no residual tumor. If a frozen section taken of the cut pancreatic and bile duct margins was positive, more tissue was taken.

### Adjuvant therapy

In this study, all patients received regular or irregular adjuvant therapy (immuno-chemotherapy, radiotherapy and/or Chinese traditional drugs) after surgery, but no patients received neoadjuvant chemotherapy or radiation therapy before surgery.

### Definitions of postoperative complications

In this study, pancreatic leakage was defined as: (1) discharge from the postpancreatic drain  $\geq 50$  mL/d after postoperative day 3, and (2) an amylase level in drainage fluid exceeding three times that of the serum concentration. Postoperative mortality was defined as death occurring in the first 30 postoperative days or before discharge from the hospital. Other complications were categorized and defined as any of the following: intra-abdominal bleeding (requiring transfusion or operative intervention); gastrointestinal bleeding (requiring transfusion or endoscopic or operative intervention); intra-abdominal abscess (fluid requiring drainage and with positive bacterial culture); wound infection (purulent drainage requiring open packing); bile leak (bilious drainage from intraoperatively placed drains or bile collection requiring drainage); wound dehiscence (partial or total disruption of the fascial or all the layers of the incision).

### Follow-up

Follow-up information was obtained through office visits and telephone contact with the patients until the time of the patients' deaths or at the end of this study. In order to confirm the dates of deaths, if any, the data were verified at the regional station of the public records department for telecommunication and computer science. Local recurrence was defined as tumor relapse within the region or presence of a pancreatic stump. Distant metastases (or dissemination) were tumor lesions in other organs (outside the pancreas under treatment) such as liver and lung or remote lymph nodes, e.g. paraaortic lymph node. Upon their discharge from hospital, the patients were seen at least four times a year, i.e. every 3 mo, within the first 5 years, and every half a year thereafter. Physical examinations, basic routine X-ray examinations and abdominal US were performed. CT of the abdomen (twice a year), and if necessary the chest or head was added.

### Statistical analysis

The statistical analysis was performed with SPSS software (version 13.0; SPSS Inc, Chicago, Ill). All results are expressed as mean  $\pm$  SD. Univariate analyses of categorical variables were performed using  $\chi^2$  tests, and

**Table 1** The relationship between resection rate and clinicopathological variables (mean  $\pm$  SD) *n* (%)

Clinicopathological variables	Radicality		
	R0 resection ( <i>n</i> = 49)	R1 + R2 resection ( <i>n</i> = 22)	Bypass or laparotomy ( <i>n</i> = 49)
Tumor size (cm)	4.8 $\pm$ 1.3 <sup>b</sup>	7.9 $\pm$ 2.2	11.0 $\pm$ 3.8
Histopathological type			
Ductal adenocarcinoma ( <i>n</i> = 60)	15 (25.0) <sup>b</sup>	10 (16.7)	35 (58.3)
Cystadenocarcinomas ( <i>n</i> = 39)	25 (64.1)	7 (17.9)	7 (17.9)
Others ( <i>n</i> = 21)	7 (33.3)	6 (28.6)	8 (38.1)
Lymph node metastasis	16 (32.7) <sup>b</sup>	19 (86.4)	43 (87.8)
Peri-organ involvement <sup>1</sup>	26 (53.1) <sup>b</sup>	18 (81.8)	40 (81.6)
TNM staging			
Stage I + II ( <i>n</i> = 17)	17 (100.0) <sup>b</sup>	0	0
Stage III ( <i>n</i> = 16)	14 (87.5)	2 (12.5)	0 (0)
Stage IVA ( <i>n</i> = 34)	18 (52.9) <sup>2</sup>	7 (20.6)	9 (26.5)
Stage IVB ( <i>n</i> = 53)	0	13 (24.5)	40 (75.5)

<sup>b</sup>*P* < 0.01 vs R1 + R2 resection and bypass or laparotomy; <sup>1</sup>Peri-organ involvement included spleen, transverse colon, left kidney, stomach and their vessels; <sup>2</sup>Six gastric malignant tumors with pancreatic invasion, one pancreatic metastasis from renal cell carcinoma after operation, 11 pancreatic cystadenocarcinomas with splenic invasion.

the multivariate analysis was performed using a non-conditional logistic regression model expressed in odds ratios. To test the independence of the risk factors, the significant variables (*P* < 0.05) in the univariate analysis were entered into a multivariate logistic regression model with likelihood ratio forward selection with a criterion of *P* < 0.05.

## RESULTS

### Resection rate and clinicopathological features

Ninety-four of 214 patients with malignant tumor of pancreatic body and tail accepted non-surgical treatment, and 120 patients underwent surgery. The overall resection rate was 59.2% (71/120), and the R0 resection rate was 40.8% (49/120). R0 resections were those where the tumors were resected with clear surgical margins, as shown by intraoperative frozen sections and confirmed by definitive histopathological examination. Twenty-two patients underwent palliative resection (R1 or R2), 49 underwent bypass or laparotomy.

Compared with patients who underwent palliative resection (R1 + R2) (7.9  $\pm$  2.2 cm) or bypass/laparotomy (11  $\pm$  3.8 cm), the tumor size (4.8  $\pm$  1.3 cm) in patients who underwent radical resection was significantly smaller (*P* < 0.01). Similar results were found in patients with pancreatic cystadenocarcinoma [25.0% (15/60) vs 64.1% (25/39) and 33.3% (7/21), *P* < 0.01], less lymph node metastasis [32.7% (16/49) vs 86.4% (19/22) and 87.8% (43/49), *P* < 0.01] or less peri-organ involvement [53.1% (26/49) vs 81.8% (18/22) and 81.6% (40/49), *P* < 0.01]. Moreover, the radical resection rates in stage I + II and stage III were 100.0% (17/17) and 87.5% (14/16), which were much higher than those in stage IVA (52.9%, 18/34) and stage IVB (0) (*P* < 0.01) (Table 1).

**Table 2** Histopathological diagnosis of 120 patients with resected pancreatic malignant tumor

Histopathological classification	Cases
Ductal adenocarcinoma	60
Invaded and metastatic tumor	7
Pancreas invaded from gastrointestinal stromal tumor	6
Pancreatic metastasis from renal cell carcinoma	1
Cystadenocarcinomas	39
Serous cystadenocarcinomas	30
Mucinous cystadenocarcinomas	9
Endocrine malignant tumor	14
Insulinoma	3
Gastrinoma	11

Definitive histology of the resected lesions revealed a cystic tumor in a solid malignancy in 67 patients (60 ductal adenocarcinoma, six circumscribed infiltration of a gastrointestinal stromal tumor, one pancreatic metastasis from renal cell carcinoma), 39 cystadenocarcinomas (30 serous cystadenocarcinomas, nine mucinous cystadenocarcinomas), and 14 malignant endocrine tumors (Table 2).

### Procedures and postoperative complications

Forty-nine of 120 patients (40.8%) underwent R0 radical operation, including 22 with combined distal pancreatectomy and splenectomy, and 27 with spleen-preserving pancreatectomy. Postoperative complications occurred in 18 patients (15.0%) with pancreatic fistula (eight patients, 6.7%) being the most common, followed by intra-abdominal bleeding (4, 3.3%), gastrointestinal bleeding (2, 1.7%), incisional infection (3, 2.5%), and intestinal fistula (1, 0.8%). There was no operative mortality (defined as any death occurring within 1 mo after surgical procedure). Moreover, no detrimental effects of postoperative complications on oncologic efficacy of R0 pancreatectomy were found in this study.

### Long-term outcomes

Long term follow-up was performed using a standardized protocol. The median follow-up time was 23.1 mo, ranging from 4 to 83 mo. Seventeen patients with pancreatic carcinoma failed to follow-up and the overall follow-up rate was 92.1%. The 1-, 3- and 5-year overall survival rates in this group were 15.7% (31/197), 7.6% (15/197) and 2.5% (5/197), respectively. Moreover, the 1-, 3- and 5-year overall survival rates in patients with R0 resection were 55.3% (26/47), 31.9% (15/47) and 10.6% (5/47), respectively, which were significantly better than those in patients with palliative resection [9.5% (2/21), 0 and 0] or those with bypass/laparotomy [1.2% (1/81), 0 and 0] (*P* < 0.01, Table 3, Figure 1A). Among 49 patients with R0 resection, there was no significant difference in survival between the combined distal pancreatectomy and splenectomy group (*n* = 22) and spleen-sparing pancreatectomy group (*n* = 27) (*P* > 0.05) (Table 4). Furthermore, the 1-, 3- and 5-year overall survival rates [15.0% (9/60), 6.7% (4/60) and 1.7% (1/60), respectively] for pancreatic adenocarcinoma patients were worse than

**Table 3** The relationship between surgical radicality and survival *n* (%)

Surgical radicality	Cases with follow-up	1-yr survival	3-yr survival	5-yr survival
R0 resection ( <i>n</i> = 49)	47	26 (55.3) <sup>b</sup>	15 (31.9) <sup>b</sup>	5 (10.6) <sup>b</sup>
R1 + R2 (palliative resection ( <i>n</i> = 22)	21	2 (9.5) <sup>b</sup>	0	0
Bypass or laparotomy ( <i>n</i> = 49)	48	6 (12.5) <sup>b</sup>	0	0
Non-surgical treatment ( <i>n</i> = 94)	81	1 (1.2) <sup>b</sup>	0	0
Total ( <i>n</i> = 214)	197	25 (12.7)	15 (7.6)	5 (2.5)

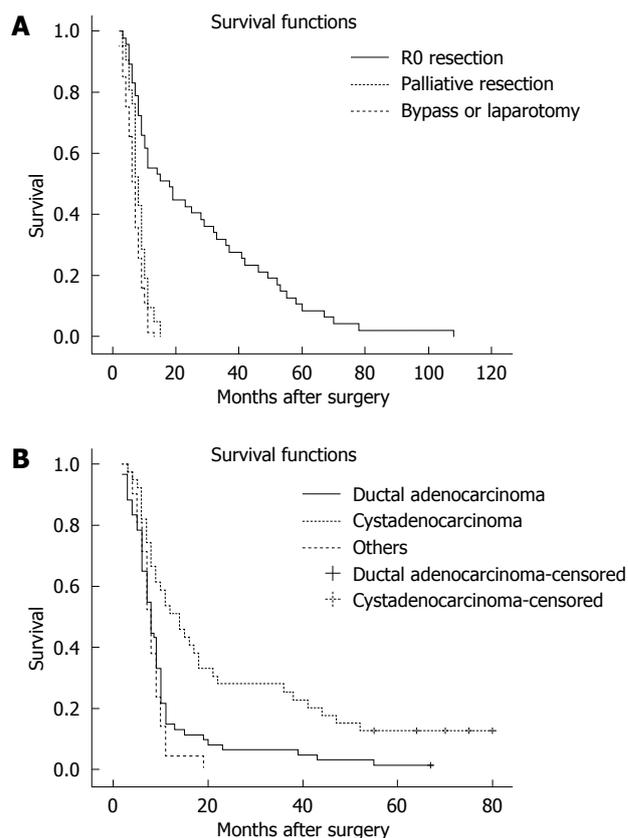
<sup>b</sup>*P* < 0.01 vs non-R0 resection, the 1-, 3- and 5-year survival rates were significantly longer.

those [53.8% (21/39), 28.2% (11/39) and 10.3% (4/39)] for pancreatic cystadenocarcinomas (Figure 1B, *P* < 0.01). In addition, the survival of patients with pancreatic adenocarcinoma of the body and tail was somewhat improved from the mid 1990s, but this improvement was not significant.

## DISCUSSION

It has been reported that less than 5% of all patients diagnosed with pancreatic carcinoma can expect to live for more than 5 years<sup>[1,2,19,23]</sup>. A radical pancreatic resection (R0) is an effective and safe method of treating various benign and malignant diseases of the pancreas. However, only 10%-20% of these individuals are candidates for surgical resection, which remains the only available chance for cure of this lethal disease<sup>[6,7,24]</sup>. Unfortunately delayed diagnosis, ineffective chemotherapy, lower radical resection rate, radiation resistance, and an intrinsic biologic aggressiveness of tumors all contribute to the poor prognosis associated with pancreatic cancer<sup>[1,2,5]</sup>.

Early detection and diagnosis are the key points to improve the outcome of pancreatic carcinoma, however almost 70% of patients with pancreatic carcinomas have unresectable disease at the time of initial diagnosis and are unable to undergo curative resection<sup>[1,5,6,25]</sup>. It has been reported that the resection rate is 20%-42.6% for pancreatic carcinoma, and the 5-year survival is 8.5%-10.6% after radical resection<sup>[1-4]</sup>. As for pancreatic carcinoma of the body and tail, the resection rate is much lower, only 10%-22%, and the prognosis is much poor because the tumor in this portion of the gland tends to invade surrounding organs and vascular structures<sup>[5,6]</sup>. Wu *et al*<sup>[5]</sup> compared clinical manifestations, pathological behavior and postoperative survival between malignant tumor of the pancreatic body and tail (*n* = 106) and malignant pancreatic head cancer (*n* = 451). The authors found postoperative median survival for resection of non-metastatic pancreatic body and tail cancer was significantly longer than similar resections in patients with metastatic disease. These results were no different than in those patients who had no resection. The overall and R0 resection rates in this study were 59.2% and 40.8%, respectively, and the 1-, 3- and 5-year overall survival rates in patients with R0



**Figure 1** Overall survival of malignant tumor of the pancreatic body and tail. A: Overall survival of 120 patients with malignant tumor of the pancreatic body and tail according to surgical radicality: Note the markedly increased survival time for patients who underwent radical resection (*P* < 0.01); B: Overall survival of 120 patients with malignant tumor of the pancreatic body and tail according to histopathological type: Note the markedly worse survival time for patients with ductal adenocarcinoma rather than cystadenocarcinomas (*P* < 0.01).

resection were 55.3%, 31.9% and 10.6%, respectively, which were better than those found in patients who had palliative resection or patients with bypass/laparotomy (*P* < 0.01). In addition, ductal adenocarcinoma patients had worse survival times than patients with pancreatic cystadenocarcinoma and other malignant tumors (*P* < 0.01). Liu *et al*<sup>[6]</sup> reported that factors influencing resection rate of pancreatic carcinoma included lymph node metastasis, tumor size and peri-pancreatic invasion, and the median survival times of radical resection, palliative resection and laparotomy for tumors of the body and tail of the pancreas were 18, 8 and 3.5 mo, respectively. Lim *et al*<sup>[26]</sup> collected a group of 396 patients aged > 65 years who were diagnosed with nonmetastatic pancreatic adenocarcinoma and found median survival was 17.6 mo, with 1- and 3-year survival rates of 60.1% and 34.3%, respectively. In this study, our findings revealed that compared with tumors which underwent palliative and bypass/laparotomy, the patients receiving R0 resections had smaller sized tumors, (*P* < 0.01), cystadenocarcinoma histopathology (*P* < 0.01), less lymph node metastasis (*P* < 0.01), less peri-pancreatic organ involvement (*P* < 0.01), and earlier stage (*P* < 0.01). These results indicate that small size, cystadenocarcinoma histopathology, lymph node metastases, and peri-

**Table 4** Influence of splenectomy on survival in patients with malignant tumors of the pancreatic body and tail after radical pancreatectomy *n* (%)

Procedures	Complications	Cases with follow-up	1-yr survival	3-yr survival	5-yr survival
Combined spleen and pancreatectomy ( <i>n</i> = 22)	7 (31.8) <sup>a</sup>	21	9 (42.9) <sup>a</sup>	6 (28.6) <sup>a</sup>	2 (9.5) <sup>a</sup>
Spleen-preserving pancreatectomy ( <i>n</i> = 27)	7 (25.9)	26	16 (61.5)	9 (34.6)	3 (11.5)
Total ( <i>n</i> = 49)	14 (26.5)	47	25 (53.2)	15 (31.9)	5 (10.6)

<sup>a</sup>*P* > 0.05.

pancreatic organ involvement were factors influencing the R0 resection rate.

Long-term survival after pancreatoduodenectomy for pancreatic carcinoma is far from excellent. The 5-year survival rate nears 10% and patients surviving more than 5 years are exceptional. It has been reported that the strongest predictors of survival are adjuvant combined chemoradiotherapy, small tumors (< 2 cm in diameter), negative lymph nodes, well-differentiated histology, undergoing surgery in a teaching hospital and high socioeconomic status<sup>[16,19,27]</sup>. A multivariate analysis of the 443 patients with periampullary adenocarcinoma from Yeo *et al*<sup>[28]</sup>, indicated that the most powerful independent predictors favoring long-term survival included a pathologic diagnosis of duodenal adenocarcinoma, tumor diameter < 3 cm, negative resection margins, absence of lymph node metastases, well-differentiated histology, and no reoperation. Schwarz *et al*<sup>[11]</sup> studied the outcomes in a group of patients (326 patients, 37 underwent splenectomy) with adenocarcinoma after distal and total pancreatectomy with or without splenectomy and concluded that splenectomy was a statistically significant unfavorable prognostic factor in survival, but not in postoperative morbidity. Shoup *et al*<sup>[4]</sup>, in a cohort with benign and low-grade malignant diseases (125 patients), reported that spleen preserving distal pancreatectomy is associated with lower infectious complications rate and reduced hospital stay, compared with distal pancreatectomy with splenectomy (*P* = 0.01 and *P* < 0.01, respectively). The median survival following resection was 15.9 mo compared to 5.8 mo in patients who were not resected (*P* < 0.0001). Actual 5- and 10-year survival rates were 22% and 18%, respectively, following extended resection, 8% and 8% following standard resection, and 0% and 0% if no resection was attempted because of locally unresectable disease. Patients undergoing extended resection for adenocarcinoma of the pancreatic body or tail have long-term survival rates similar to those for patients undergoing standard resection; they also have markedly improved long-term survival compared to those who are not considered resectable because of locally advanced disease.

Elective distal pancreatectomy is safer than pancreaticoduodenectomy but carries a high morbidity rate<sup>[5,6]</sup>. In the past decade splenectomy was associated with an increased septic complication rate<sup>[27]</sup>. Furthermore, several authors<sup>[12,24]</sup> suggested spleen preserving distal pancreatectomy in order to reduce postoperative septic complications. The technique of spleen preserving distal pancreatectomy and its absolute and relative contraindications have been described elsewhere<sup>[12,29]</sup>. In this study, the

postoperative complication rate was 15.0%, with pancreatic fistula (6.7%) being the most common, followed by intra-abdominal bleeding (3.3%), gastrointestinal bleeding (1.7%), incisional infection (2.5%), and intestinal fistula (0.8%). These are similar to previous reports<sup>[1,5,6,23]</sup>. Few retrospective studies have analyzed the influence of splenectomy in the postoperative course after distal pancreatectomy, while one study has analyzed this relationship after total pancreatectomy<sup>[29]</sup>. For example, Schwarz *et al*<sup>[11]</sup> reported that the median actuarial survival for pancreatic adenocarcinoma was 12.2 mo with splenectomy *vs* 17.8 mo without splenectomy (*P* < 0.005), and splenectomy (*P* = 0.02) as well as pathologic lymph node status (*P* = 0.0002), tumor diameter (*P* = 0.0004), and tumor differentiation (*P* = 0.007) were prognostic factors. In this study, there was no significant difference in survival between the combined distal pancreatectomy and splenectomy group and spleen-preserving pancreatectomy group.

In conclusion, early diagnosis is crucial for increasing the radical resection rate and radical resection plays an important role in the improvement of prognosis for patients with malignant tumors of pancreatic body and tail.

## COMMENTS

### Background

Pancreatic carcinoma is one of the most fatal malignant diseases and ranks fifth in cancer mortality worldwide. Survival after resection remains disappointing, with 5-year survival rates ranging from 10% to 29%. Advances in diagnostic and operative techniques and in perioperative care have increased the resectability of pancreatic cancer and have decreased rates of operative morbidity and mortality. The definition of a resectable tumor has become more clearly defined anatomically based on the availability of high-quality computed tomography scans. Such imaging now permits a precise, preoperative, noninvasive assessment of tumor resectability and adds an important level of objectivity to the staging of patients for entry into clinical trials. Importantly, the role of laparotomy is now largely restricted to patients judged "resectable" on preoperative imaging. The objective of this study was to analyze factors contributing to radical resection rate and outcome following radical resection of malignant tumors of the pancreatic body and tail.

### Research frontiers

Ways of improving the early diagnosis and radical resection are the hotspots in management of malignant tumors of the pancreatic body and tail.

### Innovations and breakthroughs

This study tries to find out the factors influencing early diagnosis and radical resection, by analysis of the factors which have effects on the resection rate of patients with malignant tumors of the pancreatic body and tail.

### Applications

By using those factors that influence early diagnosis and radical resection, we can evaluate objectively the "resectability" of each case with malignant tumors of the pancreatic body and tail.

### Terminology

This is a retrospective study on surgical treatment of malignant tumors of the pancreatic body and tail.

**Peer review**

The work is a retrospective analysis of the radical resection rate in 240 patients suffering from pancreatic cancer in two university hospitals from 1990 to 2006. The authors correctly point out that early diagnosis and curative resection is key to a positive outcome. The paper is well written and the data are adequately discussed.

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BRIEF ARTICLE

## Effectiveness and safety of splenectomy for gastric carcinoma: A meta-analysis

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### Abstract

**AIM:** To evaluate the impact of splenectomy on long-term survival, postoperative morbidity and mortality of patients with gastric cancer by performing a meta-analysis.

**METHODS:** A search of electronic databases to identify randomized controlled trials in The Cochrane Library trials register, Medline, CBMdisc (Chinese Biomedical Database) and J-STAGE, *etc* was performed. Data was extracted from the studies by 2 independent reviewers. Outcome measures were survival, postoperative morbidity and mortality and operation-related events. The meta-analyses were performed by RevMan 4.3.

**RESULTS:** Three studies comprising 466 patients were available for analysis, with 231 patients treated by gastrectomy plus splenectomy. Splenectomy could not increase the 5-year overall survival rate [RR = 1.17, 95% confidence interval (CI) 0.97-1.41]. The postoperative morbidity (RR = 1.76, 95% CI 0.82-3.80) or mortality (RR = 1.58, 95% CI 0.45-5.50) did not suggest any significant differences between the 2 groups. No significant differences were noted in terms of number of harvested lymph nodes, operation time, length of hospital stay and reoperation rate. Subgroup analyses showed splenectomy did not increase the

survival rate for proximal and whole gastric cancer. No obvious differences were observed between the 2 groups when stratified by stage. Sensitivity analyses indicated no significant differences regarding the survival rates ( $P > 0.05$ ).

**CONCLUSION:** Splenectomy did not show a beneficial effect on survival rates compared to splenic preservation. Routinely performing splenectomy should not be recommended.

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**Key words:** Gastric cancer; Splenectomy; Survival rate; Morbidity; Operative surgical procedure; Postoperative period; Treatment outcome

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Yang K, Chen XZ, Hu JK, Zhang B, Chen ZX, Chen JP. Effectiveness and safety of splenectomy for gastric carcinoma: A meta-analysis. *World J Gastroenterol* 2009; 15(42): 5352-5359 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5352.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5352>

### INTRODUCTION

Gastric cancer is a disease with a high incidence. It is estimated that approximately 21 500 new cases of gastric carcinomas and 10 880 deaths would occur in the United States in 2008<sup>[1]</sup>. There has been a trend toward proximal gastric carcinoma in Western countries<sup>[2,3]</sup>. In proximal gastric and gastroesophageal junction cancers, lymph node metastases are found more frequently in the splenic hilum<sup>[4]</sup>.

Extended lymph node dissection is regarded as essential for the treatment of gastric cancer<sup>[5]</sup>. Splenectomy is performed for the purpose of effective lymph node dissection around the splenic artery and splenic hilum and for direct invasion of the splenic hilum or spleen; however, the effect of splenectomy on the prognosis is controversial. Previous reports suggested that gastrectomy

with splenectomy resulted in better survival than gastrectomy alone in gastric cancer patients<sup>[6,7]</sup>. Some investigators have reported that splenectomy did not increase the survival rate<sup>[8-10]</sup>. In addition, the importance of the spleen as a part of the immune system and the immunological consequences of its removal have recently been stressed<sup>[11,12]</sup>.

However, recent clinical trials showed that gastrectomy with splenectomy could result in higher postoperative morbidity and mortality<sup>[13-15]</sup>.

The aim of this meta-analysis was to evaluate the impact of splenectomy on long-term survival of gastric cancer patients and to compare the postoperative morbidity and mortality of patients undergoing splenectomy with that of patients not undergoing splenectomy at the time of gastrectomy.

## MATERIALS AND METHODS

### Search strategy and study selection

We searched the electronic databases of PubMed (<http://www.ncbi.nlm.nih.gov/sites/entrez/>), the Cochrane Central Register of Controlled Trials ([http://www.mrw.interscience.wiley.com/cochrane/cochrane\\_clcentral\\_articles\\_fs.html](http://www.mrw.interscience.wiley.com/cochrane/cochrane_clcentral_articles_fs.html)), the J-STAGE Database (Japan Science and Technology Information Aggregator, Electronic) (<http://www.jstage.jst.go.jp/browse/>), and CBMdisc (Chinese Biomedical Database) (<http://dlib.edu.cnki.net/kns50/Navigator.aspx?ID=1>). Other websites and conference proceedings were searched, including those of the National Cancer Institute (<http://www.cancernet.nci.nih.gov/cancertopics>), the European Organization for Research and Treatment of Cancer (<http://www.eortc.be/>), the Southwest Oncology Group (<http://www.swog.org/>), ClinicalTrials.gov (<http://clinicaltrials.gov/>), the American Society of Clinical Oncology (<http://www.asco.org/portal/site/ASCO>). Moreover, the reference lists from relevant articles were screened for study inclusion. Eligible unpublished papers were also considered to be included, if known from consultation with Prof. Chen ZX and Prof. Chen JP.

The search strategy of Medline was as follows and was applied to other databases also: ["Stomach Neoplasms" (Mesh) AND "Carcinoma" (Mesh)] AND ["splenectomy" (MeSH) OR "spleen dissection" (textword) OR "spleen resection" (textword) OR "splenic preservation" (textword)] AND ["Comparative Study" (Publication Type) OR "follow-up studies" (Mesh) OR "Clinical Trial" (Publication Type) OR "Evaluation Studies" (Publication Type) OR "Multicenter Study" (Publication Type) OR "Random allocation" (Subheading) OR "Randomized Controlled Trial" (Publication Type/subheading) OR "Controlled Clinical Trial" (Publication Type) or "Research design" (Subheading)]. The electronic search was up to December, 2008 with no limitations regarding publication date and language.

### Inclusion and exclusion criteria

Only randomized controlled trials (RCTs) which compared the effectiveness or safety of splenectomy to

those of non-splenectomy were eligible.

The patients had been confirmed with gastric carcinoma by endoscopy and biopsy preoperatively. There was no limitation in the location of the gastric carcinoma and surgical procedure. There was no distant metastasis, the primary tumors were resectable, and the patients could tolerate the operation. Patients treated with chemotherapy, immunotherapy, *etc* perioperatively were included. There was no limitation in age, gender and race. Patients with splenectomy induced by iatrogenic injury were included because of the small number. Curative or palliative gastrectomies were included, but patients with other kinds of gastric tumors, such as lymphoma, other organ tumors or multiple gastric tumors (i.e. adenosquamous carcinoma) were excluded. Trials with uncertain or marked inequality of characteristics between groups at baseline were excluded.

### Selection, assessment and data extraction

In order to select studies for further assessment, 2 independent reviewers (Yang K, Zhang B) screened the title, abstract section and keywords of every record retrieved. Full articles were assessed if the information given suggested that the study conformed to our criteria described above. The final selection of studies was completed by 2 researchers (Yang K, Chen XZ). Any disagreements in quality assessment and data collection were discussed and resolved by a third reviewer (Hu JK) as the referee.

Data was extracted independently by 2 reviewers. Details of study sample (number in each arm), interventions (the details of splenectomy, as approach, as well as details of other treatments, such as adjuvant chemotherapy, immunotherapy, *etc*) and outcomes (5-year overall survival rate, postoperative mortality and morbidity and operation-related events) were extracted. Additionally, the year and country of study, the number and reason of withdrawals and dropouts and characteristics of patients were extracted.

If only survival curves were reported, the overall 5-year survival rates were extracted and converted from the figures as accurately as possible<sup>[16]</sup>.

When the trials had reported medians and ranges instead of means and standard deviations, we assumed medians were equal to means, and equated standard deviation to a quarter of the reported range. If neither a range nor any other measure of dispersion was reported, half of the mean or the median as standard deviation was used<sup>[17]</sup>.

Seven items relevant to the quality appraisal were used for assessment<sup>[18]</sup>: (1) whether the method of allocation was truly random; (2) whether there was proper concealment of allocation; (3) whether there was equality between the 2 groups at baseline in terms of prognostic features; (4) whether the eligibility criteria were described; (5) whether blinding of the outcome assessors was performed; (6) whether loss to follow-up in each treatment arm was demonstrated, and (7) whether intention-to-treat analysis was considered. Seven or 6 items were required for a trial to be rated as high quality, 5 or 4 items as fair quality and 3 or fewer as low quality<sup>[18]</sup>.

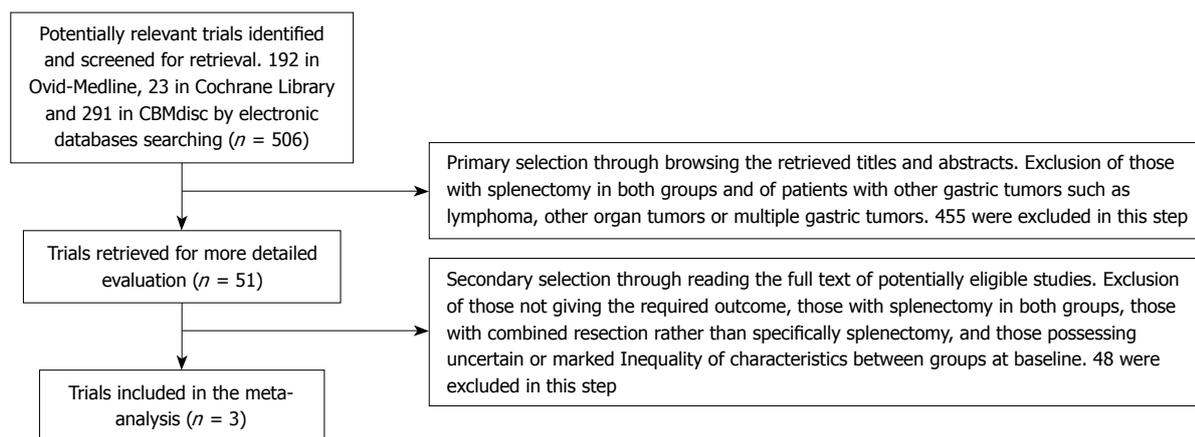


Figure 1 Flow chart showing study selection procedure.

Table 1 The characteristics of the included randomized trials

Study	Participants	Interventions	Outcomes
Csendes <i>et al</i> <sup>[63]</sup> , 2002	187 patients with gastric carcinoma entered this study. 97 patients with total gastrectomy and 90 patients with total gastrectomy and splenectomy	Total gastrectomy <i>vs</i> total gastrectomy plus splenectomy. The follow-up was at least 5 years	Five-year overall survival and survival by stage. Postoperative morbidity and mortality. Kaplan-Meier survival curve.
Toge <i>et al</i> <sup>[6]</sup> , 1985	The patients underwent total gastrectomy and had the main location of the tumor on lesser curvature region. They were divided into 2 groups at random: 41 in splenectomy (+) and 38 in splenectomy (-) groups	Splenectomy <i>vs</i> splenic preservation. The follow-up was at least 5 years	Duration of operation and hospital stay Kaplan-Meier survival curve. 5-year overall survival were from reported percentages data
Yu <i>et al</i> <sup>[64]</sup> , 2006	A total of 216 patients with proximal gastric cancer were randomized. 103 patients had the spleen preserved and 104 had a splenectomy	Splenectomy <i>vs</i> splenic preservation. Of the 207 patients, 7 were lost to follow-up (follow-up rate 96.6%) and mean duration of follow-up was 5.4 years	Harvested lymph nodes. Postoperative morbidity and mortality. Kaplan-Meier survival curve. 5-year overall survival were from reported percentages data

### Outcomes of interest and definitions

The primary outcome measures were 5-year overall survival rate, overall hospital or postoperative 30 d mortality, and overall morbidity rate, while the secondary outcome measure was operation-related events, the number of harvested lymph nodes, operation time, length of hospital stay and reoperation rate. One or more outcome measures should be available in the trials, or they were excluded.

### Statistical analysis

Weighted estimates of relative risks (RR) and weighted mean differences (WMD) with 95% confidence intervals (CI) were calculated for dichotomous data and continuous data respectively. The analyses were conducted using RevMan 4.3. A *P*-value < 0.05 was considered as statistically significant. Heterogeneities of treatment effect between trials were tested using a Chi-squared statistic with significance being set at *P* < 0.10, and the total variation across studies was estimated by I-square and divided into low, moderate and high levels, corresponding to the I-square of < 25%, 25%-50%, and > 50%<sup>[19]</sup>. If heterogeneities existed, one of the following techniques was undertaken to attempt to explain them: 1. Random effect model for meta-analyses; 2. Sub-group analyses; 3. Sensitivity analyses. Subgroup analyses stratified by the location of tumor and stage of tumor were performed.

Sensitivity analyses were performed only in high quality trials to avoid errors caused by poor quality studies<sup>[20]</sup>.

## RESULTS

### Included literature

There were 506 papers found in total (192 in Medline, 23 in Cochrane Library, 291 in CBMdisc, no new findings in other databases) and the selection was performed according to the inclusion/exclusion criteria stated above. Four hundred and fifty five trials were excluded in the primary selection through browsing the retrieved titles and abstracts and 48 trials<sup>[7-10,14-15,21-62]</sup> were excluded in the secondary selection through reading the full texts of potentially eligible studies. The flow chart of study selection is summarized in Figure 1.

Only 3 RCT trials<sup>[6,63,64]</sup> comparing the effectiveness and safety of splenectomy with gastrectomy to gastrectomy alone in patients with histologically proven gastric adenocarcinoma met the inclusion criteria. The details of included trials are listed in Tables 1 and 2.

A total of 466 patients were available for analysis, with 231 patients assigned treatment with gastrectomy plus splenectomy (treatment arm).

### Effectiveness

In this part, we used the number of patients alive as the

**Table 2** The quality of the included randomized trials

Study	Truly random	Concealed allocation	Baseline features	Eligibility criteria	Blinding assessment	Loss to follow-up	Intention to treat	Study quality
Csendes <i>et al</i> <sup>[63]</sup> , 2002	Yes	Unclear	Yes	Yes	Unclear	Yes	No	Fair
Toge <i>et al</i> <sup>[6]</sup> , 1985	Unclear	Unclear	No	No	Unclear	Unclear	Unclear	Poor
Yu <i>et al</i> <sup>[64]</sup> , 2006	Yes	Unclear	Yes	Yes	Unclear	Yes	Unclear	Fair

**Table 3** Outcomes of a meta-analysis of overall survival rates, safety, operation-related events and overall survival rates stratified by location of tumor

	No. of studies	Splenectomy (n <sup>1</sup> /N)	Splenic preservation (n <sup>1</sup> /N)	RR/WMD (95% CI)	P-value for effect size	P-value for heterogeneity	Effect model
Overall survival rate stratified by different length of follow-up	3	122/231	105/235	1.17 (0.97, 1.41)	0.1	0.85	Fixed
Postoperative morbidity and mortality							
Morbidity	1	16/104	9/103	1.76 (0.82, 3.80)	0.15	NA	Fixed
Mortality	2	6/194	4/200	1.58 (0.45, 5.50)	0.47	0.82	Fixed
Operation-related events							
No. of harvested lymph nodes	1	104 <sup>2</sup>	103 <sup>2</sup>	0.00 (-6.06, 6.06)	1	NA	Fixed
Operation time (min)	1	90 <sup>2</sup>	97 <sup>2</sup>	10.00 (-14.37, 34.37)	0.42	NA	Fixed
Length of hospital stay (d)	1	90 <sup>2</sup>	97 <sup>2</sup>	3.20 (-1.60, 8.00)	0.19	NA	Fixed
Reoperation	1	10/90	9/97	1.20 (0.51, 2.81)	0.68	NA	Fixed
Overall survival rate stratified by location of tumor (proximal and whole stomach)	2	93/190	84/197	1.14 (0.92, 1.41)	0.23	0.91	Fixed

<sup>1</sup>Represents the patients alive; <sup>2</sup>The summed number of patients in each group; RR: Relative risk; WMD: Weighted mean differences; CI: Confidence interval; NA: Not applicable.

number of events. The meta-analyses of trials showed that gastrectomy with splenectomy had no significant difference from splenic preservation on the 5-year overall survival rate, with RR of 1.17 (Table 3, Figure 2). The location and stage of the tumor had a major effect on the need for splenectomy to allow adequate hilar lymphadenectomy<sup>[10]</sup>. Thus we performed subgroup analyses with stratification by the 2 factors.

In the subgroup analyses, we also found that, for proximal and whole gastric cancer, splenectomy could not facilitate prolongation of survival. The RR of the 5-year overall survival rate was 1.14, which indicated splenectomy had no significant influence on survival rate compared to splenic preservation for proximal and whole gastric cancer (Table 3, Figure 2).

Then we analyzed the overall survival rate stratified by stage. Because of the limited number of included trials in this step, only one RCT<sup>[63]</sup> was used. The 5-year overall survival rates of patients with stage I, stage II and stage III in this RCT<sup>[63]</sup> were not significantly different between the 2 groups (all *P*-values > 0.05).

**Safety**

There was no clear and significant excess morbidity or mortality in the splenectomy group, with RR of 1.76 and 1.58 respectively, suggesting that postoperative morbidity and mortality did not occur more than in patients with splenic preservation. (Table 3, Figure 3).

**Operation-related events**

Non-significantly more lymph nodes were excised from patients undergoing splenectomy (WMD = 0.00 nodes). Operative time and length of hospital stay were not

significantly longer in the splenectomy group (WMD = 10.00 min and 3.20 d). There was no difference in reoperation rate between the 2 groups (RR = 1.20, Table 3).

**Sensitivity analysis**

The results of the sensitivity analysis, after excluding trials of low quality, are shown in Table 4. No significant differences were observed between the 2 arms in terms of the 5-year overall survival rate, and postoperative morbidity and mortality (RR = 1.14, 1.76 and 1.58, respectively).

**DISCUSSION**

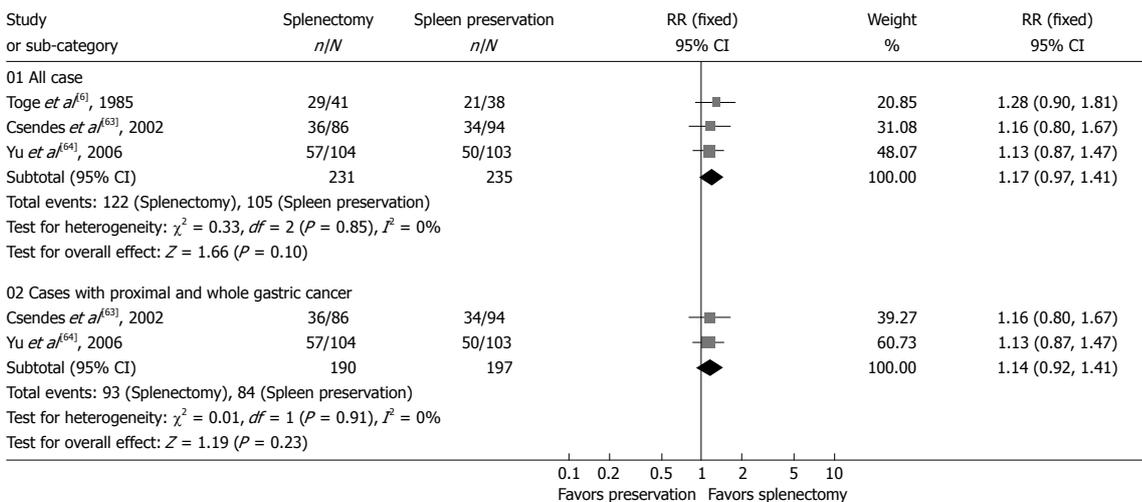
The incidence of proximal gastric cancers has increased<sup>[2,3]</sup>. Lymphography has demonstrated that the lymphatic flow from the left upper region of the stomach enters the lymph node in the splenic hilum and travels to the nodes around the celiac trunk along the splenic artery<sup>[65]</sup>. Thus it appears that splenectomy is more often performed for proximal gastric cancers<sup>[51]</sup>, and for a curative gastrectomy it is necessary to dissect the lymph nodes in the splenic hilum and the lymph nodes along the splenic artery. The frequency of metastasis to lymph nodes at the splenic hilum or along the splenic artery, which is associated with stage and tumor location, reportedly ranges from 8% to 10%<sup>[4,66]</sup>. Splenectomy has been recommended to facilitate lymph node dissection. Direct invasion of the spleen by gastric carcinoma is an exception requiring splenectomy<sup>[25]</sup>. The possibility that splenectomy could increase the survival rate of patients with gastric cancer has attracted much attention. Some prospective randomized controlled trials and retrospective analyses have been done or are

**Table 4 Sensitivity results of meta-analysis of overall survival rates and safety (excluding the trial with low quality)**

	No. of studies	Splenectomy (n <sup>1</sup> /N)	Splenic preservation (n <sup>1</sup> /N)	RR/WMD (95% CI)	P-value for effect size	P-value for heterogeneity	Effect model
Overall survival rate stratified by different length of follow-up	2	93/190	84/197	1.14 (0.92, 1.41)	0.23	0.91	Fixed
Postoperative morbidity and mortality							
Morbidity	1	16/104	9/103	1.76 (0.82, 3.80)	0.15	NA	Fixed
Mortality	2	6/194	4/200	1.58 (0.45, 5.50)	0.47	0.82	Fixed

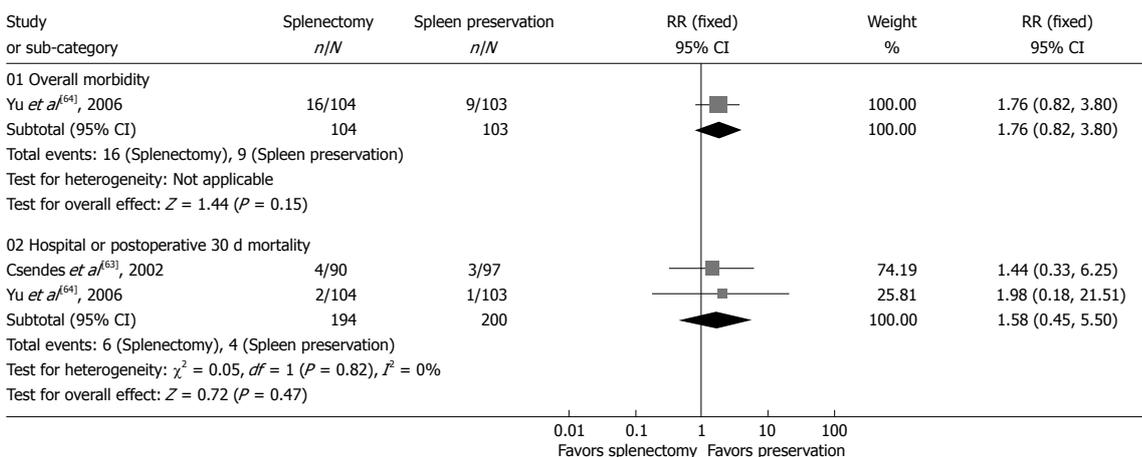
<sup>1</sup>Represents the patients alive; NA: Not applicable.

Review: Effectiveness and safety of splenectomy for gastric carcinoma: a meta-analysis  
 Comparison: 01 Survival  
 Outcome: 02 5-yr overall survival



**Figure 2 Survival rate.** Forest plot of RR of 5-year overall survival rate for all cases and cases with proximal and whole gastric carcinoma, with 95% CI. Data for a fixed-effects model are shown as there was no statistical heterogeneity.

Review: Effectiveness and safety of splenectomy for gastric carcinoma: a meta-analysis  
 Comparison: 02 Safety  
 Outcome: 01 Overall morbidity & hospital or postoperative 30 d mortality



**Figure 3 Morbidity and mortality.** Forest plot of RR of postoperative morbidity and mortality, with 95% CI. Data for a fixed-effects model are shown as there was no statistical heterogeneity.

ongoing<sup>[67]</sup>, but the main results are controversial.

When we searched for trials for the meta-analysis, we found that the search results consisted mostly of retrospective analyses with a high level of heterogeneity. From these retrospective analysis, we could see in those

who underwent gastrectomy with splenectomy, the tumor was larger, the lesion was more commonly present in the upper stomach, grossly types 3 and 4 infiltration lesions were more frequent, depth of serosa invasion was greater, the rate of lymph node involvement was higher,

and advanced stages were more frequent. Furthermore, in many retrospective papers, distal gastrectomy (naturally without splenectomy) for distal cancer and total gastrectomy with splenectomy for advanced proximal cancer were simply compared without any adjustment. So we excluded these kinds of trials for a meaningful result. With respect to the 5-year overall survival rate, our results failed to suggest that splenectomy could result in greater benefit to the patients. When stratified by proximal and whole gastric cancer, a similar result was observed. In the sensitivity analysis, after we excluded the trials of low quality, no significant differences could be detected in 5-year overall survival rates for all the cases or for cases with proximal and whole gastric cancer.

Although we included only RCTs to guarantee the reliability and validity of the results, the eligible number of patients was far too small. So we carefully selected some non-RCTs with good balanced baseline characteristics for a meta-analysis. The quality of non-randomized studies was assessed by using the Newcastle-Ottawa Scale<sup>[68]</sup> with some modifications to match the needs of this study. The quality of the studies was evaluated by examining patient selection methods, comparability of the study groups and assessment of outcome. Finally we included 7 non-RCTs with 895 patients available for analysis (453 patients were treated by splenectomy). This analysis also showed splenectomy had no significant influence on survival rates compared to splenic preservation for all the cases and for patients with proximal and whole gastric cancer, with an OR of 0.77 (95% CI: 0.57-1.04) and 0.54 (95% CI: 0.24-1.23). In the splenectomy group postoperative morbidity (OR = 3.75, 95% CI: 2.69-5.23), rather than mortality (OR = 1.38, 95% CI: 0.12-16.35), occurred more than that of splenic preservation. Based on the above results, we found splenectomy could show a trend for survival in randomized trials, and the data from non-RCTs showed an opposite trend. This discrepancy in the overall survival and morbidity between RCTs and non-RCTs may derive from the relatively uncertain quality of the non-RCTs although the included trials had balanced baselines. This arises because, in non-RCTs, splenectomy was selected for gastric cancer patients with more advanced tumors, while the spleen was preserved in earlier stage cancers. Also, in the included non-RCTs, gastric cancer requiring splenectomy was usually more extensive or originated from the gastric body. The majority were histologically diffuse type, while tumors treated by distal gastrectomy were more commonly intestinal type and had better prognosis. Furthermore, the extent of lymphadenectomy, the type of gastrectomy, the other organs resected, *etc* would affect the outcome. With respect to the availability of relatively few high quality RCTs, more well-designed RCTs are needed to explore the effectiveness of splenectomy, especially for proximal and whole gastric cancer.

Whether splenectomy could increase the survival rate in patients with lymph node metastasis at the splenic hilum or along the splenic artery, there is too little evidence. One randomized controlled trial<sup>[64]</sup> reported no patients could survive for 5 years if lymph nodes at the hilum of the spleen were positive, and the 5-year

survival rate of positive lymph node metastasis along the splenic artery in the splenectomy arm or splenic preservation arm were 23.4% and 20.0%, respectively ( $P = 0.753$ ). Zhang *et al*<sup>[52]</sup> found that splenectomy did not show superiority to splenic preservation in patients with positive No. 10 and No. 11 lymph nodes ( $P = 0.284$ ). Kodera *et al*<sup>[39]</sup> reported that in patients who had histological evidence of metastasis to the splenic hilar nodes or the nodes along the splenic artery, pancreaticosplenectomy or splenectomy did not result in improved survival. As yet, there is no evidence to support that splenectomy could increase the survival rates of patients with metastasis to the lymph nodes at the splenic hilum or along the splenic artery.

Regarding the survival rates by stage, the included analyzable trials were too few. From previous reports<sup>[58,63]</sup>, no obvious differences were observed between the 2 groups. Here, we also should note that there were distinct methods for staging at the different periods; furthermore, differences between UICC (Union Internationale Contre le Cancer) and Japanese gastric cancer parameters existed. Thus more unified trials should be done for future evaluation.

In addition, the spleen is an important component of the reticuloendothelial system and constitutes 25% of the total lymphoid mass<sup>[69]</sup>. There was a 12-fold increased risk of septicemia compared with the general population after splenectomy<sup>[63]</sup>. On the other hand, the role of the spleen in tumor immunology is still controversial<sup>[70]</sup>. Therefore the indication for splenectomy is debatable.

Recent European clinical trials of gastrectomy showed that splenectomy was an important risk factor for postoperative morbidity and mortality<sup>[13-15]</sup>. The common complications after splenectomy were pancreatitis, pleural effusion, abdominal abscess, wound infection, pancreatic leakage, ileus and anastomotic leakage<sup>[57]</sup>. Splenectomy could easily induce gastric remnant ischemia, possibly contributing to the high frequency of anastomotic leakage and mortality<sup>[25]</sup>. Resection of proximal gastric cancer was associated with a higher postoperative morbidity than that of distal gastric cancer, and splenectomy was more often performed for proximal gastric cancers<sup>[71,72]</sup>. However, our results failed to go against splenectomy in terms of postoperative morbidity and mortality. At the same time, with respect to the operation-related events, splenectomy showed no significant difference from splenic preservation in harvested lymph nodes, operation time, length of hospital stay and reoperation rate. All in all, as there were limitations in the trial quality and numbers of included trials, more high quality studies are needed.

In conclusion, splenectomy has not yet shown superiority on survival rates compared to splenic preservation. Routinely performing splenectomy should not be recommended and well-designed large-scale RCTs are required.

## COMMENTS

### Background

Splenectomy is performed for the purpose of effective lymph node dissection around the splenic artery and splenic hilum and for direct invasion of the

splenic hilum or spleen in gastric cancer; however, the effect of splenectomy on prognosis has been controversial.

### Research frontiers

Some studies compared the effectiveness or safety of splenectomy to those of non-splenectomy, but the main results were controversial. The aim of this meta-analysis was to evaluate the impact of splenectomy on long-term survival, postoperative morbidity and mortality of patients with gastric cancer.

### Innovations and breakthroughs

The current study demonstrated that splenectomy could not yet show superiority on survival rates compared to splenic preservation.

### Applications

Routinely performing splenectomy should not be recommended in gastric cancer surgery. However, well-designed large-scale RCTs are expected to investigate the effectiveness and safety of splenectomy further.

### Peer review

This is an interesting article in a controversial area. An important meta-analysis that will contribute to the literature.

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CASE REPORT

## Liver abscess and sepsis with *Bacillus pantothenicus* in an immunocompetent patient: A first case report

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### INTRODUCTION

*Bacillus* species are aerobic, or facultatively anaerobic, gram-positive or gram-variable spore-forming rods. They are ubiquitous in the environment and are found in water, dirt, air, stools, and plant surfaces. They are relatively resistant to heat, due to spore formation, and they therefore grow easily during the storage of food and produce toxins that can cause food poisoning<sup>[1-3]</sup>. The pathogenicity of *Bacillus anthracis* (*B. anthracis*) is well known in mammals. Nonanthrax species within clinical materials, which were previously considered contaminants, have increasingly been identified as pathogens<sup>[4]</sup>. One of the nonanthrax species, *B. cereus* is reported in most cases as a human pathogen<sup>[4]</sup>. Occasional reports have appeared implicating other nonanthrax species, for example, *B. thuringiensis*, *B. alvei*, *B. circulans*, *B. licheniformis*, *B. macerans*, *B. pumilus*, *B. sphaericus*, and *B. subtilis* in systemic and gastrointestinal diseases<sup>[3,4]</sup>. However, *B. pantothenicus* has not been reported as a human pathogen in any English-language literature since 1950. Here, we report a case of liver abscess and sepsis caused by *B. pantothenicus* in an immunocompetent man who was successfully treated with cefotaxime and Netilmicin, followed by oral ciprofloxacin.

### Abstract

*Bacillus* species are aerobic, gram-positive, spore forming rods that are usually found in the soil, dust, streams, and other environmental sources. Except for *Bacillus anthracis* (*B. anthracis*), most species display low virulence, and only rarely cause infections in hosts with weak or damaged immune systems. There are two case reports of *B. cereus* as a potentially serious bacterial pathogen causing a liver abscess in an immunologically competent patient. We herein report a case of liver abscess and sepsis caused by *B. pantothenicus* in an immunocompetent patient. Until now, no case of liver abscess due to *B. pantothenicus* has been reported.

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**Key words:** *Bacillus pantothenicus*; *Bacillus species*; Immunocompetence; Liver abscess and sepsis

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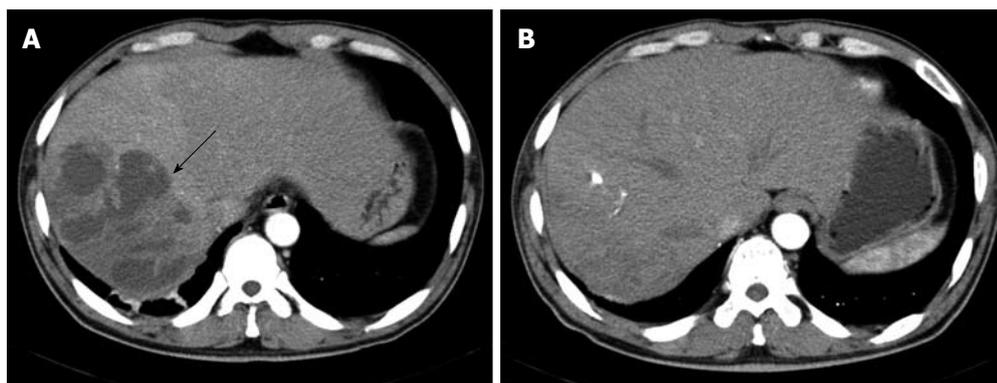
Na JS, Kim TH, Kim HS, Park SH, Song HS, Cha SW, Yoon HJ. Liver abscess and sepsis with *Bacillus pantothenicus* in an immunocompetent patient: A first case report. *World J*

### CASE REPORT

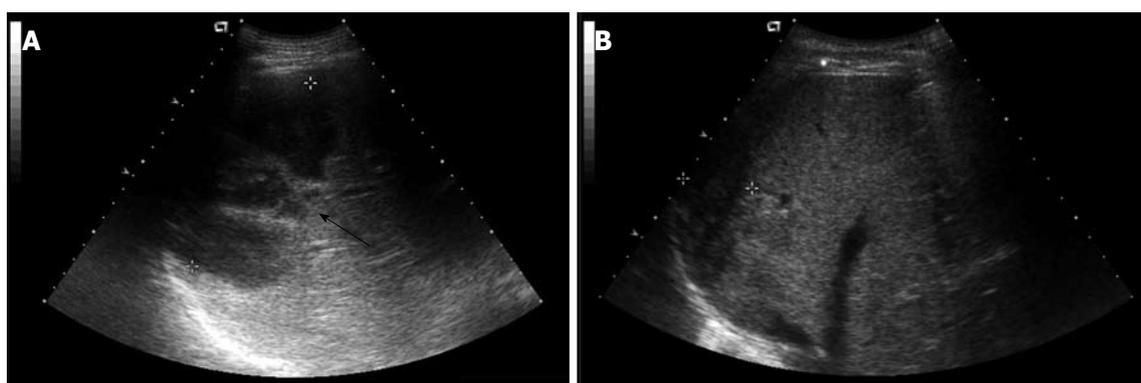
The patient, a 44-year-old man, was admitted to hospital complaining of high fever (40.2°C) and abdominal discomfort in right upper quadrant. A week before admission, he had been on a bicycle trip for two days alone and had eaten raw saltwater fish, which he had cleaned and cut himself. His past medical history was unremarkable.

His physical examination showed a body temperature of 40.2°C, blood pressure of 170/90 mmHg, a respiration rate of 20 breaths/min and a heart rate of 106 beats/min. He had tenderness on the right upper quadrant of his abdomen. Other findings were unremarkable.

Laboratory studies were as follows: hemoglobin concentration 12.6 g/dL; hematocrit 35.65%; leukocytes 16650/mm<sup>3</sup> (granulocytes 82.7%, lymphocytes 9.6%, and



**Figure 1 Contrast-enhanced computed tomography (CT) of the liver.** A: Abdominal CT scan showing a multi-loculated liver abscess (12 cm × 9 cm) in the posterior inferior (VI segment) and posterior superior segment of the right lobe (VII segment) of the liver (arrow); B: On hospital day 30, the liver abscess was almost absorbed.



**Figure 2 Abdominal ultrasonography (US) of the abdomen.** A: Abdominal US showing liver abscesses (12 cm × 9 cm) in segment 6 and 7, with multiple satellite pockets (arrow); B: Post-inflammatory change with completely healed abscess pocket after 51 d of treatment.

monocytes 5.0%); platelets 435 000/mm<sup>3</sup>; erythrocyte sedimentation rate 120 mm/h; C-reactive protein (CRP) 27.72 mg/dL; aspartate aminotransferase 121 IU/L; alanine aminotransferase 164 IU/L;  $\gamma$ -glutamyl transpeptidase 284 IU/L; alkaline phosphatase 397 IU/L; total bilirubin 1.5 mg/dL. Serologic assays for hepatitis A, B and C were negative. All other laboratory values were normal. An abdominal computed tomography (CT) scan revealed a multi-loculated liver abscess (12 cm × 9 cm) in the posterior inferior (VI segment) and posterior superior segment of the right lobe (VII segment) of the liver (Figure 1A and Figure 2A). After blood samples had been drawn for culture, empirical antibiotic therapy with cefotaxime, Netilmicin, and metronidazole was started. Two days later, a percutaneous drainage was performed. A purulent and odorous material was obtained, and it was immediately inoculated into aerobic and anaerobic culture material.

On the 6th hospital day, the results of blood cultures showed *B. pantothenicus* in all six blood sets that were sensitive to ampicillin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, oxacillin, teicoplanin, tetracycline, and vancomycin. In addition, amoeba antibodies were negative. Therefore, we discontinued metronidazole and maintained cefotaxime and Netilmicin. The culture of purulent material at drainage showed no growth of bacteria. On the 30th hospital day, a follow up CT scan was performed, which showed no abscess pocket (Figure 1B). Follow-up blood cultures grew no bacteria and he was discharged with oral ciprofloxacin. The patient has been followed up with control

visits and has shown no sign of a recurrence. Abdominal ultrasonography after 51 d of treatment showed a completely healed abscess pocket and remaining post-inflammatory changes (Figure 2B).

## DISCUSSION

*Bacillus* species, except for *B. anthracis*, rarely cause serious human infections. Occasionally, *Bacillus* species can be pathogens in certain clinical situations (e.g. patients with intravenous drug abuse, trauma, cancer, neutropenia, leukemia, receiving chemotherapy or indwelling central venous catheter) but are not usually pathogenic to immunocompetent individuals<sup>[5-7]</sup>. Clinical infections caused by *Bacillus* species (probably *B. cereus* in most cases) fall into six broad groups: (1) local infections, particularly of burns, traumatic or post-surgical wounds, and the eye; (2) bacteremia and septicemia; (3) central nervous system infections, including meningitis, abscesses, and shunt-associated infections; (4) respiratory infections; (5) endocarditis and pericarditis; and (6) food poisoning, characterized by toxin-induced emetic and diarrheagenic syndromes<sup>[2]</sup>.

*B. pantothenicus* was first described by Proom & Knight, following a nutritional analysis of mesophilic soil isolates of *Bacillus* species<sup>[8]</sup>. It was named because this strain required pantothenic acid to isolate it from different soil samples. They considered it to a species most closely resembling *B. circulans* but distinct from it, and subsequent studies confirmed the validity of the species<sup>[9-11]</sup>. Later isolations have been made from antacids, food, water, and

soil<sup>[9]</sup>. In the present case, the raw fish that the patient had eaten might have been contaminated with *B. pantothenicus*.

Organisms recovered from liver abscesses vary with the etiology. In liver infection arising from the biliary tree, enteric gram-negative aerobic bacilli and *enterococci* are common isolates<sup>[12]</sup>. Unless previous surgery has been performed, anaerobes are not generally involved in liver abscesses arising from biliary infections. In contrast, in liver abscesses arising from pelvic and other intra-peritoneal sources, a mixed flora, including aerobic and anaerobic species (especially *Bacteroides fragilis*), is common<sup>[12]</sup>. With hematogenous spread of infection, usually only a single organism is encountered; this species may be *Staphylococcus aureus* or a *streptococcal* species, such as *Streptococcus milleri*. Liver abscesses may also be caused by *Candida* species<sup>[12]</sup>. Amoebic liver abscesses remain a common clinical finding<sup>[13]</sup>.

We conducted a thorough keyword search for other cases of liver abscess due to *B. pantothenicus* in the web-based Medline database, but found none. However, there were two cases of liver abscess due to *B. cereus*. In one case, the patient was in good health<sup>[13]</sup>, but was admitted to hospital because of fever and, on CT scan, a liver abscess was shown. The treatment was started by empirical antibiotics and surgically drained, but the patient died on the 4th postoperative day due to acute peritonitis. *B. cereus* was isolated from a culture of the purulent material<sup>[13]</sup>. In the second case, the patient was diagnosed as Philadelphia chromosome-positive acute lymphoblastic leukemia (ALL) two years previously and admitted to hospital because of relapse of ALL<sup>[14]</sup>. *B. cereus* was isolated on blood culture. Multiple abscesses were found in the brain by magnetic resonant imaging (MRI) and in the liver by abdominal ultrasonography and computed tomography. *B. cereus* is susceptible to Minocycline, vancomycin, levofloxacin, and chloramphenicol and therefore they were administered to the patient. One month later, the multiple liver abscesses had disappeared but the patient died four months later due to deteriorated leukemia<sup>[14]</sup>.

The isolation of *Bacillus* species from blood cultures is clinically significant in 5%-10% of cases<sup>[15]</sup>. In the present case, *B. pantothenicus* was grown in six sets of blood cultures from different veins, with 30 min intervals between sets; thus we assume it is the true pathogen. The reason of for non-growth of *B. pantothenicus* in the purulent discharge might be the previous use of antibiotics before drainage.

Weber *et al*<sup>[16]</sup> tested 54 *B. cereus* isolates and 35 non-*cereus Bacillus* isolates for antimicrobial susceptibility and found almost uniform susceptibility to vancomycin, imipenem, gentamicin, and ciprofloxacin. Most non-*cereus Bacillus* isolates were susceptible to penicillins and cephalosporins, but almost *B. cereus* isolates were resistant to penicillins or cephalosporins, due to the presence of beta-lactamase<sup>[2,16]</sup>. Therefore, vancomycin appears to be the best treatment for *Bacillus* species infection, before the results of specific susceptibility are available<sup>[16]</sup>. However, *B. cereus* is also susceptible to clindamycin, gentamicin, chloramphenicol, and erythromycin<sup>[2,7,17-19]</sup>. In our case, empirical antimicrobial treatment with cefotaxime, Netilmicin and metronidazole for liver abscess was started before *B. pantothenicus* was iso-

lated in blood culture. After *B. pantothenicus* was identified, we discontinued metronidazole. We continued cefotaxime and Netilmicin, as most non-*cereus Bacillus* isolates are susceptible to cephalosporins. *B. pantothenicus* showed sensitivity to gentamicin and most importantly, the patient's clinical and laboratory course was improving with these antibiotics. Pyogenic liver abscesses are usually treated parenterally for two to three weeks, and for the following four to six weeks with oral agents. The patient's clinical response and follow-up imaging should be monitored to determine his response to therapy, for consideration of antibiotic duration and the need for further aspiration<sup>[12]</sup>. In this case, clinical and laboratory parameters became normalized and follow-up sonography showed complete healing of the abscess; therefore, we could stop antibiotics on the 51th hospital day.

In conclusion, we report the first case of liver abscess and sepsis caused by *B. pantothenicus* in an immunocompetent patient. The patient was successfully treated with a course of cefotaxime, Netilmicin, and metronidazole, followed by oral ciprofloxacin. This case demonstrates that unusual organisms can lead to unexpected and severe infections in patients who are previously healthy and have no obvious risk factors. It is important to consider unusual organisms as a cause of systemic infections and arrange appropriate microbiological investigations so as not to miss the clinical diagnosis.

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CASE REPORT

## Novel mutations in the *STK11* gene in Thai patients with Peutz-Jeghers syndrome

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### Abstract

Peutz-Jeghers syndrome (PJS), a rare autosomal dominant inherited disorder, is characterized by hamartomatous gastrointestinal polyps and mucocutaneous pigmentation. Patients with this syndrome have a predisposition to a variety of cancers in multiple organs. Mutations in the serine/threonine kinase 11 (*STK11*) gene have been identified as a major cause of PJS. Here we present the clinical and molecular findings of two unrelated Thai individuals with PJS. Mutation analysis by Polymerase Chain Reaction-sequencing of the entire coding region of *STK11* revealed two potentially pathogenic mutations. One harbored a single nucleotide deletion (c.182delG) in exon 1 resulting in a frameshift leading to premature

termination at codon 63 (p.Gly61AlafsX63). The other carried an in-frame 9-base-pair (bp) deletion in exon 7, c.907\_915del9 (p.Ile303\_Gln305del). Both deletions were *de novo* and have never been previously described. This study has expanded the genotypic spectrum of the *STK11* gene.

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**Key words:** Peutz-Jeghers syndrome; Serine/threonine kinase 11; Novel mutations

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Ausavarat S, Leoyklang P, Vejchapipat P, Chongsrissawat V, Suphapeetiporn K, Shotelersuk V. Novel mutations in the *STK11* gene in Thai patients with Peutz-Jeghers syndrome. *World J Gastroenterol* 2009; 15(42): 5364-5367 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5364.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5364>

### INTRODUCTION

Peutz-Jeghers syndrome (PJS) is an autosomal dominant disorder with predisposition for cancer (OMIM 175200), and is characterized by the occurrence of multiple gastrointestinal hamartomatous polyps and pigmentation of the lips, buccal mucosa and digits<sup>[1]</sup>. PJS patients also have a significantly increased risk for developing benign and malignant tumors of multiple organs both in the gastrointestinal tract and in extragastrointestinal sites<sup>[2]</sup>. Cancers of the lung, pancreas, breast, ovary and testis have been observed<sup>[3-6]</sup>. Germline mutations in the serine/threonine kinase 11 (*STK11*) gene at 19p13.3 have been demonstrated to be responsible for most PJS cases<sup>[7, 8]</sup>. The *STK11* gene consists of nine coding exons and translates into a protein of 433 amino acids<sup>[8]</sup>. *STK11* is proposed to function as a tumor suppressor that acts as an early gatekeeper<sup>[9]</sup>, regulates cell polarity<sup>[10]</sup>, and controls cell cycle arrest, cell proliferation and apoptosis<sup>[11]</sup>.

PJS is the first cancer susceptibility syndrome caused

by inactivation of the serine/threonine kinase. Previous findings suggested that the combination of germline mutations and a defect of the second allele causing loss of heterozygosity in somatic cells was responsible for the phenotypic manifestations of PJS<sup>[8]</sup>.

At least 200 different disease-causing mutations in the *STK11* gene have been described with the majority being missense/nonsense mutations. The splice-junction alterations, insertions, and nucleotide or whole gene deletions have also been reported (<http://www.hgmd.cf.ac.uk>, accessed July 2009). Most *STK11* mutations affect the catalytic kinase domain and result in inactivation or loss of protein function.

In this study, we report the clinical and molecular characterization of two unrelated Thai PJS patients. Mutation analysis revealed that both patients carried *de novo* mutations. Both mutations were also novel. This is the first report of molecularly-confirmed PJS in Thai patients.

## CASE REPORT

### Patient 1

A 14-year-old Thai boy was referred for further management of intestinal obstruction. The patient developed abdominal pain with distension and melena 3 d after receiving appendectomy. He was diagnosed with intussusception for which he underwent surgical resection. Physical examination revealed hyperpigmentation on the lower lip, buccal mucosa, and digits. Endoscopic examination revealed multiple polyps in the stomach, small bowel and colon. No other family members had any features consistent with PJS.

### Patient 2

A 14-year-old Thai girl was referred for treatment of intestinal polyps. She presented with hematochezia and prolapse of a polypoid mass. Characteristic mucocutaneous pigmentation was also observed. Endoscopic examination demonstrated multiple polyps throughout the gastrointestinal tract. She underwent polypectomy three times. The resected polyps were found to have characteristic features of Peutz-Jeghers polyps. There was no history of gastrointestinal problems in either of her parents.

### Mutation analysis

Peripheral blood samples were obtained from the probands and their available parents after written informed consent. Total RNA and genomic DNA were extracted from peripheral blood using Qiagen RNA and DNA extraction kits according to manufacturer's instructions, respectively (Qiagen, Valencia, CA, USA). Reverse transcription was performed using ImProm-IITM reverse transcriptase (Promega, Madison, WI, USA), according to the manufacturer's instructions. PCR amplification of the entire coding sequence of the *STK11* gene was performed using primers as shown in Table 1. In brief, we used 50 ng of cDNA, 1XPCR buffer (Promega), 1.5 mmol/L MgCl<sub>2</sub>, 0.2 mmol/L dNTPs, 0.2 μmol/L of

**Table 1** Oligonucleotides and PCR conditions for *STK11* mutation analysis

	Primer sequences (5' to 3')	Annealing temperature (°C)
<i>STK11</i> cDNA	F GTCGGAACACAAGGAAGGAC R AACCGGCAGGAAGACTGAGG	60
<i>STK11</i> Exon 1	F GTCGGAACACAAGGAAGGAC R CAGAACCATCAGCACCGT GA	57
<i>STK11</i> Exon 7	F ATGTCCCAGGAGTGGAGTGG R ACAGGACACTGCCCA GAGAC	60

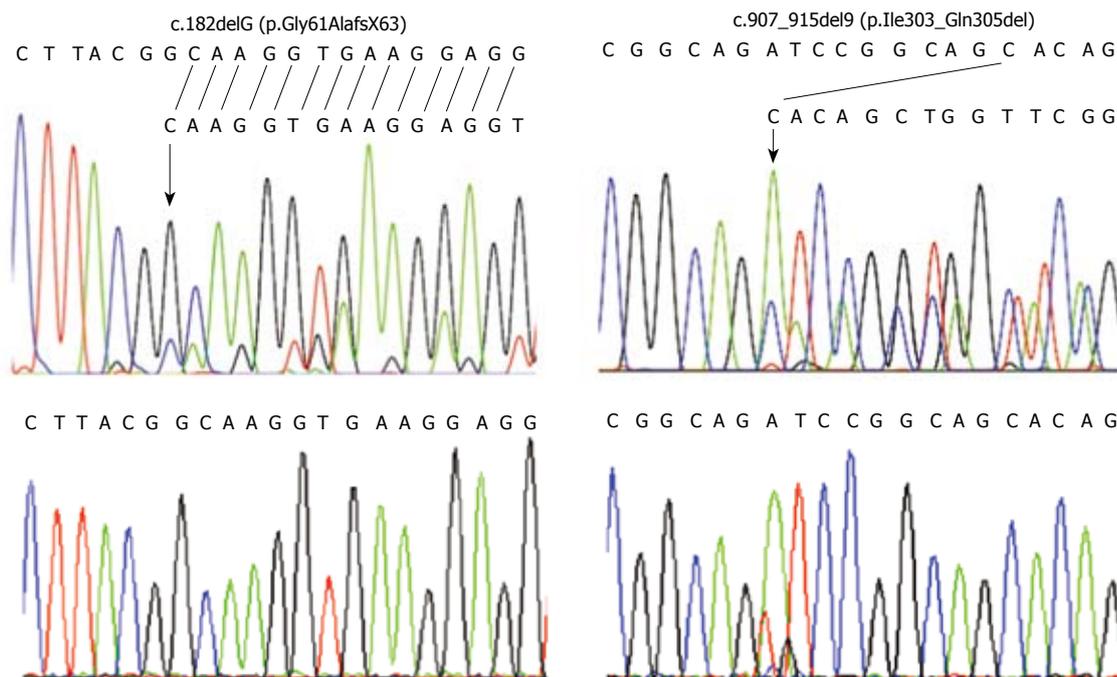
each primer and 0.5 U Taq DNA polymerase (Promega) in a volume of 20 μL using the following parameters: 35 cycles of 1 min at 94°C, 1 min at 60°C and 1 min 30 s at 72°C. PCR products were treated with ExoSAP-IT (USP Corporation, Cleveland, OH) according to the manufacturer's recommendations, and sent for direct sequencing (Macrogen Inc., Seoul, Korea). Sequence data were analyzed using Sequencher (version 4.2; Gene Codes Corporation, Ann Arbor, MI, USA). Mutations found in both patients were confirmed by direct sequencing of the genomic DNA using a set of primers and parameters according to their mutation sites (Table 1). The nucleotide position is in accordance with the *STK11* mRNA (Genbank Accession No. NM\_000455). The available parents were also tested for the identified mutation by PCR-sequencing.

## DISCUSSION

PCR-sequencing analysis of the entire coding sequence of *STK11* revealed that patient 1 was heterozygous for a novel deletion of a guanine at nucleotide position 182 (c.182delG) in exon 1 of the *STK11* gene (Figure 1, left upper panel). The loss of a guanine is expected to lead to a frameshift starting at codon 61 and introduce a premature stop codon at position 63. Sequencing his genomic DNA confirmed the presence of the c.182delG (Gly61AlafsX63) mutation. This mutation was not found in either parent (Figure 1, left lower panel).

Patient 2 was heterozygous for a novel in-frame 9-base-pair (bp) deletion (c.907\_915del9) in exon 7 (Figure 1, right upper panel). This identified mutation was confirmed by sequencing of the patient's genomic DNA. The 9-bp deletion results in absence of three amino acids in the kinase domain of the STK11 protein. The c.907\_915del9 was not detected in her mother. Her clinically unaffected father was unavailable for mutation analysis.

Both Thai patients fulfilled the clinical diagnostic criteria for PJS<sup>[12]</sup>. These include the presence of hamartomatous polyps and characteristic mucocutaneous pigmentation. Inactivating mutations in the *STK11* gene have been detected in 50%-94% of PJS patients. The highest mutation detection rate could be achieved if only patients who met the clinical criteria were considered and all mutation screening methods were used<sup>[12-15]</sup>. In this study, two different germline mutations were identified in the *STK11* gene and were found to be *de novo* as evidenced by their clinically unaffected parents



**Figure 1 Mutation analysis.** The left and right panels relate to c.182delG (p.Gly61AlafsX63) and c.907\_915del9 (p.Ile303\_Gln305del) mutations, respectively. Upper and lower panels are electropherograms of patients and unaffected parents, respectively. Each identified mutation is indicated by an arrow.

as well as molecular findings. Both mutations have never been previously described.

A novel heterozygous 1-bp deletion (c.182delG; p.Gly61AlafsX63) in exon 1 of the *STK11* gene was detected in patient 1. It resulted in a frameshift leading to premature termination of the codon at position 63. The mutation was not detected in his parents. Since codons 49-309 encode the catalytic kinase domain<sup>[16]</sup>, this truncated protein is expected to result in incomplete catalytic kinase domain leading to inactivation of the kinase activity as well as a complete loss of the C-terminal domain.

The second patient was heterozygous for a novel in-frame 9-bp deletion in exon 7 (c.907\_915del9; p.Ile303\_Gln305del). The 9-bp deletion results in absence of three amino acids in the kinase domain of the *STK11* protein. This alteration was close to another 9-bp deletion that has been previously described in PJS patients from different populations (c.908\_916del9; p.Ile303Asn; Arg304\_His306del) and involved similar residues. In addition, they were located at the conserved catalytic core of the kinase domain<sup>[7,17]</sup>. These findings confirmed an important role of these critical residues.

Several studies have been performed to analyze genotype-phenotype correlations in PJS. One such study showed that PJS patients with predominantly truncating mutations had breast carcinomas, whereas in-frame deletions in the ATP binding domain were rarely associated with cancers in PJS patients<sup>[18]</sup>. However, an analysis of the spectrum of cancers and the risk of these cancers with different types of *STK11* mutations in 419 PJS individuals suggested that there was no significant correlation between the type or site of mutation and the cancer risk<sup>[19]</sup>. Considering the correlation between

time to the onset of gastrointestinal symptoms and the mutation status, individuals with missense mutations of *STK11* had a later time to onset of the symptoms compared with those with truncating mutations<sup>[20]</sup>. A subsequent study showed a trend toward earlier age of intussusception onset in individuals with *STK11* truncating mutations; however, the difference was not statistically significant<sup>[21]</sup>. Our patient with a truncating mutation, c.182delG, in the *STK11* gene developed intussusception at the age of 14 years, close to the median time to onset of intussusception in those with *STK11* truncating mutations from both studies<sup>[20, 21]</sup>. To strengthen the genotype-phenotype correlations, larger and well-designed studies are still required.

Identification of the *STK11* mutation remains essential for a correct diagnosis of PJS and has important clinical implications. Genetic counseling and surveillance strategies which are of value in disease management could be provided for patients with PJS and their at risk family members regardless of their mutations identified.

In conclusion, we report on the clinical and molecular characterization of two unrelated Thai patients with PJS. Two novel potential pathogenic mutations, c.182delG (p.Gly61AlafsX63) and c.907\_915del9 (p.Ile303\_Gln305del) were identified expanding the mutational spectrum of *STK11*. This study demonstrates that the *STK11* gene is responsible for PJS across different populations and emphasizes the important role of genetic testing for definite diagnosis as well as appropriate genetic counseling.

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CASE REPORT

## A latent form of essential thrombocythemia presenting as portal cavernoma

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### Abstract

Essential thrombocythemia is frequently associated with abdominal thrombotic complications including portal cavernoma as a consequence of chronic portal vein thrombosis. Essential thrombocythemia in a latent form is difficult to identify at onset due to the absence of an overt disease phenotype. In the presented case report, the diagnosis of essential thrombocythemia was initially missed because the typical disease phenotype was masked by bleeding and hypersplenism. The correct diagnosis was only reached when the patient experienced persistent thrombocytosis and pseudohyperkalemia after a shunt operation.

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**Key words:** Essential thrombocythemia; Portal cavernoma; Portal vein thrombosis; Pseudohyperkalemia

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### INTRODUCTION

Portal cavernoma is the consequence of persistent portal vein thrombosis (PVT) which may be caused by a variety of conditions including cirrhosis, cancer, abdominal infectious disease and myeloproliferative disorders (MPD). Its related symptoms are gastrointestinal bleeding, splenomegaly and hypersplenism as a consequence of portal hypertension.

Essential thrombocythemia (ET) is the most prevalent MPD. It is characterized by persistent thrombocytosis with an estimated annual incidence between 0.77 and 2.53 per 100 000 population, and is relatively common in young females. The predominant clinical features are thrombosis and hemorrhage, as well as delayed occurrence of transformation into leukemia or other myeloid diseases<sup>[1]</sup>. We describe here a young woman suffering from portal cavernoma caused by latent ET, which was masked by bleeding and hypersplenism. The correct diagnosis was only reached when she experienced persistent thrombocytosis and pseudohyperkalemia after a shunt operation.

### CASE REPORT

A 36-year-old female was admitted for recurrent upper quadrant abdominal pain of one year and violent hematemesis two days before. She was found to have a low hemoglobin level, and received a transfusion in a local hospital. Physical examination revealed a non-tender and firm spleen with degree III enlargement. The patient denied the use of contraceptive agents and a history of vascular thrombosis. Laboratory investigations revealed a hemoglobin of 71 g/L, hematocrit of 26.8%, leukocyte count of  $12.4 \times 10^9/L$ , and a platelet count of  $313 \times 10^9/L$ . Viral hepatitis serology was negative. A slightly prolonged prothrombin time of 17.2/13.5 s and a partial thromboplastin time of 43.8/36.5 s were noted during coagulation screening. Liver and renal functions were within normal range. Computed tomography scan showed splenomegaly and varicose veins in the esophagus and gastric fundus (Figure 1). Portovenography showed non-visualization of the portal vein and splenic vein with the formation of lots of collaterals, confirming the presence of portal cavernoma (Figure 2).

Obvious cavernous transformation was found at the porta hepatis during surgery. Splenectomy and a mesocaval interposition shunt with prosthetic H-graft were per-



Figure 1 Computed tomography (CT) scan showed splenomegaly and varicose veins in the esophagus and gastric fundus.

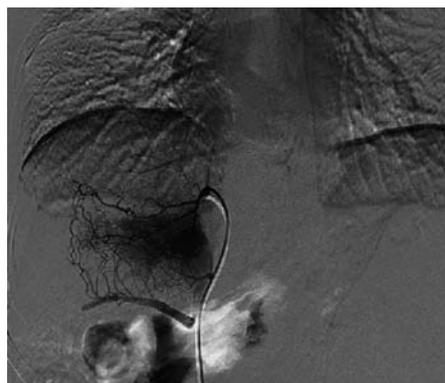


Figure 2 Portovenography showed non-visualization of the portal vein with formation of multiple collaterals in the hepatic hilum.

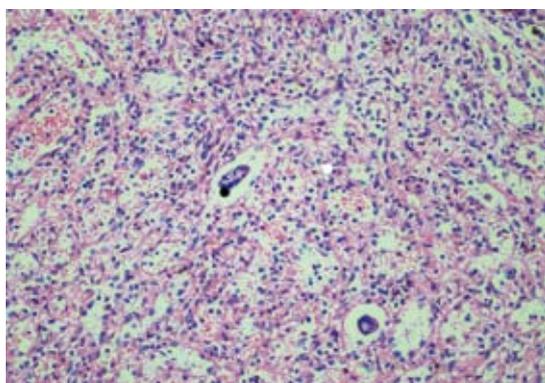


Figure 3 Spleen pathology showed chronic congestion and extramedullary hemopoiesis (magnification,  $\times 200$ ).

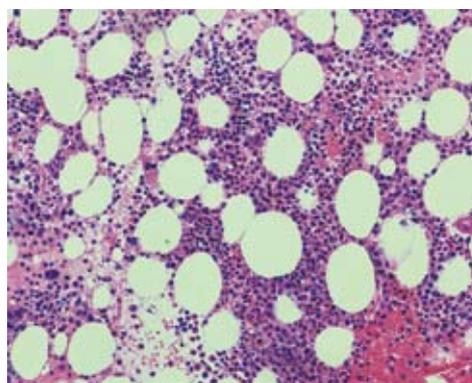


Figure 4 Bone marrow biopsy showed megakaryocytic hyperplasia (magnification,  $\times 200$ ).

formed. Pathological study of the spleen showed chronic congestion and extramedullary hemopoiesis (Figure 3). The patient received antithrombotic treatment with low-dose heparin immediately after surgery. When the platelet count progressively increased to  $1137 \times 10^9/L$  two weeks later, the patient was administered aspirin tablets 100 mg/d.

On postoperative day 18, routine laboratory investigations revealed hyperkalemia of 6.1 mmol/L, hemoglobin of 10.3 g/L, leukocyte count of  $14.7 \times 10^9/L$ , and platelet count of  $1551 \times 10^9/L$ . She denied any complaints and electrocardiogram was normal. Hyperkalemia repeatedly occurred despite the use of exchange resin and insulin. Pseudohyperkalemia was suspected after all the causes of hyperkalemia were excluded through detailed examination. Plasma potassium concentration was found to be within the normal range, while the serum concentration remained high. The diagnosis of pseudohyperkalemia was confirmed and the medical treatment for hyperkalemia was stopped. One month after surgery, the platelet count reached  $2246 \times 10^9/L$ . A hematology consultation was obtained to perform a bone marrow biopsy, which signified megakaryocytic hyperplasia (Figure 4) and negative Bcr/abl confluence gene. The diagnosis of essential thrombocythemia was established and the patient was started on hydroxyurea therapy under close hematological monitoring. After discharge from hospital, she was asymptomatic, and the platelet count decreased to  $629 \times 10^9/L$  within three months of surgery.

## DISCUSSION

Cavernous transformation of portal vein refers to the development of a network of tortuous collateral vessels bypassing the obstructive area due to chronic PVT. Once the liver blood supply decreases significantly, the compensatory mechanism is activated and collaterals begin to form within a few days of the obstruction and organize into a cavernomatous transformation in 3-5 wk. Symptom development is often insidious and related to the progression of portal hypertension. Cirrhosis and cancer are common causes in adults. Other possible etiological factors include abdominal surgery and trauma, pancreatic disease, splanchnic infection, other hypercoagulability associated disease, and the use of oral contraceptive medicines. Ogren *et al*<sup>[2]</sup> studied 23 796 autopsies in Sweden and found the population prevalence of PVT was 1.0%. They reported that 28% of PVT patients had cirrhosis, 23% primary and 44% secondary hepatobiliary malignancy, 10% major abdominal infectious or inflammatory disease and 3% had a myeloproliferative disorder. MPD with a combination of several prothrombotic factors constitute the most common identifiable causes among non-cirrhotic and non-tumoral PVT cases in the West with an estimated prevalence of 30%-60%<sup>[3,4]</sup>.

ET is frequently associated with thrombotic complications in the large abdominal vessels. Gangat *et al*<sup>[5]</sup> reported a prevalence rate of 4% for abdominal vein thrombosis

in 460 consecutive patients with ET, but did not provide detailed information on portal vein thrombosis. The risk factors for thrombosis in ET patients include age, thrombotic history, cardiovascular risk factors and genetic or acquired thrombophilia<sup>[6]</sup>. It seems likely that elevated platelet counts are implicated in thrombotic events in ET, however, the degree of elevation does not appear to be important. It is well documented that thrombosis may occur at relatively low platelet counts<sup>[7]</sup>. ET usually carries the best prognosis among the MPD, but abdominal vein thrombosis was identified as a risk factor for poor survival and appeared to be the result of excess death from leukemic or fibrotic transformation and hepatic failure<sup>[5]</sup>.

Treatment options for ET include aspirin (or an equivalent therapy) and cytoreductive therapy to control the platelet count such as hydroxyurea, anagrelide and interferon, however, there is still a lot of controversy regarding the role of anticoagulation in patients with chronic PVT. The management of bleeding varices due to MPD is not different from cirrhotic or cancer patients, but the prognosis is unquestionably better in the former cases<sup>[8]</sup>. It seems that the mortality rate from variceal bleeding in patients without liver disease is primarily related to underlying diseases other than bleeding itself<sup>[9]</sup>.

In our case, the diagnosis of cavernous transformation was definite on admission. MPD was not considered because the platelet count was only slightly elevated. After splenectomy, the platelet count increased very quickly, and even exceeded  $2000 \times 10^9/L$ . This suggested that the features of ET in peripheral blood cell counts are masked by hypersplenism. For patients with extreme thrombocytosis, active research is important to exclude latent MPD, although reactive thrombocytosis is usually a more common cause than ET. Recently the discovery of a mutation in V617F of the *Janus Kinase 2* gene in a significant proportion of ET patients provides a new strategy for molecular diagnosis<sup>[7]</sup>.

For patients with non-cirrhotic portal hypertension, in particular with extrahepatic portal vein thrombosis, portosystemic shunt surgery provides efficient and permanent decompression of the portal venous system without liver function deterioration or encephalopathy. Several authors have advocated preserving the spleen whenever possible especially in MPD, because the spleen may become a place for extramedullary marrow formation<sup>[9]</sup>. During surgery, we planned to perform a spleen-preserving splenorenal side-to-side shunt, but failed to find normal splenic veins, so converted to splenectomy and a mesocaval interposition (H-graft) shunt.

In the present case, the patient developed elevated serum potassium of 6.1 mmol/L with extreme thrombocytosis of  $1551 \times 10^9/L$  on postoperative day 18. Active research failed to find a cause of hyperkalemia, therefore

pseudohyperkalemia was suspected and plasma potassium level was measured to confirm the diagnosis. The phenomenon of pseudohyperkalemia was first reported in 1955, and has been commonly observed in patients with severe thrombocytosis, erythrocytosis and the presence of activated platelets<sup>[10]</sup>. It is characterized by a marked elevation of serum potassium concentration with normal plasma potassium level. Patients with pseudohyperkalemia are usually without any clinical manifestation of electrolyte imbalance. The main mechanism causing this phenomenon is the release of potassium from excessive cells *in vitro* during clotting of the sample. Pseudohyperkalemia has no harmful consequences and an aggressive therapeutic approach is unnecessary.

In summary, for patients with portal cavernoma, if no common causes such as cirrhosis and neoplasm are found, investigations into an uncommon cause of MPD is indicated using bone marrow biopsy and coagulation profile. It is important to realize that the features of ET in peripheral blood cell counts could be masked by hypersplenism.

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## Hepatocellular carcinoma and evidence-based surgery

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### Abstract

Transplantation cannot be considered the most important therapeutic procedure for hepatocellular carcinoma (HCC). In France, no more than 2% of patients with HCC undergo a transplantation. Randomized controlled trial must assess the benefit to risk ratio of various potentially "curative" treatment procedures (transplantation, resection, radio-frequency ablation).

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### TO THE EDITOR

Rampone *et al*<sup>[1]</sup> stated that hepatocellular carcinoma (HCC) still remains a considerable challenge for sur-

geons and that transplantation is the most important therapeutic procedure. However, facts seem different for an evidence-based medicine adept<sup>[2]</sup>.

There is a challenge for the patient because yet no randomized controlled trial (RCT) has assessed the benefit to risk ratio of various potentially "curative" treatment procedures (transplantation, resection, radio-frequency ablation). RCT is feasible. Benefit of chemoembolization to patients with unresectable HCC and sorafenib in a palliative indication are evidence-based from RCT. Recruiting is not an issue since HCC is the fifth most common cause of cancer.

Transplantation cannot be considered the most important therapeutic approach. In France with 66 000 000 inhabitants, 7500 inhabitants die of HCC per year. In 2007, 1061 transplantations were performed for various conditions and 6% of the candidates died while on waiting list due to the shortage of organs<sup>[3]</sup>. In Europe, 15% of transplantations are performed for HCC<sup>[4]</sup>. Therefore, in France, no more than 2% of patients with HCC undergo a transplantation. Nevertheless, more than one out of four exceeds the Milan criteria, a situation which does not improve the results<sup>[4]</sup>. Again, data are missing, and the national agency in charge of transplantation does not publish survival after transplantation according to indications. However, they exist.

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## Meetings

### Events Calendar 2009

January 12-15, 2009  
Hyatt Regency San Francisco, San Francisco, CA  
Mouse Models of Cancer

January 21-24, 2009  
Westin San Diego Hotel, San Diego, CA  
Advances in Prostate Cancer Research

February 3-6, 2009  
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)  
Second AACR Conference  
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009  
Hyatt Regency Boston, Boston, MA  
Translation of the Cancer Genome

February 8-11, 2009  
Westin New Orleans Canal Place, New Orleans, LA  
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009  
Hong Kong Convention and Exhibition Centre, Hong Kong, China  
19th Conference of the APASL  
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009  
Orlando, Florida  
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009  
Vienna, Austria  
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease  
[www.easl.ch/vienna2009](http://www.easl.ch/vienna2009)

March 13-14, 2009  
Phoenix, Arizona  
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009  
Marriott Wardman Park Hotel  
Washington, DC  
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009  
Glasgow, Scotland  
British Society of Gastroenterology (BSG) Annual Meeting  
Email: [bsg@mailbox.ulcc.ac.uk](mailto:bsg@mailbox.ulcc.ac.uk)

April 8-9, 2009  
Silver Spring, Maryland  
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009  
Colorado Convention Center, Denver, CO  
AACR 100th Annual Meeting 2009

April 22-26, 2009  
Copenhagen, Denmark  
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)  
<http://www.easl.ch/>

May 17-20, 2009  
Denver, Colorado, USA  
Digestive Disease Week 2009

May 29-June 2, 2009  
Orange County Convention Center  
Orlando, Florida  
45th ASCO Annual Meeting  
[www.asco.org/annualmeeting](http://www.asco.org/annualmeeting)

May 30, 2009  
Chicago, Illinois  
Endpoints Workshop: NASH

May 30-June 4, 2009  
McCormick Place, Chicago, IL  
DDW 2009  
<http://www.ddw.org>

June 17-19, 2009  
North Bethesda, MD  
Accelerating Anticancer Agent Development

June 20-26, 2009  
Flims, Switzerland  
Methods in Clinical Cancer Research (Europe)

June 24-27 2009  
Barcelona, Spain  
ESMO Conference: 11th World Congress on Gastrointestinal Cancer  
[www.worldgicancer.com](http://www.worldgicancer.com)

June 25-28, 2009  
Beijing International Convention Center (BICC), Beijing, China  
World Conference on Interventional Oncology  
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009  
Snowmass, CO, United States  
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009  
Aspen, CO, United States  
Molecular Biology in Clinical Oncology

August 1-7, 2009  
Vail Marriott Mountain Resort, Vail, CO, United States  
Methods in Clinical Cancer Research

August 14-16, 2009  
Bell Harbor Conference Center, Seattle, Washington, United States  
Practical Solutions for Successful Management  
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009  
Beijing International Convention Center (BICC), Beijing, China  
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists (IASGO)  
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009  
Taipei, China  
Asian Pacific Digestive Week  
<http://www.apdwcongress.org/2009/index.shtml>

October 7-11, 2009  
Boston Park Plaza Hotel and Towers, Boston, MA, United States  
Frontiers in Basic Cancer Research

October 13-16, 2009  
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States  
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009  
Versailles, France  
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009  
Boston, MA, United States  
The Liver Meeting

November 15-19, 2009  
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States  
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009  
London, UK  
Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

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The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of

balancing selection in *Arabidopsis*. *Proc Natl Acad Sci USA* 2006; In press

#### Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

#### Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

## Books

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 15 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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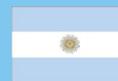
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<sup>[1]</sup>Passed away on October 20, 2007

<sup>[2]</sup>Passed away on June 14, 2008



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## Genome-wide association studies - A summary for the clinical gastroenterologist

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### Abstract

Genome-wide association studies (GWAS) have been applied to various gastrointestinal and liver diseases in recent years. A large number of susceptibility genes and key biological pathways in disease development have been identified. So far, studies in inflammatory bowel diseases, and in particular Crohn's disease, have been especially successful in defining new susceptibility loci using the GWAS design. The identification of associations related to autophagy as well as several genes involved in immunological response will be important to future research on Crohn's disease. In this review, key methodological aspects of GWAS, the importance of proper cohort collection, genotyping issues and statistical methods are summarized. Ways of addressing the shortcomings of the GWAS design, when it comes to rare variants, are also discussed. For each of the relevant conditions, findings from the various GWAS are summarized with a focus on the affected biological systems.

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**Key words:** Genome-wide association studies; Inflammatory bowel disease; Gastroenterology; Hepatology

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### INTRODUCTION

The genetic epidemiology of gastrointestinal diseases has been hard to unravel, despite the fact that many of the diseases have a high sibling recurrence risk pointing to genetic risk factors<sup>[1,2]</sup>. However, over the last three years, with the recent advent of genome-wide association studies (GWAS), a wealth of susceptibility loci have been discovered. Several hundred GWAS have been reported to this end, with thousands of reports on novel disease genes and loci. Interestingly, inflammatory bowel disease (IBD) has been leading the race<sup>[3-17]</sup>. The genes and polymorphisms identified have revealed several biological pathways by which gastroenterological diseases develop and may eventually be treated. Although most of the associations are weak in a statistical sense [odds ratios (OR) of risk variants in the range of 1.1-1.2]<sup>[10,18]</sup>, they point to loci involved in biological systems worth investigating further with other methodologies. This current review includes studies published or available early online up to August 20, 2009.

The most important milestone in the development of GWAS has clearly been the HapMap project which involved genotyping of 1 million single nucleotide polymorphisms (SNPs) of the human genome in the first phase<sup>[19]</sup> and 3.1 million in the second phase<sup>[20]</sup>. Since the frequency of genetic variants varies between different geographical regions, genotyping was performed in populations from Nigeria (Yoruba), Japan, China and the United States [residents with ancestry from Northern and Western Europe, collected in 1980 by the Centre d'Etude du Polymorphisme Humain (CEPH) and used for other human genetic maps]. The HapMap website ([www.hapmap.org](http://www.hapmap.org)) reports on the frequencies of the SNPs and their correlation through linkage disequilibrium (LD). The HapMap resource has become an invaluable tool for genetic research<sup>[21]</sup>. By taking into account the information regarding LD in HapMap, companies have been able to select SNPs for the most recent genome-wide genotyping arrays that provide information on more than 90% of the

genetic variation in HapMap. In addition to SNPs selected *via* LD, modern genotyping arrays also contain markers with a high likelihood of biological relevance (e.g. non-synonymous SNPs leading to an amino acid change in the encoded protein), as well as probes designed to detect other types of genetic variation (e.g. deletions, insertions and duplications of DNA segments). Once completed, the currently ongoing 1000 genomes project (<http://www.1000genomes.org/>), which aims at sequencing 1000 individuals, will further add to the publicly available catalogues of genetic variants and aid in the design of novel genotyping products.

The GWAS design contrasts with the traditional hypothesis-driven studies of biomedical research. Initial criticism with regard to this design has been replaced by appraisal as the scientific community has realized that hypothesis-free data mining performed in a systematic manner<sup>[22]</sup> represents a powerful tool to pin-point biological systems and generate hypotheses for further research.

## PHASES OF A GWAS

Table 1 summarizes the different parts in the study design that make up a GWAS. In the following paragraphs, detailed points are discussed regarding the GWAS study design, both for the researcher planning to embark on a GWAS and the casual reader trying to interpret the findings of an existing study.

### Cohort collection

To maximize the power to detect even low-effect risk variants, the study panels of a GWAS should preferably contain DNA from as many patients and healthy controls as possible. However, this ideal is limited by several pragmatic factors such as the logistics of identifying and classifying patients, recruitment formalities and financial constraints. Given the significant investment, with a considerably higher price per sample than for genotyping of a few markers, great care should be taken in the sample collection process. The “lowest hanging fruits” with the highest risk effects were easily identified in the first round of GWAS and further studies in the same phenotypes will require larger study panels to identify the genes associated with a more modest risk.

The phenotype of the cases used in the study should be very homogeneous. For instance, for IBD it would be advisable to only include patients for whom long-lasting follow-up data is available in order to ensure the correct diagnosis was made (e.g. for IBD, 10% of the patients change diagnosis during the first year of the disease course<sup>[23]</sup>). Although a perfect phenotype description is the aim, a small misascertainment seems to only slightly reduce power<sup>[24]</sup>. If the alternative is not to do a study at all, slight uncertainties in phenotyping can probably be accepted and other aspects for increasing power applied instead.

To enrich the sample collection with risk alleles and thereby increase power, familiar cases<sup>[25]</sup>, which are believed to have the respective disease due to a larger

contribution of genetic factors, can be used. The risk estimates achieved in such a cohort should not be used to judge the risk effect of the allele in the general population, both due to enrichment of the associated alleles in these types of panels and preferential selection of the most associated markers (the so called ‘winner’s curse’)<sup>[26]</sup>. Therefore, replication of findings and final effect size calculations need to be performed in separate study panels sampled without bias.

In rare diseases or in meta-analyses (see separate section on this subject below), case-control cohorts recruited in different countries and even continents<sup>[18,27]</sup> might be necessary to make the study panels large enough to perform sensible genome-wide association analyses. The main challenge that arises when several case-control cohorts are combined is differences in allele frequencies between populations; a problem that is particularly pronounced in African populations<sup>[28]</sup>, but also needs to be taken into account when combining study panels of European descent<sup>[29]</sup>. Recently, analytical tools utilizing genome-wide SNP data for correction of population heterogeneity/stratification (e.g. the EIGENSTRAT software<sup>[30]</sup> and similar approaches<sup>[31]</sup>) have become available to generate non-inflated population stratification-corrected test statistics.

Healthy control data can be shared between groups working in different diseases, thereby reducing the cost of genotyping. Increasing the control-to-case ratio increases the power. This was recently done to an extreme degree in an Icelandic study with 37 196 controls and 192 cases<sup>[32]</sup>. It is important to keep in mind that the gain in power is minute when increasing the control-to-case ratio above 4. For example, the power at a *P*-value of  $5 \times 10^{-7}$  in a study including 1000 cases increases from 77% to 87% for an allele with a frequency of 0.2 and an OR of 1.4, by increasing the number of controls from 4000 to 10 000.

### Genotyping chips

The high density genotyping chips now available have the potential to assay up to 1 million markers (Affymetrix SNP 6.0 and Illumina 1M). In essence, the chips consist of dense arrays of specific labeled probes for given DNA sequences that will emit a light signal in the case of hybridization (binding of a matching DNA sequence in the investigated sample).

Since the price is lower, it has recently been suggested that the most cost-effective way to perform a GWAS is to continue using the older and cheaper arrays with medium density (300 000-500 000 SNPs) and then computationally determine untyped SNPs in the remainder of the genome by means of the HapMap reference (so called imputation)<sup>[33]</sup>. An even cheaper approach is to employ DNA-pooling<sup>[34]</sup>. This means that the DNA samples from several individuals are pooled together and subsequently used for genotyping. However, this design only yields estimated allele frequencies across all case or control samples instead of individual genotypes. Therefore, analyses relying on

**Table 1** Phases in the initiation and analysis of a genome-wide association study

Sample panel building
Cases and healthy controls of same ethnicity (for power estimates see figure 2)
Enrichment with early onset cases and/or familial cases
Keep variability in phenotype at a minimum
Establish replication cohort(s) after the same principles. Other, yet similar, ethnicities may be included, although matched healthy controls should be collected
Genotyping
Sample preparation (DNA extraction, calibration)
Genotyping chip (cost <i>vs</i> number of samples)
Genetic coverage
Initial quality control
Exclude samples failing platform-specific QC measures
Exclude samples with low call-rate
Exclude SNPs with a low genotyping rate
Exclude SNPs with a low minor allele frequency and those grossly out of Hardy-Weinberg equilibrium (e.g. $P < 10^{-4}$ )
Statistical analysis
Imputation of non-genotyped SNPs using HapMap as the reference
Single-point association analysis, if needed include covariates of interest in the present study (e.g. gender, sex, smoking, imputation uncertainties <i>etc.</i> )
Manually inspect cluster plots for highly significant SNPs that should be followed-up
Select 1-2 SNPs from each associated locus to take forward in replication
Replication
Genotype (preferentially independent technology) in a panel of cases and healthy controls that are properly sized to detect effects in the same range as seen in the discovery panel
Follow-up experiments
Highly depends on results, i.e. nature of genetic finding, and normally not part of the GWAS design

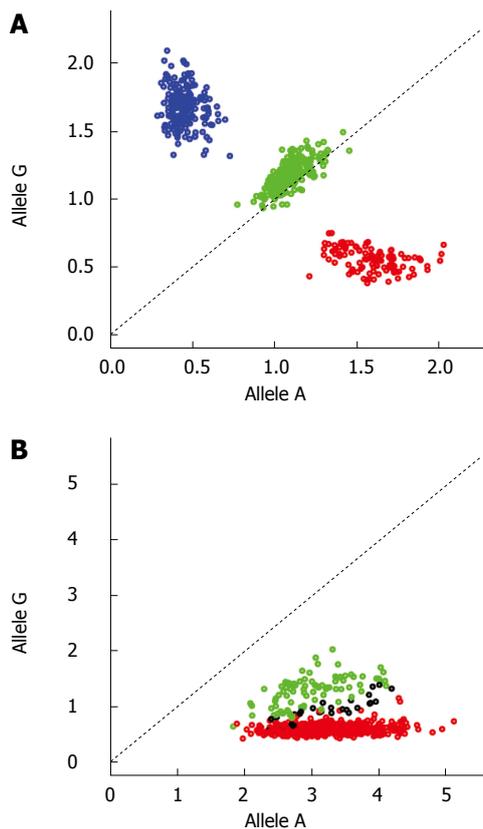
individuals' genotypes such as phenotype associations and imputation are not possible.

### Genetic coverage

As opposed to a candidate gene study, a GWAS aims to, as the name implies, assay the whole genome. Therefore, the genetic coverage of the employed genotyping array is of crucial importance. At present, the dbSNP database (<http://www.ncbi.nlm.nih.gov/sites/entrez>) of known SNPs includes over 12 million entries<sup>[35]</sup>, and of these almost 9 million are annotated as validated (for validation criteria see [http://www.ncbi.nlm.nih.gov/projects/SNP/snp\\_legend.cgi?legend=validation](http://www.ncbi.nlm.nih.gov/projects/SNP/snp_legend.cgi?legend=validation)). Due to LD, assaying all these SNPs in a GWAS is not necessary to achieve complete genome-wide coverage. The genetic information obtained at SNPs can depending on the rate of recombination within this genomic region - precisely predict the alleles of closely linked, un-genotyped markers. There are substantial differences in the coverage between the different commercially available SNP arrays, but simulation has demonstrated that this does not necessarily translate into an increased power for detecting disease-associated variants<sup>[36]</sup>. It is also important to keep in mind that a high overall coverage does not necessarily mean that an individual gene is well covered, and at the gene level, there are large differences in coverage between different genotyping platforms<sup>[37]</sup>. Coverage estimations also tend to be biased, as in most cases the HapMap is used as the reference which is, by itself, assumed to be partially biased in terms of selected SNPs and in terms of included populations. Therefore, SNP arrays that mostly include HapMap tagging SNPs will have a higher genetic coverage compared to arrays that include random SNPs when basing assumptions on the HapMap.

### Dataset quality

The sizes of modern genome-wide datasets are larger by many magnitudes than what was typical for a candidate gene study. For instance, in a GWAS with 1000 cases and 1000 controls using the Affymetrix SNP 6.0, the number of genotypes generated is  $1.8 \times 10^9$ . This huge amount of data renders automatic and semi-automatic procedures necessary, since manual processing is simply not feasible. In principle, this process can be divided into two steps (1) Exclusion of samples (low-performance samples, related individuals and population outliers) and (2) Exclusion of SNPs with evidence of bad performance. Partly this process has to be done iteratively as (a) influences (b) and vice-versa. Firstly, measures should be taken to ensure that the PCR and hybridization reaction are performed properly, and samples where this is not the case should be discarded at this stage or processed over again. Next, platform-specific quality measures should be applied (e.g. the QC-contrast for the Affymetrix SNP 6.0 chip<sup>[38]</sup>) to make sure the samples are within acceptable limits for the experiment as a whole. After this, genotype calling is performed, preferably in batches of similarly handled samples as batch effects can have an impact on the results<sup>[39]</sup>. Next, samples with a low call-rate (typically < 95%) and samples where there are mismatches between the gender recorded and the gender calculated based on the genotype data of the X chromosome are detected and should be excluded, since both of these measures can relate to poor performance on particular chips. The latter also can be due to sample mix-up. To avoid poor performing probes within otherwise acceptable arrays, SNPs with study-wise low genotyping rate (< 95%) should be removed along with SNPs with a low (< 1%) minor allele frequency. SNPs with a low minor



**Figure 1** Example of cluster plots for two SNPs. Plot A shows the plotting of the normalized intensity values for a SNP with good clustering. Each color represents the respective genotype (blue for GG, green for AG and red for AA). Plot B demonstrates the cluster plot for a SNP with bad clustering (same color coding as in A). Disease-associated SNPs demonstrating cluster plots as in B should be discarded as the significant associations at such SNPs are most likely technical artifacts.

allele frequency have a tendency to be mis-called by the clustering algorithm, since most likely only two instead of three clusters will be present in the signal intensity plots, with a significantly smaller heterozygote cloud. Even when one plans to use robust statistical tests, SNPs showing deviation (normal cut-off  $P$ -values  $10^{-4}$ - $10^{-7}$ ) from Hardy-Weinberg Equilibrium in the healthy controls (not in the case/patient panel) should be removed, since this is also an indication of low genotyping quality<sup>[40]</sup>. After these measures have been applied, the resulting SNP set can be forwarded to the initial association analysis for removal of duplicates, related individuals software and ethnic outliers with, for instance, PLINK<sup>[41]</sup> and EIGENSTRAT<sup>[30]</sup>. After the removal of duplicates, related individuals and ethnic outliers, the SNP-specific quality measures should be performed over again on a fresh dataset without these samples. As the genotyping is an automatic process with little user input, one should go back to the raw data after the chosen statistical test has been performed to manually inspect the clustering plots of top hits. An example of two typical cluster plots, one good and one bad, can be found in Figure 1. The SNPs with evidence of bad clustering should be discarded and not followed up.

### Imputation

To increase the number of SNPs to test for association,

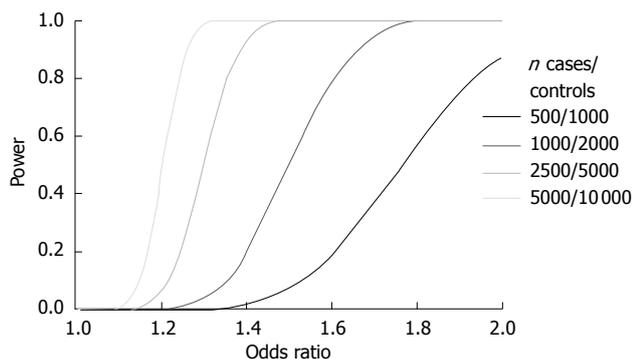
the use of imputation to estimate genotypes at un-genotyped loci has become popular. Imputation will also increase call rates at all typed SNPs to 100%. Given the former feature, researchers may choose less dense and therefore cheaper arrays. Another advantage is that the chance to be closer to the possible functional variant, and hence statistical power - increases with a denser SNP data set.

In brief, imputation relies on the LD and haplotype information contained in a reference dataset. The HapMap datasets are most commonly used, which the algorithm aligns with the genotyped SNPs to use LD information to estimate the genotype at the target locus. Imputation has already been available in software packages such as PHASE and fastPHASE<sup>[42,43]</sup>, however recent implementations such as MACH (<http://www.sph.umich.edu/csg/abecasis/MaCH/>), IMPUTE<sup>[44]</sup> and Beagle<sup>[45]</sup> specifically aimed at GWAS data are recommended<sup>[46]</sup> and have been shown to produce accurate and reliable results<sup>[47]</sup>. There are some differences between the software packages, with MACH and IMPUTE having the edge<sup>[47,48]</sup>, but in general, they produce comparable results.

### Statistical analysis

The goal of a GWAS is to identify the genetic variants that are statistically associated with the disease or trait in question. The first step to achieve this is normally to perform a single-locus association test, i.e. only a single SNP is considered at a time. As up to 1 million SNPs are assessed, it is impossible to have an *a priori* hypothesis about the genetic model expected. The statistical test used should therefore be robust and powerful to detect different genetic models (e.g. dominant, recessive and allele-dosage). As both the allele count and genotype count  $\chi^2$  tests do not meet these requirements, a trend-based test is normally recommended, for instance the Cochran-Armitage trend test. If it is sensible to include co-variables in the disease model (e.g. sex, age, BMI *etc.*), the best way is to use a logistic regression procedure and add these as covariates. For quantitative traits (e.g. enzyme levels), normal linear regression is applicable and could also include co-variables.

After the initial test statistics have been calculated, the genomic inflation factor should be determined (i.e. the median  $\chi^2$  test statistics observed divided by the expected test statistics) and a quantile-quantile plot (a plot of the observed *vs* the expected test statistics or the negative logarithm of the  $P$ -values) should be examined. The quantile-quantile plot should not, in the setting of a non-stratified dataset, show large deviations from the expected distribution in the lower tail. In the upper tail, deviation indicates possible disease association. A genomic inflation factor above 1 typically indicates the presence of population stratification (or a differential bias in genotyping)<sup>[49]</sup>. If the genomic inflation factor is above 1.1, methods for correcting for population heterogeneity should be considered. It is, however, important to note that a small increase in the inflation factor can be caused by disease-associated markers.



**Figure 2 Power calculations for different case-control study panel sizes.** Power calculations for different case-control study panel sizes using an allelic based association test and a  $P$ -value of  $P < 5 \times 10^{-7}$ . All calculations assume a minor allele frequency of 30% and 1:2 ratio of cases vs controls.

Most of the analytical methods described above are implemented in the software package, PLINK<sup>[41]</sup>. The analysis of a small number of cases and controls could in principle be analyzed on a standard desktop computer. However, for more computationally challenging analyses, e.g. imputation, and/or a large number of cases and controls, the use of a high-performance computing cluster with a comfortable batch job submission environment is desirable.

Methods looking at haplotypes<sup>[43,50]</sup> and gene-gene or SNP-SNP interactions (so called epistasis)<sup>[51]</sup> should also be applied. Recently, pathway-oriented analyses, i.e. analyzing SNPs that belong to genes in a common biological pathway<sup>[52-54]</sup>, have been proposed. We would recommend that these methods are first applied after the main associations in the dataset have been explored and replicated.

### Replication

Applying the traditional  $P$ -value cut-off of  $< 0.05$  for statistical significance to a GWAS leads to the fact, even in the presence of no association, that 5% of the tested SNPs are reported as statistically significant. In a typical GWAS where around 650 000 SNPs are tested, this means that 30 000-35 000 SNPs are reported to associate significantly with disease. To avoid this very large number of false-positive results, a very conservative  $P$ -value cut-off that is robust to correction using Bonferroni's method has been described, namely the term of a "genome-wide significant  $P$ -value". In the Wellcome Trust case-control consortium landmark paper<sup>[5]</sup> this level was set to  $P < 5 \times 10^{-7}$ . However, with new chips assaying over 1 million markers and even imputed results with more than 2 million markers, this genome-wide significance threshold might not be conservative enough. On the other hand, simulation studies have suggested the effective number of tests to be around  $10^{6[55]}$ ; the reason for this number being lower than the actual number of tested SNPs being the correlation between SNPs due to LD. In Figure 2 the power estimates for different sample sizes for different ORs are shown. A few studies have already, in the first round of analyses, been able to identify novel disease

loci at genome-wide significance<sup>[56-58]</sup>. As most studies do not achieve such robust associations in their initial phase, a two-staged design is applied. This means that the strongest associated SNPs are carried forward to another study panel and again tested for association. Corrections for multiple testing must be applied to the association results obtained in the second stage. Even when applying strict criteria for selection of SNPs for replication, normally only a few SNPs will replicate. While most often signifying that the original associations were due to type I errors, this can also be due to different effects of a disease variant in different populations, e.g. due to interaction with other genetic variants; so called epistasis<sup>[59]</sup>.

## BOWEL DISEASES (SEE TABLE 2 FOR DETAILS)

### Inflammatory bowel diseases - ulcerative colitis and Crohn's disease

Ulcerative colitis (UC) and Crohn's disease are the two major phenotypes of IBD with a combined incidence rate of 2.2 to 28.9 per 100 000 person-years in Caucasian populations<sup>[60]</sup>, giving rise to inflammation in the colon and the entire intestinal tract, respectively<sup>[61]</sup>. The sibling recurrence risks are estimated to be 15-35 for Crohn's disease and 6-9 for UC<sup>[1]</sup>. Besides the associations seen within the human leukocyte antigen (HLA)-complex<sup>[62-64]</sup>, there was one particularly notable and reproducible genetic discovery in gastroenterology before the advent of GWAS; the association of Crohn's disease with the *NOD2* (also known as *CARD15*) gene<sup>[65,66]</sup>. A thorough review of the relevant genetics, including the *NOD2* gene in Crohn's disease before the GWAS era, can be found elsewhere<sup>[67]</sup>.

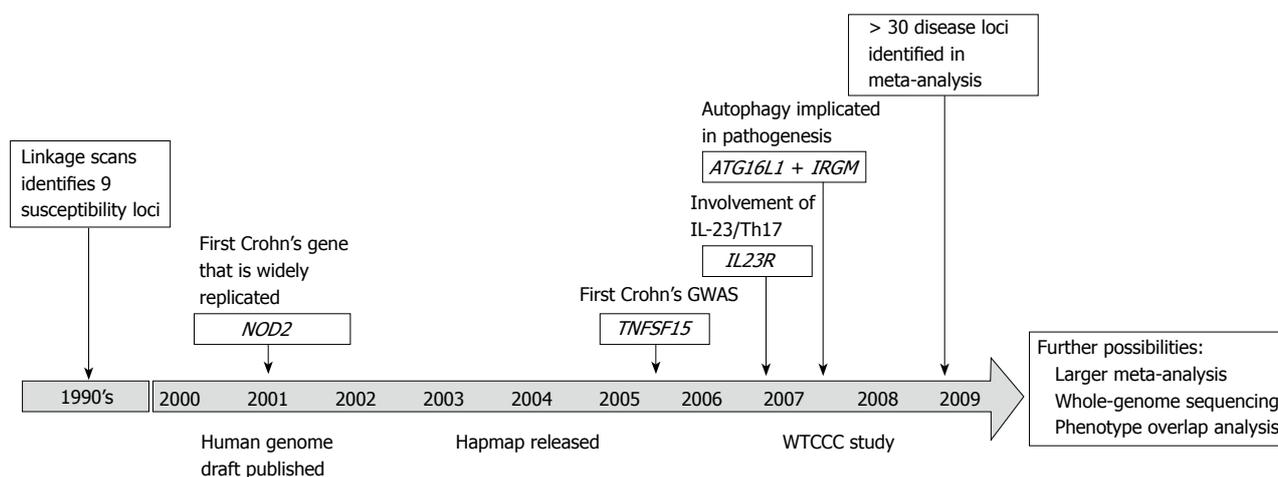
Figure 3 shows the development of Crohn's disease genetics over the last 10 years. The eight GWAS performed in Crohn's disease<sup>[3-9,15,17]</sup> have identified several loci influencing disease susceptibility and a recent meta-analysis implicated 20-30 additional loci<sup>[10]</sup>. It should be noted that the discovery panel and the replication panel used in the meta-analysis overlap with the previous studies and some of the confirmatory associations are biased, as the original study panel is also included. Many of the new findings in Crohn's disease segregate into particular biological pathways and functions. Two of the key pathways are autophagy and the IL-23/Th17 pathway. Autophagy is responsible for recycling of cellular organelles and long-lived proteins, and plays an important role in tissue homeostasis and in the processing of intracellular bacteria, which is also known as xenophagy. ATG16L1 may participate in this process *via* the regulation of Paneth cells<sup>[68]</sup>, whereas IRGM mediates autophagy of intracellular bacteria<sup>[69]</sup>. IL-23 stimulates the Th17 cell population to produce IL-17 and other pro-inflammatory cytokines involved in intestinal inflammation<sup>[70,71]</sup>. Interestingly, one of the first reported IBD genes by GWAS, *IL-23R*<sup>[4]</sup>, participates in the IL-23/Th17 pathway. Through pathway analyses<sup>[53]</sup>

Table 2 Genome-wide association studies in bowel disease

Disease	Genotyping platform	Number of cases/controls in discovery panel	Number of cases/controls in replication panel(s)	Novel Loci reported	Ref.	Independently replicated
Celiac disease	Illumina HumanHap300	778/1422	991/1489	<i>KIAA1109-TENR-IL2-IL21</i> (4q27)	[112]	[165,166]
Celiac disease Two papers extending the study above with follow-up of more markers	Illumina HumanHap300	767/1422	Paper 1: 1643/3406 Paper 2: 2220/3851	Paper 1: <i>1q31 (RGS1), 3p21 (CCR1, CCR2, CCRL2, CCR3, CCR5 and XCR1), 2q12.1 (IL18RAP), 3q25-3q26 (IL12A), 3q28 (LPP), 6q25 (TAGAP), 12q24 (LTK and ATXN2), Paper 2: 6q23.3 (TNFAIP3) REL</i>	[113,114]	( <i>1q31, 3p21, 3q25-26, 3q28</i> ) <sup>[167]</sup> ( <i>3q25-26, 12q24, 1q31, 3q28</i> ) <sup>[166]</sup> ( <i>IL18RAP</i> ) <sup>[168]</sup>
Crohn's disease	Custom array (80k SNPs)	94/752	347 trios and 233 multiplex families (Crohn's disease and UC)	<i>TNFSF15</i>	[15]	For CD: <sup>[10,77,169-171]</sup> CD and UC: <sup>[14]</sup>
Crohn's disease	Custom SNPlex panel of non-synonymous SNPs (16k)	735/368	498/1032 and 380 trios	<i>ATG16L1</i>	[3]	[5,8-10,14,73,80,84,172-188]
Crohn's disease	Illumina HumanHap300	547/548	401/433 and 883 families (Crohn's disease and UC)	<i>IL23R</i>	[4]	For CD: <sup>[5,9,13,14,17,73,79,82-84,173,174,178,180,182,184,185,188-197]</sup> For UC: <sup>[12,13,73,173,178,180,184,185,188,190,191,195,195]</sup>
Crohn's disease Published in two articles; WTCDC main paper and separate Crohn's disease paper	Affymetrix 500k	1748/2938	1182/2024	<i>IRGM</i> <i>PTPN2</i> <i>NKX2-3</i> <i>MST1</i> <i>1q24</i> <i>1q31</i> <i>IL12B</i> <i>FLJ45139</i>	[5,6]	( <i>IRGM, NKX2-3, 1q24</i> ) <sup>[184]</sup> ( <i>IRGM, IL12B, MST1, NKX2-3, PTPN2, 1q24</i> ) <sup>[73]</sup> ( <i>MST1</i> ) <sup>[17]</sup> ( <i>IRGM</i> ) <sup>[187]</sup> ( <i>IRGM</i> ) <sup>[198]</sup> ( <i>NKX2-3</i> ) <sup>[199]</sup> ( <i>IRGM</i> ) <sup>[200]</sup> ( <i>1q24, IRGM, IL12B, MST1, NKX2-3, PTPN2</i> ) <sup>[73]</sup> ( <i>MST1</i> ) <sup>[201]</sup> ( <i>IL12B, MST1, IRGM, 1q24</i> ) <sup>[84]</sup>
Crohn's disease	Perlegen array 164279 markers	382 trios	752/828 and 521 trios	Replication of already known loci	[17]	
Crohn's disease	Affymetrix 100k	393/399	1861/1961 and 829 trios	<i>NELL1</i>	[7]	
Crohn's disease Joint analysis of the individuals in study above with Sarcoidosis patients	Affymetrix 100k	382/394 and 398 Sarcoidosis patients	1549/3361 and 924 Sarcoidosis patients	<i>10p12</i>	[16]	
Crohn's disease	Illumina HumanHap300	946/977	353/207 and 530 trios	<i>10q21</i> <i>PHOX2B</i> <i>NCF4</i> <i>FAM92B</i>	[8]	( <i>NCF</i> ) <sup>[200]</sup> ( <i>10q21</i> ) <sup>[199]</sup> ( <i>10q21</i> ) <sup>[184]</sup> ( <i>10q21</i> ) <sup>[84]</sup> ( <i>10q21</i> ) <sup>[73]</sup> [6,7,73,184]
Crohn's disease	Illumina HumanHap300	547/928	1266/559 and 428 trios	<i>5p13.1</i>	[9]	
Crohn's disease	Illumina HumanHap300 and Affymetrix 500k	3230/4829	2325/1809 and 1339 trios	<i>PTPN22, ITLN1, 1q32, CDKAL1, 6q21, CCR6, 7p12, 8q24, JAK2, 10p11, C11orf30, LRRK2/MUC19, 13q14, ORMDL3, STAT3, 21q21, ICOSLG</i> <i>ECM1</i>	[10]	( <i>1q32, LRRK2/MUC19, 7p12, 8q24</i> ) <sup>[84]</sup>
Ulcerative colitis	Custom Infinium array (11k SNPs)	905/1465	936/1470		[13]	
Ulcerative colitis	Affymetrix SNP 5.0	1167/777	1855/3091	<i>IL-10, ARPC2, HLA-BTNL2</i>	[11]	( <i>HLA-BTNL2</i> ) <sup>[13]</sup> , ( <i>HLA-BTNL2</i> ) <sup>[12]</sup>
Ulcerative colitis	Illumina HumanHap300 and HumanHap550	1052/2571	1387/1115	<i>1p36, 12q15</i>	[12]	

Pediatric IBD (Crohn's and Ulcerative colitis)	Illumina HumanHap550	1011/4250	173/3481 + WTCCC Crohn's cohort	20q13 ( <i>TNFRSF6B</i> ), 21q22	[14]	(20q13) <sup>[202]</sup>
Colorectal carcinoma	Affymetrix 100k + Affymetrix 10k + Custom panel	960/984 (numbers for Affymetrix 100k, slightly more for the two other technologies)	4024/4042	8q24 (published simultaneously as study below)	[91]	[87-90,92-99]
Colorectal carcinoma	Illumina HumanHap550	930/960	7334/5246	8q24 (published simultaneously as study above)	[87]	[88-99]
Colorectal carcinoma Same discovery panel as study above, published separately	Illumina HumanHap550	930/960	7473/5984	<i>SMAD7</i>	[110]	[88]
Colorectal carcinoma Extension of the two studies above with a staged design	Illumina HumanHap550	922/927	Phase 2: 2854/2822 Phase 3: 4287/3743 Phase 4: 10731/10961	10p14, 8q23	[25]	
Colorectal carcinoma	Illumina HumanHap300 and HumanHap240S	1012/1012	Phase 2: 2 024/2 092 Phase 3: 14 500/13 294	11q23	[88]	[203]
Colorectal carcinoma	Illumina HumanHap550 + HumanHap300 and HumanHap240S	Phase 1: 1902/1929	Phase 2 - 39k SNPs: 4878/4914 13 406/14 012 (Total number, all markers not genotyped in entire panel)	<i>BMP4, CDH1, RHPN2, 20p12.3</i>	[18]	
Hirschsprung's disease	Affymetrix 500k	181/346	190/510	<i>NRG1</i>	[120]	

If the authors mainly reported one region with several candidate genes the region is listed with the candidates in the region in parentheses, while if a specific gene was reported this is listed. Where more than one panel was used, but jointly analyzed, the sum of cases and controls in these panels is listed.



**Figure 3 Historical milestones in Crohn's disease genetics.** Developments in the knowledge of the genetics of Crohn's disease. Only milestone gene discoveries are shown. With the publication of the Crohn's disease meta-analysis in 2009 the number of replicated loci is now greater than 30.

of other components of this pathway the importance has been further demonstrated<sup>[54,72]</sup>. Importantly, a “pathway-based” analysis approach also takes into account variants that do not pass the formal threshold for being taken forward for replication in a traditional GWAS.

Several genes originally shown to associate with Crohn's disease were recently shown to confer risk also for ulcerative colitis<sup>[73,74]</sup>. This indicates the presence of

shared pathogenetic mechanisms in these closely-related conditions. However, disease genes that are specific for UC also exist, such as variants in the *IL-10* gene<sup>[11]</sup>. This specific finding is supported by the fact that *il-10* *-/-* mice develop colitis<sup>[75]</sup> closely resembling human UC. Other UC-specific regions are found at chromosome 1p36 and 12q15<sup>[12]</sup>, however, at these loci, the exact disease genes remain to be identified. The association of classical HLA alleles and UC is well known<sup>[63]</sup>, and also

the GWAS SNPs near the HLA class II genes are among the most prominent findings<sup>[11,12]</sup>. Functional enquiries are needed to clarify how the general IBD genes and phenotype-specific UC and Crohn's disease genes operate in defining the IBD phenotype and even in affecting extraintestinal manifestations such as primary sclerosing cholangitis<sup>[64]</sup>.

Except for one study regarding Crohn's disease<sup>[15]</sup>, which identified the *TNFSF15* gene in a Japanese population, all GWAS in IBD have so far been performed in populations of Caucasian descent. Follow-up studies of *TNFSF15* have demonstrated differences in the effect across different populations<sup>[76,77]</sup>. This mirrors differences in IBD epidemiology in Asian populations compared to Caucasians<sup>[78]</sup>, and further GWAS in Asia are likely to yield insight into differences in genetic susceptibility to IBD between these populations.

Notably, Crohn's disease genes identified in adult patient populations show associations also in pediatric populations of the same disease<sup>[14,79-84]</sup>.

### Colorectal cancer

Colorectal cancer is one of the most important malignancies world-wide, responsible for approximately 500 000 deaths annually<sup>[85]</sup>. The relative risk in siblings is estimated to be between 2-7, depending on the site in the colon, with the right colon showing the highest heritability<sup>[86]</sup>.

The chromosome *8q24* locus is the most widely-replicated region for colorectal cancer discovered by GWAS<sup>[87-99]</sup>. This region has proven to harbor variants that predispose to several cancer types (e.g. prostate cancer and breast cancer<sup>[100-103]</sup>). As suggested by a recent publication<sup>[98]</sup>, there is most likely more than one cancer-associated mutation at this locus. It was recently shown that one of the lead SNPs (rs6983267) is related to *MYC* expression and the activity of key Wnt signaling pathways<sup>[104,105]</sup>. In prostate cancer, it has been noted that the effect sizes of the risk variants differ among populations, necessitating the need for further characterization, e.g. systematic re-sequencing<sup>[106]</sup> of this locus, to be performed in multiple ethnicities in parallel<sup>[107]</sup>. The association between colorectal cancer and genetic variants at chromosome *18q21*, most probably in the *SMAD7* gene, has been subjected to extensive characterization. This characterization has led to the identification of a novel SNP which influences expression levels of *SMAD7*, highlighting the importance of *SMAD7* expression in colorectal carcinogenesis<sup>[108]</sup>. Interestingly, a variant at chromosome *5p15* (rs401681), originally shown to confer risk for basal cell carcinoma, was recently reported to protect against colorectal carcinoma<sup>[109]</sup>. Most likely the causative variant(s) at this latter locus remain(s) to be defined. This finding is an example of the complexity of allelic associations at a disease locus.

Most of the disease loci discovered in colorectal cancer exhibit low ORs (~1.1-1.2). Identification of further disease loci would therefore necessitate a large number of cases and controls to achieve a sufficient

power. In a recent meta-analysis of two colorectal cancer GWAS studies<sup>[88,110]</sup>, totaling 13 315 individuals, and subsequent replication in 27 418 individuals<sup>[18]</sup>, as little as four novel loci (at chromosomes *14q22.2*, *16q22.1*, *19q13.1* and *20p12.3*, respectively) were identified. This picture contrasts with the situation in Crohn's disease where smaller study panels have detected more than 30 disease loci, and highlights the challenges of detecting disease loci in conditions with a low degree of heritability.

### Celiac disease

When exposed to gluten, a protein found in wheat, rye and barley, celiac disease patients develop inflammatory lesions with villi destruction in the small intestine<sup>[111]</sup>. The main genetic factor predisposing to celiac disease, the HLA-DQB1\*0201 variant, has been known for 20 years<sup>[62]</sup>. However, since this HLA allele is present at high prevalence also in individuals who do not develop celiac disease<sup>[62]</sup>, other genetic risk factors are likely to exist. One GWAS in celiac disease has been performed and published in three stages<sup>[112-114]</sup>. The first of these publications demonstrated that, besides markers in the HLA-complex, variants in the *IL2-IL21* region are associated<sup>[112]</sup>. Evidence concerning the involvement of a specific variant in this region was not possible due to strong LD in the region. Interestingly, this region has also been shown to associate with type I diabetes, rheumatoid arthritis and recently UC, hinting at the presence of a common factor for immune-mediated diseases<sup>[115-117]</sup>. To increase the likelihood of disease-gene identification in this GWAS data, additional non-HLA SNPs were subsequently subjected to genotyping in an even larger replication panel<sup>[113,114]</sup>. Solid evidence for association at several novel regions was obtained, several of which harbor genes of relevance to immunological components of celiac disease pathogenesis (such as the chemokine-receptor cluster at chromosome *3p21*, the *IL12A* locus at *3q25-3q26* and *TNFAIP3*). For other associations, e.g. the *LPP* gene at chromosome *3q28*, further studies are required to define how defective gene function could contribute to the pathogenesis. As for the *IL2-IL21* region, several of the loci also show associations with type I diabetes<sup>[118]</sup>.

### Hirschsprung's disease

For Hirschsprung's disease, a disease characterized by lack of ganglia in the colon, there are large ethnic differences in incidence and clear cases of family aggregation, both of which are good indicators of a genetic contribution to pathogenesis. For a large proportion of familial cases and a considerable amount of sporadic cases, it has long been known that mutations in the *RET* gene are important<sup>[119]</sup>. In a small GWAS, Garcia-Barcelo *et al*<sup>[120]</sup> recently made several important discoveries. Firstly, they confirmed the associations previously detected at the *RET* locus. Secondly, they identified strong associations at the *NRG1* gene. Thirdly, they reported a significant and strong interaction effect between *NRG1* and *RET* polymorphisms in the

Table 3 Genome-wide association studies in liver disease

Disease/trait	Genotyping platform	Number of cases/ controls in discovery panel	Number of cases/ controls in replication panel(s)	Novel Loci reported	Ref.	Independently replicated
Gallstone Chronic hepatitis B	Affymetrix 500k Illumina HumanHap550	280/360 179/934	2000/1202 Second stage: 607/1267 (12k SNPs) Third stage: 1300/2100	<i>ABCG8</i> <i>HLA-DPA/B</i>	[140] [132]	[142]
Liver enzymes	Affymetrix 500k (5636 samples) Illumina HumanHap550 (1200 samples), Two different Perlegen, custom arrays (879 samples)	7715	4704	ALT: <i>CPN1-ERLIN1- CHUK</i> <i>PNPLA3-SAMM50</i> ALP: <i>ALPL, GPLD, JMJD1C-REEP</i> GGT: <i>HNF1A</i> <i>PNPLA3</i>	[122]	
Non-alcoholic fatty liver disease	Perlegen Custom array (12k)	2111		<i>PNPLA3</i>	[127]	
Primary Biliary Cir- rhosis	Illumina HumanHap370 and Illumina, HumanHap300	505/1507	526/1206	<i>IL12A, IL12RB2</i>	[27]	
Drug-induced liver injury due to Flu- cloxacillin	Illumina 1M	51/282	23 cases (only HLA typed)	<i>HLA-B*5701</i>	[124]	
Response to hepati- tis C treatment	Illumina HumanHap610	1671		<i>IL28B</i>	[137]	

If the authors mainly reported one region with several candidate genes the region is listed with the candidates in the region in parentheses, while if a specific gene was reported this is listed. Where more than one panel was used, but jointly analyzed, the sum of cases and controls in these panels is listed.

combined discovery and replication panel. The *NRG1* gene is probably important in the development of the enteric nervous system and is thus a plausible biological candidate for a Hirschsprung's disease gene. Another interesting aspect of this study is that robust gene findings are possible even in small patient panels. This is probably more likely when the phenotype is clearly defined and shows early life debut, i.e. environmental factors are less likely to be influential.

## LIVER DISEASES (SEE TABLE 3 FOR DETAILS)

### Biochemical markers of liver disease

One of the most important clinical tools for detecting, diagnosing and monitoring liver diseases is the use of different biochemical parameters (often called 'liver enzymes'). It has long been known that there are genetic factors influencing the level of these enzymes, described in genetic terms as a genuine quantitative trait<sup>[121]</sup> that could either be due to (a) variants influencing the levels without any pathological condition present and/or (b) variants that are truly associated with liver disease and which indicates that undiagnosed cases of disease are the cause of the increased blood levels. Both of these entities are important to discover as: (a) may have practical implications for the handling of apparently increased levels in healthy individuals, while (b) may serve as early markers for liver disease in apparently healthy individuals. Yuan *et al.*<sup>[122]</sup> were able to identify 6 different loci associated with the levels of these enzymes (ALT: *CPN1-ERLIN1-CHUK* and *PNPLA3-SAMM50*, ALP: *ALPL, GPLD* and *JMJD1C-REEP*, GGT: *HNF1A*). A large meta-analysis of several GWAS

datasets recently suggested highly significant associations with bilirubin levels and variants at the *UGT1A1* and *SLCO1B1* loci<sup>[123]</sup>.

### Drug-induced liver injury

In a very small genome-wide analysis in terms of sample size, a highly significant association of variants at the *HLA-B* locus was detected in 51 cases of flucloxacillin-induced liver injury<sup>[124]</sup>. The identified SNP was in LD with the *HLA-B\*5701* allele and subsequent direct genotyping confirmed this with an effect size equaling an OR of 80.6 (95% CI: 22.8-284.9). Although no formal replication panel was investigated, as many as 20 out of 23 additional cases of flucloxacillin-induced liver injury were *HLA-B\*5701* positive. This shows that development of drug-induced liver injury is clearly dependent upon host factors of which HLA variants are probably key determinants<sup>[125]</sup>.

### Non-alcoholic fatty liver disease (NAFLD)

There is an increased number of subjects with NAFLD in siblings of overweight children with NAFLD, indicating the presence of genetic risk factors in this condition<sup>[126]</sup>. In a study evaluating the genetics of NAFLD using a quantitative measure (hepatic fat content measured by magnetic resonance imaging) an association with markers in the *PNPLA3* gene was recently reported<sup>[127]</sup>. Importantly, the association was independent of key confounders such as body mass index, alcohol consumption and diabetes. Noteworthy is that the low number of SNPs assayed in the GWAS (approximately 10 000) was sufficient to generate this highly interesting finding<sup>[128]</sup>. Whereas the function of the *PNPLA3* gene is not known, the finding is

substantiated by differential regulation of the gene in different metabolic states<sup>[129]</sup>. Since NAFLD is an increasingly common condition, it is interesting to note that this locus also shows associations with alanine transaminase levels in the liver enzyme population-based study mentioned above<sup>[122]</sup>.

### **Viral hepatitis**

Hepatitis B is one of the major causes of world-wide liver morbidity and mortality<sup>[130]</sup>. Part of the variability in clinical course<sup>[131]</sup> is related to viral properties, but there is strong reason to believe that host genetics are of importance. In a three-staged GWAS design, particular variants of the class II *HLA-DPA* and *HLA-DPB* genes were recently shown to be associated with chronic hepatitis B infection in Asian populations<sup>[132]</sup>. The HLA-complex is characterized by strong LD<sup>[133]</sup> and, as hinted at by other studies, distinct HLA genes may prove to be important<sup>[134]</sup>. To clarify this, verification of the Asian findings in Caucasian populations and supplementary mechanistic studies are needed. Chronic hepatitis C is a progressive liver disease, complicated by development of cirrhosis and hepatocellular carcinoma<sup>[135]</sup>. Treatment with pegylated interferon and ribavirin offers the potential of viral eradication in up to 80% of the patients<sup>[136]</sup>. In a GWAS of treatment response, variants in the *IL28B* gene were found to be associated in the three (European-Americans, African-Americans and Hispanics) ethnicities tested<sup>[137]</sup>, shedding light on the biological mechanisms operating to define treatment success.

### **Gallstones**

Defects of bile formation are likely to be important in the development of biliary calculi<sup>[138]</sup>, inspiring candidate gene studies of important transport proteins involved in this process<sup>[139]</sup>. Given this *a priori* knowledge, it was not surprising that the main disease locus in a German gallstone GWAS was *ABCG8*, encoding a component of the cholesterol transporter heterodimer ABCG5/G8<sup>[140]</sup>. Interestingly, the findings in previous linkage studies support the GWAS results<sup>[141]</sup>. The *ABCG8* effect is not specific to Caucasian populations, as is evident from a Taiwanese report<sup>[142]</sup>.

### **Primary biliary cirrhosis**

Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease characterized by autoimmune destruction of the biliary canaliculi. There is evidence for genetic factors being involved in pathogenesis with a sibling recurrence risk reported at approximately 10.5<sup>[2]</sup>. PBC has long been known to exhibit strong HLA associations<sup>[143,144]</sup>, so it was not surprising that SNPs in the HLA-complex were among the top hits of a recent GWAS<sup>[27]</sup>. Of particular interest were associations detected at the *IL12A* and *IL12RB* loci, strongly supporting the presence of autoimmune mechanisms in PBC pathogenesis. Genetic defects of the IL-12 pathway have been proposed in other autoimmune conditions and the findings in PBC

are thus in line with a paradigm where the majority of GWAS findings in these conditions seem to be common denominators rather than disease specific-findings<sup>[145]</sup>.

## **FURTHER POSSIBILITIES WITH THE GWAS APPROACH**

### **Meta-analysis**

The advent of imputation has facilitated integration of data from different genotyping platforms<sup>[10,146,147]</sup>. A meta-analysis of GWAS data sets in type 2 diabetes has highlighted the importance of risk variants with low effect sizes<sup>[148,149]</sup>. In this condition, one of the loci with a low effect size (OR < 1.2) is the *PPARG* locus, where the biological implications in terms of glitazone therapeutics in type II diabetes has long been proven. In gastroenterology, a large GWAS meta-analysis has so far only been performed in Crohn's disease, where more than 30 loci were detected<sup>[10]</sup>. There is also a potential for combining different but related phenotypes to increase power in discovering factors common to both entities. A common risk factor for Crohn's disease and sarcoidosis, both of which are characterized by granulomas, was discovered with such an approach<sup>[16]</sup>, and similar approaches can be defined for other clinical features of otherwise unrelated conditions<sup>[109,115]</sup>.

In this way, GWAS studies also herald collaboration between research groups in different countries and across scientific traditions, a trend which can possibly generate scientific initiatives and discoveries, even beyond the meta-analyses. In the Genetic Association Information Network (GAIN) consortium (<http://www.genome.gov/19518664>), the collaboration has been formalized and has led to a series of successful publications<sup>[150]</sup>. Interestingly, researchers have also released their datasets into the public domain, partly due to requirements from their funding sources (e.g. NIH). The release of data has been facilitated through the dbGAP interface<sup>[151]</sup>; however caution needs to be exercised in terms of data protection and privacy issues<sup>[152]</sup>.

### **Copy number variants (CNVs)**

In the present review we have focused on the typical findings of a GWAS; the associations between particular SNPs and a disease trait. The importance of further genetic and functional characterization of SNP findings is highlighted by the association between a deletion polymorphism at the *IRGM* locus and Crohn's disease<sup>[153]</sup>, a mutation which was later shown to alter the expression level of *IRGM*<sup>[154]</sup>. Interestingly, this deletion was perfectly correlated with a SNP in the first study reporting an association in this region<sup>[6]</sup>. With dense SNP arrays and the aid of imputation of un-genotyped markers, there is a good chance of detecting CNV associations through LD in this manner. In addition, the genotyping chips have separate probes for CNVs and specific computer algorithms can use these probes (and even the intensities from the SNP probes) to generate genotypes for CNVs at a given position. Most likely we

will see a number of studies, even in gastroenterology and hepatology, where the application of these algorithms will lead to the identification of disease-associated CNVs<sup>[155,156]</sup>.

### Deep re-sequencing

Due to the inherent design of the SNP arrays and the available SNPs in the HapMap, rare variants (frequency < 1%-10%) are not easily detected in a GWAS analysis<sup>[157]</sup>. Also for statistical reasons, the power for detecting such variants in the unbiased GWAS design is low. In addition to common variants influencing gene expression or protein function, a disease locus can be constituted by a multitude of rare variants with a high penetrance. This is a well-known phenomenon in monogenic diseases such as cystic fibrosis, where hundreds of rare disease-causing mutations have been defined<sup>[158]</sup>. Probably the typical situation at a disease locus is a combination of common and rare disease variants, as highlighted for the *NOD2* locus (20% rare, 80% common)<sup>[159]</sup>. Only the common variants are “visible” in the GWAS design, but this does not thus exclude the presence of additional disease-causing variants at a locus only detectable by careful investigation of the identified disease genes. The identification of a disease-related variant with a functional effect, even if only present in singular patients<sup>[160]</sup>, can yield important insight into the pathogenesis of a condition. Over the last few years second generation sequencing technology (also called next-generation) has become available. This technology is able to resequence large regions of DNA or even complete human individual genomes<sup>[161]</sup>. There are now three commercial platforms available (SOLiD from Applied Biosystems, 454/FLX from Roche and Genome Analyzer from Illumina), all of which offer unprecedented throughput when compared to traditional Sanger sequencing (which has been the gold standard since 1977<sup>[162]</sup>). The application of a combined re-sequencing/association study approach was recently demonstrated in type 1 diabetes with the identification of *IFIH1* variants with likely functional effects<sup>[163]</sup>. No such extensive re-sequencing studies have so far been performed in gastroenterology or hepatology.

### CONCLUSION

GWAS have proven to be an important tool for dissecting the genetic architecture of common diseases. The genotyping arrays and statistical tools are now mature and their application has been a huge success in gastroenterology. In crude numbers, in only 2-3 years, the approach has delivered many times the number of genes that were discovered during the first 15-20 years of gastroenterological disease genetics. As illustrated by several of the findings we have summarized in this review, the effect sizes are often low and can only be discovered with large case-control collections. Clearly, for some diseases (such as primary sclerosing cholangitis) this situation may not be achievable and complementary approaches (e.g. pathway analyses<sup>[52-54]</sup>) may be needed to

identify genetically defined disease mechanisms.

As we have argued in the present review, and which is also a view shared by the editors of prominent journals, the size of the study panels is important. Interestingly, however, several of the genes so far confirmed were initially detected in what was an apparently under-powered discovery panel (e.g. *TNFSF15* discovered in 94 cases<sup>[15]</sup>). In addition, clinically highly important findings have been made in small case-control panels<sup>[57]</sup>. Therefore, GWAS in smaller disease populations should also be welcomed, since identifying as little as a single disease gene can open broad avenues for future mechanistic studies<sup>[127]</sup>.

In addition to the results from recent GWAS, it is worth mentioning a gene identified through a classical candidate gene approach. After identification of the role of *XBP1* in endoplasmic reticulum stress in a mouse *xbp1* knock-out model, polymorphisms in this gene were tested in a large panel of IBD patients<sup>[164]</sup>. In terms of *P*-values, several of the IBD associations detected at this locus are below the detection limit of the “GWAS radar” and highlight why hypothesis-based candidate gene approaches still have a role in disease genetics.

Even in the case of large effect sizes, the new GWAS findings may not have immediate implications for clinical practice<sup>[124]</sup>, however, and what seems to be the bottom line of the present role of these studies in biology serves as a glimpse of the intricate pathology involved. Whereas few, if any, of the GWAS have provided a comprehensive mechanistic explanation as to how the detected polymorphisms affect disease risk, they have defined the priorities for basic research for decades to come. Ultimately, the challenge and goal for this research will be to define relevant diagnostic and prognostic markers as well as novel therapeutic options.

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## Peutz-Jeghers syndrome: Diagnostic and therapeutic approach

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### Abstract

Peutz-Jeghers syndrome (PJS) is an inherited, autosomal dominant disorder distinguished by hamartomatous polyps in the gastrointestinal tract and pigmented mucocutaneous lesions. Prevalence of PJS is estimated from 1 in 8300 to 1 in 280000 individuals. PJS predisposes sufferers to various malignancies (gastrointestinal, pancreatic, lung, breast, uterine, ovarian and testicular tumors). Bleeding, obstruction and intussusception are common complications in patients with PJS. Double balloon enteroscopy (DBE) allows examination and treatment of the small bowel. Polypectomy using DBE may obviate the need for repeated urgent operations and small bowel resection that leads to short bowel syndrome. Prophylaxis and polypectomy of the entire small bowel is the gold standard in PJS patients. Intraoperative enteroscopy (IOE) was the only possibility for endoscopic treatment of patients with PJS before the DBE era. Both DBE and IOE facilitate exploration and treatment of the small intestine. DBE is less invasive and more convenient for the patient. Both procedures are generally safe and useful. An overall recommendation for PJS patients includes not only gastrointestinal multiple polyp resolution, but also regular lifelong cancer screening (colonoscopy, upper endoscopy, computed tomography, magnetic resonance imaging or ultrasound of the pancreas, chest X-ray, mammography and pelvic examination

with ultrasound in women, and testicular examination in men). Although the incidence of PJS is low, it is important for clinicians to recognize these disorders to prevent morbidity and mortality in these patients, and to perform presymptomatic testing in the first-degree relatives of PJS patients.

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**Key words:** Gastrointestinal endoscopy; Intraoperative period; Peutz-Jeghers syndrome; Hamartoma

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### INTRODUCTION

Peutz-Jeghers syndrome (PJS) belongs among the most important familial hamartomatous polyposis syndromes, and is associated with significant morbidity, variable clinical course and considerable predisposition to malignancy. This editorial will attempt to give an overview of up-to-date knowledge on diagnostic and therapeutic aspects of this disease.

### DEFINITION AND HISTORY OF PJS

PJS is an inherited, autosomal dominant disorder with variable inheritance, characterized by hamartomatous polyps in the gastrointestinal tract, mostly in the small bowel, and pigmented mucocutaneous lesions. PJS was first reported in a pair of identical twins with melanotic macules described by Connor in 1895 and illustrated by Hutchinson in 1896<sup>[1,2]</sup>. Later in life, the twins developed what are now known to be additional features of PJS; one died of intussusception at age 20 years, and the other died of breast cancer at the age of 52 years<sup>[3,4]</sup>.

The primary description of PJS was published by Peutz in 1921 in one Dutch family (the Harrisburg family)

as a gastrointestinal familial polyposis with pigmentations. Simultaneously occurring nasal polyposis was described in the original report by Peutz<sup>[5]</sup>. The pedigree of this original Dutch family continues to be followed<sup>[6,7]</sup>. Jeghers specified the description in 10 cases from different families in his work in 1949, and defined the relations between pigmented lesions, gastrointestinal polyposis and increased risk of carcinoma; approximately half of his patients suffered from gastrointestinal malignancy<sup>[3]</sup>. The eponym PJS was first used in 1954<sup>[8]</sup>. The first histological description of hamartomatous polyps was made in 1957 by Horrilleno and colleagues<sup>[9]</sup>. Since this time, descriptions have appeared of several different syndromes with the propensity to develop these polyps in the upper and lower gastrointestinal tracts. The hamartomatous polyposis syndromes are a heterogeneous group of disorders, which are inherited in autosomal dominant fashion. These syndromes include familial juvenile polyposis syndrome, PJS, phosphatase and tensin homolog gene (PTEN) hamartoma tumour syndromes (Cowden's and Bannayan-Riley-Ruvalcaba syndromes), multiple endocrine neoplasia syndrome 2B, hereditary mixed polyposis syndrome, Cronkhite-Canada syndrome, basal cell nevus syndrome, and neurofibromatosis 1. The hamartomatous polyposis syndromes represent only a small number (< 1%) of the inherited gastrointestinal cancer predisposition syndromes<sup>[10,11]</sup>.

A history of PJS with biographies of Peutz and Jeghers has been published<sup>[12]</sup>, and many early PJS papers have been made available online by the Jeghers Medical Index ([www.jeghers.com/pj\\_pubmed.aspx](http://www.jeghers.com/pj_pubmed.aspx)). The website [www.peutz-jeghers.com](http://www.peutz-jeghers.com) is another resource for PJS patients and healthcare providers.

Association of Cancer Online Resources (ACOR) is a large collection of cancer-related internet mailing lists, which have delivered over 1.5 million e-mail messages per week to subscribers across the globe. In addition to supporting the mailing lists, ACOR develops and hosts state-of-the-art internet-based knowledge systems that allow the public to find and use credible information relevant to their illness (PJS Online Support Group, <http://listserv.acor.org/archives/pjs.html>).

## PREVALENCE

The estimation of population prevalence of PJS differs between studies. The widest estimated range is from 1 in 8300 to 1 in 280000 individuals<sup>[11,13-18]</sup>. Probable prevalence is around 1 in 100000 people. The disease has variable penetrance, even within families; some members will only manifest hyperpigmentation, while others may manifest pigmentations and hamartomatous polyps.

Very important data are available thanks to different registries of polyposis. The St. Mark's Polyposis Registry ([www.polyposisregistry.org.uk](http://www.polyposisregistry.org.uk)) is the oldest in the world. This was started in 1924 by Dr. Cuthbert Dukes and Mr JP Lockhart Mummery. The statement made by Dr. Dukes in 1958 remains true today: *"It would be difficult to find a more promising field for the exercise of cancer control than a*

*polyposis family, because both diagnosis and treatment are possible in the precancerous stage and because the results of surgical treatment are excellent"*.

The Danish Polyposis Register was established in 1971 and the register became national in 1975.

American Family History Registries are also available on the net: [www.fascrs.org/patients/family\\_history\\_registries/](http://www.fascrs.org/patients/family_history_registries/).

## GENETICS AND PATHOPHYSIOLOGY

Genetic testing is suitable for confirmation of PJS. Nowadays, the only identifiable mutations causing PJS affect the STK11 (serine/threonine-protein kinase 11 alias LKB1) gene, located on chromosome 19p13.3. STK11 is the official designation for LKB1 by the Human Genome Organisation (HUGO); [www.genenames.org/data/hgnc\\_data.php?hgnc\\_id=11389](http://www.genenames.org/data/hgnc_data.php?hgnc_id=11389). This gene was identified in 1998. It encodes for a multifunctional serine-threonine kinase, important in second messenger signal transduction. The serine-threonine kinase modulates cellular proliferation, controls cell polarity, and seems to have an important role in responding to low cellular energy levels<sup>[15,19]</sup>. In the performance of this last role, the STK11 protein is involved in the inhibition of AMP-activated protein kinase (AMPK), and signals downstream to inhibit the mammalian target of rapamycin (mTOR; also known as FKBP12-rapamycin complex-associated protein or FRAP) pathway; the mTOR pathway is dysregulated in patients with PJS<sup>[15]</sup>. Although the exact mechanism of action of STK11 has not been outlined completely, the function of this protein product is likely to be important in growth inhibition. Genetic alterations in STK11 may represent loss of heterozygosity at a tumor suppressor gene locus.

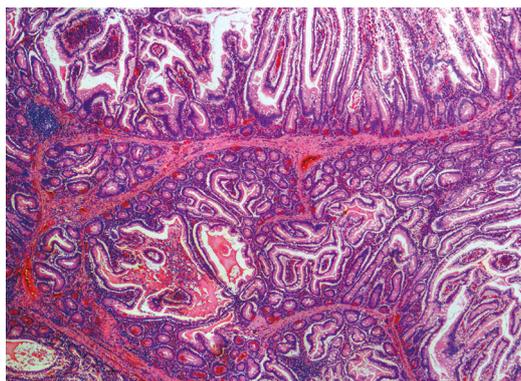
Recent studies have suggested the involvement of STK11 also in more common human disorders including diabetes mellitus and in a significant fraction of lung adenocarcinomas. These observations have increased the interest towards the signaling pathways of this tumor suppressor kinase<sup>[20]</sup>.

Genetic testing for STK11 mutations is available but they have variable sensitivity; in familial cases, 70%, in sporadic cases, from 30% to 67%. A significant proportion of familial and sporadic PJS may result from mutations in genes other than STK11 or so far unidentified means of LKB1 inactivation. In total, 91% of the studied families have shown LKB1 inactivation<sup>[11,13,14,16,17,19,21-23]</sup>. Variable penetrance and clinical heterogeneity make it difficult to determine the exact frequency of PJS<sup>[24]</sup>.

Germline mutations in the tumor suppressor gene PTEN (10q22-23) are responsible for a group of phenotypically diverse conditions, which have collectively been called the PTEN hamartoma tumor syndrome. These are rare autosomal dominant conditions different from PJS<sup>[15,22]</sup>.

## PATHOLOGY

The hamartomatous polyposis syndromes are chara-



**Figure 1** Hamartoma, a typical PJS polyp demonstrating the arborizing pattern of smooth-muscle proliferation. HE staining, magnification 100 ×. (Courtesy of Professor A. Ryska, MD, PhD).



**Figure 3** Hamartoma of the jejunum.

characterized by an overgrowth of cells native to the area in which they normally occur. It is important to note that there is an overgrowth of cells or tissues, at least initially, with no presumed neoplastic potential<sup>[14,22]</sup>. Hamartomatous polyps are composed of the normal cellular elements of the gastrointestinal tract, but have a markedly distorted architecture<sup>[11,15]</sup> (Figure 1). Microscopically, extensive smooth-muscle proliferation, with an elongated, arborized pattern of polyp formation, can be seen<sup>[15,16]</sup>. PJS-associated polyps can be differentiated from sporadic hamartomatous polyps and hamartomatous polyps associated with other syndromes by a unique smooth muscle core that arborizes throughout the polyp. PJS-type polyps do not have specific endoscopic features and can only reliably be distinguished from other types of polyps by histopathology. The unique PJS polyp pathology is best appreciated in PJS small intestine polyps<sup>[25]</sup>. The histopathology of PJS-associated gastric polyps can be similar to hyperplastic gastric polyps.

Larger hamartomas often contain foci of adenomatous tissue; a malignant development in a hamartomatous polyp has been described<sup>[7,26-28]</sup>.

## CLINICAL FEATURES AND NATURAL HISTORY OF THE DISEASE

Hyperpigmentation is present as mucocutaneous macules



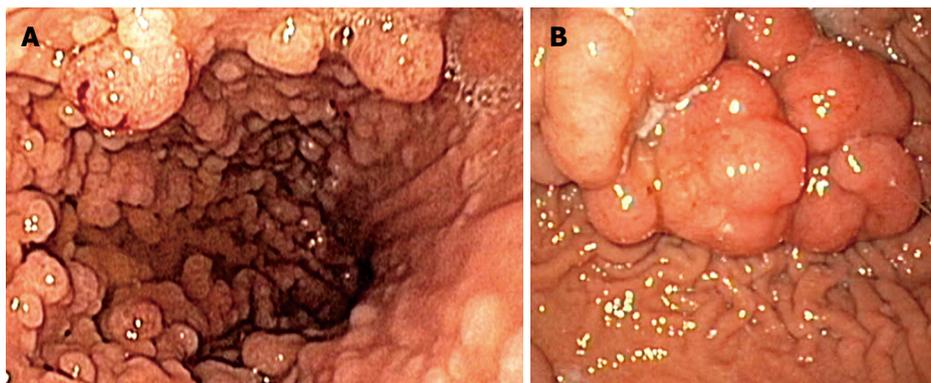
**Figure 2** Pigmentations of the lips and oral mucosa.

on the lips (Figure 2) and around the mouth, eyes, nostrils, and on the buccal mucosa; and sparsely on the fingers, soles of the feet, palms, anal area and intestinal mucosa. Characteristic pigmentations are present in 95% of the patients and are caused by pigment-laden macrophages in the dermis. They are typically flat, blue-gray to brown spots 1-5 mm in size. Malignant degeneration of these lesions is extremely rare. These macules can be distinguished from common freckles as the latter never appear in the oral cavity, are sparse near the lips and nostrils, and absent at birth. Hyperpigmentations can even disappear during adolescence. Diagnosis is defined by the presence of histopathologically confirmed hamartomatous polyps (Figure 1) and at least two of the following clinical criteria: family history, hyperpigmentation and polyps in the small bowel<sup>[11,13-17,21,22,29]</sup> (Figure 3).

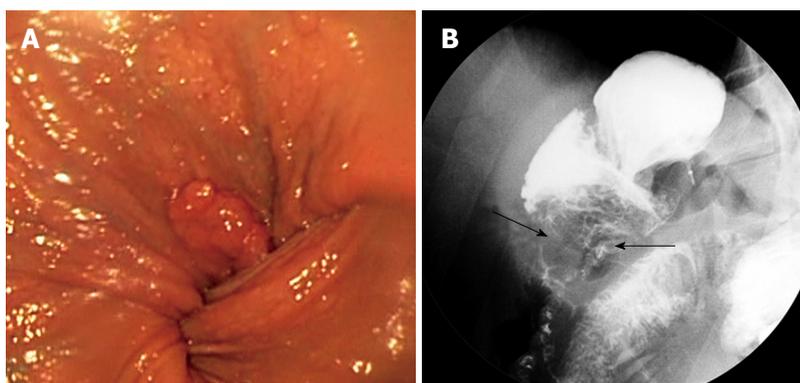
The median time to first presentation with polyps is about 11-13 years of age, and approximately 50% will have experienced symptoms by the age of 20 years<sup>[13,14]</sup>. During the first three decades of life, anemia, rectal bleeding, abdominal pain, obstruction and/or intussusception are common complications in patients with PJS<sup>[13,15]</sup>. Nearly half of the patients experience an intussusception during their lifetime, most often in the small intestine<sup>[30]</sup>.

The setting of 222 patients with PJS was presented by Utsunomiya *et al.*<sup>[31]</sup> in 1975. They presented patients with PJS in Japan ascertained between 1961 and 1974. The average age at diagnosis was 23 years in men and 26 years in women, with a male to female ratio of 1:1.13. Obstruction was observed in 42.8%, intussusception in 46.9%, most often in the small intestine, and rectal bleeding in 13.5% of the patients. The polyps occurred in the stomach in 48.6% (Figure 4A and B), small intestine 64%, colon 53.2%, and rectum 32%<sup>[31]</sup>. This study demonstrated the natural history of the disease despite this being based on surveys, and the study suffering some imperfections (e.g. cancer was found only in 28 of 222 patients).

Hearle *et al.*<sup>[32]</sup> have published a study of STK11 status and intussusception risk in PJS. According to this study, 135 (60%) of the probands possessed a germline STK11 mutation, and 109 (48%) probands had a history of intussusception at a median age of 15.0 years but with wide variability (range: 3.7-45.4 years). Median



**Figure 4** Hamartomas of the stomach. A: Multiple; B: Voluminous.



**Figure 5** Gastric outlet obstruction (same patient as seen in Figure 4B). A: Endoscopic view; B: fluoroscopy, mass of polyps marked by arrows.

time to onset of intussusception was not significantly different between those with identified mutations and those with no mutation detected, at 14.7 and 16.4 years, respectively. Similarly, no differences were observed between patient groups on the basis of the type or site of STK11 mutation<sup>[32]</sup>.

A quite rare but possible complication is gastric outlet obstruction caused by a giant gastric polyp<sup>[33-35]</sup> (Figure 4B and Figure 5). Children with PJS have a risk of numerous laparotomies due to the complications. Gastrointestinal screening of the small bowel should be started from childhood and when polyps develop they should be managed if possible by endoscopic resection<sup>[29,36]</sup>. PJS-related polyps occur most frequently in the small intestine. Over 90% of affected individuals will develop polyps in the small intestine during their lifetime. The incidence within the small intestine is greatest in the jejunum and progressively decreases in the ileum and duodenum<sup>[13,37,38]</sup>. Involvement of the colon (53% of patients), stomach (49%), and rectum (32%) is also seen<sup>[15,33-35]</sup>. Well-timed polypectomy may obviate the need for repeated urgent operations and extensive small bowel resections leading to short bowel syndrome<sup>[39,40]</sup>.

Extraintestinal polyps are also reported. Nasal polyposis is thought to be a rare complication. de Leng *et al*<sup>[41]</sup> have observed nasal polyposis in eight (15%) of 52 Dutch patients with PJS. The loss of heterozygosity, and the absence of eosinophilia suggest a distinct pathogenesis compared with sporadic nasal polyposis. In the same study, 11 of 12 PJS-associated nasal polyps were found to express cyclooxygenase-2 (COX-2) compared with 19 of 28 sporadic nasal polyps<sup>[41]</sup>. Six of 22 members of the

original Peutz pedigree have been diagnosed with nasal polyposis<sup>[6]</sup>. Three PJS patients have been reported with nasopharyngeal carcinoma<sup>[6,42]</sup>.

In a series of 72 PJS patients, three (4.1%) had gallbladder polyps<sup>[43]</sup>. Also reported are one PJS patient who required cholecystectomy for gallbladder obstruction by polyps, and one with common bile duct obstruction caused by polyps<sup>[44]</sup>. Two PJS patients have been reported with gallbladder cancer; one of whom had gallbladder cancer arising near but not in hamartomatous gallbladder polyps<sup>[31,45]</sup>. Several PJS patients have been reported with bile duct cancer (cholangiocarcinoma)<sup>[46,47]</sup>. Hamartomatous polyps in PJS patients have also been reported in the ureter<sup>[48]</sup>, respiratory tract<sup>[49,50]</sup> and on the tonsils<sup>[51]</sup>.

PJS is associated with specific genetic mutations and an increased lifetime risk of both gastrointestinal and extraintestinal malignancies<sup>[22]</sup>; i.e. pancreatic, lung, breast, uterine, ovarian and testicular malignancies (Sertoli cell tumors secrete estrogen and can lead to gynecomastia). Inherited forms of gastrointestinal cancer have attracted the attention of scientists over the past two decades, putting the accent on prevention. The possibility of developing any cancer by age 65 years was 37% in accordance with data from the St. Mark's Polyposis Registry<sup>[52]</sup>. Hearle *et al*<sup>[53]</sup> have analyzed the incidence of cancer in 419 PJS patients, and 297 of them had documented STK11/LKB1 mutations. Ninety-six cancers were found among these patients. The risk for developing cancer at ages 20, 30, 40, 50, 60 and 70 years was 2%, 5%, 17%, 31%, 60% and 85%, respectively. The most common cancers represented in this analysis were

of gastrointestinal origin. In non-gastrointestinal tumors, breast cancer was the most common, with the risk being 8% and 31% at ages 40 and 60 years, respectively. The cancer risk was similar in the STK11/LKB1 mutation positive and negative group<sup>[53]</sup>.

Mehenni *et al.*<sup>[42]</sup> have followed the cumulative incidence of cancer in 149 PJS patients with germline mutation(s) in LKB1, this being estimated using Kaplan-Meier analysis of time to cancer onset, and compared between relevant subgroups with log rank tests. Thirty-two cancers were found in LKB1 mutation carriers. Overall cancer risks at ages 30, 40, 50, 60 and 70 years were 6%, 18%, 31%, 41% and 67%, respectively. There were similar overall cancer risks between male and female carriers. However, there were overall cancer risk differences for exon 6 mutation carriers *vs* non-exon 6 mutation carriers. Most (22/32) of the cancers occurred in the gastrointestinal tract, and the overall gastrointestinal cancer risks at ages 40, 50, 60 and 70 years were 12%, 24%, 34% and 63%, respectively. In female patients, the risk of developing gynecological cancer at age 40 and 50 years was 13% and 18%, respectively. Mutations in exon 6 of LKB1 were associated with a higher cancer risk than mutations within other regions of the gene<sup>[42]</sup>.

In a meta-analysis of Giardiello *et al.*<sup>[54]</sup>, the cumulative risk of developing any cancer in PJS was 93%. Of all tumors associated with PJS, breast cancer poses the greatest risk, affecting 32%-54% of patients<sup>[54]</sup>. In their systematic study, Boardman *et al.*<sup>[55]</sup> have determined that the relative risk for all cancers in PJS patients significantly increased (RR = 9.9). The relative risk for gynecological and breast cancers in women was 20.3, and for gastrointestinal cancers, 50.3<sup>[55]</sup>. Screening recommendations for PJS patients includes not only gastrointestinal multiple polyp resolution, but also regular cancer screening.

## TREATMENT

There are two basic modalities in diagnosis and treatment of small bowel hamartomas: intra-operative enteroscopy (IOE) and double balloon enteroscopy (DBE).

DBE is a new enteroscopy method that allows examination and treatment of the jejunum and ileum in almost all patients. The system consists of a 200-cm enteroscope and a 145-cm over-tube which have soft latex balloons at their tips. By using these balloons to grip the intestinal wall, the endoscope can be inserted further without forming redundant loops of intestine<sup>[56]</sup>.

IOE is a combination of laparotomy (or laparoscopy) with endoscopy. It allows manipulation to ensure the entire small bowel is visualized and nearly all polyps are removed in an endoscopic or surgical manner<sup>[17]</sup>.

IOE was accepted as the ultimate diagnostic and/or therapeutic procedure for complete investigation of the small bowel, especially before the DBE era. A surveillance program published in the *British Journal of Surgery* in 1995 recommended IOE for small bowel polyps or abdominal pain in patients with PJS<sup>[22]</sup>. At that time,

IOE improved polyp clearance without the need for additional enterotomy and helped to reduce the frequency of laparotomy<sup>[56]</sup> (Figure 6).

The first clinical application of DBE occurred in 1999 and was reported 2001 by its inventor Yamamoto *et al.*<sup>[57]</sup>. DBE was established into clinical practice in 2003 and has taken the place of IOE for most indications. DBE is in most cases a suitable replacement for push enteroscopy, IOE, and to some extent, small bowel follow-through and computed tomography (CT)<sup>[58]</sup>. In spite of this, there is a small group of patients unsuitable for the DBE method. There could be some small intestinal adhesions after previous surgery making the DBE method impossible. It is necessary to inform all patients before DBE of possible surgical laparotomy with IOE or small bowel resection in case of failure of the DBE method<sup>[39]</sup>.

It is necessary to clear away all small intestinal polyps as far as it is possible (at least all polyps greater than 5 mm). Oncl *et al.*<sup>[59]</sup> have compared two groups of PJS patients. The first group of eight patients had only problem-focused surgery because of bleeding or obstruction. These patients required 23 further operations within 87 patient-follow-up-years (2.64 operations per year). The second group of three patients were operated upon using IOE, with removal of all small intestinal polyps. These patients did not require any further surgery within 21 patient-follow-up-years<sup>[59]</sup>. Timely clearance of all small intestinal polyps and careful screening is the gold standard for PJS patients.

## IOE

IOE is still a useful method for a specific group of patients (e.g. failure of DBE, adhesions, multiple small transmural lesions unresolvable by endoscopic methods, carcinoids, and blue rubber bleb nevus syndrome) and therefore it is necessary to be able to use this method. We started with IOE in our department in 1995, where more than 7000 gastrointestinal endoscopies are accomplished per year. Over the course of the following years, the average need for this investigation stabilized to 6-8 per year. After launching DBE in March 2006 in our endoscopy unit, the need for IOE dropped to 1-2 cases per year.

IOE still remains a unique method of small bowel investigation and a solution for some pathological findings. The investigation is invasive, therefore, precise indication is imperative. The procedure takes place in the operating theater with close cooperation with surgical team. One of the advantages of IOE is that there is a surgeon on the other side of the small bowel wall. This allows the endoscopist to be more invasive and calmer. Perfect teamwork by the surgeon, endoscopist and anesthesiologist is indispensable.

The day before the investigation, a standard colonoscopy preparation of the bowel is performed (oral sodium phosphate or macrogolum solution). All patients are investigated under general anesthesia and intubated. Over-inflation of the stomach is prevented by introducing

a nasogastric tube for permanent suction at the beginning of the procedure. The physician performing the endoscopy becomes a member of the operating team. All our IOEs are performed using standard laparotomy with a mid-small bowel enterotomy. Polyps are removed preferentially in an endoscopic manner using a polypectomy snare. Some bigger polyps were removed by surgical excision because it was easier and less time-consuming. The characteristic technical details of IOE have already been presented<sup>[39]</sup>.

### DBE

DBE has an accessory channel and good maneuverability in the distal small intestine, and it enables endoscopic treatment, including polypectomy. DBE is also useful for cases of difficult colonoscopy, providing success rates of total colonoscopy between 88%-100%. Although it has been a few years since its development, the usefulness of DBE is now well recognized. This challenging procedure has rapidly become popular and currently is used in many countries<sup>[60]</sup>. DBE is clinically useful for obtaining the diagnosis, starting treatment and providing therapeutic endoscopy. DBE is a useful and safe method of obtaining tissue for diagnosis and carrying out polypectomy<sup>[61]</sup>.

The investigation is performed after overnight fasting using oral procedures, or after bowel preparation using standard colonoscopy preparation (oral sodium phosphate or macrogolum solution) and anal (retrograde) procedures. Total inspection by DBE usually is achieved by a combination of sequential oral and anal intubation; success rates are reported to be 40%-80%<sup>[60]</sup>. To confirm total enteroscopy, we use Indian ink tattooing of the small bowel. Total enteroscopy is also confirmed in the case of reaching the cecum by an oral approach, which is achieved in 10% of all oral DBEs in our setting.

We have used CO<sub>2</sub> insufflation in DBE procedures regularly since 2007. We have had no complications with hyperinflation, the comfort of the patient rapidly increases, and this type of insufflation is helpful for easier and deeper insertion of the endoscope, because the absorption of CO<sub>2</sub> is 150 times faster than absorption of air in the bowel. Combination of water with simethicone is used routinely to do away with bubbles in the intestine.

Venous access is obtained before the procedure. All patients are monitored during the procedure, for oxygen saturation, heart rate, and blood pressure. Intravenous crystalloids are administered during DBE. Conscious sedation seems to be much better in DBE in comparison with general anesthesia. Abdominal pain is a very important warning signal, and it is necessary to terminate the procedure immediately in such cases. Intense pain may be a sign of inadequate pressure on the pancreas and high risk of post-DBE pancreatitis<sup>[62]</sup>. We use small intravenous repetitive doses of midazolam and pentazocine for conscious sedation (batch-wise).

The oral approach is preferred in all patients, because the number of polyps is significantly higher in the jejunum than the ileum. If panenteroscopy is not achieved by the oral approach, we perform Indian ink marking of the

small bowel. Control capsule enteroscopy is performed subsequently to locate possible additional polyps in an uninvestigated part of the small bowel (in the distal small bowel below the Indian ink tattooing). After finding such additional polyps, we continue with oral DBE and complete the polypectomy.

Endoscopic therapy in the small intestine, whose wall is very thin, should be performed with special care to avoid complications such as bleeding and perforation<sup>[63]</sup>. To prevent bleeding, we use only pure coagulation for cutting of hamartomas with a long stalk. If the stalk is long enough to prevent hypercoagulation of the intestinal wall, this method is safe and very useful. However, this method must be abandoned for polyps with a short stalk or for sessile polyps. The use of too much coagulation could lead to hypercoagulation of the intestinal wall and perforation within a few days after the procedure.

### Chemoprevention

Some recent studies have demonstrated the chemopreventive efficacy of rapamycin on PJS in a mouse model<sup>[64-66]</sup>. Rapamycin (sirolimus) is a macrolide compound with immunosuppressant properties that is obtained from *Streptomyces hydropiscus*. Rapamycin treatment led to a dramatic reduction in polyp burden and size. A significant reduction in microvessel density was seen in polyps from the rapamycin-treated mice compared to those from the control group. The antiangiogenic effect of rapamycin may play a role in polyp reduction<sup>[66]</sup>.

The potent antiproliferative activity of the macrolide antibiotic rapamycin is known to involve binding of the drug to its cytosolic receptor, FKBP12, and subsequent interaction with targets of rapamycin, resulting in inhibition of p70 S6 kinase. However, the downstream events that lead to inhibition of cell cycle progression remain to be elucidated. The antiproliferative effects of rapamycin are associated with prevention of mitogen-induced downregulation of the cyclin-dependent kinase inhibitor p27Kip1, which suggests that the latter plays an important role in the growth pathway targeted by rapamycin. Murine BC3H1 cells, selected for resistance to growth inhibition by rapamycin, exhibited an intact p70 S6 kinase pathway but had abnormally low p27 levels that were no longer responsive to mitogens or rapamycin. Fibroblasts and T lymphocytes from mice with a targeted disruption of the p27Kip1 gene had impaired growth-inhibitory responses to rapamycin. These results suggest that the ability to regulate p27Kip1 levels is important for rapamycin to exert its antiproliferative effects<sup>[67]</sup>.

A pilot open-label study starts this year in the University of Utah and recruiting of participants *via* the internet ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)). Rapamycin analogs, which are already FDA-approved and are being used in more than 50 ongoing clinical trials, could one day be used for the treatment of PJS patients.

De Leng *et al*<sup>[68]</sup> have studied the presence of COX-2 in PJS. Moderate or high levels of epithelial COX-2 were present in 25% of hamartomas, including two hamartomas

with dysplastic changes, and 64% of carcinomas. The presence of COX-2 expression in PJS carcinomas and dysplastic hamartomas provides a rationale for chemoprevention with nonsteroidal anti-inflammatory drugs or COX-2 inhibitors. Focal immunohistochemical changes, which may indicate premalignant potential, were present in some nondysplastic PJS hamartomas. Molecular changes in carcinomas and dysplastic hamartomas in PJS are distinct from the usual adenoma-carcinoma sequence<sup>[68]</sup>.

The study of Udd *et al*<sup>[69]</sup> was designed to determine whether COX-2 inhibition reduced tumor burden in Lkb1(+/-) mice or PJS patients. Genetic interactions between COX-2 and Lkb1 in polyp formation were analyzed in mice with combined deficiencies in these genes. Pharmacological inhibition of COX-2 was achieved by supplementing the diet of Lkb1(+/-) mice with celecoxib. In PJS patients, COX-2 was inhibited with a daily dose of 2 × 200 mg celecoxib for 6 mo. Total polyp burden in Lkb1(+/-) mice was significantly reduced in a COX-2(+/-) (53%) and in a COX-2(-/-) (54%) background. Celecoxib treatment initiated before polyposis (3.5-10 mo) led to a dramatic reduction in tumor burden (86%) and was associated with decreased vascularity of the polyps. Late treatment (6.5-10 mo) also led to a significant reduction in large polyps. In a pilot clinical study, a subset of PJS patients (2/6) responded favorably to celecoxib with reduced gastric polyposis<sup>[69]</sup>. Both studies suggest that COX-2 chemoprevention is beneficial in the treatment of PJS.

Metformin has been shown to inhibit mTOR activity in breast cancer cells but is unable to inhibit mTOR in cells that lack STK11<sup>[70,71]</sup>. Based on these data, it is unclear how effective metformin would be in PJS patients who are germline haplo-insufficient for STK11 and in PJS neoplastic tissue that does not express STK11.

Lifestyle factors including excess weight, lack of exercise, smoking, and alcohol use are risk factors for the cancers associated with PJS. Although no study of PJS patients has shown that modification of these risk factors reduces cancer risk, all PJS patients should be advised to adopt a healthy lifestyle.

## OVERALL EXPERIENCE OF PJS PATIENTS

New endoscopic technology may improve management of intestinal polyposis<sup>[24,37]</sup>. Nowadays, DBE in combination with capsule enteroscopy are the gold standard for diagnosis and treatment of the small bowel<sup>[72]</sup>. These methods have replaced IOE in nearly all patients with PJS. Until only recently, primary surgical resection and IOE were the only available possibilities for treating polyps in the mid-small bowel in patients with PJS<sup>[73-76]</sup>. DBE and video capsule enteroscopy have changed this approach and it is now possible to not only to perform endoscopic surveillance and diagnose these lesions, but also to resect them<sup>[57,58,73,77,78]</sup>. Many reports on DBE have suggested that this new method may be able to replace at least IOE in many circumstances<sup>[63,79,80]</sup>.

Indications of IOE have diminished over recent

**Table 1** Benefits and drawbacks of endoscopic methods<sup>[39,77]</sup>

Method	Intra-operative enteroscopy	Double balloon enteroscopy
Benefits	All polyps removed in one procedure	Less invasive
	Partnership with a surgeon	Well tolerated
	Less time-consuming for endoscopist	Shorter sick leave
Drawbacks	Necessity of laparotomy	Often more than one procedure required
	Possibility of adhesions formation	Not feasible in some patients (because of adhesions)
	Longer convalescence	Longer procedure

years because of the development of DBE<sup>[57]</sup>. Despite its current introduction into clinical practice<sup>[80-82]</sup>, IOE is reserved for those cases in which DBE cannot be performed or fails to investigate the entire small intestine, especially to prevent excessive bowel resection. The advantages and disadvantages of IOE and DBE methods are summarized in Table 1.

Pennazio *et al*<sup>[83]</sup> have followed seven patients during a 10-year period. During the first period, five patients underwent emergency small bowel resection (two were operated on twice). Subsequently, three of four patients with diffuse polyposis underwent IOE during which, on average, 16 polyps per patient were removed (range: 10-25 polyps; mean diameter: 16 mm, range: 3-50 mm). The other three patients with polyps only in the proximal jejunum underwent periodic push enteroscopy alone (mean: three per patient), during which, a mean of 12 polyps per patient were removed (range: 7-15 polyps; mean diameter: 11 mm, range: 3-40 mm)<sup>[83]</sup>.

From 1999 to 2006 we performed seven IOEs in seven patients (four women and three men) in our single tertiary center. A total number of 182 polyps were removed during IOE; 179 by an endoscopist and three by a surgeon. From six to 75 polyps were removed per session (mean: 26). The largest hamartoma measured 4 cm in diameter. The age of the patients ranged from 20 to 50 years (mean: 31 years). The mean time of the endoscopic part of the procedure was not measured exactly; it was estimated at 60 min. We had no serious complications in the IOE group.

From 2006, we accomplished 11 DBEs in another seven PJS patients (five women and two men). In our DBE group, a total of 205 polyps were removed. The age of the patients ranged from 12 to 48 years (mean: 25 years). From one to 37 polyps were removed per session (mean: 13). All polyps were resolved using the endoscopic method (snare polypectomy). The largest hamartoma was 6 cm in diameter and was removed by a piecemeal technique without complications. The mean time required to carry out the DBE procedure was 113 min (range: 20-270 min). All patients from the DBE group were resolved by means of endoscopic polypectomy; none of them had to be operated on. We had no serious complications in the DBE group.



**Figure 6** Intra-operative enteroscopy. Polypectomy snare over a small polyp shines through the intestinal wall.

The endoscopist performing DBE should be trained not only in endoscopy, but also in polypectomy. He/she must be experienced and be able to resolve complications<sup>[73,77]</sup>. Moreover, it is necessary to have close collaboration with a surgeon in case of complications. Complete polypectomy in IOE or DBE can provide a longer symptom-free interval<sup>[39,40,77]</sup>.

In the series published to date, complications of DBE solely relating to the diagnostic procedure are rare. A recent retrospective multicenter survey in four countries has indicated a complication rate of 0.8% (13/1728) in diagnostic procedures and 4.3% (27/634) for therapeutic DBE, and there were no fatal cases<sup>[63,84,85]</sup>.

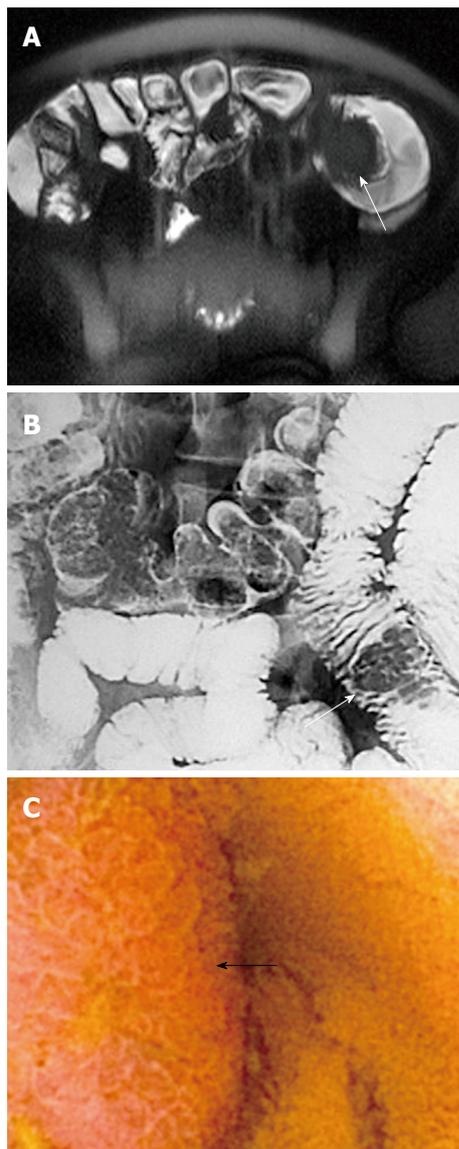
During DBE, careful attention must be paid not to insufflate too much air, because this induces intestinal loop formation and impedes deep advancement<sup>[73]</sup>. With CO<sub>2</sub> insufflation, we solved this problem completely. Hyperinflation in combination with oxygen desaturation is a common complication according to the literature<sup>[73]</sup>. Continual monitoring during the procedure is indispensable.

Quite a common complication could be bleeding after polypectomy<sup>[73]</sup>. We do not consider it to be serious if the patient's blood count does not drop and if the bleeding is resolved by the endoscopist without surgical support in the case of DBE.

A feared complication is perforation after polypectomy. Perforation has been reported in 2%-6.5% of patients<sup>[74,75]</sup>. Polypectomy of large polyps appears to be the procedure associated with the highest risk<sup>[74]</sup>.

## FOLLOW-UP AND MALIGNANCY SCREENING

It is necessary to find the optimal method for screening of small intestinal polyps. Previously, the only method was enteroclysis (Figure 7A). Recently, wireless capsule endoscopy has been found to detect more polyps than small bowel radiographic studies (Figure 7B). Capsule endoscopy is a feasible, safe and sensitive test for small bowel surveillance in patients with PJS, even in children. It is significantly more comfortable than barium



**Figure 7** Hamartoma of the jejunum (arrow). A: Magnetic resonance imaging; B: Enteroclysis; C: Capsule endoscopy.

enterography<sup>[86,87]</sup>. The use of capsule endoscopy permits earlier diagnosis, therefore, the clinical course of intestinal polyps has changed. These polyps are now seen before the occurrence of obstructive signs. Capsule endoscopy can detect polyps in the entire length of the small intestine, with a higher diagnostic yield compared to CT and magnetic resonance imaging (MRI) with enteroclysis, particularly for lesions less than 1 cm in diameter<sup>[88]</sup>. CT is another option for screening. CT with oral contrast has demonstrated greater sensitivity than small bowel radiography<sup>[15]</sup>. In our opinion, MRI with oral contrast is superior to CT, not only to prevent high doses of radiation in young people during CT, but also for higher accuracy of MRI enteroclysis. Unlike capsule endoscopy, MRI enteroclysis can determine the exact size of bigger polyps (Figure 7C). On the other hand, capsule endoscopy is more successful in finding small polyps. That is why we prefer a combination of both these methods, as do many others<sup>[73,87,89]</sup>.

All PJS sufferers have to be enrolled for lifelong

Table 2 Cancer screening in PJS patients<sup>[13-17,22]</sup>

Site of possible tumour	Age of screening initiation	Periodicity	Method of screening
Colon	15 yr <sup>1</sup>	2 yr <sup>2</sup>	Colonoscopy, CA 19-9
Oral parts of GIT	15 yr <sup>1</sup>	2 yr <sup>2</sup>	Gastroscopy
Small intestine	15 yr <sup>1</sup>	2 yr <sup>2</sup>	Capsule enteroscopy or MRI scan
Breast	21 yr	Monthly	Self-examination
		6-12 mo	Sonography or mammography, CA 125
Thyroid gland	18 yr	1 yr	Sonography, clinical examination
Pancreas	18 yr	1 yr	Sonography or CT or MRI scan
Uterus and ovaria	18 yr	1 yr	Sonography, clinical examination
Testes	18 yr	1 yr	Sonography, clinical examination
Lung	18 yr	5 yr?	X-ray, clinical examination

<sup>1</sup>Earlier in symptomatic persons; <sup>2</sup>Endoscopy each year as long as polyps are present, subsequently prolong intervals. PJS: Peutz-Jeghers syndrome.

screening because of the high risk of different carcinomas (Table 2). There have been some case reports in the literature of the occurrence of malignancy in very young patients, even in children<sup>[38,90]</sup>.

There is no consensus or organization-approved guidelines for cancer surveillance in PJS patients. Different protocols are used at Johns Hopkins Hospital<sup>[91]</sup>, St. Mark's Hospital<sup>[92]</sup>, the Mayo Clinic<sup>[93]</sup>, the University of Edinburgh<sup>[94]</sup>, Danish Polyposis Registry<sup>[95]</sup>, and the University of Newcastle (Australia)<sup>[96]</sup>. There are many differences between the protocols, but whichever protocol is used, it should be modified according to available resources, the individual patient's disease manifestations, psychosocial situation, and personal preferences.

It is necessary to perform consistent screening for possible malignancies in all patients with PJS: colonoscopy, upper endoscopy, CT, MRI or ultrasound of the pancreas, chest X-ray, mammography and pelvic examination with ultrasound in women, testicular examination in men, carbohydrate antigen 19-9 (CA-19-9), and cancer antigen (CA 125)<sup>[52-55]</sup>.

DBE together with capsule endoscopy are essential modalities for the management of small intestinal diseases. IOE is the ultimate method in those patients in whom complete DBE investigation is impossible because of adhesions or other technical complications. IOE will be reserved for those cases in which DBE could not be performed or fails to investigate the entire small intestine, especially to prevent excessive bowel resection<sup>[39,77]</sup>.

We currently perform all of these methods in our department: push-enteroscopy, DBE, IOE and wireless capsule endoscopy. We do not consider these methods competitive but complementary for proper indications.

Last but not least, the psychosocial impact of PJS is very important and deserves special attention. Results have shown that PJS patients suffer from mild depression even though physically they did not feel any impact from their condition compared to the general population. However, having PJS causes them to alter many important life decisions. This fact is important in developing a plan for care of these patients regarding genetic counseling and surveillance strategies for PJS patients<sup>[16,97]</sup>.

It is even possible to treat benign hyperpigmentation

of the face by means of laser therapy. Q-switched lasers are the preferred method. The treatment is effective<sup>[98]</sup>, but this is only complementary cosmetic therapy.

## CONCLUSION

In summary, timely polypectomy, preferably using the DBE method, are essential for patients with PJS. Follow-up of gastrointestinal polyps is necessary. The best combination of methods is capsule enteroscopy and MR enteroclysis. Lifelong screening of malignancies is indispensable on a regular basis. It is necessary to investigate all first-degree relatives of the patient. Although the incidence of PJS is low, it is important for clinicians to recognize these disorders to prevent morbidity and mortality in these patients, and to perform presymptomatic testing in patients at risk.

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## Gastric ghrelin in relation to gender, stomach topography and *Helicobacter pylori* in dyspeptic patients

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### Abstract

**AIM:** To investigate the level of gastric ghrelin in stomach mucosa of dyspeptic patients in relation to *Helicobacter pylori* (*H pylori*) infection, bacterial cytotoxicity, topography and gender.

**METHODS:** The study comprised 40 premenopausal women (19 *H pylori* positive) and 48 men (17 *H pylori* positive) with functional dyspepsia. All gastric biopsy specimens revealed normal mucosa or non-atrophic gastritis. Gastric ghrelin concentration was determined by Enzyme linked immunosorbent assay. The *cagA* and *vacA* strains of bacterial DNA were identified by multiplex polymerase chain reaction.

**RESULTS:** In general, infection with *H pylori* caused

an increase in gastric ghrelin level regardless of gender and stomach topography. Significantly more hormone was present in both, non-infected and *H pylori* positive female samples, as compared to males. The distribution of bacterial strains showed *cagA*(+) *vacA* s1m1 and *cagA*(-) *vacA* s2m2 genotypes as the most common infections in the studied population. A tendency to higher ghrelin levels was observed in less cytotoxic (*cagA* negative) strain-containing specimens from the antrum and corpus of both gender groups (without statistical significance).

**CONCLUSION:** An increase in gastric ghrelin levels at the stage of non-atrophic gastritis in *H pylori* positive patients, especially in those infected with *cagA*(-) strains, can exert a gastroprotective effect.

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**Key words:** Dyspepsia; Gastric ghrelin; *Helicobacter pylori*; Gender

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### INTRODUCTION

Ghrelin is a peptide hormone that plays an important role in food intake, energy homeostasis and body-weight regulation. It is expressed in multiple tissues and exerts both, endocrine and paracrine/autocrine effects. This 28-amino acid peptide was discovered by Kojima *et al*<sup>[1]</sup> in 1999. Approximately 70% of the body pool of this hormone is produced by gastric X/A cells located in the corpus and fundus of the stomach and is secreted into the extracellular matrix and into the blood. Normal ghrelin levels in serum range from 145-160 fmol/mL

and rapidly drop after gastrectomy<sup>[2]</sup>. Ghrelin stimulates growth hormone (GH) release *via* the GHRH-dependent (growth hormone releasing hormone) mechanism acting on the growth hormone secretagogue receptor (GHS-R). Released GH and its mediator, insulin-like growth factor 1, affect a range of physiological processes<sup>[3]</sup>. It has been suggested that ghrelin exerts a gastroprotective role. Its level is significantly lower in gastric tumours than in normal gastric mucosa<sup>[4]</sup>.

Ghrelin possesses anti-proliferative effects on breast, lung and thyroid cell lines and exerts protective actions on the gastric mucosa<sup>[5]</sup>. In the alimentary tract, ghrelin increases acid secretion and stimulates gastric emptying *via* vagal activation<sup>[6]</sup>. Ghrelin levels change significantly depending on the body's energy requirements<sup>[7]</sup>. It is known that exogenous ghrelin significantly inhibits the activation of nuclear factor (NF)- $\kappa$ B and plasma tumor necrosis factor- $\alpha$ <sup>[8]</sup>. It is well known that *Helicobacter pylori* (*H pylori*)-mediated gastric inflammation critically depends on the efficient recruitment and activation of macrophages, with sufficient NF- $\kappa$ B activation<sup>[9]</sup>. This transcription factor plays an important role in activation of expression of proinflammatory cytokines. Studies are being conducted to determine the effect of sociological and environmental factors on ghrelin release.

There is a great deal of interest in the role of *H pylori* infection in oncogenesis. *H pylori* infection always induces chronic active gastritis, which, in the presence of other factors, can lead to the development of gastric cancer (GC). Thus, based on evidence that infections increase the risk of GC, *H pylori* was categorized as a class I carcinogen more than 10 years ago<sup>[10]</sup>. Only 15% of those colonized develop the disease, and the pathogenesis depends upon strain virulence, host genetic susceptibility, and environmental cofactors. Virulent factors include the *cag* pathogenicity island (PAI), which induces proinflammatory and proliferative epithelial cell signaling. The expression of various products encoded in the *cag* PAI is known to be involved in inducing inflammation, ulceration and carcinogenesis. Each *H pylori* strain possesses one *vacA* gene, which due to its genetic variability, can be either of type s1 or s2 in the polymorphic signal region or type m1 or m2 in the polymorphic mid-region<sup>[11]</sup>. Another PAI-associated gene is the *cagA* gene, expressed by the majority of *H pylori* strains, irrespective of the geographic origin and clinical diagnosis<sup>[12]</sup>. *H pylori vacA* s1m1 *cagA* virulent strains are significantly more frequent in patients with GC<sup>[13]</sup>. An increased predisposition to GC is thought to be associated with the presence of *H pylori cagA* strains<sup>[14]</sup>.

The aim of this study was to determine whether there is any correlation between gastric ghrelin level and *H pylori* infection in relation to bacterial strain cytotoxicity, patients' gender and stomach topography.

## MATERIALS AND METHODS

### Patients

The study comprised 88 patients with functional dyspepsia (classification according to Rome III criteria)<sup>[15]</sup>. Exclusion criteria included chronic or psychiatric illness, preg-

nancy, drug and alcohol abuse - defined as consumption of more than two alcoholic drinks per day and NSAIDs or PPIs administration during the 14 d prior to the study. Patients with histopathological changes higher than those of non-atrophic gastritis (according to Sydney System Scale update<sup>[16]</sup>) and with a body mass index (BMI) below 18 and above 29 kg/m<sup>2</sup> were excluded from the study. The study comprised 40 premenopausal women (mean age 42  $\pm$  13 years), of whom 19 were *H pylori* positive and 48 men (mean age 35  $\pm$  10 years), of whom 17 were *H pylori* positive. Initially, all the selected subjects had their BMI calculated (as body weight in kilograms divided by the square of their height in meters)<sup>[17]</sup>. The physical examinations were performed at fasting in the morning. Subjects underwent a routine endoscopic evaluation at enrolment (a gastroscopy with biopsy). From each patient, five biopsy specimens from the antrum and five from the corpus were taken. One of the samples was used to perform a urease test, and a second sample was used for histological assessment. Another sample was used to determine the bacterial strain, and the remaining two specimens, taken from the anterior and posterior wall of the antrum or corpus were used for ghrelin level determination.

Histological assessments were performed using haematoxylin-eosin and Giemsa staining. Two paraffin-embedded tissue blocks (from the antrum and corpus) were used for microscopic section preparation. From each paraffin block, two sections were obtained. The microscopic gastric mucosa assessment was based on criteria according to the updated Sydney System Classification<sup>[16]</sup>.

### Protein fraction isolation

Biopsy specimens were frozen in physiological saline at -20°C. DNA, RNA and protein fractions were extracted using a TriPure Isolation Reagent (Roche Diagnostic GmbH, Mannheim, Germany). Protein concentrations were assessed with the Biorad Protein Assay (BIO-RAD Laboratories, Hercules, CA, USA). Purified protein fractions were used to evaluate ghrelin levels in biopsy specimens by the immunoenzymatic enzyme linked immunosorbent assay (ELISA) method.

### Multiplex polymerase chain reaction (PCR)

Multiplex PCR reactions were performed with the use of specific primers for amplification of the *cagA* gene and subtypes of the *vacA* (s1/2 and m1/2) gene in biopsy specimens<sup>[18]</sup>. The tissue samples were suspended in lysis buffer (10 mmol/L Tris-HCl, 0.1 mol/L EDTA, 5% SDS, 100-200  $\mu$ g/mL proteinase K). The mixture was incubated at 50°C for 2 h, and then DNA was isolated by extraction with a mixture of phenol, chloroform and isoamyl alcohol and precipitated with ethanol. PCR products of the lengths: 350 base pairs (bp) (*cagA*), 259 bp (*vacA* s1), 286 bp (*vacA* s2), 567 bp (*vacA* m1) and 642 bp (*vacA* m2) were subjected to electrophoretic analysis on a 2% agarose gel.

### ELISA test

Ghrelin levels in gastric biopsy specimens were measured using the indirect ELISA "sandwich" test. A 96-well

Table 1 Characteristics of the patients included to the study (mean  $\pm$  SD)

	<i>Hp</i> (-)			<i>Hp</i> (+)		
Number of patients (female/male)	21/31			19/17		
Age (yr, female/male)	41.3 $\pm$ 15.0/36.4 $\pm$ 11.5			45.0 $\pm$ 11.6/38.4 $\pm$ 13.8		
BMI (female/male)	23.9 $\pm$ 3.2/25.1 $\pm$ 2.6			23.3 $\pm$ 3.0/24.0 $\pm$ 3.4		
Alcohol drinking habit (% of group population, female/male)	ND (45.0/28.6)	1/m (50.0/50.0)	1/w (5.0/21.4)	ND (57.0/33.3)	1/m (43.0/44.4)	1/w (0.0/22.3)

*Hp*: *Helicobacter pylori*; (-): No infection; (+): Positive; ND: Not drinking; 1/w: Once a week; 1/m: Once a month.

MaxiSorp plate (NUNC Thermo Fisher Scientific, Roskilde, Denmark) was coated with chicken monoclonal antibodies against the C-terminal domain of human ghrelin (Abcam Ltd., Cambridge, UK) in carbonate buffer (pH 9.6). Plate wells were rinsed 5 times with PBS buffer and blocked with 1% BSA-PBS (SERVA Electrophoresis GmbH, Heidelberg, Germany) and protein fraction was added to the wells at a final amount of 1 mg per well. The plates were incubated at room temperature for 2 h. The wells were then rinsed five times with PBS, and rabbit polyclonal IgG antibodies against the N-terminal end of human ghrelin were added. The plates were incubated overnight at 4°C. HRP goat polyclonal antibodies against rabbit IgG (Abcam) were applied. The reaction was revealed by ABTS substrate (BIOMOL GmbH, Hamburg, Germany) at a 1 mg/mL concentration in phosphate-citrate buffer with H<sub>2</sub>O<sub>2</sub> (Chempur, Piekary Slaskie, Poland) for 20 min in darkness. Then, the absorbance was measured at 414 nm by the Synergy HT (BioTek, Winooski, VT, USA) plate reader. The measurements allowed us to obtain the values of ghrelin absorbance after subtraction of the background (the absorbance value of the well without gastric biopsy proteins). Test normalization was performed by analyzing a model curve obtained using commercially available hormone. The mean value of ghrelin levels determined in two specimens, taken from the anterior and posterior wall of the antrum or corpus of each patient was taken for further evaluations.

### Ethics

The study was conducted in accordance with the Declaration of Helsinki and with principles of the Good Clinical Practice. These studies were approved by the Ethical Commission of the Medical University of Lodz, Poland. Each patient before being enrolled into the research program was acquainted with the aim of the study and gave conscious, written consent to participate in the study.

### Statistical analysis

The Shapiro-Wilks'  $W$  test was used to analyze the normality of the distribution. The differences between median values of gastric ghrelin in two independent groups were calculated by the use of the nonparametric Mann-Whitney  $U$  test. In order to test the statistical significance of differences between the three groups we used the non-parametric Kruskal-Willis test. The gastric ghrelin level is shown as a box around the midpoint

(median) which represents the 25th and 75th percentiles. The whiskers outside of the box represent the non outlier data. Outlier data points are also plotted. The statistical significance of the difference for each test was identified by a two-tailed probability ( $P$ ).  $P$  values of less than 0.05 were considered significant.

## RESULTS

### Selection of patients

A total of 88 patients with functional dyspepsia, having normal mucosa or non-atrophic gastritis were enrolled in the study. Full characteristics of the patients including the mean age, BMI and alcohol consumption are given in Table 1.

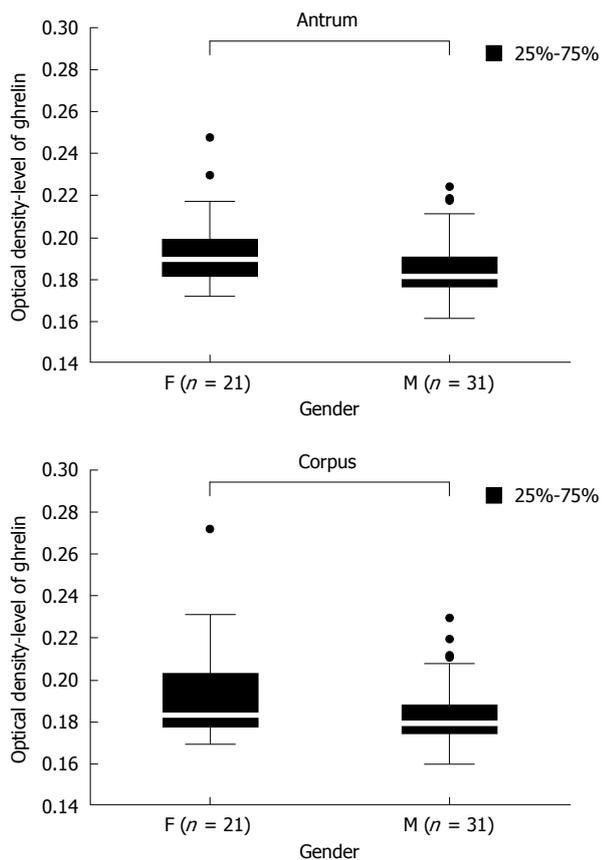
### Gastric ghrelin and gender in non-infected patients

There are no clear data concerning the dependence of gastric ghrelin level on patients' gender. Therefore the first groups analyzed were the controls, without *H pylori* infection, consisting of 21 premenopausal women (mean age 41  $\pm$  13 years) and 31 men (mean age 36  $\pm$  11 years). The levels of gastric ghrelin in the antrum and corpus were determined for each patient and then the mean values were calculated for the analyzed groups of patients. The results are presented in Figure 1 and show statistically significant [antrum: 0.189250 (F) *vs* 0.181000 (M),  $P = 0.004610$  and corpus: 0.183000 (F) *vs* 0.179000 (M),  $P = 0.017149$ ] higher levels of this hormone in female samples (F) compared to male samples (M), independent of gastric topography.

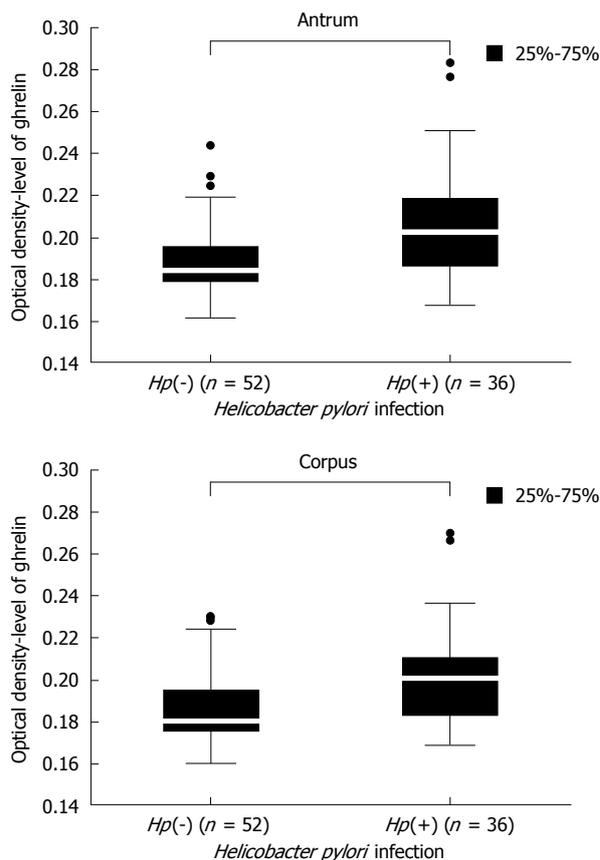
### Gastric ghrelin and gender in patients with *H pylori* infection

The first question was to clarify the influence *H pylori* infection on the level of gastric ghrelin. Data concerning this issue are not consistent and therefore we compared the levels of gastric ghrelin in samples from the antrum and corpus of *H pylori* negative [*Hp*(-)] and *H pylori* positive [*Hp*(+)] patients. These results are shown in Figure 2 and demonstrate that, independent of stomach topography, the levels of this hormone were elevated in infected specimens. Moreover, the observed differences between infected and non-infected groups of patients were statistically significant {antrum: 0.184000 [*Hp*(-)] *vs* 0.202500 [*Hp*(+)],  $P = 0.000000$  and corpus: 0.178000 [*Hp*(-)] *vs* 0.201000 [*Hp*(+)],  $P = 0.000000$ } (Figure 2).

Because the level of gastric ghrelin was higher in the stomach mucosa of women than in men, we performed a detailed analysis of ghrelin levels depending on *H pylori*



**Figure 1** Ghrelin levels in gastric biopsy specimens from the antrum and corpus of patients without *H. pylori* infection. The numbers (n) of female (F) and male (M) patients are given.



**Figure 2** Comparison of ghrelin level in *H. pylori* negative [Hp(-)] and *H. pylori* positive [Hp(+)] samples from the antrum and corpus of all screened patients. Population numbers are given.

**Table 2** Distribution of *Helicobacter pylori* strains in the study population of 19 women and 17 men in relation to gastric topography

<i>H. pylori</i> genotype	No. of patients (female/male)	
	Antrum	Corpus
<i>cagA</i> (-) <i>vacA</i> s2 m2	8 (5/3)	9 (6/3)
<i>cagA</i> (+) <i>vacA</i> s1 m1	17 (11/6)	17 (11/6)
<i>cagA</i> (+) <i>vacA</i> s1 m2	1 (1/0)	1 (1/0)
<i>cagA</i> (+) <i>vacA</i> s2 m2	2 (1/1)	1 (0/1)
<i>cagA</i> (+) <i>vacA</i> (+) genotype ndt	3 (0/3)	3 (0/3)
<i>cagA</i> (-) <i>vacA</i> (+) genotype ndt	5 (1/4)	5 (1/5)

For 8 patients the genotype of *vacA*(+) strains was not determined (ndt).

infection and sex. As shown in Figure 3, in both men and women, the levels of gastric ghrelin were higher, as expected, in *H. pylori* positive biopsy specimens {women antrum: 0.189250 [Hp(-)] vs 0.214667 [Hp(+)],  $P = 0.000002$ ; women corpus: 0.183000 [Hp(-)] vs 0.209250 [Hp(+)],  $P = 0.000214$ ; men antrum: 0.181000 [Hp(-)] vs 0.192250 [Hp(+)],  $P = 0.000664$ ; men corpus: 0.179000 [Hp(-)] vs 0.193000 [Hp(+)],  $P = 0.001514$ }, regardless of gastric topography. However, the differences in hormone levels between *H. pylori* negative and *H. pylori* positive patients were more prominent in female samples.

Next, we compared the levels of gastric ghrelin in *H. pylori* positive female and male samples. The screened groups (19 women and 17 men) were sufficient to obtain

statistically significant data, and indicated that in female samples the levels of hormone were higher than in male samples [antrum: 0.214667 (F) vs 0.192250 (M),  $P = 0.000375$  and corpus: 0.209250 (F) vs 0.193000 (M),  $P = 0.000292$ ] independent of stomach topography (antrum vs corpus) (Figure 4).

**Gastric ghrelin and *H. pylori* cytotoxicity**

The distribution of selected bacterial strains *vacA* and *cagA* in the study population was evaluated by multiplex PCR amplification of bacterial DNA (Table 2). The most common infections, with *cagA*(+) *vacA* s1m1 and *cagA*(-) *vacA* s2m2 strains, were observed in specimens from the antrum and from the corpus of both study groups. In contrast, the *cagA*(+) *vacA* s1m2 and *cagA*(+) *vacA* s2m2 strains were present in a minor number of patients only. Interestingly, in one case (a female patient), two different strains were detected: *cagA*(-) *vacA* s2m2 (in the corpus) and *cagA*(+) *vacA* s2m2 (in the antrum). Because of the varied distribution of *H. pylori* strains and the small number of patients within these groups, *H. pylori* infected subjects were combined into two subgroups: those without cytotoxin CagA gene [*cagA*(-)], and those with cytotoxin CagA(+) gene [*cagA*(+)].

As expected, all the evaluated *H. pylori* strains expressed *vacA* but differed with respect to the subtype combinations of s1/s2 and m1/m2. The percentage distribution of all studied subgroups (regardless of the

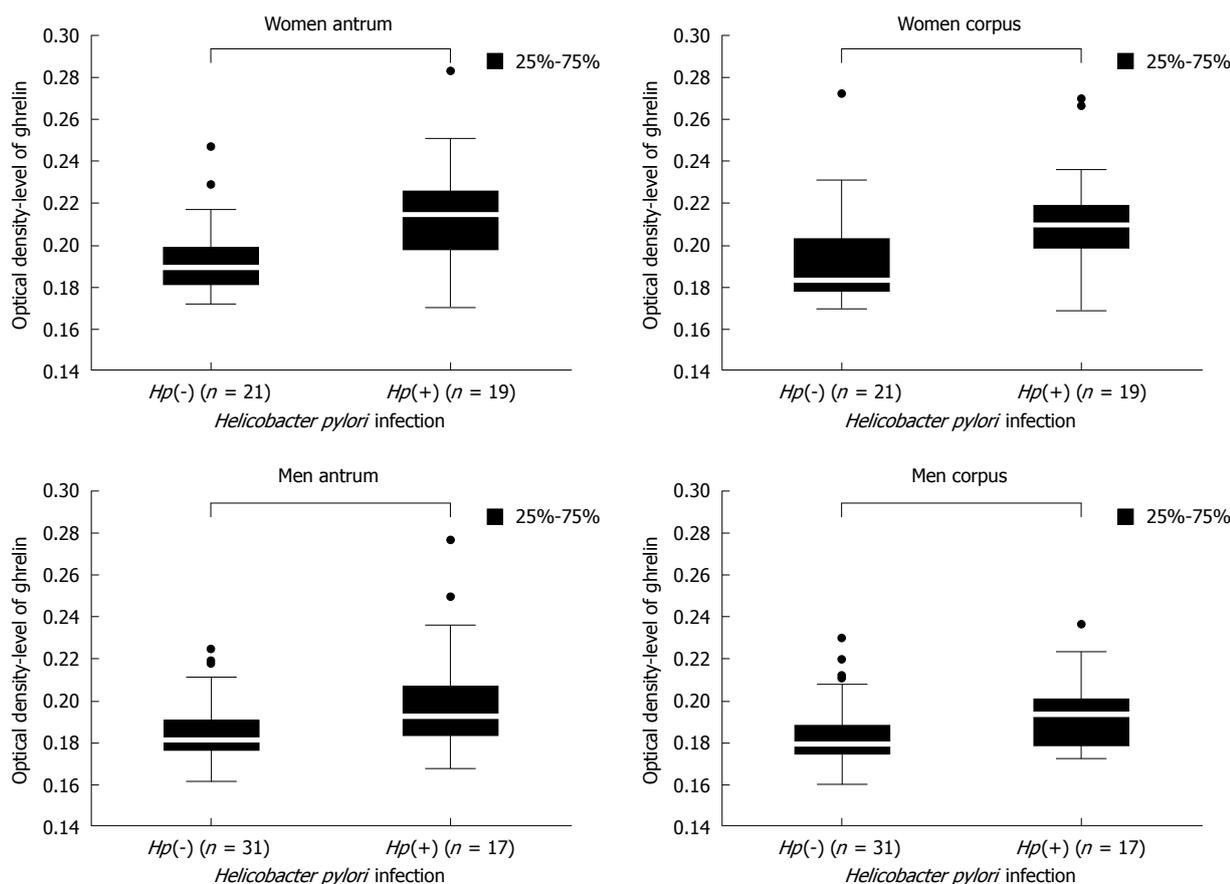


Figure 3 Comparison of ghrelin levels in *H pylori* negative [*Hp*(-)] and *H pylori* positive [*Hp*(+)] samples taken from the antrum and corpus of female and male patients. Population numbers are given.

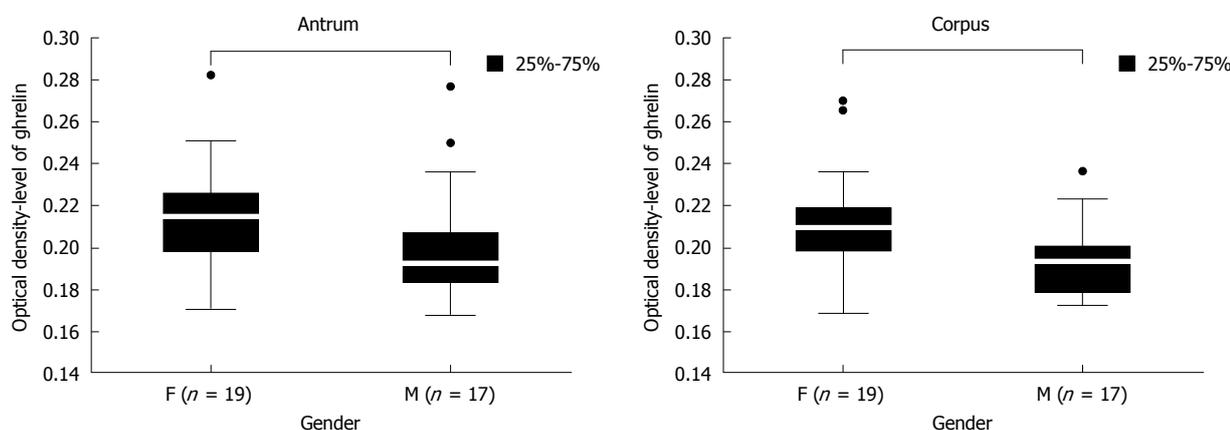


Figure 4 Ghrelin levels in gastric biopsy specimens taken from the antrum and corpus of female (F) and male (M) patients with *H pylori* infection. The numbers in both gender groups are given.

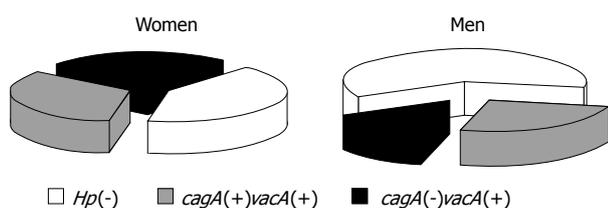
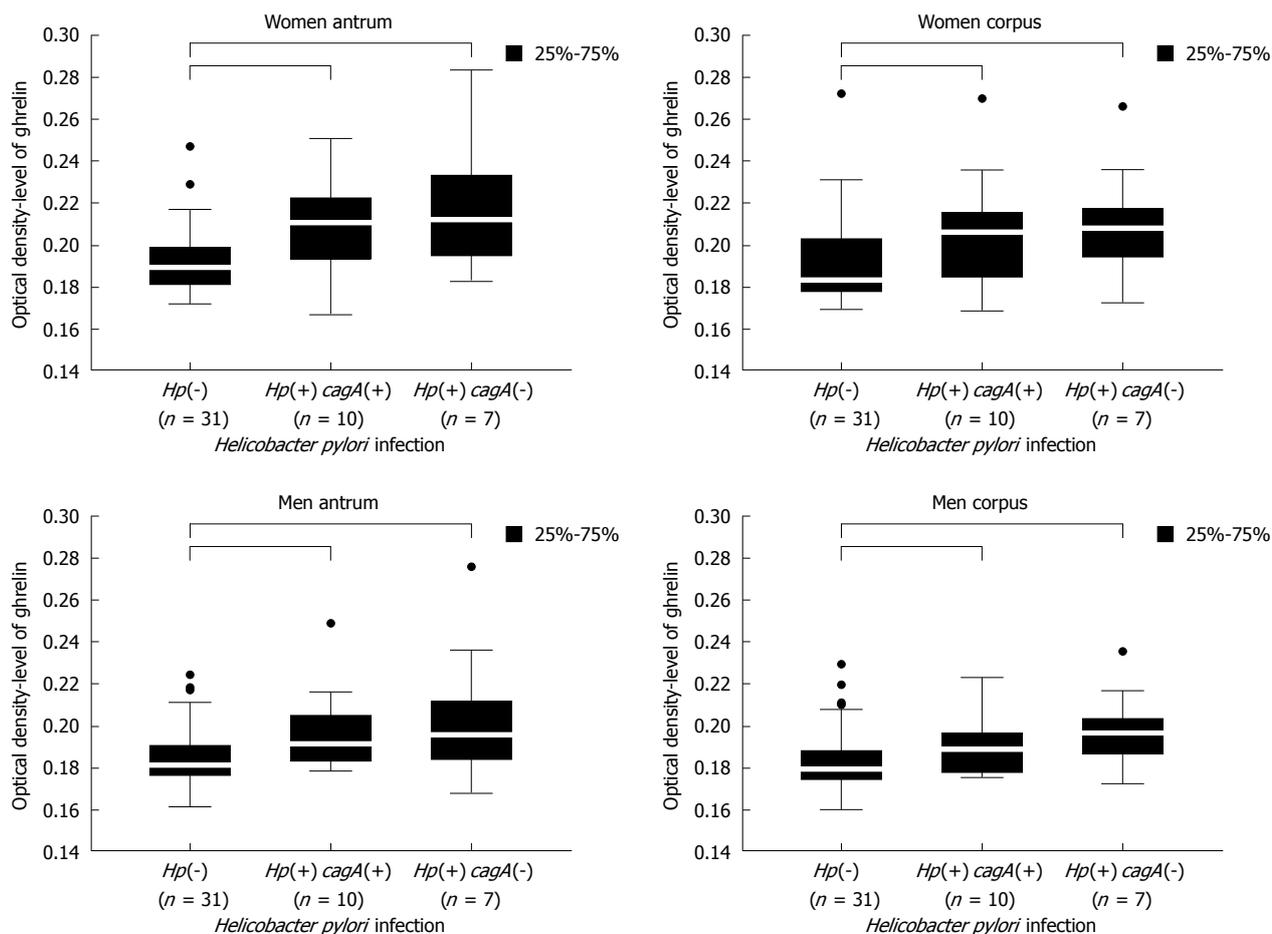


Figure 5 Distribution of subjects in each study group of female and male patients. Subjects without *H pylori* infection are marked as *Hp*(-), while the remaining subjects are *H pylori* positive with and without *cagA* expression (*cagA*(+)*vacA*(+) and *cagA*(-)*vacA*(+), respectively).

subtypes) is presented in Figure 5. The data showed that the biggest group of subjects was composed of those without *H pylori* infection. The percentage distribution of patients according to genotype varied according to gender. In men, the least common *H pylori* strain was *cagA*(-)*vacA*(+), whereas in women, the percentage distribution of all the genotypes was similar.

Due to the observed differences in ghrelin levels in the samples from women and men, the relationship between the hormone levels and *H pylori* cytotoxicity was evaluated separately for each gender.



**Figure 6** Ghrelin levels in female and male antrum and corpus gastric biopsies in relation to *H. pylori* strain cytotoxicity. The numbers in the patient populations are given.

**Female samples:** The results obtained for female samples (Figure 6) showed that ghrelin levels were higher in women infected with both, *cagA(+)/vacA(+)* and *cagA(-)/vacA(+)* strains compared to women without *H. pylori* infection {women antrum: 0.189250 [*Hp(-)*] vs 0.210500 [*Hp(+ cagA(+))*] vs 0.212000 [*Hp(+ cagA(-))*],  $P = 0.0002$  and women corpus: 0.183000 [*Hp(-)*] vs 0.206000 [*Hp(+ cagA(+))*] vs 0.208000 [*Hp(+ cagA(-))*],  $P = 0.0027$ }. No statistical significance was noted for differences in ghrelin levels in relation to strain cytotoxicity, although a tendency for higher amounts of hormone in the samples infected with *cagA(-)/vacA(+)* strains was observed.

**Male samples:** In *H. pylori* infected men the observed changes in gastric ghrelin levels were similar to the changes observed in female samples, but varied depending on gastric topography. Statistically significant increases in ghrelin levels were noted in the antrum {men antrum: 0.181000 [*Hp(-)*] vs 0.191250 [*Hp(+ cagA(+))*] vs 0.195500 [*Hp(+ cagA(-))*],  $P = 0.0028$  and men corpus: 0.179000 [*Hp(-)*] vs 0.188500 [*Hp(+ cagA(+))*] vs 0.196333 [*Hp(+ cagA(-))*],  $P = 0.0023$ }, however, the differences between ghrelin levels in relation to strain cytotoxicity were not significant (Figure 6). In corpus samples, an increase in ghrelin level was only caused by *cagA(-)/vacA(+)*, however, this increase was not statistically significant. Moreover, the

differences between hormone levels in samples carrying both studied *H. pylori* genotypes did not reach  $P < 0.05$ .

## DISCUSSION

The data available in the literature concerning both, the effects of *H. pylori* infection and patients' gender on ghrelin secretion are not consistent. Some authors claim that the level of circulating ghrelin is higher in women than in men<sup>[19]</sup>, but others maintain that it is similar in both sexes<sup>[20]</sup> and does not vary from childhood to adulthood<sup>[21]</sup> or undergo an age-related decrease<sup>[22]</sup>. In our studies we clearly demonstrated that in the case of gastric ghrelin, the hormone level was much higher in pre-menopausal women than in men (Figure 1). It was interesting to note that this relationship could also be seen in *H. pylori* infected patients (Figure 4), where the amount of ghrelin was higher in female specimens. Studies by Gualillo *et al.*<sup>[23]</sup> performed on rat stomach showed that gonadal hormones did not alter ghrelin mRNA, and suggested that ghrelin level was age- rather than gender-dependent. Therefore, one might conclude that higher hormone level in female mucosa might originate from altered hormone release rather than from a change in level of expression. Higher ghrelin level in pre-menopausal women positively correlates with lower testosterone level

when compared to that in postmenopausal women<sup>[19]</sup>, in whom testosterone level is higher. We speculate that higher gastric ghrelin levels in the premenopausal study group could exert a protective effect on gastric mucosa. GC incidence increases with age, and this malignant tumour is relatively rare in males and females under 45 years. In general, the incidence rates in females at a given age are equivalent to the rates in males at an age 10 years younger. The consistency of these differences has never been adequately explained although theories have been proposed that sex-specific hormones in females play a protective role against this malignancy<sup>[24]</sup>.

The influence of *H pylori* infection on gastric ghrelin level requires discussion. Most studies show that *H pylori* infection leads to decreased ghrelin secretion<sup>[25-27]</sup>. However, other reports suggest that serum ghrelin levels are similar in infected and non-infected patients<sup>[25]</sup>. In our studies, *H pylori* infected mucosa had more ghrelin than non-infected mucosa, regardless of gender and stomach topography (Figures 2 and 3). Moreover, we determined whether *H pylori* cytotoxicity could influence the level of ghrelin in gastric mucosa, since there is only very limited published information on the changes in ghrelin levels in relation to virulence of *H pylori* strains<sup>[28]</sup>. It is well known that the strains with higher virulence produce both CagA and vacuolating cytotoxin VacA. CagA protein is the product of *cagA* gene expression, which belongs to the group of genes defined as the “cytotoxicity island”. The *cagB*, *cagC*, *picA*, and *picB* genes also belong to this group. The *cagA* gene is present in about 60%-70% of *H pylori* strains. It is associated with prominent inflammatory and atrophic changes and increases the risk (3-6 times) of developing a peptic ulcer, gastric adenocarcinoma and lymphoma<sup>[29-32]</sup>. *H pylori* infection results in an increase in proliferative activity and affects the apoptotic processes of the glandular epithelium of the gastric mucosa. Although there are a variety of proposed mechanisms by which *H pylori* infection increases the risk of GC, it is thought that the primary mode of action involves the induction of long-term chronic inflammation<sup>[33]</sup>.

Because of the varied distribution of *H pylori* strains and the small number of patients with *vacA* s1m2 and s2m1 genotype in our studies (Table 2), the analysis comprised two groups of subjects - those infected with *H pylori* strains of *cagA*(-)*vacA*(+) genotype and those of *cagA*(+)*vacA*(+) genotype. We showed that bacterial strains of both genotypes increased ghrelin secretion in infected women and men compared to non-infected subjects (Figure 6), however, infection with *cagA*(-)*vacA*(+) strains showed a tendency to increase gastric ghrelin level more than infection with *cagA*(+)*vacA*(+) strains, although no statistical significance was reached.

It is worth noting that the results of the study discussed above and the conclusions drawn from them apply only to patients without advanced histopathological changes in the gastric mucosa. It is likely that *H pylori* infection leads to the early stage of inflammatory changes described as non-atrophic gastritis, which causes activation of protective mechanisms in the mucosa and results in an increase in gastric ghrelin expression. This increase

was more prominent in the gastric mucosa of both male and female patients infected with *cagA*(-)*vacA*(+) bacterial strains of lower cytotoxicity (Figure 6).

From the literature it can be concluded that ghrelin levels in the gastric mucosa significantly decrease with more advanced histopathological changes, especially after the atrophic gastritis stage<sup>[34,35]</sup>. It has been suggested that the level of this hormone can be considered as one of the markers of gastric mucosal change progression and, at the same time, can be one of the indications for gastroscopy.

The results of the study performed by Osawa *et al*<sup>[36]</sup> showed a significant decrease in gastric preproghrelin mRNA level 12 wk after *H pylori* eradication. These observations are inconsistent with our results. The discrepancies between our results (higher gastric ghrelin level in *H pylori* positive patients) and the previously published data<sup>[36-38]</sup> could have arisen due to different stages of gastric mucosa inflammatory changes (chronic, severe atrophic gastritis, peptic ulcer) in subjects enrolled in the study. Moreover, earlier reports did not analyze results for women and men separately, which, as our study suggests, is an important factor influencing gastric ghrelin level. The study on the impact of *H pylori* infection on ghrelin level is part of our research on the relationship between these bacteria and biosynthesis of the proteins possessing proapoptotic and antiproliferative activities, which are important in the process of carcinogenesis<sup>[39-41]</sup>.

In conclusion, based on the results obtained in our study, we may assume that an increase in gastric ghrelin levels at the stage of non-atrophic gastritis in *H pylori* positive patients, especially those infected with *cagA* negative strains can exert a gastroprotective effect. Future studies are needed to delineate the role of gastric ghrelin in pathological processes induced by *H pylori*.

## COMMENTS

### Background

Ghrelin is a peptide hormone that plays an important role in food intake, energy homeostasis and body-weight regulation. Ghrelin possesses anti-proliferative effects on breast, lung and thyroid cell lines and exerts a protective action on gastric mucosa. Its level is significantly lower in gastric tumors than in normal gastric mucosa. The published data on the effects of sociological and environmental factors on gastric ghrelin level are not consistent. One of these factors is *Helicobacter pylori* (*H pylori*) infection which always induces chronic active gastritis, and in the presence of other factors, can lead to the development of gastric cancer.

### Research frontiers

Investigation on the mechanisms involved in oncogenesis in the digestive tract-environmental factors related to bacterial infection.

### Innovations and breakthroughs

The authors demonstrate, for the first time, that premenopausal women have higher gastric ghrelin levels than men. The level of this appetite hormone increases in *H pylori* infection, and was higher in female samples. More cytotoxic *H pylori* strains expressing the *cagA*(+) gene had less of an effect on gastric ghrelin level. Expression of this hormone was also dependent on histopathological changes, and at the stage of non-atrophic gastritis in *H pylori* positive patients, especially those infected with *cagA* negative strains, may exert a gastroprotective effect.

### Applications

The data discussed in this paper are of value since they univocally demonstrate the influence of selected environmental and sociological factors on the level of expression of gastric ghrelin. The results may be used in future research to cor-

relate the level of gastric ghrelin with plasma ghrelin and to discuss the effect of selected factors on the hormone release process. Future studies are needed to delineate the role of gastric ghrelin in pathological processes induced by *H pylori*.

### Peer review

The authors demonstrate the increased protein levels of ghrelin in gastric biopsy specimens of *H pylori*-positive subjects compared to *H pylori*-negative subjects, particularly in virulent types of *H pylori*-carrying patients, and suggest a protective role of ghrelin. The authors also demonstrate the gender difference in this respect. The findings are interesting, but this reviewer strongly suggests that the authors should provide additional data on mRNA expression of ghrelin in gastric specimens or plasma levels of ghrelin, since increased ghrelin concentration in the stomach will be produced by either increased synthesis or decreased secretion from the stomach.

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ORIGINAL ARTICLE

## Effect of growth hormone on small intestinal homeostasis relation to cellular mediators IGF-I and IGFBP-3

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### Abstract

**AIM:** To evaluate the effects of growth hormone (GH) on the histology of small intestines which might be related to the role of insulin like growth factor (IGF)-I, IGF-binding protein 3 (IGFBP-3) and its receptors.

**METHODS:** Twelve week-old adult male Wistar albino rats were divided into two groups. The study group ( $n = 10$ ), received recombinant human growth hormone (rGH) at a dose of 2 mg/kg per day subcutaneously for 14 d and the control group ( $n = 10$ ) received physiologic serum. Paraffin sections of jejunum were stained with periodic acid shift (PAS) and hematoxylin and eosin (HE) for light microscopy. They were also examined for IGF-I, IGFBP-3 and IGF-receptor immunoreactivities. Staining intensity was graded semi-quantitatively using the HS-CORE.

**RESULTS:** Goblet cells and the cells in crypt epithelia

were significantly increased in the study group compared to that of the control group. We have demonstrated an increase of IGF-I and IGFBP-3 immunoreactivities in surface epithelium of the small intestine by GH application. IGF-I receptor immunoreactivities of crypt, villous columnar cells, enteroendocrine cells and muscularis mucosae were also more strongly positive in the study group compared to those of in the control group.

**CONCLUSION:** These findings confirm the important trophic and protective role of GH in the homeostasis of the small intestine. The trophic effect is mediated by an increase in IGF-I synthesis in the small intestine, but the protective effect is not related to IGF-I.

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**Key words:** Growth hormone; Small intestine; Like growth factor-1; Insulin like growth factor binding protein 3

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### INTRODUCTION

Growth hormone (GH) is a pituitary-derived polypeptide hormone that has diverse physiologic effects including the regulation of growth in long bones, carbohydrate, lipid metabolism, and metabolic functions of the liver<sup>[1]</sup>. The mitogenic effect of GH in the crypts of Lieberkuhn of the duodenum in hypophysectomized rats was first described by Leblond and Carriere in 1955<sup>[2]</sup>. Small intestine that is lined by epithelial cells with a rapid turnover rate has been considered as a potential target of GH<sup>[3,4]</sup>. Systemic administration of GH markedly increases trophic action in the small intestine and enhances the remnant bowel morphological and proliferative adaptation<sup>[3]</sup>.

Circulating GH binds to the GH receptor (GHR) in

target cells and stimulates the production of insulin like growth factor (IGF)-I in the liver and other target tissues including the intestine. The GHR is expressed throughout the epithelium and in the lamina propria, muscularis mucosa, submucosa, and muscularis propria. The presence of GH receptors in crypt and villus epithelial cells of rats suggests a direct cellular effect of GH on small intestinal growth<sup>[5]</sup>. GH action has traditionally been believed to be mediated by GH dependent hepatic production of IGF-I. However, with the discovery of various extrahepatic IGF-I synthesis sites, some of which are GH dependent, a paracrine/autocrine mechanism of action has been proposed<sup>[6]</sup>. IGF-I receptors have also been immunolocalized in the gastrointestinal tract of rats<sup>[7,8]</sup>. Locally expressed IGF binding proteins (IGFBPs) are known to modulate IGF-I action. Of six known high-affinity IGFBPs, IGFBP-3 has been of interest with respect to a role in regulating intestinal growth. Systemically administered IGF-I is known to increase the expression of IGFBP-3 in rat small intestine<sup>[9]</sup> and colon<sup>[10]</sup>, although there are segmental differences in the effects of circulating IGF-I on locally expressed IGFBPs, which correlate with segment-specific growth effects<sup>[9,10]</sup>.

GH action is primarily mediated by IGF-I, although both growth factors show tissue-selective effects. Specific binding sites for IGF-I, and GH are present in the small intestine and evidence suggests that circulating IGF-I and GH can interact with their respective functional intestinal receptors<sup>[2,11,12]</sup>. GH administration stimulates mucosal growth in normal rats<sup>[2]</sup>. GH action has been attributed to be the result of both a direct effect of GH, and an indirect stimulating effect on the target cells *via* IGF-I released locally or from the liver<sup>[3]</sup>. Specific GH receptors (GHR), IGF-I receptors, and local production of IGF-I have been shown throughout the epithelial and mesenchymal derived elements of the gastrointestinal tract<sup>[4,5]</sup>, implicating a complex interaction between direct and indirect effects of GH<sup>[12-14]</sup>. It has not yet been shown whether GH affects the small intestine directly or *via* IGF-I. Therefore, the objective of this study was to evaluate the morphological effects of GH on normal small intestines due to changes occurring in IGF-Rs and IGFBP-3 by administration of GH.

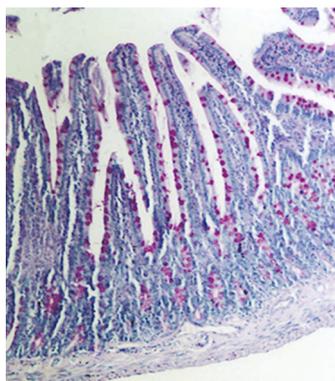
## MATERIALS AND METHODS

The animal facilities and protocols used in the present study were approved by the Animal Research Ethics Committee of the University of Celal Bayar. This study was performed in the Department of Histology and Embryology and Department of Pediatrics, between 2001 and 2002. Twelve week-old adult male Wistar albino rats (250-300 g) were included in the study. All animals were kept under standard conditions on a 12 h light/dark cycle and maintained at optimal temperature. The animals were fed with a standard diet and given free access to water. They were randomized into two groups. The study group ( $n = 10$ ) received recombinant human GH (Norditropin-Novo Nordisk, Denmark) at a dose of 2 mg/kg per day subcutaneously for 14 d and the control

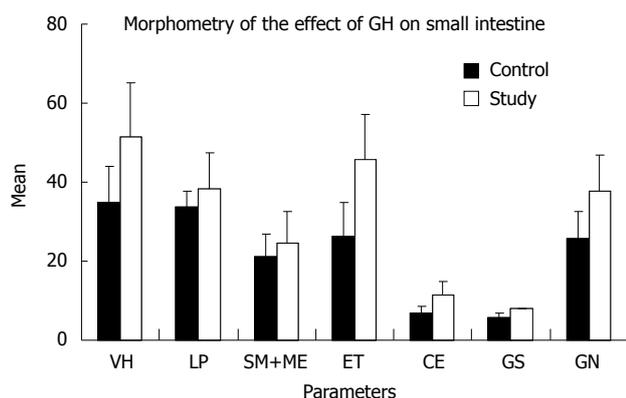
group ( $n = 10$ ) received the same volume of serum physiologic intracutaneously. Anesthesia was achieved by intraperitoneal injection of pentobarbitone. Animals were perfused intracardially with phosphate buffered saline (PBS) and subsequently with 10% formalin solution. The antrum of the jejunum was dissected and postfixed in 10% formalin solution for 24 h at room temperature. The samples were washed overnight with tap water and were dehydrated through a graded series of ethanol. They were incubated in xylene and then embedded in paraffin. Serial sections (5  $\mu$ m) were taken from both groups and collected onto gelatin-coated slides. Sections were deparaffinized at 60°C overnight, immersed in xylene and rehydrated through a graded series of ethanol. They were then washed in tap water and stained using either histochemical (H-E or PAS) or immunohistochemical (IGF-I, IGF-R, IGFBP-3) methods according to their routine protocols. H-E or PAS stained slides were mounted using entellan covered with glass cover slips prior to viewing and photographed under the Olympus BX-40 (Olympus, Tokyo, Japan) light microscope. Morphometric parameters were measured by three blinded observers using the Olympus BX-40 microscope with a video camera (JVC-TK-C 601, Tokyo, Japan) for digital imaging. Villus height as a distance from the tip of the villus to the villus-crypt junction, total epithelial thickness, villus/crypt ratio, and the size and the number of goblet cell were measured at 15 sites of the jejunum<sup>[15,16]</sup>.

An indirect immunofluorescence method was used to determine the immunoreactivities of IGF-I and IGFBP-3. Prior to preincubation in non-immune serum for 1 h, all slides were washed in PBS three times. Sections were then incubated with the primary antibody (Goat-anti-human IGF-I: DSL-2800 IRMA and anti-IGFBP-3; DSL-6600 IRMA, Diagnostic system Laboratories-Texas, USA) in a humid, sealed chamber at 4°C for overnight. Slides were then washed three times in PBS and the site of antigen-antibody reaction was revealed by incubation with fluorescence isothiocyanate (FITC)-conjugated anti-goat diluted 1:100, for 2 h at 4°C. After incubation, sections were rinsed and mounted. Control staining included omission of the primary antibody and replacement with rabbit nonimmune serum. Sections were examined in a fluorescence microscope (Olympus, Tokyo, Japan) equipped with filter setting for viewing FITC fluorescence.

An indirect immunoperoxidase method was used to determine the immunoreactivity of IGF-I Receptor. Deparaffinized sections were washed with PBS and treated with 0.1% trypsin solution. After that, they were washed with PBS and treated with 0.3% hydrogen peroxide for 10 min at room temperature to inactivate endogenous peroxidase activity. These sections were then washed in PBS and they were incubated with the primary antibody mouse monoclonal anti-IGF-I Receptor diluted 1:100 (Calbiochem, CA, USA) in a humid chamber overnight at 4°C. They were then incubated with avidin-biotin-horseradish peroxidase complex (anti-mouse immunoperoxidase antibody: Universal DAKO LSAB2 Kit, CA, USA) for 1 h. The colour reaction was developed using AEC Substrate System-containing 3-amino-9-ethylcar-



**Figure 1** Photomicrograph of the tunica mucosa (M) and muscularis mucosa (MM) of the small intestine in the GH-administered group. Villous hypertrophy and increased goblet cells were seen in the small intestine. PAS,  $\times 100$  (Original magnification).



**Figure 2** Morphometric criteria to determine the effects of growth hormone on small intestine. VH: The height of villus; LP: Lamina Propria; SM: Submucosa; ME: Muscularis externa; ET: Thickness of epithelium; CE: Crypt epithelial cell; GS: The size of goblet cell; GN: The number of goblet cells per villus. Height of the villi, epithelial thickness, and crypt epithelial cell number rats given GH were significantly increased ( $P < 0.01$ ). The size and the number of goblet cells were also significantly increased ( $P < 0.05$ ).

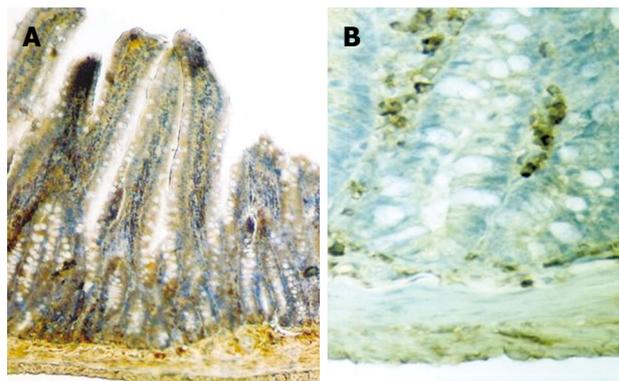
bazole (Dako, Glostrup, Denmark). Between each step, sections were washed three times in PBS. Sections were counterstained with Mayer's hematoxylin, dehydrated, and covered with mounting medium. Normal mouse serum was used as a negative control.

Three observers blinded to clinical information evaluated the immunohistochemical staining scores independently. Staining intensity was graded semi-quantitatively using the H-SCORE<sup>[17]</sup> which was calculated with the following equation:  $H\text{-SCORE} = \sum P_i (i + 1)$ , where  $i$  = intensity of staining with a value of 1, 2 or 3 (mild, moderate, or strong, respectively) and  $P_i$  is the percentage of epithelial cells stained with different intensity, varying between 0%-100%. Results were expressed as mean  $\pm$  SE. Differences between groups were statistically analyzed with one-way ANOVA.  $P$  value of  $< 0.05$  was considered significant.

## RESULTS

### Histological examination

Rats that were given GH displayed hypertrophy in the small intestine (Figure 1). Height of the villi ( $34.9 \pm 9.2$  vs  $51.6 \pm 13.8$ ), epithelial thickness ( $26.3 \pm 8.3$  vs  $45.7 \pm 11.7$ ), and crypt epithelial cell number ( $6.7 \pm 2.1$  vs  $11.4 \pm 3.7$ ) of these rats were significantly increased ( $P <$



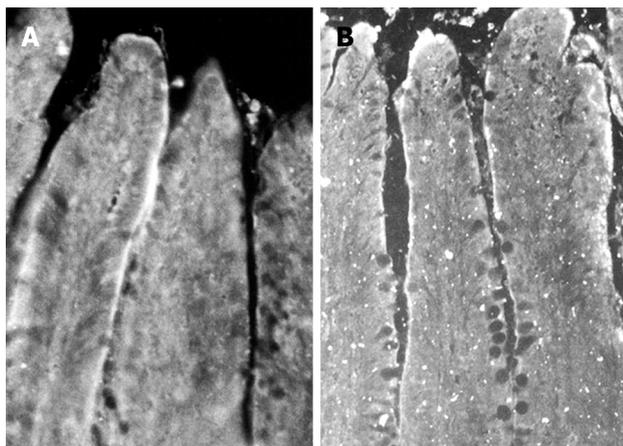
**Figure 3** Like growth factor (IGF)-R immunoreactivity pattern in small intestines of the rats in the study group given growth hormone (A) and control group (B) by using the immuno-peroxidase technique. While weak to moderate IGF-R immunostaining was observed in the epithelial cells of the small intestines in the control group, an increase in the immunostaining was seen in the study group. Note the strong immunoreactivity in the epithelial component and the moderately immunostained smooth muscle of the muscularis mucosa. Goblet cells display little or no immunoreactivity, in contrast to the dense immunoreaction in columnar cells of the study group.  $\times 100$  (Original magnifications).

$0.01$ ). The size ( $5.7 \pm 1.2$  vs  $7.8 \pm 1$ ) and the number ( $25.6 \pm 6.8$  vs  $37.9 \pm 9$ ) of goblet cells were also significantly increased ( $P < 0.05$ ). The morphometric results of the groups according to histologic criteria are shown in Figure 2.

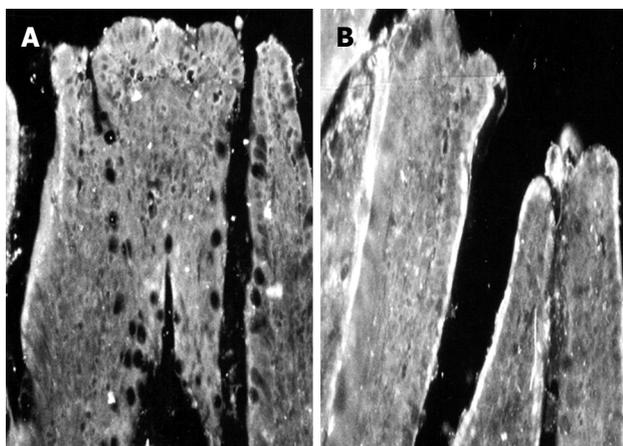
### Localization of IGF-R immunoreactivity

Immunohistochemical staining for the IGF-R showed specific staining in the crypts as well as the apical and basolateral membranes of villus epithelial cells of the control group. Immunoreactivity was primarily observed in the cytoplasm. All staining was similar to each other and they were considered to be of moderate intensity (++) staining. Crypt and villous columnar cells of the small intestine displayed moderate immunoreactivity, whereas goblet cells were weakly immunostained. No immunostaining was evident in occasional large goblet cells of the small intestine. Enteroendocrine cells of the intestinal mucosa were positively immunostained. Muscularis mucosae, muscularis externa and medial smooth muscle cells of vessels exhibited moderate immunoreactivity. Macrophages in the lamina propria and submucosa exhibited positive immunoreactivity in the control group (Figure 3).

There appeared to be a greater intensity of staining for the IGF-R in the study group compared with the control group, as shown in Figure 3 (intensity +++). Crypt epithelial cells and enteroendocrine cells of the intestinal mucosa were strongly positive immunostained (intensity +++). Weak or no immunostaining was evident in large goblet cells of the small intestine. Muscularis mucosae, muscularis externa and medial smooth muscle cells of vessels exhibited moderate immunoreactivity. Scattered macrophages in the lamina propria and submucosa exhibited intense immunoreactivity in the study group. There was also an increase in the intensity of IGF-R immunoreactivity as well as the number of



**Figure 4** Immunoreactivity of IGF-I was seen in the small intestine of the control group (A) and the study group (B) by using an immunofluorescence technique. Increased immunoreactivity was seen in the study group.  $\times 200$  (Original magnifications).



**Figure 5** Immunoreactivity of IGF-binding protein 3 (IGFBP-3) was seen in the small intestines by using an immunofluorescence technique in the control group (A) and the study group (B). Increased immunoreactivity was seen in the study group.  $\times 200$  (Original magnifications).

immunoreactive cells in general. The morphometry of immunoreactivities is shown in Table 1.

#### Localizations of IGF-I and IGFBP-3 Immunoreactivity

In the control group, both the immunoreactivities of IGF-I (Figure 4A) and IGFBP-3 (Figure 5A) were considered moderate in the small intestine (intensity +/++, respectively). Immunoreactivity was mainly localized on the surface epithelium. Rats that were given GH showed a statistically significant ( $P < 0.001$ ) increase in the immunoreactivities of IGF-I (Figure 4B,  $85.33 \pm 11.06$  vs  $195.00 \pm 9.00$ ) and IGFBP-3 (Figure 5B,  $91.00 \pm 16.82$  vs  $201.33 \pm 18.04$ ) which were determined by H-SCORE (Figure 6).

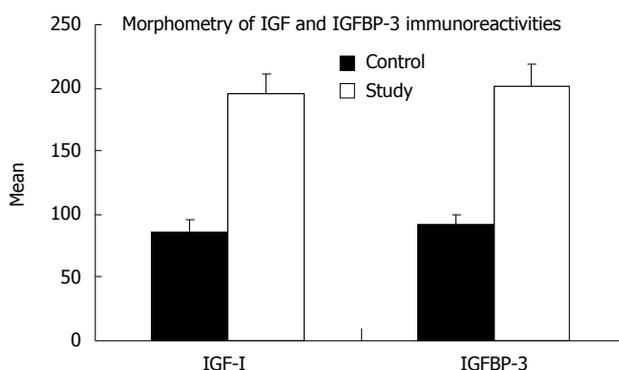
## DISCUSSION

In our study, histological examination revealed an increase in the wall thickness, villus height and crypt cell proliferation of the small intestine after GH admi-

**Table 1** Intensity of cellular localization of IGF-receptor in small intestines of the rats in the control and study groups

Cells	Control group	Study group
Crypt and villous columnar cells	+ / ++	+++
Enteroendocrine cells	++	+++
Goblet cells	- / +	- / +
Occasional large Goblet cells	-	- / +
Paneth cells	-	-
Muscularis mucosae and muscularis externa	++	+++

Weak immunostaining: +; Moderate immunostaining: ++; Strong immunostaining: +++. IGF: Like growth factor.



**Figure 6** IGFBP-3 and IGF-I immunoreactivities in rat small intestine epithelium in the control and the study groups using the indirect immunofluorescence method. (Weak immunostaining: +; Moderate immunostaining: ++; Strong immunostaining: +++). In rats that were given growth hormone, there was a statistically significant increase in the immunoreactivities of IGF-I.

nistration. Our findings have shown that systemic administration of GH increases intestinal growth by stimulating crypt cell production as has been reported by previous studies<sup>[10,17]</sup>. Trophic and promoting effects of GH on the small intestine as shown in human and animal studies have suggested that this peptide could be used in the treatment of patients with malabsorption and malnutrition caused by extensive disease or resection of the small bowel, as is the case in patients with the short bowel syndrome<sup>[10,17-19]</sup>. Exogenous GH has been particularly useful in gastrointestinal rehabilitation of patients with short bowel syndrome in some series<sup>[20-22]</sup>, although not all<sup>[23]</sup>.

In our study, a significant increase in the number of goblet cells was observed in the study group compared to that in the control group. Gastrointestinal epithelium is covered by a protective mucus gel composed predominantly of mucin glycoproteins which are synthesized and secreted by goblet cells<sup>[24]</sup>. This epithelium provides a barrier against potential injury threats of luminal acids, enzymes, bacteria and toxins<sup>[25]</sup>. This result suggests that GH could play a protective role on intestinal mucosa by increasing mucus-producing cells. A previous study has shown the protective effects of GH in rats receiving abdominal radiotherapy<sup>[26]</sup>. However, its functional role had not been clearly assessed *in vivo*.

Effects of GH could be mediated *via* the regulation of intermediate factors like IGF-I, the best known and most intensively studied among the factors. IGF-I is pro-

duced in the liver and other tissues in response to GH stimulation<sup>[27]</sup>. As expected, this had led the investigators to conclude that the local production and function of IGF-I were dependent on GH. However, it is now clear that IGF-I could have functions independent from GH. The results of our study have shown that IGF-I receptor immunoreactivity in crypt and villous columnar cells, enteroendocrine cells and muscularis mucosae was stronger in the study group compared to the control group. Immunoreactivity of Paneth cells was found negative and goblet cells displayed faint to moderate immunoreactivity. These results suggest that the effects of GH on crypt, villous columnar cell, enteroendocrine cells and muscularis mucosae can be mediated *via* IGF-I. In transgenic mouse studies, overexpression of the gene encoding IGF-I has been found to be associated with increased small bowel length, mucosal mass and crypt cell proliferation<sup>[28,29]</sup>. Wheeler *et al*<sup>[3]</sup> have shown that the effects of GH/IGF-I on crypt epithelial cell proliferation in human duodenal mucosa *in vitro*, were similar to the effects of IGF-I alone and were greater than GH alone, suggesting an action mediated solely by IGF-I. Sigalet *et al*<sup>[30]</sup> had demonstrated significant alteration in intestinal morphology of the rat by IGF-I treatment. It has been shown that systemic administration of GH increases trophic activity of the small intestinal mucosa and enhances morphologic and proliferative adaptation of the remnant bowel after surgical resection, both through a direct effect *via* GH receptor present in the gastrointestinal tract and through augmentation of IGF-I synthesis and activity<sup>[31,32]</sup>.

We found that goblet cells were weakly immunostained for IGF-I receptor both in the study and the control group. In another study that detected GH receptor, goblet cells showed faint to moderate immunoreactivity or were immunonegative<sup>[2]</sup>. These results suggested that GH could have direct effects on goblet cell proliferation of the rat small intestine. In the present study, enteroendocrine cells were positive for IGF-I receptor immunostaining in small intestines of rats given GH which may stimulate local or systemic IGF-I production. In addition to its trophic action, IGF-I receptor appears to be extremely important not only for IGF-I but also for modulation of the effects of other growth factors<sup>[33,34]</sup>. Overexpression of IGF-I receptor may promote trophic actions of other growth factors on small intestinal mucosa. Moreover, IGF-I, due to its trophic action, may increase the secretion of gastrointestinal hormones from enteroendocrine cells.

GH increases circulating levels of IGF-I and seems to enhance IGF-I expression locally in the intestinal mucosa<sup>[35]</sup>. A characteristic feature of IGF-I is its ability to bind high affinity IGF-BPs which are thought to modulate its actions<sup>[9,36]</sup>. One of the major functions of IGF-BPs is to control access to the receptors and consequently to modulate biologic responses of cells to IGF-I. IGF-BP-3 has the highest affinity for IGF and is the most abundant IGF-BP in plasma<sup>[6]</sup>. In this study, an increase in the immunoreactivities of IGF-I and IGF-BP-3 were observed in small intestines of GH-administered rats. This result

suggested that GH causes an increase in both IGF-I and IGF-BP-3 production in the small intestine. The plasma concentration of IGF-BP-3 is regulated by GH. IGF-BP-3 concentration that is low in GH deficient patients increases with GH treatment<sup>[6,36]</sup>. This increase is attributed to a direct effect of GH on IGF-BP-3 synthesis as well as the prolongation of the half-life of IGF-BP-3 by binding to other proteins<sup>[7]</sup>. Systemic administration of GH may cause binding of IGF-I and IGF-BP-3 to microvilli on the small intestinal epithelium of rats and may increase their synthesis directly. Thus, IGF-BP-3 may regulate tissue localization, distribution and access to IGF-I receptor that modulates the cell response to IGF-I.

Indeed, even in GH transgenic animals, only transient effects of GH excess on crypt proliferation were observed at weaning and were not sustained in adult animals despite maintained increases in mucosal mass through adulthood. This contrasts with IGF-I, in which a majority of studies in IGF-I transgenic mice and multiple *in vivo* models of IGF-I induction, including TPN and resection models, have demonstrated potent trophic actions of IGF-I accompanied by proliferative and anti-apoptotic actions. While these trophic actions of IGF-I may be beneficial to increase the mass of functional intestinal mucosa, they may also increase the risk of intestinal tumors. Despite the long-held view that GH is a trophic hormone and the proliferative effects of which are mediated by IGF-I, evidence in the healthy intestine suggests that IGF-I is a more potent enterotrophic factor than GH. This is found even when GH elevates plasma IGF-I to levels similar to those found in models of IGF-I infusion. Available evidence has suggested that the local expression of IGF-I occurs primarily in mesenchymal cells of the lamina propria of the normal intestine. IGF-I also has autocrine actions that increase the proliferation and growth of mesenchymal cells. Collectively, these results suggest that IGF-I derived from intestinal mesenchymal cells regulates the growth and function of neighboring epithelial cells, as well as mesenchymal cells themselves. Further support for this hypothesis comes from observations that levels of local IGF-I mRNA expression correlate with bowel growth during periods of altered nutrient status, resection, and disease<sup>[3,4,37]</sup>.

In summary, systemic administration of GH has caused proliferation of crypt epithelial cells, goblet cells and enteroendocrine cells in the small intestinal mucosa of rats. The number of IGF-I receptors in small intestinal mucosa, particularly in crypt epithelial and enteroendocrine cells, was increased. However, there was not an increase in the number of IGF-I receptors in goblet cells. Increases observed in mucosal height may be mediated through local rather than systemic IGF-I production, as IGF-I and IGF-BP-3 were found to increase in rats' small intestine particularly in the surface epithelium. Increased IGF-I expression has been observed after GH administration and GH exerts some of its effects through stimulation of IGF-I and IGF-BP-3 expression. GH improved villous morphology and small intestine homeostasis by affecting the cells through mediators. These

findings provide a basis for further studies on the role of GH in the regulation of gastrointestinal function and growth, in health and in colorectal diseases.

In conclusion, GH exerts trophic and protective effects on small intestinal mucosa by increasing IGF-I synthesis. It seems to exert its trophic actions *via* IGF-I by increasing number of IGF-I receptors on crypt and epithelial cells. IGF-I by itself does not have a role in protective action. Further research into the mechanism of action of GH and IGF-I is also needed to fully define the clinical appropriateness of these growth factors in particular settings.

## COMMENTS

### Background

Growth hormone (GH) has a lot of diverse physiologic effects. Systemic administration of GH markedly increases trophic action in the small intestine. The peripheral effects of GH are mediated by IGF-1. IGFBP-3 is a GH-dependent molecule and can be an important indicator of conditions associated with altered GH secretion and action. It is not known whether effects of GH on small intestine are direct or mediated *via* IGF-1.

### Research frontiers

To demonstrate histologic changes induced by GH in the small intestine and to define mechanisms of trophic and protective effects of GH on small intestine.

### Innovations and breakthroughs

This is the first study reporting the importance of IGF-1 as a mediator for protective and trophic effects of GH on small intestines. The authors' study demonstrated that trophic effects are related to IGF-1 while protective effects are not.

### Applications

Better understanding of trophic and protective effects of GH on small intestines provide researchers with an opportunity to observe the effectiveness of treatment of intestinal diseases like small bowel syndrome. Also IGF-1 treatment may be considered as a therapeutic option in such diseases.

### Peer review

The authors evaluate the effects of growth hormone on the histology of small intestines which might be related to the role of IGF-I, IGF-binding protein 3 (IGFBP-3) and its receptors. They find trophic effect is mediated by an increase in IGF-I synthesis in small intestine, but the protective effect is not related to IGF-I.

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## Expression and localization of *Wolfram syndrome 1* gene in the developing rat pancreas

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involved in some aspects of pancreatic development and further research on *WFS1* may provide new evidences to prove the interactions between mesenchyma and epithelia at the same time.

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**Key words:** *Wolfram syndrome 1*; Pancreas; Morphogenesis; Mesenchyme; Pancreatic beta cells

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### Abstract

**AIM:** To investigate the expression and function of *Wolfram syndrome 1* gene (*WFS1*) during the development of normal pancreas.

**METHODS:** Pancreas from Sprague-Dawley rat fetuses, embryos, young and adult animals were used in this study. Expression levels of *WFS1* in pancreas of different development stages were detected by reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting. To identify the cell location of *WFS1* in the developing rat pancreas, double-immunofluorescent staining was performed using antibodies to specific cell markers and *WFS1*, respectively.

**RESULTS:** Compared to E15.5, the highest level of *WFS1* mRNA was detected at E18.5, the level of *WFS1* mRNA in E15.5 and P0 was less, and at a lowest at adult ( $P < 0.05$  vs P0 and adult), respectively. Compare to E15.5, the highest level of *WFS1* was at P14 and lowest at P21 ( $P < 0.05$  vs P14 and P21), respectively. The *WFS1* positive staining is expressed in the normal developing rat pancreas mainly in the islet beta-cells and mesenchyme at each stage tested.

**CONCLUSION:** These results indicate that *WFS1* may be

### INTRODUCTION

The pancreas is a complex organ composed of 2 different cell populations, exocrine and endocrine cells. The acini and ducts form the exocrine pancreas, which produces and transports digestive enzymes into the duodenum. The endocrine component contains 4 types of cells that secrete hormones to regulate glucose metabolism and other physiological processes<sup>[1]</sup>. In rats, the pancreas develops from the foregut endoderm in 3 major steps: endoderm formation [embryonic day (E) 7.5], pancreatic morphogenesis (E10.5), and differentiation of endocrine and exocrine cells (E13.5). At approximately E16, the islet progenitor cells leave the contiguous epithelium, migrate through the adjacent extracellular matrix into the surrounding mesenchyme, and aggregate to form the islets of Langerhans. The islets are not completely formed until shortly before birth on E18-E19<sup>[2-4]</sup> and undergo further remodeling and maturation for 2-3 wk after birth<sup>[5]</sup>. Thus, the developing pancreas presents a challenge for developmental biologists because of the complex morphogenetic processes underlying the development of this organ. Until now, the factors that control the progressive development of pancreatic architecture and function have remained unclear. To

identify these functional factors we performed a gene array at E15.5, E18.5, birth and adult stages. The *Wolfram syndrome 1* gene (*WFS1*), was one of the factors found to be abundantly expressed in islet cells at E15.5 and E18.5 (data not shown). Wolfram syndrome (WS) is an autosomal recessive, progressive, neurodegenerative disorder characterized by diabetes mellitus and optic atrophy<sup>[6]</sup>. This disorder is also associated with diabetes insipidus and deafness, hence the acronym DIDMOAD (diabetes insipidus, diabetes mellitus, optic atrophy, and deafness). Other manifestations such as atonic bladder, ataxia, nystagmus and predisposition to psychiatric illness may also be present<sup>[7,8]</sup>. *WFS1*, discovered in 1998, was mapped to chromosome 4p16.1 by positional cloning<sup>[9,10]</sup>. The gene is composed of 8 exons spanning 33.4 kb of genomic DNA. The 3.6 kb mRNA encodes an 890-amino acid polypeptide named wolframin<sup>[9,11]</sup> with 9 predicted transmembrane domains, and belongs to a novel gene family. Biochemical studies in cultured cells indicate that *WFS1* protein is an integral, endoglycosidase H-sensitive membrane glycoprotein primarily localized in the endoplasmic reticulum<sup>[12]</sup>. Postmortem studies of the pancreas from subjects with WS have shown  $\beta$ -cell loss<sup>[10]</sup>. Mice with a disrupted *WFS1* were also reported to exhibit impaired glucose homeostasis accompanied by a progressive reduction of  $\beta$ -cell mass<sup>[13]</sup>. Thus, *WFS1* seems to play a role in the normal function of  $\beta$  cells, but little is known about its function during embryogenesis. To the best of our knowledge, no study has investigated the relationship between *WFS1* expression and pancreas development, and the expression of *WFS1* during pancreatic development in rats is poorly understood. Knowledge of the regional and temporal expression of *WFS1* will be useful in understanding its potential roles in pancreatic development. Therefore, we further examined the expression patterns of *WFS1* in rat pancreas during development.

## MATERIALS AND METHODS

### **Animals and preparation of rat pancreatic tissue**

Sprague-Dawley (SD) rats were purchased from the Animal Center of Nanjing Medical University (Nanjing, China). SD rats (2:1, male:female) were mated overnight. At noon the next day, if a vaginal plug was discovered, it was considered as Day 0.5 of gestation (E0.5). Embryos were removed at E15.5 and E18.5 from the uterus of pregnant rats, which were sacrificed by cervical dislocation. Pancreata from E15.5 and E18.5 rat embryos were isolated as previously described<sup>[14]</sup> under a stereomicroscope. Rat pancreata at postnatal (P) days 0, 7, 14, 21 and from adults, were directly isolated by the unaided eye. All experiments were conducted in accordance with the Chinese Law for Animal Protection and were approved by the local animal care committee. Five rats were used at each age stage. Dissected tissues were immediately rinsed 3 times with phosphate-buffered saline (PBS) to remove serum proteins, and fixed with 4% paraformaldehyde in PBS overnight for histology, or frozen in liquid nitrogen for RNA and protein isolation.

### **RNA extraction, reverse transcription-polymerase chain reaction (RT-PCR)**

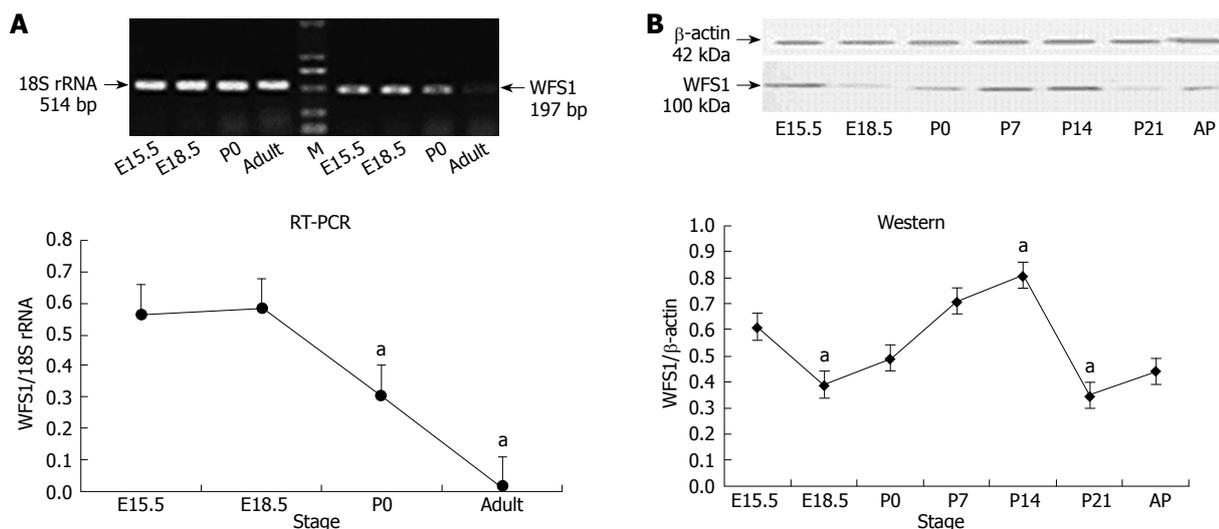
Total RNA was extracted from pancreata at each time point with TRIZOL reagent (Invitrogen Life Technologies, Burlington, Ontario, Canada), according to the manufacturer's instructions. The quality of the RNA was verified by agarose gel electrophoresis using ethidium bromide staining. For each PCR, 2  $\mu$ g of DNA-free total RNA with oligo (deoxythymidine) primers and reverse transcriptase were used. PCR was performed in 25  $\mu$ L reactions containing 25 ng of cDNA, 0.2 nmol of each primer pair, and 0.3  $\mu$ L of Taq DNA polymerase. PCR was carried out in a T-gradient Biometra PCR thermal cycler (Montreal Biotech Inc., Kirkland, Quebec, Canada) to determine the annealing temperature for *WFS1* primers. We used the following primer pairs: *WFS1*, forward: 5'-CTGCTCTTTTGCTGGTTCT-3', reverse: 5'-GATGTCCTTGGTGATGTCG-3' (497 bp); and 18S rRNA, forward: 5'-ACGAACCAGAGCCGAAAGC-3', reverse: 5'-GGACATCTAAGGGCATCACAG-3' (514 bp). PCR conditions were as follows: 2 min at 94°C, followed by up to 35 cycles of 94°C for 30 s, 54.3°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. To estimate the linear range of the nested reactions, we analyzed the PCR products for 10, 15, 20, 25, 30, and 35 cycles. The amplified products were analyzed on 1% agarose gels and visualized by ethidium bromide staining. The data were normalized for 18S rRNA expression.

### **Preparation of protein samples**

The pancreata was homogenized in a detergent lysis buffer containing 8 mol/L urea, 2% CHAPS, 40 mmol/L Tris, 65 mmol/L DTT and 2% IPG buffer. The lysate was then centrifuged at 15000 *g* for 1 h at 4°C. The total protein concentration of each sample was analyzed using a modified Bradford assay. All samples were stored at -80°C prior to the electrophoresis.

### **Western blotting analysis**

An equal amount of protein samples (50  $\mu$ g protein in 20  $\mu$ L buffer) from each time point were boiled in 3  $\times$  loading buffer (10 mmol/L Tris-HCl, pH 6.8 including 3% SDS, 5%  $\beta$ -mercaptoethanol, 20% glycerol and 0.6% bromophenol blue) for 3 min and separated by 12.5% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). For blocking, membranes were incubated with 5% fat-free milk in Tris-buffered saline plus 0.05% Tween-20 (TBST) overnight at 4°C. The membranes were then incubated with the primary antibody (NB100-1918, rabbit polyclonal antibody to *WFS1*, diluted 1:1000, Novus Biologicals Littleton, CO, USA) (sc-47778 mouse polyclonal antibody to  $\beta$ -actin, diluted 1:1000, Santa Cruz Biotechnology Inc., USA) for 2 h at room temperature. After washing in TBST, the membranes were incubated with the peroxidase-linked goat anti-rabbit IgG conjugates (PIERCE, Prod 1858415) or peroxidase-linked goat anti-mouse IgG conjugates (sc-2055, Santa Cruz Biotechnology Inc. USA) for 1 h at room temperature. The membranes were then washed



**Figure 1** *Wolfram syndrome 1 (WFS1)* mRNA (A) and protein expression (B) in the developing rat pancreas. A: *WFS1* mRNA measured by reverse transcription-polymerase chain reaction (RT-PCR). upper: from embryonic day (E) 15.5 to adulthood: *WFS1* mRNA expression was highest at E15.5 and E18.5. M: Marker; lower: *WFS1* mRNA expression was analyzed and normalized to 18S rRNA. Results are indicated in percentage above the 18sRNA value and are representative of three independent experiments. Compared to E15.5, the highest level of *WFS1* mRNA was detected at E18.5, the E15.5 and P0 was less, and at a lowest at adult ( $P < 0.05$  vs P0 and adult), respectively; B: Western blotting of *WFS1* protein in the pancreas of E15.5, E18.5, P0, P14, P21 and adult rats. upper: expression of *WFS1* protein (100 kDa) with  $\beta$ -actin (42 kDa) as a loading control. The position of the molecular weight markers are indicated on the left; lower: the expression of *WFS1* protein shows dynamic changes during rat pancreas development. Results are indicated in percentage above the e15.5 value and are representative of three independent experiments. Compare to E15.5, The highest level of *WFS1* was at P14 and lowest at P21 ( $P < 0.05$  vs E18.5, P14 and P21), respectively.

again in TBST, incubated in enhanced chemiluminescence reagents (ECL, Amersham Life Science, Cleveland, OH, USA) for 1 min, and exposed to VA 711 B Blue Sensitive X-ray films. Densitometric quantification of bands at sub-saturating levels was performed using the Syngenetool gel analysis software (Syngene, Cambridge, UK).

**Double fluorescence immunohistochemistry**

Tissues were fixed in 4% paraformaldehyde overnight at 4°C followed by a standard protocol of dehydration and paraffin embedding, and 5  $\mu$ m sections were cut. The paraffin sections were deparaffinized in xylene and rehydrated in graded ethanol and distilled water. The non-specific binding sites were blocked in 1% bovine serum albumin for 30 min. For *WFS1* protein and insulin, glucagon or vimentin double immunofluorescence, the rabbit anti-*WFS1* primary polyclonal antibody was applied and revealed using FITC-labeled Rabbit anti-goat IgG (1:400, sc-2777, Santa Cruz Biotechnology Inc., USA). Mouse anti-insulin primary polyclonal antibody (1:1000, Santa Cruz Biotechnology Inc., USA) or mouse anti-vimentin primary monoclonal antibody (1:1000, Chemicon Temecula, CA, USA) was then applied and revealed by cy3-labeled anti-mouse IgG (1:400, Chemicon Temecula, CA, USA). Sections were placed in Gel Mount Aqueous Mounting Medium (G0918, Sigma, USA) with a cover glass, and were examined under an Olympus BX51 microscope (Olympus Optical, Tokyo, Japan). Images were taken at a magnification  $\times$  400.

**Statistical analysis**

Analysis of the experimental data was performed using PD Quest 7.0 software and the paired Student *t*-test.  $P < 0.05$  was considered statistically significant. Data are presented as the mean  $\pm$  SD.

**RESULTS**

***mRNA expression of WFS1 in rat pancreas at different developmental stages***

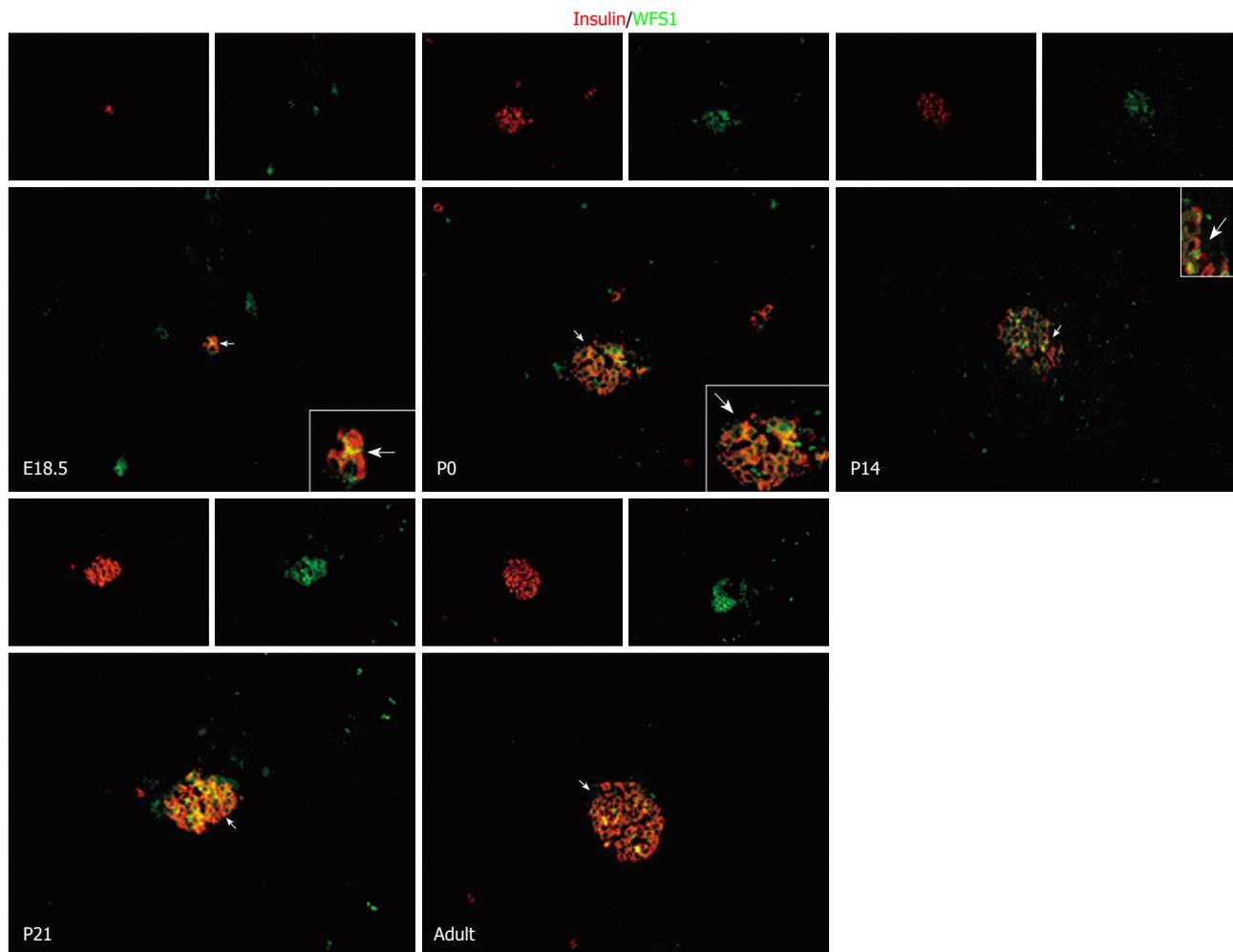
The mRNA expression levels of *WFS1* in rat pancreas during its development were examined by RT-PCR. 18S RNA as used as an internal control and assessed under the same conditions. As shown in Figure 1A, RT-PCR for the *WFS1* specific region yielded a band of the expected size (197 bp) in rat pancreas tissue at different developmental stages. The mRNA expression of *WFS1* was high at E15.5 and E18.5, started to decrease at birth, and continued to decline to adulthood.

***Protein expression of WFS1 in rat pancreas at different developmental stages***

We performed Western blotting using total protein samples extracted from pancreata obtained at E15.5, E18.5, P0, P7, P14 and P21 and from adult rats (Figure 1B).  $\beta$ -actin (42 kDa) was used as an internal control under the same conditions. Analysis of the blots showed that *WFS1* (100 kDa) protein was expressed in the pancreas. Densitometric quantification of each band showed that the expression of *WFS1* protein was high at E15.5, and then decreased until birth (P0). Subsequently, the level of *WFS1* protein increased gradually and peaked at P14. The expression of *WFS1* protein was lowest at P21. The level of *WFS1* protein was relatively high in the adult rat.

***Regional localization of WFS1 protein in rat pancreas at different developmental stages***

To determine the localization of *WFS1* protein during pancreatic development, serial pancreas sections (obtained at E18.5, P0, P14, P21 and in adulthood) underwent



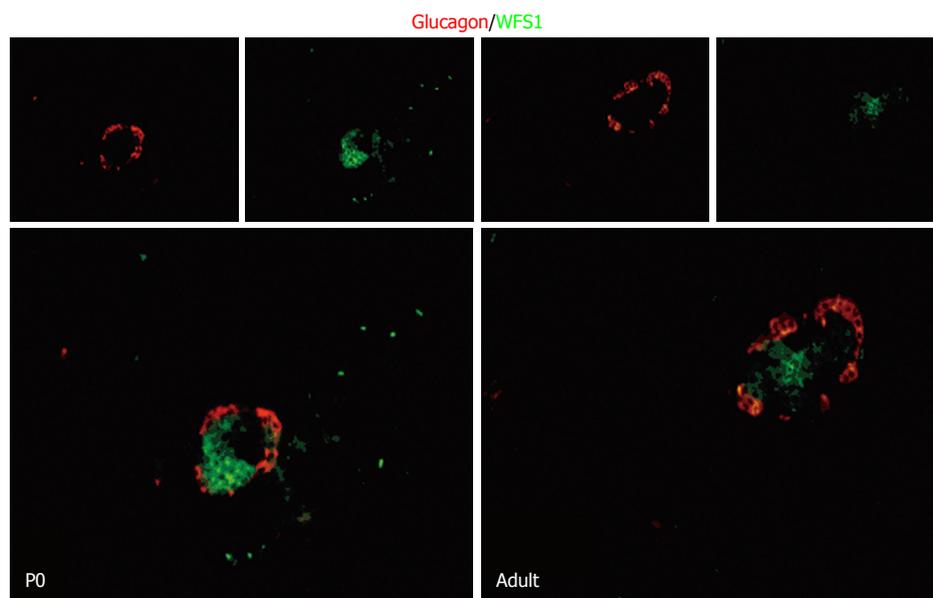
**Figure 2** Immunofluorescent localization of WFS1 protein and insulin in the pancreas of E18.5, P0, P14, P21 and adult rats. The WFS1 antibody was detected with an FITC (green)-labeled secondary antibody and the insulin antibody was detected with a Cy3 (red)-labeled secondary antibody. An overlap between WFS1 (green) and insulin (red) labeling is colored orange. Modest WFS1 staining is seen in the developing islets from E18.5 to adulthood, and overlaps insulin-positive  $\beta$ -cells. A few WFS1-labeled cells are scattered away from the islets. Primary objective magnification,  $\times 400$ . Insets, higher magnifications of the areas indicated by arrows for E18.5, P0 and P14.

double immunofluorescence. We found that WFS1 protein was co-expressed with insulin and could not be detected in  $\alpha$ -cells. As shown in Figure 2, there was a consistent overlap between the WFS1 protein-positive cells and cells labeled with insulin at each stage tested. This means that, during pancreatic development and remodeling, the WFS1 protein is continually expressed in  $\beta$  cells. As shown in Figure 3, there was little overlap between the WFS1 protein-positive cells and cells labeled with glucagon at any of the stages tested. This indicates that WFS1 protein is not expressed in  $\alpha$ -cells during development. Interestingly, we also found that WFS1-positive cells were scattered throughout the pancreas. Therefore, we performed further double immunofluorescence, which confirmed that WFS1 protein was co-expressed with vimentin in mesenchymal cells (Figure 4).

## DISCUSSION

Organogenesis involves 2 processes: morphogenesis, which is the shaping of an organ, and cytodifferentiation, which is the acquisition and expression of specialized

cellular functions within that organ<sup>[15]</sup>. During pancreatic development, specialized cell types are generated from progenitor cell populations and are precisely organized into the elaborate structure of the adult organ. Previously, most researchers focused on deriving functional islets from stem cells or other cell types. Gu *et al*<sup>[16]</sup> generated transcriptional profiles of enriched cells isolated from pancreata at 4 biologically significant stages of endocrine pancreas development (endoderm before pancreas specification, early pancreatic progenitor cells, endocrine progenitor cells and adult islets of Langerhans) to identify new endocrine regulatory genes and markers. With temporal and spatial analysis of genes expressed in embryonic endocrine cells, a database of potential progenitor cell markers was generated. In the present study, using a gene array, *WFS1* was found to be one of the genes that were specifically and highly expressed in islet cells at E15.5 and E18.5. Then, we used RT-PCR and Western blotting to analyze the expression of *WFS1* at 6 biologically significant stages of rat pancreas development: E15.5, E18.5, P0, P14, P21 and in adult rats. During the later embryonic period, the pancreatic cells



**Figure 3** Immunofluorescent localization of *WFS1* protein and glucagon in the pancreas of P0 and adult rats. The *WFS1* antibody was detected with an FITC (green)-labeled secondary antibody and the glucagon antibody (mouse monoclonal antibody) was detected with a Cy3 (red)-labeled secondary antibody on the same section. The overlap between *WFS1* (green) and glucagon (red) labeling is colored orange. *WFS1*-positive cells and glucagon-positive cells can be seen in 2 pancreas sections tested. Double-labeling with antibodies to *WFS1* and glucagon reveal that the cells labeled with *WFS1* are surrounded by  $\alpha$ -cells. Primary objective magnification,  $\times 400$ .

exhibit dramatic growth, differentiation and proliferation. Accordingly, it may not just be a concomitant phenomenon that the expression of *WFS1* protein is greater during the later embryonic period. The present study confirmed the changes in *WFS1* mRNA levels in the rat pancreas during development by RT-PCR.

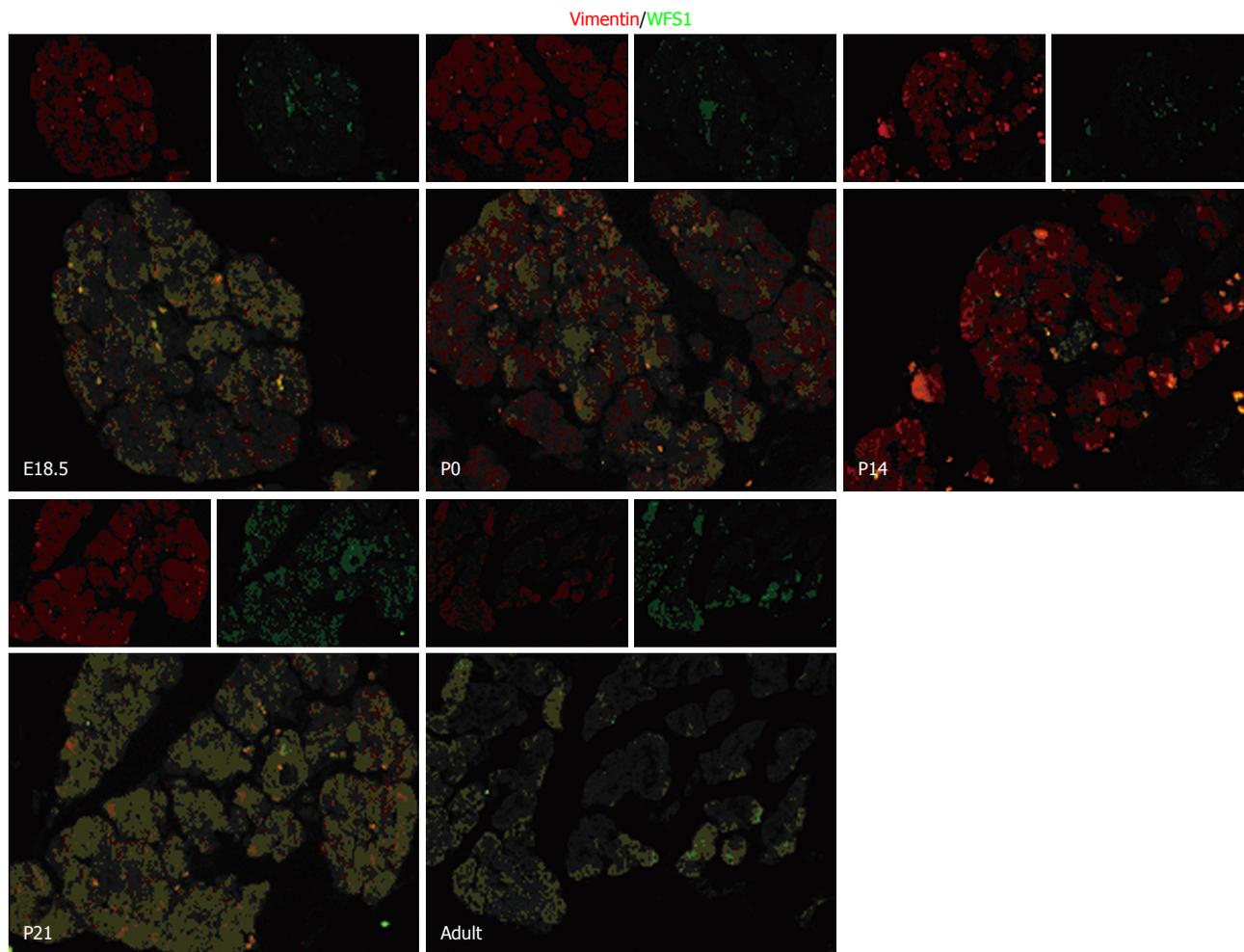
The architecture of the islet is completely formed shortly before birth; however, the function of the islet is not fully developed at this stage. It has been reported that the pancreas of neonatal rat undergoes further remodeling and maturation for 2-3 wk after birth<sup>[17]</sup>. The remodeling process includes a decrease in  $\beta$ -cell mass and enhanced islet neogenesis. The number of  $\beta$ -cells is mainly regulated by apoptosis and cell replication<sup>[18]</sup>. The results of Western blotting revealed that the *WFS1* protein level was highest during the postneonatal period, particularly P14. Although the function of wolframin is still not completely known, recent studies have revealed its localization to the endoplasmic reticulum and that it may play an important role in endoplasmic reticulum (ER) homeostasis by regulating  $\text{Ca}^{2+}$ <sup>[11,19]</sup>. During islet development, particularly in the ER at E15.5 and P14, secretory and transmembrane proteins involved in development regulation fold into their native conformation and undergo posttranslational modifications important for their activity and structure. Consequently, ER homeostasis and the *WFS1* protein may be important in islet development. It is well known that *WFS1* protein induces cation channel activity in ER membranes<sup>[11]</sup> and regulates calcium levels in the ER<sup>[19]</sup>. *WFS1* protein also plays a role in stimulus-secretion coupling for insulin exocytosis in pancreatic  $\beta$  cells<sup>[13,16,19,20]</sup>. Therefore, enhanced expression of *WFS1* protein in adults suggests that *WFS1* may retain its function in adult islets.

Surprisingly, the pattern of *WFS1* protein expression was not consistent with the pattern of *WFS1* mRNA

expression, as described above. This difference may result from post-transcriptional control, although the mechanisms need further investigation. RNA binding protein controls the various steps and the rate of transcription of microRNA, which regulates the destruction of RNA and changes in chromatin structure may be involved in the regulation of *WFS1* expression.

During remodeling in the perinatal period, apoptosis is an important mechanism that decreases  $\beta$ -cell mass. Apoptosis of  $\beta$ -cells is significantly increased in neonates compared with adult rats, and peaks at 2 wk of age<sup>[18]</sup>, while the highest level of *WFS1* protein was detected at the same time. It has been reported that ER stress induces *WFS1* expression in pancreatic  $\beta$ -cells<sup>[21]</sup> and the expression of *WFS1* is regulated by inositol requiring  $1\alpha$  (*Ire1* $\alpha$ ) and PKR-like ER kinase (PERK), central regulators of the unfolded protein response<sup>[22]</sup>. It has also been reported that *WFS1*-deficient pancreatic  $\beta$ -cells exhibit increased phosphorylation of RNA-dependent protein kinase-like ER kinase, chaperone gene expression and active XBP1 protein levels, indicating an enhanced ER stress response. Furthermore, the increased ER stress response was accompanied by impaired  $\beta$ -cell proliferation and increased caspase-3 cleavage<sup>[21]</sup>. Since the *WFS1* protein attenuates ER stress, maintains cell cycle progression and represses the apoptotic pathway, specifically in pancreatic  $\beta$ -cells, it may play an important role in the maintenance of  $\beta$ -cell mass by balancing  $\beta$ -cell growth (differentiation and proliferation) and  $\beta$ -cell death (apoptosis) during pancreatic remodeling.

During organogenesis, specialized cell types are generated from progenitor cell populations and are precisely organized into the elaborate structure of the adult organ. Numerous cell-cell communications and the initiation of complex inter-regulating genetic networks are involved to ensure the fidelity of organogenesis. Several



**Figure 4** Immunofluorescent localization of WFS1 protein and vimentin in the pancreas of E18.5, P0, P14, P21 and adult rats. The WFS1 antibody was detected with an FITC (green)-labeled secondary antibody and the vimentin antibody was detected with a Cy3 (red)-labeled secondary antibody on the same section. The overlap between AFP (green) and vimentin (red) labeling is colored orange. WFS1-positive cells can be detected in pancreas sections from E18.5 to adult. Double-labeling with antibodies to WFS1 and to vimentin revealed colocalization of these proteins in the same cells from E18.5 to adult. Primary objective magnification,  $\times 400$ .

studies have reported the role of mesenchymal and/or epithelial interactions in pancreatic development and suggested that multiple diffusible mesenchyme-derived factors may act on the developing epithelia to influence cell division, cell fate, differentiation, and to determine the proportion of endocrine vs exocrine tissue<sup>[23-26]</sup>. Examination of the ontogeny of *WFS1* in the developing rat pancreas revealed an intimate relationship between WFS1 protein and the mesenchymal and/or epithelial interactions<sup>[27]</sup> in pancreatic development. Vimentin is a marker for mesenchymal cells<sup>[28,29]</sup>. In the present study, the results of double immunofluorescence revealed that WFS1 protein was co-expressed with vimentin. This indicates that WFS1 protein is localized to the pancreatic mesenchyme shortly before birth on E18.5 and after birth. Previous studies revealed that the late embryonic stage is a key phase in late pancreatic organogenesis, in which a dramatic increase in the number of endocrine and exocrine cells with high levels of insulin and exocrine enzymes are observed. The islets are fully formed shortly before birth on E18.5 and undergo further remodeling and maturation after birth<sup>[26]</sup>. Considering the previous

reports of WFS1 protein function and the results of our experiments, we suggest that WFS1 protein plays an important role in many aspects of pancreas development. However, the underlying mechanism is not fully understood and needs further research, which may also provide new evidence to confirm the interactions between mesenchymal and epithelial cells.

In summary, our present study has, for the first time, demonstrated that WFS1 protein is localized to the mesenchyme in the rat pancreas by immunofluorescence. The dynamic expression of *WFS1* in the various developmental stages of the pancreas indicates that *WFS1* may be involved in many aspects of pancreatic development.

## COMMENTS

### Background

Wolfram syndrome (WS), caused by the mutant of the *Wolfram syndrome 1* gene (*WFS1*), is an autosomal recessive, progressive, neurodegenerative disorder accompanied by diabetes mellitus and optic atrophy. Although it has been reported that WFS1 protein is found to be enriched in endocrine progenitors vs non-endocrine progenitor cells at embryonic day (E) 13.5, its expression and function during the development of normal pancreas remain unknown.

**Research frontiers**

It is thought that the pancreatic endocrine cells including beta cells arise from duct epithelial cells, however much research has been done to explore a different source. Novel strategies would obviously benefit from the use of beta stem cells/progenitor cells.

**Innovations and breakthroughs**

*WFS1* expression has never been assessed in the developing pancreas and it is highly expressed in P14 on which the apoptosis rate of beta cells is at a high point. *WFS1* protein has been proved to be a downstream factor in the endoplasmic reticulum stress signal pathway.

**Applications**

This study reveals the possible mechanism of apoptosis in pancreas remodeling and may lead to new possibilities for diabetes therapy.

**Peer review**

This is a very interesting study that provides novel information that will be useful for the understanding of the role *WFS1* in pancreatic development as well as disease.

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ORIGINAL ARTICLE

## Effects of angiotensin-1 on attachment and metastasis of human gastric cancer cell line BGC-823

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### Abstract

**AIM:** To evaluate the effects of angiotensin-1 (Ang-1) on adhesion of gastric cancer cell line BGC-823 and expression of integrin  $\beta$ 1, CD44V6, urokinase-type plasminogen activator (uPA) and matrix metalloproteinase-2 (MMP-2).

**METHODS:** BGC-823 cells were transfected transiently with adenovirus-Ang-1 (Ad-Ang-1). Cells transfected transiently with adenovirus-green fluorescent protein (Ad-GFP) and untransfected cells were used as a negative and blank control group, respectively. The cell adhesion rate between cell and extracellular matrix (ECM) was determined by cell adhesion assay. To investigate whether Ang-1 could reinforce gastric carcinoma metastasis, we performed migration and invasion assays in BGC-823 cells. The mRNA and protein expression of integrin  $\beta$ 1, CD44V6, uPA and MMP-2 were detected by reverse transcription polymerase

chain reaction and Western blotting, respectively. The expression of integrin  $\beta$ 1 and CD44V6 was measured by immunohistochemistry.

**RESULTS:** BGC-823 cells were transfected successfully. The adhesion rate increased significantly in the Ad-Ang-1 group ( $P < 0.05$ ). The Ad-Ang-1-transfected group had a significant increase in migration and invasion compared with that of the mock-transfected and Ad-GFP groups. The mRNA and protein expression of integrin  $\beta$ 1, CD44V6, uPA and MMP-2 in the Ad-Ang-1 group was higher than that in the Ad-GFP and blank control groups ( $P < 0.05$ ). Compared with mock-transfected and Ad-GFP groups, integrin  $\beta$ 1 and CD44V6 expression intensity greatly increased ( $P < 0.05$ ).

**CONCLUSION:** Transfection of Ang-1 into human gastric cancer cell line BGC-823 can significantly increase expression of integrin  $\beta$ 1 and CD44V6, by which cell adhesion and metastasis to the ECM are promoted.

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**Key words:** Angiotensin-1; CD44V6; Cell adhesion; Gastric cancer; Integrin  $\beta$ 1; Matrix metalloproteinase-2; Neoplasm metastasis; Urokinase-type plasminogen activator

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### INTRODUCTION

Angiotensin-1 (Ang-1) has been identified *via* secretion trap and homology cloning techniques as a family of structurally related proteins that bind with similar specificity and affinity to a common endothelial-cell-specific receptor tyrosine kinase, Tie2<sup>[1-3]</sup>. Ang-1 is

required for correct organization and maturation of newly formed vessels and promotes quiescence and structural integrity of adult vasculature. However, its functions and mechanisms in tumors are not clear<sup>[4,5]</sup>. In addition to Tie receptors, Ang-1 has been found to bind integrins. Experiments with blocking antibodies, as well as cells deficient in certain integrins, suggest Ang-1 can bind several different integrins, including  $\alpha 2\beta 1$ ,  $\alpha 5\beta 1$ ,  $\alpha v\beta 3$  and  $\alpha v\beta 5$ <sup>[6-9]</sup>. Also, some researchers have confirmed that Ang-1 can support cell adhesion mediated by integrins<sup>[7]</sup>. Whether there is a relationship between Ang-1 and integrin  $\beta 1$  has not been established. In addition, Ang-1 can affect cell adhesion, and CD44V6 is a member of the cell adhesion family, therefore, we can infer that there is a relationship between Ang-1 and CD44V6. At the same time, in primary adenocarcinoma, cancer cell invasion is facilitated by interaction with the local stromal compartment, whereas metastatic growth requires the ability of the cancer cell to interact with the new host tissue before the cancer cells can invade and destroy the target organ. The local stromal compartment, which comprises inflammatory cells and fibroblasts, facilitates the process by secreting extracellular matrix (ECM)-degrading proteases, including matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (uPA). MMP-2 (also known as gelatinase A and the 72-kDa type IV collagenase) has the ability to degrade type IV collagen in the basement membrane, and it is one of the major stroma-derived MMPs. One proposed mechanism of MMP-2 activation is through the plasminogen activator/plasmin system, in which pro-uPA binds to uPA receptor (uPAR), through a specific amino-terminal sequence of its non-catalytic chain. This binding result in uPA activation, accelerates the conversion of plasminogen to plasmin on the cell surface, and localizes these enzymes to focal contact sites. Many studies have indicated that MMP-2 plays an important role in tumor invasion of the basement membrane. Therefore, we used adenovirus as a vector to transfect the cell line BGC-823, to explore the effects of Ang-1 on integrin  $\beta 1$ , CD44V6, uPA and MMP-2 of human gastric cancer cell line BGC-823, and study the mechanism of adhesion to the ECM and metastasis of gastric cancer.

## MATERIALS AND METHODS

### **BGC-823 cell line and cell culture**

Human gastric adenocarcinoma cell line BGC-823 (purchased by Chinese Academy of Sciences) was cultured in RPMI 1640 medium (Gibco) that contained 10% fetal bovine serum (FBS) and 10% penicillin/streptomycin, and maintained under an atmosphere of 5% CO<sub>2</sub> with humidity at 37°C.

### **Preparation of adenoviral vectors and the determination of multiplicity of infection (MOI)**

The recombinant adenovirus vector that carried Ang-1 and control adenoviral vector that carried green fluorescent protein (GFP) were constructed and kindly

provided by The First Affiliated Hospital of Nanjing Medical University<sup>[10]</sup>. The recombinant adenoviral vectors that expressed Ang-1 and GFP were amplified by infection of 293 cells. A suspension of 293 cells ( $5 \times 10^8$  cells/L) was added into the culture capsule. The linearized recombinant adenoviral vectors that expressed Ang-1 or GFP genes were co-transfected with Lipofectamine 2000 (Gibco) into 293 cells. The supernatant fluid that contained adenoviral vectors was transfected into more 293 cells after occurrence of a cytopathic effect. The viral extracts were propagated in 293 cells and were purified by CsCl (Sigma) density purification, and dialyzed and stored in dialysis buffer (Spectrum) with 10% glycerol at -70°C. Subsequently, the titer of each viral stock was measured by TCID<sub>50</sub>. 293 cells were seeded in 96-well plates ( $5 \times 10^7$  cells/L) for 24 h, and 100  $\mu$ L dilute supernatant fluid that contained adenoviral vectors ( $10^{-1}$ - $10^{-12}$ ) was added respectively. Then, we calculated the titer of each viral stock after the cells were incubated with the complexes for 10 d. Subsequently, BGC-823 cells were seeded in 96-well plates ( $1 \times 10^4$  cells/well). After 8 h exposure to control adenoviral vectors that contained medium at different MOI (10, 20, 30, 50 or 100) at 37°C, the medium was substituted with RPMI 1640 that contained 10% FBS. Forty-eight hours later, the cells that expressed GFP were counted by fluorescence microscopy to calculate efficiency of transfection. An MOI of 20 was the best.

### **Cell transfection**

BGC-823 cells were seeded in 96-well plates ( $5 \times 10^7$  cells/L) 24 h before adenoviral transfection at 60%-70% confluence in medium that contained 10% FBS. The cells were infected at an MOI of 20 in serum-free medium with Ad-Ang-1 and Ad-GFP as a control adenoviral vector. Except for the above two groups, one additional control group was used, which consisted of uninfected cells.

### **Cell adhesion assay**

Ninety-six-well plates were coated with 50  $\mu$ L 10 mg/L fibronectin at 4°C. At the same time, 96-well plates were coated with 50  $\mu$ L 10 mg/L BSA at 4°C as a control ECM. A total of  $1 \times 10^5$  cells in 100  $\mu$ L of the medium were plated in 96-well plates at 37°C. After 1 h, 96-well plates were washed with PBS, and the medium was replaced with 50  $\mu$ L 0.05% MTT solution and 150  $\mu$ L RPMI 1640, and incubated for 4 h. After incubation, the MTT solution was removed, and the cells were suspended in 150  $\mu$ L DMSO (Sigma). Absorbance was measured at 570 nm using a microplate reader (Bio-Rad Laboratories).

### **Invasion assays**

The ability of BGC-823 cells to migrate through Matrigel-coated filters was measured using Transwell chambers with 8- $\mu$ m pore polycarbonate filters coated with 300  $\mu$ g Matrigel in the top side of the filter. BGC-823 cells were mock-transfected or transfected with Ad-GFP or Ad-Ang-1 (MOI = 20). After 36 h, the cells were trypsinized, resuspended in serum-free medium, seeded on the top

compartment of the chamber, and incubated for 24 h. At the end of incubation, the cells were stained, and the cells and Matrigel on the top surface of the filter were removed carefully with a cotton swab. The invasive cells that adhered to the bottom surface of the filter were quantified under a light microscope ( $\times 200$ ) (Nikon, Japan). The data were presented as the average number of cells attached to the bottom surface from randomly chosen fields. Each treatment condition was assayed using triplicate filters, and filters were counted at five areas.

### Migration assay

The migration and invasiveness of BGC-823 cells were evaluated in six-well transwell chambers with upper and lower culture compartments that were separated by polycarbonate membranes with 8- $\mu\text{m}$  sized pores. The three types of cells were detached, washed twice in PBS, and resuspended in serum-free RPMI 1640 medium. A total of  $1 \times 10^5$  cells in 500  $\mu\text{L}$  were placed in the upper chamber of a Millicell Insert (Millipore, USA), and the lower chamber was filled with 1.6 mL RPMI 1640/10% FBS. After a 4 h incubation period, the cells in the upper chamber that did not migrate were scraped away gently, and adherent cells on the lower surface of the insert were stained with hematoxylin and eosin and photographed. Triplicate assays were performed for each group of cells.

### Reverse transcription polymerase chain reaction (RT-PCR) of integrin $\beta 1$ , CD44V6, uPA and MMP-2

Total RNA was isolated from BGC-823 cells using Trizol reagents (Invitrogen) according to the manufacturer's instructions, and utilized for RT-PCR. Primers designed for integrin  $\beta 1$  were as follows: forward, 5'-AATGAA GGGCGTGTGGTAG-3', and reverse, 5'-AGACAC CACTCGCAGATG-3'. Primers designed for CD44V6 were as follows: forward, 5'-GGCAACTCC TAGTAGTACAAC-3', and reverse, 5'-CAGCTGTCC CTGTTGTTCGAAT-3'. Primers designed for uPA were as follows: forward, 5'-TCTGTGTGTGGGACTGAT GC-3', and reverse, 5'-GCCCTGACCTGAATCACA AT-3'. Primers designed for MMP-2 were as follows: forward, 5'-CTAGACAAGGGCCACAGACC-3', and reverse, 5'-GAGGAAGCAAACCTCGAACA-3'. Primers designed for  $\beta$ -actin, which was used as a loading control for the RT-PCR, were as follows: forward, 5'-CCACCC ATGGCAAATTCATGGCA-3', and reverse, 5'-TCTAG ACGGCAGGTCAGGTCCAC-3'. After visualization of the PCR products by 20 g/L agarose gel electrophoresis with ethidium bromide staining gel, images were obtained and the densities of the products were quantified using a digital gel image analysis system (Pharmacia Biotech). The PCR fragments were identified according to their molecular mass using a DNA marker (DL 600 and 1200 marker, TaKaRa Biotech).

### Western blotting

Western blotting was utilized for the detection of integrin  $\beta 1$  and CD44V6. Cell lysates were prepared and separated by 15% SDS-PAGE and transferred to nitro-

cellulose membranes. Membranes were blocked for 1 h at room temperature in 5% milk solution. PAGE was detected by incubating the transferred membrane overnight at 4°C with anti-human rabbit monoclonal antibody (Cell Signaling Technology, USA) at 1:100 dilution in 5% milk solution. Secondary antibody, anti-rabbit IgG/horseradish peroxidase, was added and cells were incubated for 2 h at room temperature. The signal was detected by the ECL detection system (Amersham), according to the manufacturer's protocol, and imaged digitally by Kodak Formatter (Kodak, Japan).

### Immunocytochemistry for integrin $\beta 1$ and CD44V6

BGC-823 cells were prepared as a suspension that contained  $1 \times 10^8$  cells/L and were added to a six-well plate that contained many small glass slides. The cells were infected with recombinant adenovirus vector as before at 70% confluence on the glass slides. These small glass slides were taken out after 36 h. Expression of integrin  $\beta 1$  and CD44V6 was determined immunohistochemically with an anti-integrin  $\beta 1$  (Beijing Zhongshan Jinqiao Biotech), anti-CD44V6 mouse monoclonal antibody (Beijing Zhongshan Jinqiao Biotech), respectively, at a dilution of 1:100, and using the streptavidin-peroxidase technique with the SPtm kit (SP-9000; Maixin). Negative controls were prepared by substituting PBS for the primary antibodies, and known positive controls were included in each staining run. Finally, positive expression was measured *in situ* by Image-Proplus 4.5.

### Statistical analysis

Data are presented as mean  $\pm$  SE. Means among multiple groups were detected by one way ANOVA. Statistically significant differences were determined by  $Q$  test and were defined as  $P < 0.05$ .

## RESULTS

### Transfection efficiency of the adenovirus vector in BGC-823 cells

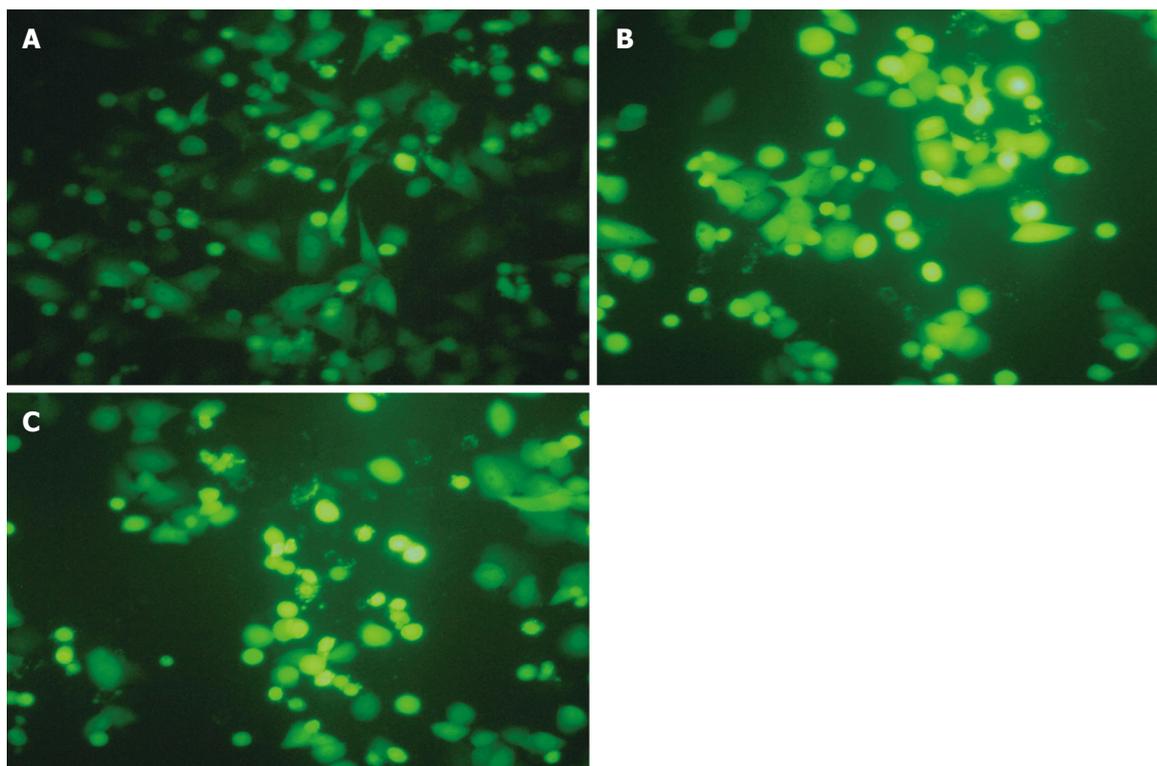
As MOI increased, the number of BGC-823 cells expressing GFP protein increased. None of cells could be transfected when MOI was 0 (Data was not shown). Nearly all the cells could be transfected when MOI was 20 (Figure 1A). However, became detached and died when MOI was 30 or 50 (Figure 1B and C).

### Cell adhesion assay

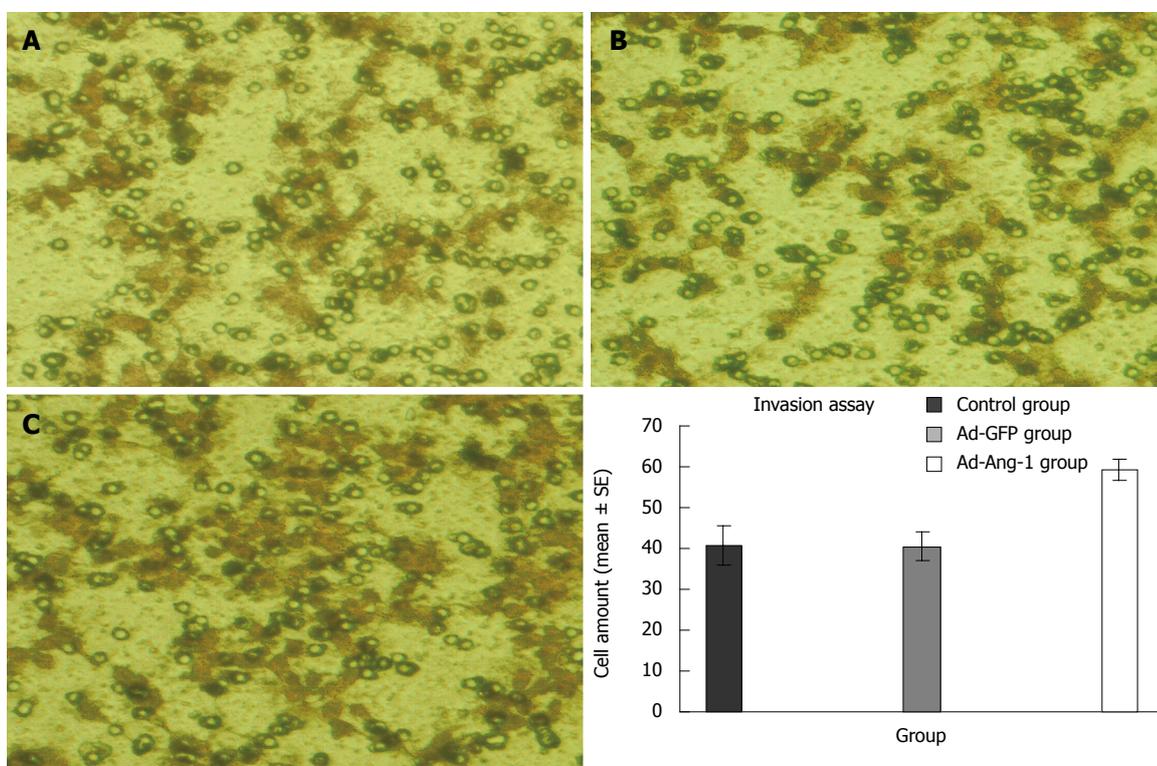
We analyzed the effects of BGC-823 cells on cell adhesion after transfection with Ang-1. Compared to control group, the rate of adhesion of integrin  $\beta 1$  and CD44V6 in the Ad-Ang-1 group greatly increased ( $P < 0.05$ ) (Table 1).

### Invasion assay

Overexpression of Ang-1 was induced by Ad-Ang-1. There was no difference between the control and Ad-GFP groups (Figure 2A and B). The Ad-Ang-1-transfected cells showed significantly greater invasion than the mock-transfected and Ad-GFP groups (Figure 2C). These results



**Figure 1** Expression of fluorescence in BGC-823 cells ( $\times 200$ ). As MOI increased, so did the number of BGC-823 cells that expressed GFP protein. A: Nearly all the cells could be transfected when MOI was 20; B: Most cells became detached and died at MOI = 30; C: Most cells became detached and died at MOI = 50. Diagrams are representative of at least three independent experiments.

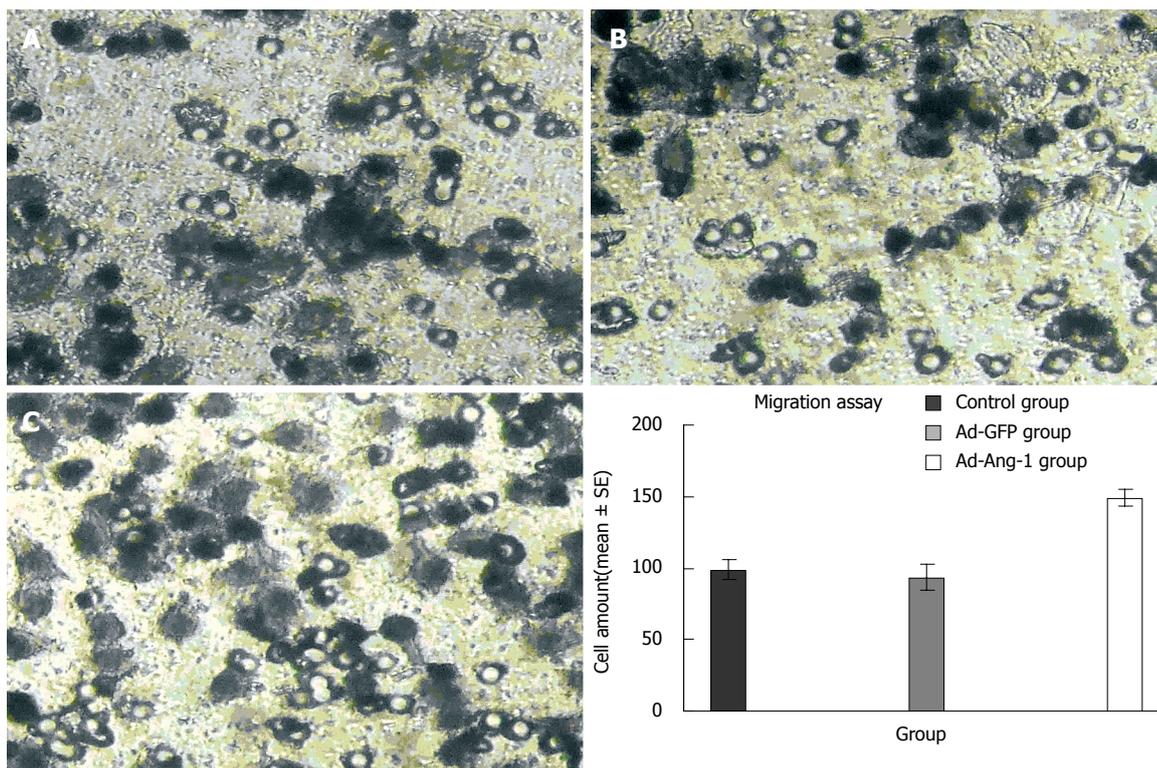


**Figure 2** Invasion assay in BGC-823 cells ( $\times 200$ ). A: Control group; B: Ad-GFP-transfected group; C: Ad-Ang-1-transfected group. There was no difference between the control and Ad-GFP groups. Ad-Ang-1-transfected cells had a significant increase in invasion compared with the mock-transfected and Ad-GFP groups ( $P < 0.05$ ). Diagrams are representative of at least three independent experiments.

provide evidence that Ang-1 enhances gastric carcinoma invasion ( $P < 0.05$ ).

**Migration assay**

Cell migration was greater in the Ad-Ang-1 group than



**Figure 3** Migration assay in BGC-823 cells ( $\times 200$ ). A: Control group; B: Ad-GFP-transfected group; C: Ad-Ang-1-transfected group. There was no difference between the control and Ad-GFP groups. In the Ad-Ang-1 group, there were more cells than in the control and Ad-GFP groups ( $P < 0.05$ ). Diagrams are representative of at least three independent experiments.

**Table 1** Cell adhesion assay (mean  $\pm$  SE)

Group	Experiment group cell A <sub>570</sub> /BSA group cell A <sub>570</sub>				Cell adhesion rate (%)
	1	2	3	4	
Control group	1.23 $\pm$ 0.071	1.28 $\pm$ 0.025	1.29 $\pm$ 0.032	1.28 $\pm$ 0.010	27.20
Ad-GFP group	1.172 $\pm$ 0.006	1.342 $\pm$ 0.036	1.264 $\pm$ 0.006	1.294 $\pm$ 0.016	26.80
Ad-Ang-1 group	1.756 $\pm$ 0.020	1.589 $\pm$ 0.007	1.502 $\pm$ 0.011	1.957 $\pm$ 0.005 <sup>a</sup>	70.10

<sup>a</sup>Compared to control group and Ad-GFP group  $P < 0.05$ . Cell adhesion rate = (experiment group cell A<sub>570</sub>/BSA group cell A<sub>570</sub>-1)  $\times$  100%. There was no difference between the control and Ad-GFP groups. Compared to the control and Ad-GFP groups, the rate of adhesion of integrin  $\beta$ 1 and CD44V6 in the Ad-Ang-1 group greatly increased ( $P < 0.05$ ).

in the control and Ad-GFP groups (Figure 3C), but there was no difference between the latter two groups (Figure 3A and B). These results demonstrate that Ang-1 enhances gastric carcinoma migration ( $P < 0.05$ ).

**RT-PCR of integrin  $\beta$ 1, CD44V6, uPA and MMP-2**

RT-PCR revealed that the mRNA expression of integrin  $\beta$ 1, CD44V6, uPA and MMP-2 was increased in the Ad-Ang-1-transfected group ( $P < 0.05$ ) (Figure 4).

**Western blotting of integrin  $\beta$ 1, CD44V6, uPA and MMP-2**

Western blotting revealed that protein expression of integrin  $\beta$ 1, CD44V6, uPA and MMP-2 was increased in the Ad-Ang-1-transfected group ( $P < 0.05$ ) (Figure 5).

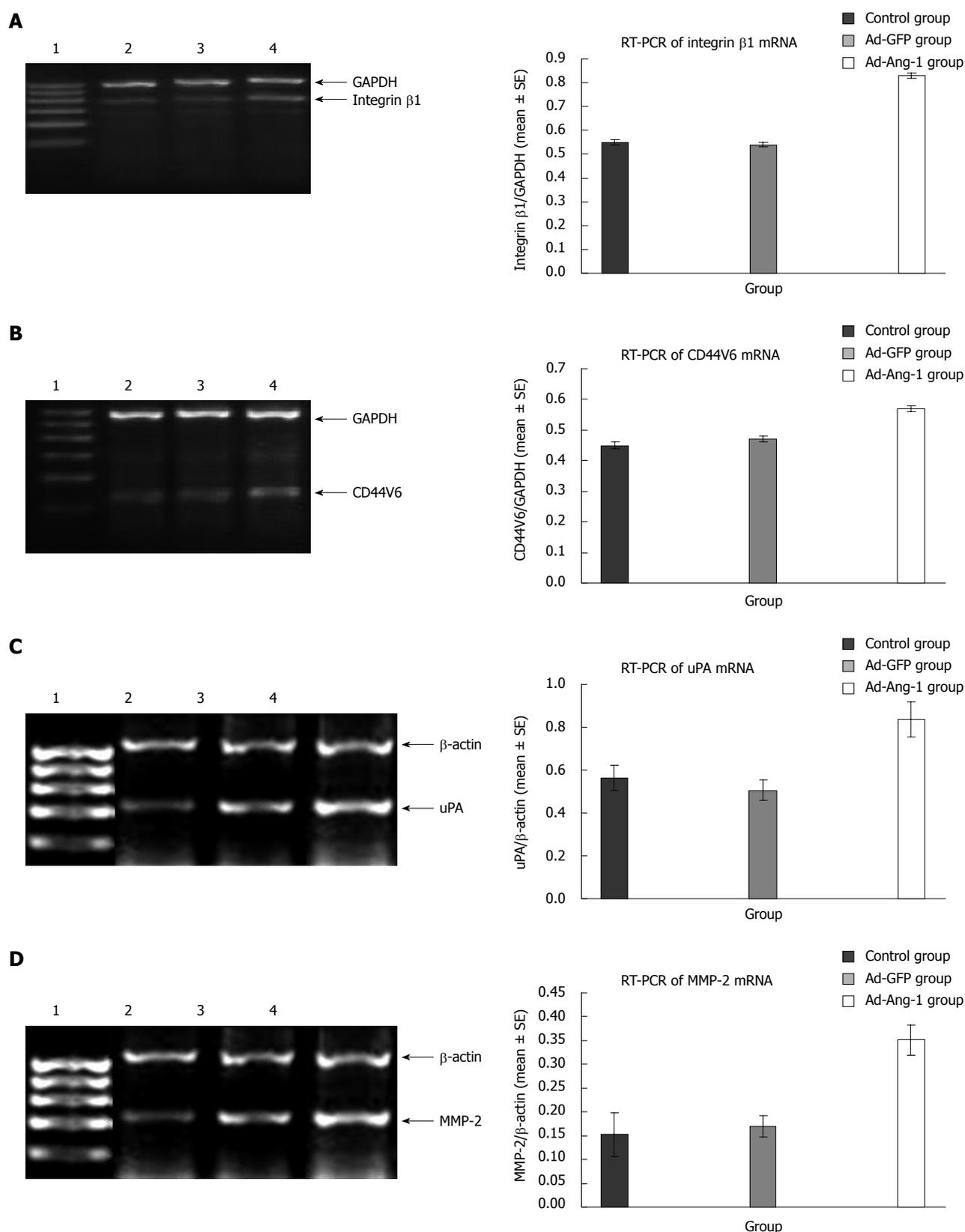
**Expression of integrin  $\beta$ 1 and CD44V6 protein**

Integrin  $\beta$ 1 and CD44V6 protein were both detected in BGC-823 cells in all three groups by using their

respective antibodies. The staining intensity of integrin  $\beta$ 1 in the Ad-Ang-1-transfected group was  $0.183 \pm 0.014$ , which was significantly higher than that in the control ( $0.114 \pm 0.023$ ,  $P < 0.05$ ) and Ad-GFP-transfected ( $0.149 \pm 0.013$ ,  $P < 0.05$ ) groups (Figure 6A). Compared with the other groups, expression of CD44V6 was also up-regulated (Figure 6B) ( $P < 0.05$ ). The staining intensity of this protein in the Ad-Ang-1-transfected group ( $0.147 \pm 0.011$ ) was higher than that in the control ( $0.089 \pm 0.007$ ) and Ad-GFP-transfected ( $0.065 \pm 0.021$ ) groups.

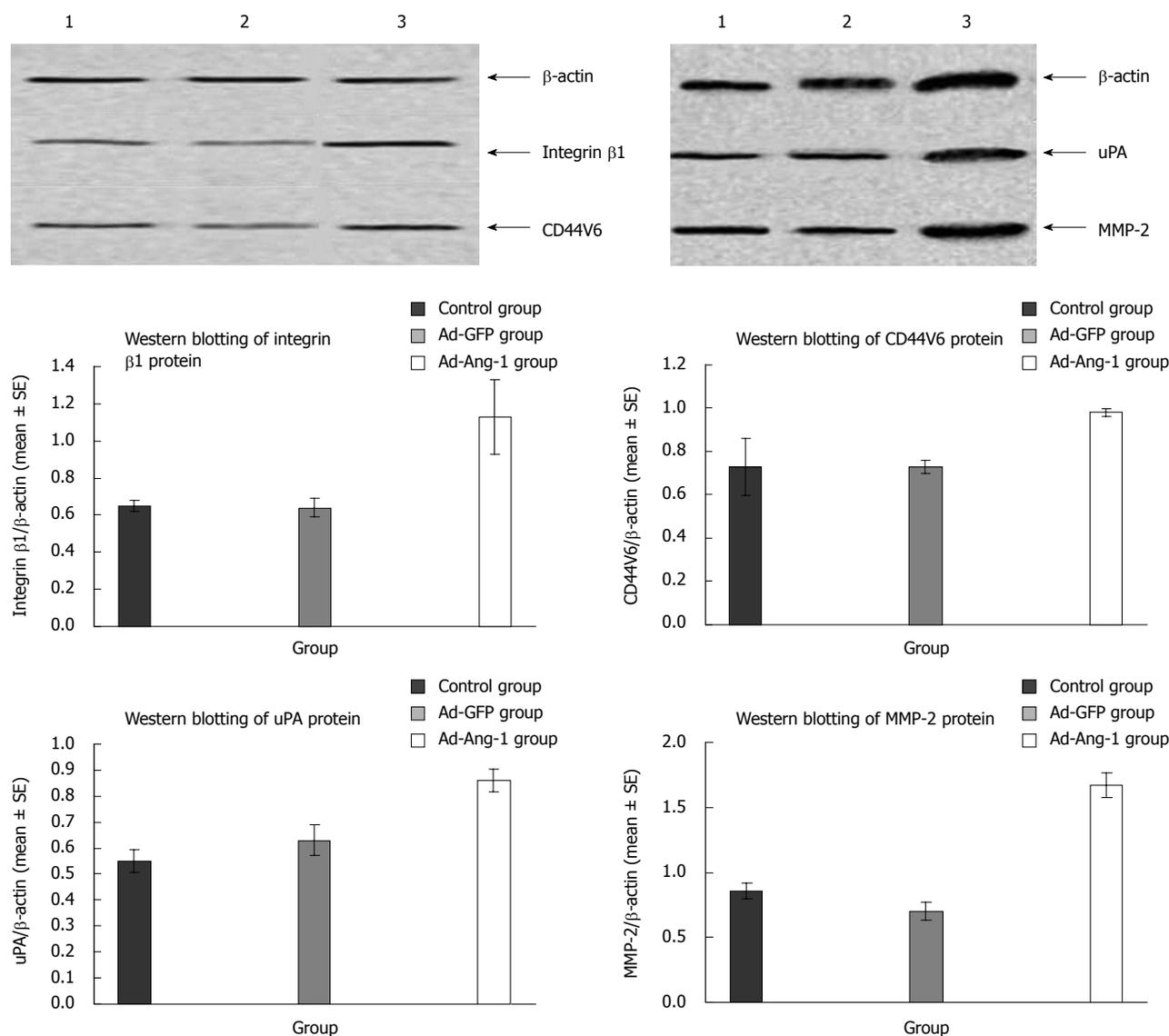
**DISCUSSION**

The angiopoietin family of morphogens has an essential function in vascular and lymphatic growth and remodeling<sup>[11]</sup>. The role of Ang-1 is controversial. Ang-1 is sequestered by peri-endothelial and vascular smooth muscle cells, and acts specifically on endothelial cells



**Figure 4** Expression of integrin  $\beta 1$  (A), CD44V6 (B), uPA (C) and MMP-2 (D) mRNA. 1: Markers: 100, 200, 300, 400, 500 and 600 bp; 2: Control group; 3: Ad-GFP-transfected group; 4: Ad-Ang-1-transfected group. A: Ang-1 group:  $0.830 \pm 0.010$ ; control group and Ad-GFP group:  $0.551 \pm 0.009$  and  $0.543 \pm 0.010$ , respectively; B: Ang-1 group:  $0.570 \pm 0.012$ ; control group and Ad-GFP group:  $0.451 \pm 0.014$  and  $0.472 \pm 0.013$ , respectively; C: Ang-1 group:  $0.837 \pm 0.081$ ; control group and Ad-GFP group:  $0.563 \pm 0.059$  and  $0.506 \pm 0.048$ , respectively; D: Ang-1 group:  $0.351 \pm 0.032$ ; control group and Ad-GFP group:  $0.153 \pm 0.046$  and  $0.170 \pm 0.023$ , respectively. These results show that the expression of integrin  $\beta 1$ , CD44V6, uPA and MMP-2 mRNA was increased in the Ang-1 group ( $P < 0.05$ ). Diagrams are representative of at least three independent experiments.

(ECs) through binding and activation of the cell surface tyrosine kinase receptor Tie2<sup>[12]</sup>. The angiotensin family



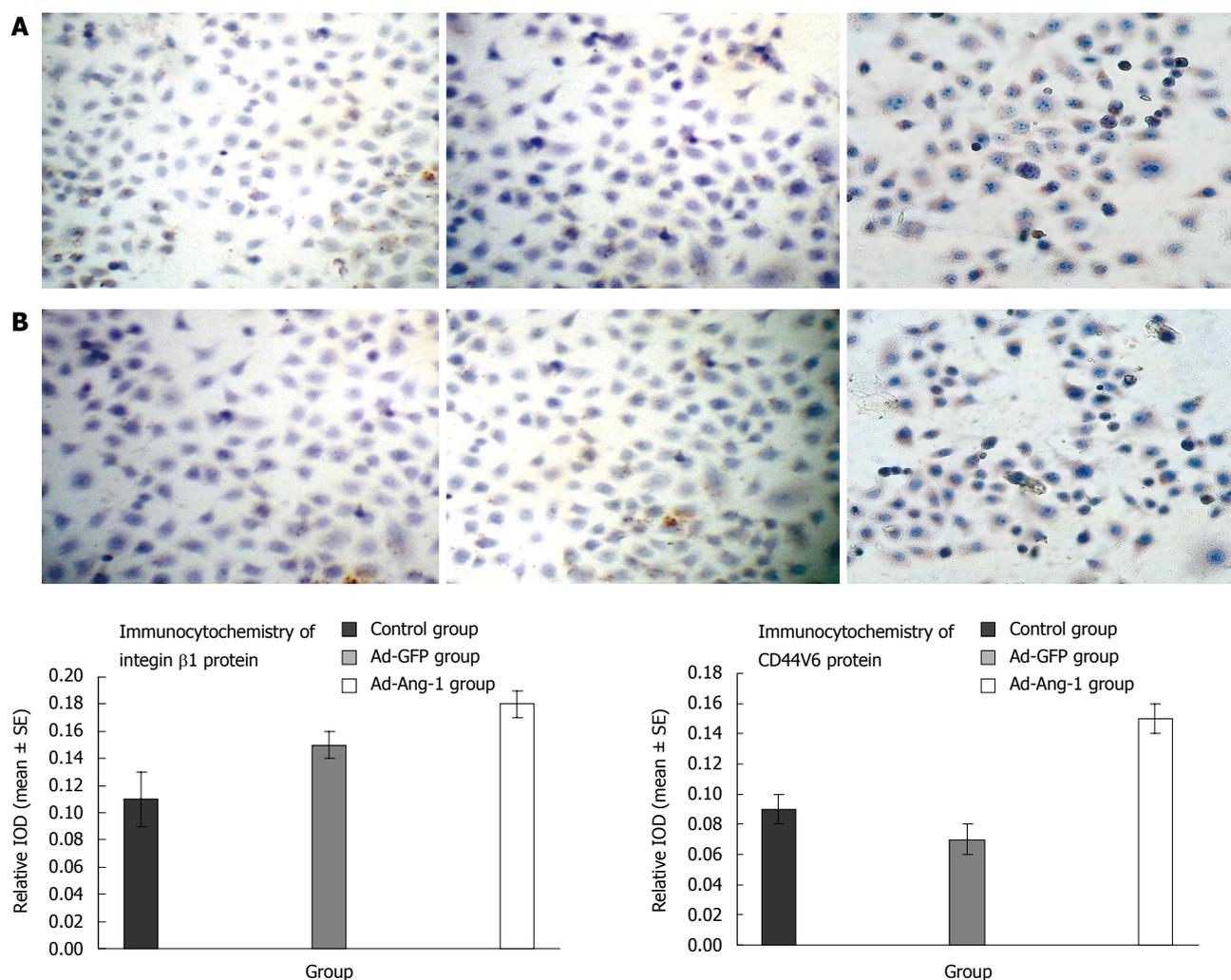
**Figure 5** Expression of integrin  $\beta$ 1 and CD44V6, uPA and MMP-2 protein. 1: Control group; 2: Ad-GFP-transfected group; 3: Ad-Ang-1-transfected group. After transfection with Ang-1, the protein level was greater than that in the control and Ad-GFP groups, but there was no difference between the control and Ad-GFP group. Diagrams are representative of at least three independent experiments.

of proteins contain an N-terminal coiled-coil domain, as well as a C-terminal fibrinogen-like domain that shares a high degree of homology to the analogous domains in the ECM proteins tenascin C and fibrinogen  $\gamma$  and  $\beta$ <sup>[11,2,13]</sup>. In a study of a pro-B cell line that stably expressed Tie2, it has been found that addition of Ang-1 to the culture medium enhanced cell adhesion to fibronectin<sup>[14]</sup>. In another study, fluorescence-activated cell-sorter Tie2-positive hematopoietic cells also responded to Ang-1 treatment with increased adherence to a fibronectin-coated substrate<sup>[15]</sup>. It has been shown recently that Ang-1 promotes cell adhesion<sup>[16]</sup>, and that this process is mediated by  $\alpha$ 5-integrin in ECs<sup>[17]</sup>. Moreover, the finding that Ang-1 can bind ECM extracts from carcinoma cells<sup>[17]</sup> has offered new insights into understanding the role of Ang-1 in modulating the angiogenic microenvironment.

Cancer progression depends on an accumulation of metastasis-supporting genetic modifications and physiological alterations. Often, these physiological

changes are regulated by cell signaling molecules, which target signal transduction pathways and ultimately, gene expression. Cell adhesion is the main step in cancer metastasis, and it is mediated by integrin heterodimers<sup>[18]</sup>. Cross-talk between integrins and growth factor receptors has been shown to coordinate biological processes through the regulation of downstream and inside-out signaling pathways<sup>[19-23]</sup>. Tyrosine kinase receptors and integrins share many downstream effectors.

Integrins have crucial roles in angiogenesis<sup>[24]</sup> and allow vascular cells to adapt their adhesive machinery to the so-called "provisional" ECM components, such as fibronectin, collagen and vitronectin, that are exposed by basement degradation around sprouting vessels<sup>[25]</sup>. Integrins  $\alpha$ v $\beta$ 3,  $\alpha$ v $\beta$ 5,  $\alpha$ 2 $\beta$ 1 and  $\alpha$ 5 $\beta$ 1 are upregulated in newly formed blood vessels<sup>[26,27]</sup>, and  $\alpha$ v $\beta$ 3 and  $\alpha$ v $\beta$ 5 antagonists inhibit angiogenesis *in vitro* and *in vivo*<sup>[28,29]</sup>. Integrins can exist in different functional states that regulate their biological functions<sup>[30]</sup>. Additionally,



**Figure 6** Expression of integrin  $\beta 1$  (A) and CD44V6 (B) protein ( $\times 200$ ). A: Expression of integrin  $\beta 1$  in the Ad-Ang-1-transfected group was  $0.183 \pm 0.014$ , which was significantly higher than that in the control group ( $0.114 \pm 0.023$ ,  $P < 0.05$ ) and Ad-GFP-transfected group ( $0.149 \pm 0.013$ ,  $P < 0.05$ ); B: Compared with the other groups, expression of CD44V6 was also up-regulated. The staining intensity for this protein in the Ad-Ang-1-transfected group ( $0.147 \pm 0.011$ ) was higher than that in the control group ( $0.089 \pm 0.007$ ,  $P < 0.05$ ) and Ad-GFP-transfected group ( $0.065 \pm 0.021$ ,  $P < 0.05$ ). Diagrams are representative of at least three independent experiments.

integrin  $\beta 1$  plays a key role in gastric cancer, and it can mediate cell adhesion.

Integrin  $\beta 1$  is a receptor of Ang-1, therefore, we speculate whether there is a relationship between integrin  $\beta 1$  and Ang-1. In addition, CD44 is a member of the immunoglobulin superfamily that is expressed on most epithelial and non-epithelial cells. Functionally, CD44 binds hyaluronate in the ECM to maintain tissue/organ structure, promote cell aggregation and mediate cell movement, and CD44 variant isoforms, especially CD44v6, have been identified as protein markers for metastasis in hepatocellular, breast, colorectal and gastric cancer<sup>[31-34]</sup>. Therefore, we speculate whether Ang-1 can affect the expression of CD44v6. Moreover, the expression of MMP-2 was stronger in metastatic tissues than in primary tumors. Up regulation of MMP-2 in neoplastic foci might be helpful to gastric carcinogenesis and metastasis<sup>[35]</sup>. In this model, the development of angiogenesis was highly dependent upon MMP-2 expression<sup>[36]</sup>. It has been reported that Ang-1 contributes to increased secretion of MMP-2 and decreased secretion of tissue inhibitors of metalloproteinase-2. Vascular

endothelial growth factor (VEGF) and Ang-1 have an immunomodulatory role in airway remodeling<sup>[37]</sup>. uPA is a serine protease that functions in the conversion of the circulating zymogen plasminogen to the active, broad-spectrum serine protease plasmin. Plasmin, in turn, mediates the pericellular proteolysis of ECM components, and activates other proteases such as MMPs and collagenases, which leads to further degradation and remodeling of the ECM. It has been found that uPA levels are elevated in various malignancies, including breast, pancreatic, gastric, lung and colorectal carcinoma<sup>[38]</sup>. uPA expression is correlated with enhanced VEGF-induced tumor angiogenesis and may play a role in invasion and nodal metastasis of gastric carcinoma, thereby serving as a prognostic marker of gastric cancer<sup>[39]</sup>. Human gastric cancer cell lines express uPA mRNA and activity, which correlates with their peritoneal seeding potential<sup>[40]</sup>. Therefore, we also detected the expression of uPA and MMP-2. We explored the effects of Ang-1 on integrin  $\beta 1$ , CD44V6, uPA and MMP-2 in the human gastric cancer cell line BGC-823, and studied the mechanism of adhesion and metastasis in gastric cancer.

We found that cell adhesion increased clearly in the Ad-Ang-1-transfected group. Therefore, we considered whether Ang-1 can affect the expression of integrin  $\beta$ 1 and CD44V6 through cell adhesion. We found that mRNA and protein expression of integrin  $\beta$ 1 and CD44V6 in BGC823 cells was enhanced, using RT-PCR, Western blotting and immunocytochemistry. We also found that Ang-1 affected the expression of uPA and MMP-2 to increase the invasiveness of gastric cancer. We suggest that there is a signaling pathway between Ang-1, integrin  $\beta$ 1, CD44V6, uPA and MMP-2, and through this pathway, Ang-1 can affect the attachment and metastasis of carcinoma.

## COMMENTS

### Background

Gastric carcinoma is one of the most common cancers worldwide. The mortality of gastric carcinoma is currently rising faster than for any other cancer in China. About 60% of patients die from metastasis of gastric cancer. Progression of this disease may be associated with overexpression of angiopoietin-1 (Ang-1).

### Innovations and breakthroughs

Recent reports have highlighted that Ang-1 has been found to bind integrin  $\beta$ 1 and matrix metalloproteinase-2 (MMP-2). Ang-1 may influence cell adhesion, invasion and migration. This is believed to be the first study to show that overexpression of Ang-1 can affect adhesion, invasion and migration of BGC823 cells through the expression of integrin  $\beta$ 1, CD44V6, MMP-2 and urokinase-type plasminogen activator (uPA).

### Applications

By understanding how Ang-1 affects attachment and metastasis, this study may represent a future strategy for therapeutic intervention in the treatment of patients with gastric carcinoma.

### Terminology

Ang-1 is a member of the angiopoietin family. It has essential functions in vascular and lymphatic growth and remodeling. Integrin  $\beta$ 1 and CD44V6 are members of the cell adhesion family. They mediated cell and extracellular matrix adhesion. MMP-2 and uPA are associated with tumor invasion and migration.

### Peer review

The present study demonstrates a role for Ang-1 in adhesion, migration and invasion of human gastric cancer cells. Specifically, transient transfection of Ang-1 increases attachment and migration of this cell line. The experimental design is well thought out and the results are intriguing.

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ORIGINAL ARTICLE

## Stable knockdown of heparanase expression in gastric cancer cells *in vitro*

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### Abstract

**AIM:** To develop short hairpin RNA (shRNA) against heparanase, and to determine its effects on heparanase expression and the malignant characteristics of gastric cancer cells.

**METHODS:** Heparanase-specific shRNA was constructed and transferred into cultured the gastric cancer cell line SGC-7901. Stable subclonal cells were screened by G418 selection. Heparanase expression was measured by reverse transcriptase-polymerase chain reaction (RT-PCR), real-time quantitative PCR and Western blotting. Cell proliferation was detected by 2-(4,5-dimethyltriazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetry and colony formation assay. The *in vitro* invasiveness and metastasis of cancer cells were measured by cell adhesion assay, wound healing

assay and matrigel invasion assay. The angiogenesis capabilities of cancer cells were measured by tube formation of endothelial cells.

**RESULTS:** Stable transfection of heparanase-specific shRNA, but not of scrambled shRNA and mock vector, resulted in reduced mRNA and protein levels of heparanase. The shRNA-mediated knockdown of heparanase did not affect the cellular proliferation of SGC-7901 cells. However, the *in vitro* invasiveness and metastasis of cancer cells were decreased after knockdown of heparanase. Moreover, transfection of heparanase-specific shRNA decreased the *in vitro* angiogenesis capabilities of SGC-7901 cells.

**CONCLUSION:** Stable knockdown of heparanase can efficiently decrease the invasiveness, metastasis and angiogenesis of human gastric cancer cells. In contrast, stable knockdown of heparanase does not affect the cell proliferation.

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**Key words:** Gastric cancer; Heparanase; RNA interference; Invasion; Metastasis

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### INTRODUCTION

Gastric cancer, a serious health problem, remains the second most common type of fatal cancer worldwide because of the invasiveness and metastasis of cancer cells<sup>[1,2]</sup>. Metastasis of tumor cells involves a multistep process, including detachment, invasion, migration, angiogenesis, adhesion to endothelial cells, extravasation, and regrowth in distant organs<sup>[3]</sup>. It has been established that the basement membrane (BM) and extracellular

matrix (ECM) act as a barrier to prevent tumor cells from invasion and metastasis<sup>[4]</sup>. Heparanase (HPA), a mammalian endo-beta-D-glucuronidase, is capable of degrading the heparan sulfate proteoglycans (HSPGs) in the ECM and BM, and is proposed to have a promoting role in cancer invasion, angiogenesis and metastasis<sup>[5]</sup>. In advanced gastric cancer, expression of HPA was frequently observed and correlated with histopathological parameters reflecting the invasiveness, metastatic potential and prognosis of gastric cancers<sup>[6,7]</sup>. Thus HPA may represent an important target for treatment of gastric cancer.

Currently, a variety of HPA inhibitors have been developed, including competitive heparin sulfate-mimicking compounds, neutralizing anti-HPA antibodies, modified non-anticoagulant species of heparin, and inhibitory small molecules<sup>[8]</sup>. It has been reported that inhibiting the expression of HPA can lead to inhibition of tumor invasiveness, metastasis and angiogenesis<sup>[9,10]</sup>. However, because of the multiple biologic activities of these compounds, the mechanism of their antitumor activity and its relation to HPA inhibition are not straightforward<sup>[8-11]</sup>. Moreover, the pleiotropic interactions of these compounds with the ECM and the cell surface might produce nonspecific and undesirable effects<sup>[8-11]</sup>. Thus, novel approaches are needed to reduce the role of HPA in cancer progression<sup>[8]</sup>.

In previous studies, genetic approaches targeting HPA have been regarded as a promising alternative. Antisense oligonucleotides, ribozymes, and short interference RNA (siRNA) have been developed to suppress HPA expression<sup>[12-15]</sup>. However, it still remains unknown whether stable transfection of short hairpin RNA (shRNA) can knockdown the HPA expression and decrease the invasiveness and metastasis of cancer cells. In the current study, the HPA-specific shRNA was constructed and transferred into cultured gastric cancer cells. We demonstrated, for the first time, that stable knockdown of HPA expression decreased the *in vitro* invasive, metastatic and angiogenic capabilities of gastric cancer cells.

## MATERIALS AND METHODS

### Cell culture

Human gastric cancer cell line SGC-7901 and human umbilical endothelial cell line (HUVEC) were purchased from the American Type Culture Collection and grown in RPMI1640 medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Inc.), penicillin (100 U/mL) and streptomycin (100 g/mL). Cells were maintained at 37°C in a humidified atmosphere of 50 mL/L CO<sub>2</sub>.

### shRNA construct for heparanase knockdown and transfection

Oligonucleotides encoding a shRNA specific for the heparanase encoding sequence were subcloned into pGenesil-1 (Genesil Biotechnology, Wuhan, China). Annealed oligonucleotides were cloned downstream of the U6 promoter (primer 1 sequence, 5'-GATCCGGA

ATCAACCTTTGAAGAGCTATGGACACCTTAGT TGGAAACTTCTCTTTTGGAGCTCA-3'; primer 2 sequence, 5'-AGCTTGAGCTGAAAAAAGGAATCA ACCTTTGAAGAGTGTCCATAGCCTTAGTTGGA AACTTCTCG-3'). The sequence 5'-GATCCAGCAUC GUACGUAGGCCA GCTATGGACATCGTAGCAT GCATCCGGTCTTTTGGAGCTCA-3' (sense), and 5'-AGCTTGAGCTGAAAAAAGCAUCGUACGUAG GCCAGTGTCCATAGTCGTAGCATGCATCCGGTC G-3' (antisense) were used as a scrambled RNAi control. The constructs were verified by DNA sequencing. The plasmids pGenesil-1, pGenesil-scrambled, and pGenesil-HPA were transfected with Genesilencer Transfection Reagent (Genlantis, San Diego, CA), according to the manufacturer's instructions. Stable cell lines were screened by administration of G418 (Invitrogen, Carlsbad, CA).

### Real-time quantitative polymerase chain reaction (PCR)

Total RNA was isolated with RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA). The reverse transcription reactions were conducted with Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN, USA). The PCR primers were designed by Premier Primer 5.0 software as the following: for human HPA 5'-GAATGGACGGACTGCTAC-3' and 5'-CCAAAGA ATACTTGCCTCA-3' amplifying a 261-bp fragment; for human GAPDH 5'-AGAAGGCTGGGGCTCATT G-3' and 5'-AGGGGCCATCCACAGTCTTC-3' amplifying a 258-bp fragment. Real-time PCR with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was performed using ABI Prism 7700 Sequence Detector (Applied Biosystems). The fluorescent signals were collected during the extension phase, Ct values of the sample were calculated, and HPA transcript levels were analyzed by 2<sup>-ΔΔCt</sup> method.

### Western blotting

Cellular protein was extracted with 1 × cell lysis buffer (Promega, Madison, WI, USA). Protein (50 μg) from each sample was subjected to 4%-20% pre-cast polyacrylamide gel (Bio-Rad, Hercules, CA, USA) electrophoresis and transferred to nitrocellulose membranes (Bio-Rad). For HPA (InSight Company, Rehovot, Israel) and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) detection, the primary antibody dilutions were 1:500 and 1:1000, respectively, followed by 1:3000 dilution of goat anti-rabbit horseradish peroxidase-labeled antibody (Bio-Rad). ECL substrate kit (Amersham, Piscataway, NJ, USA) was used for the chemiluminescent detection of signals with autoradiographic film (Amersham).

### Measurement of cell viability

Cell viability was monitored by the 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, St. Louis, MO, USA) colorimetric assay. Briefly, 20 μL of MTT (5 mg/mL) was added to each well. After 4 h incubation at 37°C, the cell supernatants were discarded, MTT crystals were dissolved with DMSO and the absorbance measured at 570 nm. Percent viability was defined as the relative absorbance of treated *vs* untreated

control cells. All experiments were done with 6-8 wells per experiment and repeated at least 3 times.

### **Colony formation assay**

The cells were seeded at a density of 300/mL on 35-mm dishes. Colonies were allowed to grow for 10-14 d. The medium was discarded and each well was carefully washed twice with phosphate buffered saline (PBS). The cells were fixed in methanol for 15 min, then stained with crystal violet for 20 min. Finally, positive colony formation ( $> 50$  cells/colony) was counted. The survival fraction for cells was expressed as the ratio of plating efficiency of treated cells to that of untreated control cells.

### **Cell adhesion assay**

$2 \times 10^4$  cells were inoculated into each ECM-coated well of 96-well plates that were precoated with 100  $\mu$ L of 20  $\mu$ g/mL matrigel (BD Biosciences, Franklin Lakes, NJ, USA), and incubated at 37°C in serum-free complete medium (pH 7.2) for 2 h. After incubation, the wells were washed 3 times with PBS and the remaining cells were fixed in 4% paraformaldehyde for 20 min at room temperature. The cells were stained with 0.1% crystal violet and washed 3 times with PBS to remove free dye. After extraction with 10% acetic acid, absorbance of the samples was measured at 570 nm.

### **Wound healing assay**

The cells were scraped with the fine end of 1-mL pipette tips (time 0). Plates were washed twice with PBS to remove detached cells, and incubated with the complete growth medium. Cell migration into the wound empty space was followed after 24 h and photographed.

### **Matrigel invasion assay**

The Boyden chamber technique (Transwell analysis) was performed. Briefly, the 8- $\mu$ m pore size filters were coated with 100  $\mu$ L of 1 mg/mL matrigel (dissolved in serum-free RPMI1640 medium). 600  $\mu$ L of RPMI1640 medium containing 10% FBS was added to the lower chambers. Homogeneous single cell suspensions ( $1 \times 10^5$  cells/well) were added to the upper chambers and allowed to invade for 24 h at 37°C in a CO<sub>2</sub> incubator. Migration was allowed to proceed for 24 h at 37°C. Cells remaining attached to the upper surface of the filters were carefully removed with cotton swabs. Migrated cells were stained with 0.1% crystal violet for 10 min at room temperature and examined by light microscopy. Quantification of migrated cells was performed according to published criteria<sup>[16]</sup>.

### **Tube formation assay**

Fifty microliters of growth factor-reduced matrigel was polymerized on 96-well plates. HUVEC cells were serum starved in RPMI1640 medium for 2 h. The endothelial cells were suspended in RPMI1640 medium preconditioned with subclonal SGC-7901 cells, added to the matrigel-coated wells at the density of  $5 \times 10^4$  cells/well,

and incubated at 37°C for 18 h. Tube formation was visualized using a Leitz inverted microscope equipped with a Sony color digital DXC-S500 camera. Quantification of antiangiogenic activity was calculated by measuring the length of tube walls formed between discrete endothelial cells in each well relative to the control.

### **Statistical analysis**

Unless otherwise stated, all data are shown as mean  $\pm$  standard error of the mean (SEM). Statistical significance ( $P < 0.05$ ) was determined by the Student *t*-test or analysis of variance (ANOVA) followed by assessment of differences using SPSS 12.0 software (SPSS Inc., Chicago, IL, USA).

## **RESULTS**

### **shRNA decreased HPA expression in gastric cancer cells**

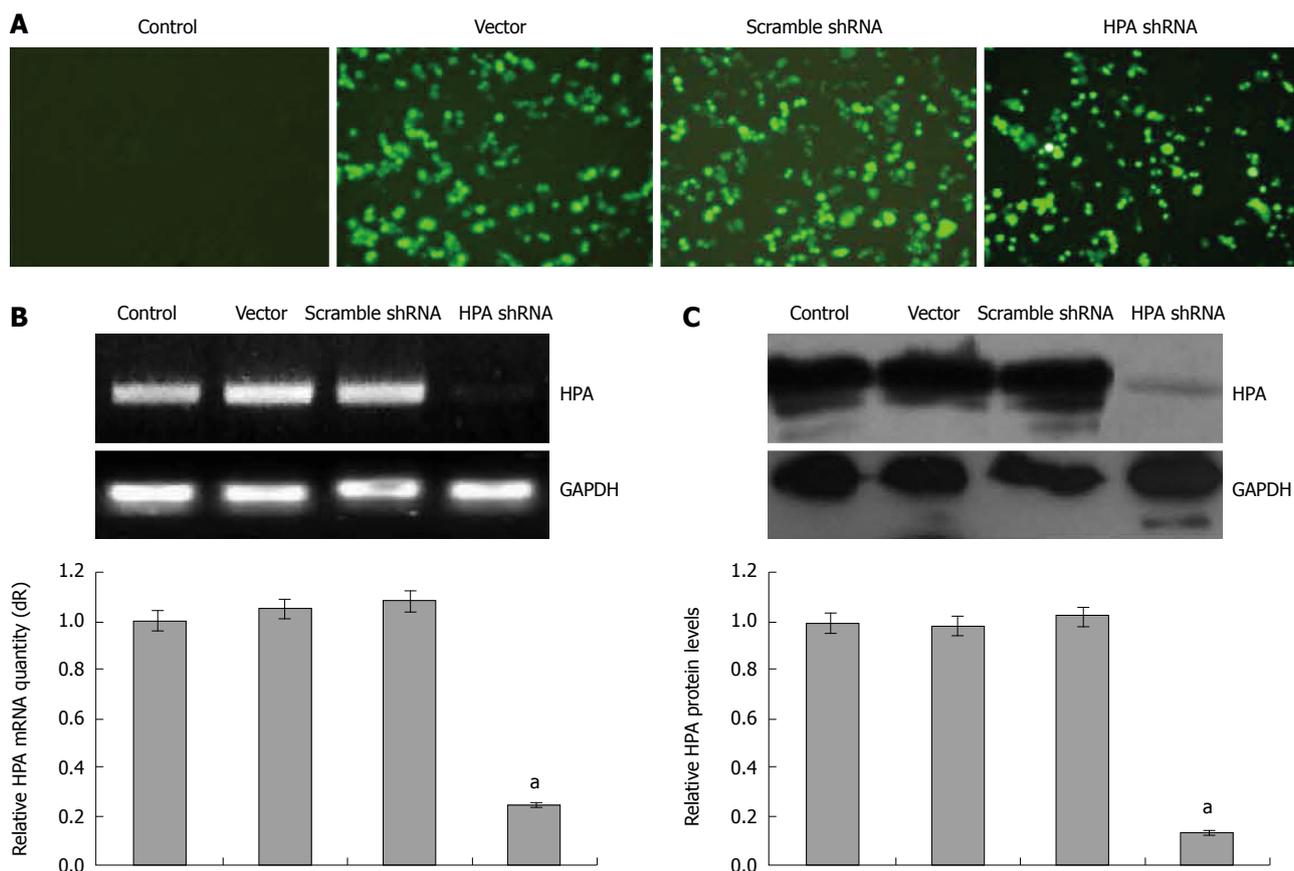
To examine the effects of shRNA on the expression of human HPA, gastric cancer SGC-7901 cells were transfected with pGenesil-1, pGenesil-scrambled, or pGenesil-HPA. The transfection efficiency was monitored by the enhanced green fluorescent protein (EGFP) reporter within these vectors. As shown in Figure 1A, 24 h after the Genesilencer-mediated transfection, EGFP was expressed within the cytoplasm of cancer cells. The subclonal cells were established by G418 selection. The mRNA and protein expression of HPA were examined by reverse transcriptase (RT)-PCR, real-time quantitative PCR and Western blot. As shown in Figure 1B and C, the HPA mRNA and protein could be detected in the parental cells, and stable transfection of the empty vector and scrambled shRNA did not affect the expression level of HPA. However, HPA was significantly decreased in the stable HPA shRNA-transfected cells. These results indicated that the HPA-specific shRNA used in this study was efficient in knockdown of the expression of HPA in gastric cancer cells.

### **Knockdown of HPA did not affect the *in vitro* cell proliferation of gastric cancer cells**

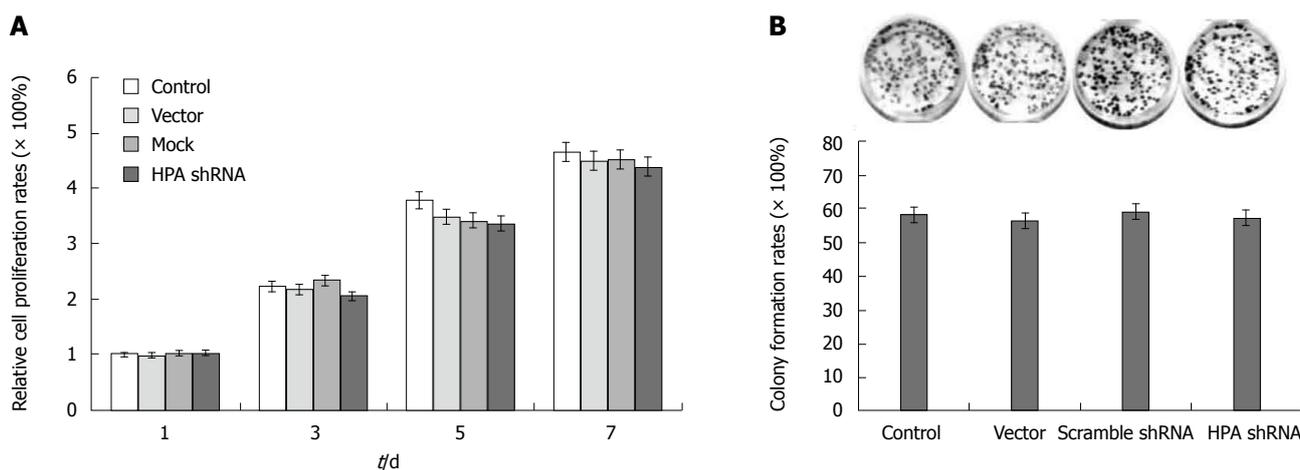
The effects of stable transfection of HPA shRNA on cell proliferation of SGC-7901 cells were measured by MTT colorimetric assay. We found that stable transfection of HPA shRNA or scrambled shRNA, did not affect cell proliferation, when compared to the parental cells and mock group (Figure 2A). In addition, the colony formation assay further revealed that stable transfection of HPA shRNA did not influence the cell proliferation of cultured SGC-7901 cells (Figure 2B). These results indicated that knockdown of HPA did not affect the *in vitro* cell proliferation of gastric cancer cells.

### **Knockdown of HPA decreased adhesion, migration and invasiveness of gastric cancer cells *in vitro***

As cell adhesion, migration and invasiveness are 3 critical steps involved in metastasis, and combined with the evidence that HPA plays critical roles in invasiveness



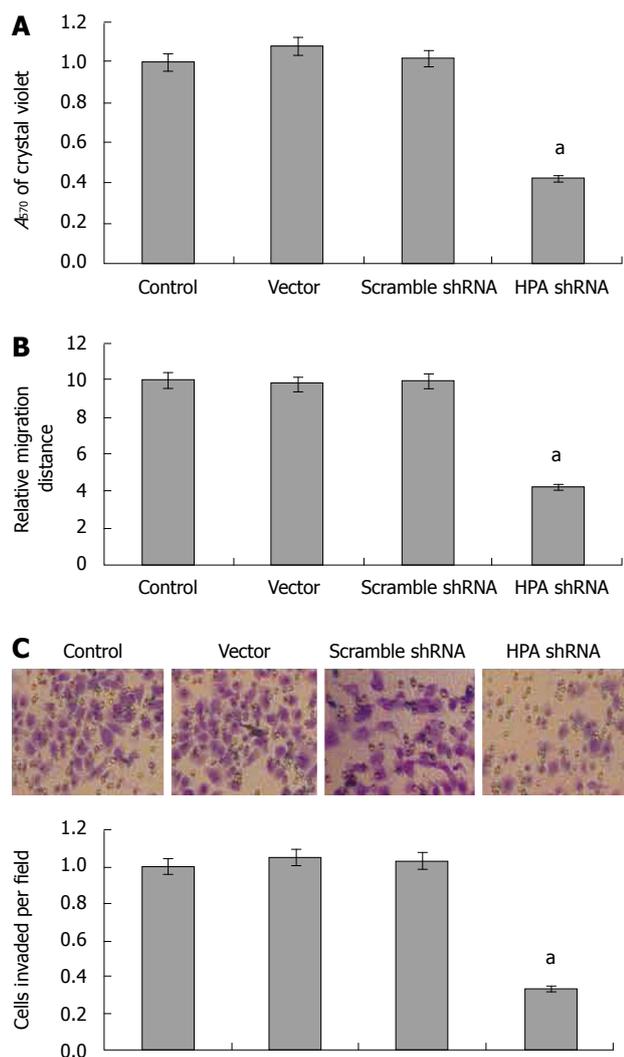
**Figure 1** shRNA suppresses heparanase expression in gastric cancer cells. A: The gastric cancer SGC-7901 cells were transfected with pGenesil-1, pGenesil-scrambled, and pGenesil-HPA. The transfection efficiency was monitored by the enhanced green fluorescent protein (EGFP) reporter within these vectors. The stable subclonal cells were screened by G418 selection; B: RT-PCR and real-time quantitative PCR indicated that stable transfection of empty vector (mock) and scrambled shRNA did not affect the mRNA level of heparanase. However, heparanase mRNA was significantly decreased in the stable shRNA-transfected cells; C: Western blot indicated that stable transfection of heparanase-specific shRNA, but not of scrambled shRNA or vector (mock), resulted in decreased heparanase protein expression in SGC-7901 cells. The symbol (\*) indicates a significant ( $P < 0.05$ ) decrease from parental cells. Experiments were performed in triplicate with essentially identical results.



**Figure 2** Stable knockdown of heparanase does not affect the *in vitro* cell proliferation of gastric cancer cells. A: MTT colorimetric assay indicated that stable transfection of shRNA did not affect the proliferation of SGC-7901 cells, when compared to the parental cells and mock group; B: In the colony formation assay, stable transfection of heparanase-specific shRNA, scrambled shRNA or vector (mock), did not influence the cell proliferation of cultured SGC-7901 cells. Experiments were performed in triplicate with essentially identical results.

and metastasis of cancer cells, we examined the effects of HPA shRNA on these characteristics of gastric cancer cells. In the adhesion assay, SGC-7901 cells stably transfected with HPA shRNA exhibited markedly reduced ability to adhere to the precoated matrigel,

when compared to parental cells and the mock group (Figure 3A). However, the cells stably transfected with scrambled shRNA and vector (mock) had similar ability to adhere as parental cells (Figure 3A). In addition, stable transfection of HPA shRNA into SGC-7901 cells resulted

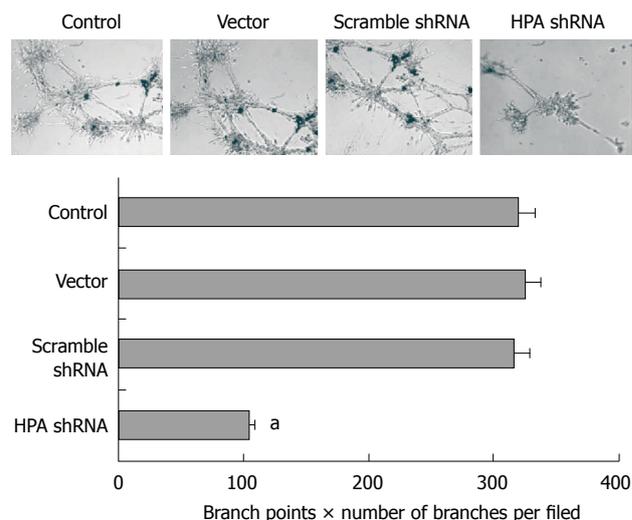


**Figure 3** Stable knockdown of heparanase abolishes the adhesion, migration and invasiveness of gastric cancer cells *in vitro*. A: In the adhesion assay, SGC-7901 cells stably transfected with shRNA exhibited a markedly reduced ability into adhere to the precoated matrigel, when compared to parental cells and vector (mock) group. However, the cells stably transfected with scrambled shRNA had similar ability for adhesion as parental cells; B: In the wound healing assay, the cells stably transfected with heparanase-specific shRNA demonstrated an impaired migration capacity when compared to the parental cells and vector (mock) group; C: In transwell analysis, stable transfection of shRNA abolished the invasive capabilities of SGC-7901 cells, when compared to the parental cells and vector (mock) group. The symbol (<sup>a</sup>) indicates a significant ( $P < 0.05$ ) decrease compared to parental cells. Experiments were performed in triplicate with essentially identical results.

in an impaired migration capacity, when compared to the parental cells and mock group as evidenced by the scratch migration assay (Figure 3B). Moreover, stable transfection of HPA shRNA decreased the invasive capabilities of SGC-7901 cells, when compared to the parental cells and mock group as evidenced by transwell analysis (Figure 3C). These results suggested that HPA-specific shRNA decreased the adhesion, invasiveness and metastasis of gastric cancer cells *in vitro*.

#### Knockdown of HPA inhibited the *in vitro* angiogenesis of gastric cancer cells

We further investigated the effects of HPA-specific



**Figure 4** Stable knockdown of heparanase inhibits *in vitro* angiogenesis of gastric cancer cells. Extensive tube formation of endothelial cells was observed in parental and vector (mock) groups. However, when the endothelial cells were treated by the medium preconditioned with stable heparanase shRNA transfected SGC-7901 cells, tube formation was suppressed. The symbol (<sup>a</sup>) indicates a significant ( $P < 0.05$ ) decrease compared to parental cells. Experiments were performed in triplicate with essentially identical results.

shRNA on the *in vitro* angiogenesis capabilities of SGC-7901 cells. As shown in Figure 4, extensive tube formation of endothelial cells was observed in parental and mock cells. However, when the endothelial cells were treated with medium preconditioned with stable HPA shRNA-transfected SGC-7901 cells, tube formation was significantly decreased (Figure 4). These results indicate that transfection of HPA shRNA markedly decreased the angiogenesis of gastric cancer cells *in vitro*.

## DISCUSSION

Invasiveness and metastasis is one of the characteristics of malignant cells<sup>[17]</sup>. Metastatic cancer cells invade parenchymal tissue and penetrate vascular channels to form satellite tumors in distant organs<sup>[17,18]</sup>. The ECM and BM provide a physical barrier to the migration of cancer cells<sup>[4]</sup>. HSPGs are the essential structural component of ECM and cell surfaces, and are composed of a protein core covalently linked to sulfated glycosaminoglycan<sup>[19]</sup>. The heparan sulfate side chains of HSPGs interact with other components of ECM, such as fibronectin, collagen, and laminin, to provide matrix assembly and stability<sup>[19]</sup>. HPA is the predominant enzyme responsible for degradation of heparan sulfate activity, which is involved in fundamental biologic processes associated with ECM remodeling and cell migration, such as development and morphogenesis, inflammation, angiogenesis, and cancer metastasis<sup>[20-22]</sup>. Many kinds of cells express HPA, such as metastatic tumor cells, proliferating endothelial cells, and activated leukocytes<sup>[23]</sup>. Numerous studies have indicated that HPA is highly expressed in tumors at both the mRNA and protein levels<sup>[24]</sup>. In addition, HPA plays an important role in sustaining the pathology of malignant tumors<sup>[24]</sup>. Expression of HPA is associated with the

aggressiveness of tumor cell lines<sup>[24]</sup>. Increased levels of HPA were detected in the sera of animals bearing metastatic tumors, in the sera of patients with cancer, and in the urine of some patients with aggressive metastatic disease<sup>[25]</sup>, reflecting an association between abnormal HPA secretion and metastatic disease. Thus, HPA is one of the targets for cancer therapy.

It has been established that many potent inhibitors, such as sulfated polysaccharides and heparin-mimicking polyanionic molecules, are capable of inhibiting HPA activity<sup>[8]</sup>. One representative of these inhibitors, PI-88, is being evaluated in a multicentre phase II clinical trial<sup>[26]</sup>. Heparin, other sulfated polysaccharides, and heparin-mimicking molecules that inhibit HPA enzymatic activity also reduce the incidence of metastasis in experimental animals<sup>[21]</sup>. However, because of the potential non-specific activity of these inhibitors and insufficient knowledge of the functional mechanisms of HPA, the application of HPA inhibitors in clinical trials may require further investigation, such as identification of the sugar residues in heparan sulfate adjacent to the HPA cleavage site and the crystallization and analysis of the 3-dimensional structure of the enzyme<sup>[8,27-30]</sup>. In hepatocellular carcinoma cell lines, antisense oligonucleotides decreased HPA expression and inhibited the invasiveness and angiogenesis of cancer cells<sup>[13]</sup>. Uno *et al.*<sup>[31]</sup> constructed an adenoviral vector carrying an antisense full-length human HPA cDNA, and found that HPA expression, and the *in vitro* invasiveness and metastasis of human lung cancer A549 cells were specifically inhibited. Downregulation of HPA by overexpression of either anti-HPA ribozyme or RNA silencing vector has been shown to not only inhibit HPA activity but also impair tumor invasiveness and metastasis, resulting in improved survival of tumor-bearing mice<sup>[14,15]</sup>. In highly metastatic B16-BL6 mouse melanoma cells, transfection of mouse HPA-specific siRNA resulted in a decrease in the invasiveness and metastasis of tumor cells<sup>[14]</sup>.

Previous studies indicated that the expression of HPA was frequently observed in advanced gastric cancers, and the frequency was significantly correlated with histopathological parameters reflecting invasive and metastatic potentials and prognosis of gastric cancers<sup>[6,7]</sup>. Our studies also demonstrated the overexpression of HPA protein in advanced gastric cancer (data not shown). However, it still remains largely unknown whether knockdown of HPA expression can decrease the invasiveness and metastasis of gastric cancer cells. In this study, we constructed HPA-specific shRNA and transfected into cultured SGC-7901 cells. The subclonal cells were generated for stable knockdown of HPA. We found that shRNA decreased HPA mRNA and protein in gastric cancer cells. Stable knockdown of HPA expression decreased the *in vitro* migration, invasiveness and metastasis of gastric cancer cells. However, stable knockdown of HPA expression did not affect the proliferation of SGC-7901 cells, which was consistent with previous findings in human SMMC7721 liver cancer cells and mouse *B16 L6* melanoma cells<sup>[13,14]</sup>.

Previous evidence showed that the cleavage of HSPGs

by HPA may release heparan sulfate-bound cytokines and growth factors from cell surface or from the ECM, such as basic fibroblast growth factor and vascular endothelial growth factor<sup>[29,32]</sup>. Thus, HPA may facilitate tumor cell invasiveness and neovascularization, both critical steps in tumor progression<sup>[33]</sup>. A pronounced correlation between HPA expression and tumor microvessel density has been reported<sup>[34]</sup>. In this study, we found that the angiogenesis of gastric cancer cells *in vitro* was decreased by stable shRNA transfection. To our knowledge, the data described here represent the first successful application of shRNA-mediated gene silencing to stably reduce the levels of HPA in gastric cancer.

In summary, we have demonstrated that stable knockdown of HPA expression can efficiently inhibit the invasiveness, metastasis and angiogenesis of human gastric cancer cells. It is likely that the inhibition of HPA expression possibly depresses the degradation of ECM and BM, thus inhibiting the invasiveness and metastasis of gastric cancer. Therefore, our study suggests that HPA-specific shRNA may be of potential value as a novel therapeutic strategy in human gastric cancer. Combined with our identical results of stable transfection of HPA-specific shRNA into bladder cancer cell lines (data not shown), we believe that this HPA-specific shRNA may be also applicable for the therapies of other cancers overexpressing HPA.

## COMMENTS

### Background

Previous studies have indicated that the heparanase (HPA) is correlated with histopathological parameters and poor prognosis of gastric cancers. Although their efficiencies in inhibiting the expression of HPA, the traditional HPA inhibitors may produce nonspecific and undesirable effects. Thus, novel approaches are needed to inhibit the role of HPA in cancer progression.

### Research frontiers

In recent years, genetic approaches targeting HPA have been regarded as a promising alternative. Antisense oligonucleotides, ribozyme, and small RNA interference (siRNA) have been developed to decrease the HPA expression. However, it remains unknown whether stable transfection of short hairpin RNA (shRNA) can knockdown the HPA expression and decrease the invasiveness and metastasis of gastric cancer cells.

### Innovations and breakthroughs

In the current study, the HPA-specific shRNA was constructed and transferred into cultured gastric cancer cells. The authors demonstrated, for the first time, that stable knockdown of HPA expression decreased the *in vitro* invasive, metastatic and angiogenic capabilities of gastric cancer cells. However, stable knockdown of heparanase does not affect the proliferation of gastric cancer cells.

### Applications

By understanding the effects of HPA-specific shRNA on the *in vitro* invasiveness, metastasis and angiogenesis capabilities of gastric cancer cells, this study may represent a future strategy for therapeutic intervention in the treatment of patients with gastric cancer. In addition, the HPA-specific shRNA may be also applicable for the therapies of other cancers overexpressing HPA.

### Terminology

HPA is a mammalian endo- $\beta$ -D-glucuronidase that is capable of degrading the heparan sulfate proteoglycans in basement membrane and extracellular matrix, and plays important roles in cancer invasion, angiogenesis and metastasis.

### Peer review

The authors examined the effects of HPA-specific shRNA on the cultured gastric cancer cells. It revealed that stable transfection of HPA-specific shRNA decreased the mRNA and protein levels of HPA in SGC-7901 cells.

The shRNA against HPA efficiently decreased the invasiveness, metastasis and angiogenesis of gastric cancer cells. The results are interesting and may represent a novel strategy for the treatment of gastric cancer.

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## G-CSF in Peg-IFN induced neutropenia in liver transplanted patients with HCV recurrence

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### Abstract

**AIM:** To evaluate the efficacy of granulocyte colony stimulating factors (G-CSF) in liver transplanted patients with hepatitis C (HCV) recurrence and Pegylated-IFN  $\alpha$ -2b induced neutropenia, and to evaluate the impact of G-CSF administration on virological response.

**METHODS:** Sixty-eight patients undergoing antiviral treatment for post-liver transplantation (OLT) HCV recurrence were enrolled. All patients developing neutropenia received G-CSF.

**RESULTS:** Twenty three (34%) received G-CSF. Mean neutrophil count at the onset of neutropenia was 700/mm<sup>3</sup> (range 400-750/mm<sup>3</sup>); after 1 mo of G-CSF it increased to 1210/mm<sup>3</sup> (range 300-5590/mm<sup>3</sup>) ( $P < 0.0001$ ). Three patients did not respond to G-CSF. Treatment duration was similar in neutropenic and non-neutropenic patients. No differences in the rate of discontinuation, infections or virological response were observed between the two groups. G-CSF was protective for the onset of *de novo* autoimmune hepatitis ( $P < 0.003$ ).

**CONCLUSION:** G-CSF administration is effective in the case of Peg-IFN induced neutropenia increasing

neutrophil count, prolonging treatment and leading to sustained virological response (SVR) rates comparable to non-neutropenic patients. It prevents the occurrence of *de novo* autoimmune hepatitis.

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**Key words:** Granulocyte colony stimulating factors; Liver transplantation; Hepatitis C virus recurrence; Antiviral treatment

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### INTRODUCTION

Both interferons (IFNs) and pegylated interferons (Peg-IFNs) may induce neutropenia<sup>[1-4]</sup>. This side effect may limit adherence to treatment which is one of the most important factors related to virological response<sup>[1,5-7]</sup>.

In immunocompetent patients, neutropenia has not been associated with infections. However, in oncological immunodepressed patients neutropenia is associated with infections<sup>[8-10]</sup> and liver transplanted patients are immunosuppressed. In fact, liver transplanted patients have a high rate of infections reaching 56% within the first year post transplantation<sup>[11-13]</sup>. This ease at infections coupled to the baseline leucopenia induced by the immunosuppressive regimens challenging the management of these patients by the clinician.

There are no guidelines on the use of granulocyte colony stimulating factors (G-CSF) for the treatment of IFN induced neutropenia. Moreover, the impact of G-CSF administration during antiviral therapy for chronic hepatitis C has not been determined yet.

Nevertheless, the use G-CSF is becoming a standard of care in this setting, especially in liver transplanted patients, and is recommended by several authors<sup>[5,14-19]</sup>.

A recent study by our group showed that G-CSF administration has a protective effect for the development of *de novo* autoimmune hepatitis during antiviral therapy in transplanted patients<sup>[20]</sup>.

This effect is not surprising as G-CSF has been shown to have several immunological properties: induces T-regulator (T-regs) mobilization and activity, both directly and through the expansion of tolerogenic myeloid precursor and type 2 dendritic cells mobilization; moreover it skews the cytokine profile, inducing tolerogenic dendritic cells and T-regs, which finally suppresses T cell activity<sup>[21-26]</sup>.

The aims of the present study were to evaluate the efficacy of G-CSF use in liver transplanted patients with hepatitis C virus (HCV) recurrence and Peg-IFN  $\alpha$ -2b induced neutropenia, and to evaluate the impact of G-CSF administration on virological response.

## MATERIALS AND METHODS

Patients undergoing antiviral treatment for post orthotopic liver transplantation (OLT) HCV recurrence were consecutively enrolled in Bologna Liver Transplantation Centre between October 2001 and April 2005. All patients received Peg-IFN  $\alpha$ -2b at the dose of 1.0 mcg/kg once weekly (Peg-Intron®, Schering-Plough, Italy), and Ribavirin (Rebetol®, Schering-Plough, Italy) at a dose of 8-10 mg/kg per day. Transplanted patients had to fulfil the following criteria for antiviral treatment: detectable HCV-RNA by PCR, elevated ( $> 1.0 \times$ ) serum alanine aminotransferase (ALT) levels and histological features of HCV hepatitis on liver biopsy. Exclusion criteria were: evidence of decompensated liver disease, histological evidence of rejection and drug-related injury, HBsAg positivity, human immunodeficiency virus (HIV) positivity, moderate to severe anemia (Hb  $< 10$  g/dL), neutropenia (neutrophil count  $< 1000/\text{mm}^3$ ), thrombocytopenia (PLT  $< 50000/\text{mm}^3$ ), impaired renal function (creatinine clearance  $< 50$  mL/min), significant history of cardiovascular and psychiatric diseases, ongoing alcohol abuse and previous post-LT treatment with PEG-IFN. Hematologic determinations were carried out using conventional tests at baseline and weekly for the first month, then monthly until the end of the study.

All patients developing neutropenia during antiviral treatment received Granulocyte Colony-Stimulating Factor (G-CSF) (Granulokine®, Roche, Italy). Below 750/mmc neutrophils, G-CSF 300  $\mu\text{g}/\text{wk}$  was administered and in case of non significant response the dose was increased to 600  $\mu\text{g}/\text{wk}$ . When the neutrophil count did not increase satisfactorily, despite G-CSF administration, Peg-IFN dose was reduced. When neutrophils fell below 500/mmc despite G-CSF administration, antiviral treatment was discontinued. G-CSF treatment was continued until restoration of neutrophil count to values comparable

Table 1 Baseline characteristics of patients at enrolment

Sex (M/F)	46/22
Age, median (range)	59 (22-68)
BMI, median (range)	24.2 (15.5-40.5)
Months since LT, median (range)	15.5 (1-151)
Previous acute cellular rejection <i>n</i> (%)	13 (19)
Days since OLT, mean	36.4
Previous CMV infection <i>n</i> (%)	14 (21)
Genotype <i>n</i> (%)	
1	53 (76.5)
2	6 (9)
3	4 (6)
4	5 (7)
Viral load (MEq/mL), median (range)	10.6 (0.009-40)
ALT (IU/L), median (range)	136 (52-945)
Neutrophil count ( $\times 10^3/\text{mmc}$ ) mean $\pm$ SD	3 $\pm$ 1.2
Hemoglobin (g/dL) mean $\pm$ SD	12.9 $\pm$ 1.5
Cirrhosis <i>n</i> (%)	10 (15)
Pre-LT antiviral treatment <i>n</i> (%)	29 (45)
Post-LT antiviral treatment <i>n</i> (%)	15 (22)
Induction immunosuppression <i>n</i> (%)	
Monoclonal antibodies	10 (14)
Steroids	49 (72)
Other	3 (4)
NA	6 (10)
Maintenance immunosuppression <i>n</i> (%)	
Cyclosporine	30 (44)
Tacrolimus	22 (32)
Cyclosporine + steroids	6 (9)
Tacrolimus + steroids	9 (13)
Other	1 (1.5)

LT: Liver transplantation; OLT: Orthotopic liver transplantation; CMV: Cytomegalovirus.

to the patient's baseline. None of the patients received azathioprine or mycophenolate mofetil.

All patients gave written informed consent according to the Ethical Committee Procedures of our Hospital for the administration of off label drugs.

## Statistical analysis

Data were analyzed on an intention-to-treat-basis. Results are presented as median (range). Non parametric tests were used to compare variables between groups (Wilcoxon,  $\chi^2$  test). All  $P < 0.05$  by the two-tailed test were considered significant. All data analyses were conducted using the MedCalc Package.

## RESULTS

Sixty-eight patients (46 males and 22 females), median age 59 (22-68 years) were enrolled in the study (Table 1). Ten patients were cirrhotic at enrolment. Twenty three (34%) received G-CSF according to our study design. Table 2 shows the baseline characteristics of patients developing neutropenia or not and of all patients together. The only factor related to neutropenia development was pre-treatment neutrophil count, which was significantly lower in patients who later developed neutropenia and were treated with G-CSF. Median neutrophil count at the onset of neutropenia was 700/mmc (range 400-750/mmc) and after one month

Table 2 Characteristics of patients developing neutropenia

	G-CSF	No G-CSF	P
Sex (M/F)	10/13	Dec-33	NS
Age, median (range)	60 (41-68)	58 (22-67)	NS
Months since LT, median (range)	15 (2-151)	19 (1-148)	
Genotype n (%)			
1	17 (73)	36 (80)	NS
2	2 (8.6)	4 (8.8)	NS
3	2 (8.6)	2 (4.4)	NS
4	2 (8.6)	3 (6.6)	NS
Neutrophil count ( $\times 10^3/\text{mmc}$ )	2.23 $\pm$ 0.96	3.14 $\pm$ 1.22	0.0021
mean $\pm$ SD			
Cirrhosis n (%)	10 (15)		
Induction immunosuppression n (%)			
Monoclonal antibodies	4 (17)	6 (13)	NS
Steroids	17 (73)	32 (71)	NS
Other	1 (3)	2 (4)	NS
NA	1 (3)	5 (1)	NS
Maintenance immunosuppression n (%)			
Cyclosporine	10 (43)	20 (44)	NS
Tacrolimus	8 (34)	14 (31)	NS
Cyclosporine + steroids	1 (4)	5 (11)	NS
Tacrolimus + steroids	3 (13)	6 (13)	NS
Other	1 (4)	0	NS

G-CSF: Granulocyte colony stimulating factors.

Table 3 Peg-IFN and RBV dose reductions n (%)

	G-CSF	No G-CSF	P
RBV reduction	6 (26)	17 (37)	0.4
RBV withdrawal	4 (17)	3 (6)	0.2
Peg-IFN + RBV reduction	2 (8)	1 (2)	0.2

Peg-IFN: Pegylated interferons; RBV: Ribavirin.

of G-CSF administration it increased to 1210/mmc (range 300-5590/mmc) ( $P < 0.0001$ ) (Figure 1). Mean G-CSF treatment duration was  $4.9 \pm 3.6$  mo. Three patients did not respond to G-CSF administration after one month; two patients had an improved neutrophil count with an increased dose of G-CSF and a reduction of Peg-IFN dose and continued treatment until the end of the planned 48 wk period, the other discontinued treatment. No patient had to reduce their Peg-IFN dose in the non-neutropenic group. Table 3 shows Ribavirin (RBV) and Peg-IFN dose modification during treatment in both groups. At multivariate analysis, several factors were evaluated (age, sex, time from OLT, type of immunosuppression, presence of cirrhosis, basal neutrophil count) but none was associated with non response to G-CSF.

The mean treatment duration was similar in neutropenic and non-neutropenic patients regardless of G-CSF administration and genotype (Figure 2A).

Causes of premature discontinuation are shown in Table 4. No significant differences in the rate of discontinuation were observed between the two groups (neutropenic and non-neutropenic) (Figure 2B).

Two severe infections were observed in the G-CSF group (1 pneumonia and 1 urinary infection) and 5 in the non-neutropenic patients (3 pneumonias, 1 liver abscess and 1 cytomegalovirus) ( $P =$  No significance) (Figure

Table 4 Causes of premature discontinuation in the two groups

Discontinuation cause	G-CSF group	Non neutropenic group
Liver decompensation	3	3
Severe asthenia	2	1
Neutropenia	1	
Anemia	1	
Toxic hepatitis	1	
Non response		1
Liver abscess		1
<i>De novo</i> AIH		9
<i>De novo</i> HBV infection		1

AIH: Autoimmune hepatitis; HBV: Hepatitis B virus.

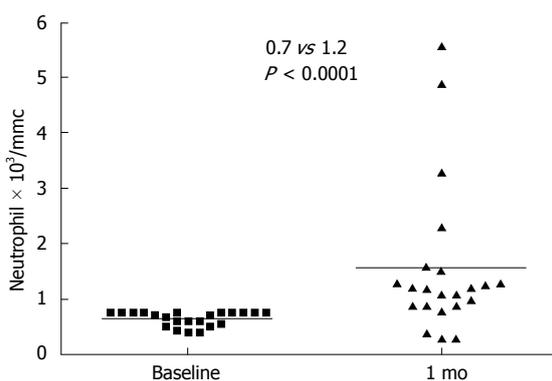


Figure 1 Neutrophil count response after 1 mo of granulocyte colony stimulating factors administration.

2C). Among neutropenic patients, the neutrophil counts were  $700/\text{mm}^3$  and  $900/\text{mm}^3$  respectively at the onset of infections. In non-neutropenic patients developing infections, the neutrophil count was  $> 1000/\text{mm}^3$  in all at the onset of neutropenia.

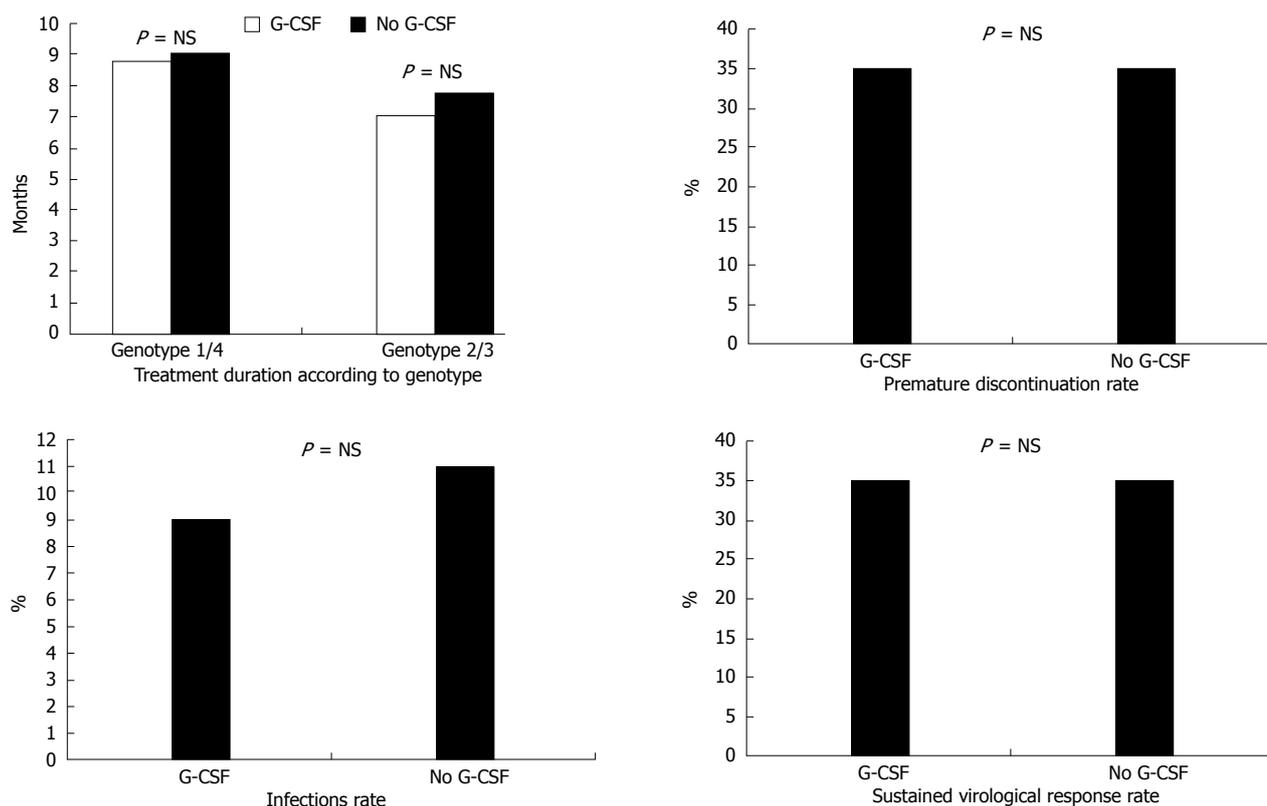
### Viral response

Sustained virological response was 30% in the G-CSF group versus 35% in the non-neutropenic patients ( $P =$  NS) (Figure 2D).

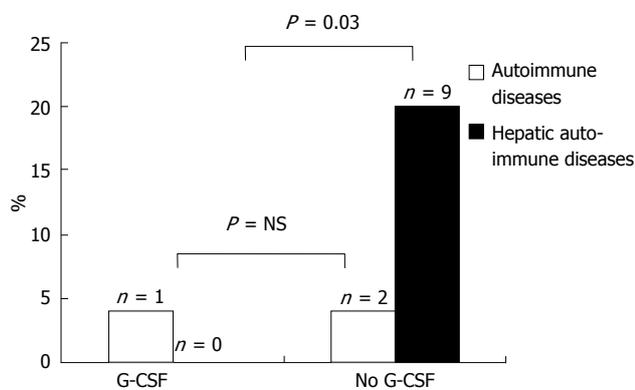
### Autoimmune diseases

In the G-CSF group no *de novo* hepatic autoimmune disease was observed, while 9 patients in the other group developed autoimmune hepatitis ( $P < 0.03$ ) (Figure 3). This pathological entity has already been extensively described by our group<sup>[20]</sup> and therefore won't be discussed in detail here. *De novo* autoimmune hepatitis diagnosis was performed in patients with an unexplained cause of graft dysfunction, after the exclusion of other known causes (infections, anastomoses complications, acute or chronic rejection) and the application of the International Autoimmune Hepatitis Group score<sup>[27]</sup>. This disease was related to severe patient and graft outcome; in fact two patients died and two had a graft failure with one patient re-enlisted for transplant. In our series no rejection episodes were observed.

The incidence of non hepatic autoimmune manifestations was similar in the two groups. One patient had autoimmune thyroiditis and another had systemic lupus erythematosus (SLE) responding to



**Figure 2** Comparison of treatment duration, premature discontinuation rate, infections rate and sustained virological response (SVR) rate between neutropenic patients treated with G-CSF and untreated non-neutropenic patients.



**Figure 3** Incidence of hepatic and systemic autoimmune diseases in neutropenic patients treated with G-CSF and untreated non-neutropenic patients.

steroid treatment<sup>[28]</sup> in the G-CSF group, while among non-neutropenic patients, one had autoimmune gastritis.

## DISCUSSION

Our data shows that G-CSF administration is effective in resolving neutropenia in transplanted patients treated with Peg-IFN  $\alpha$ -2b for HCV recurrence. The improvement or the resolution of neutropenia allowed us to prolong treatment duration to a time which is comparable to that of non-neutropenic patients and presumably contributed to patients reaching an SVR rate which was comparable in the two groups. G-CSF administration did not affect the rate of infections or the

occurrence of non hepatic autoimmune diseases, while it was a protective factor for the development of *de novo* autoimmune hepatitis. The only predictive factor related to the development of neutropenia during antiviral treatment was pre-treatment neutrophil count.

Clinical experience, mainly from studies in immunocompetent patients, suggests that maintenance of optimal dosages of Peg-IFN and RBV results in higher rates of SVR. Previous studies on liver transplanted patients have shown that, overall, a SVR between 30% and 45% is achievable<sup>[29-35]</sup>. Therefore, in our opinion, adjunctive treatments allowing the maintenance of optimal dosages of Peg-IFN and RBV during treatment are advisable. In our series the rate of SVR was not significantly different among patients treated and not treated with G-CSF. The lack of a statistically significant difference could be affected by the small number of patients, but the rate of SVR in neutropenic patients treated with G-CSF is comparable to what is reported in literature. Moreover, although not statistically significant, a larger proportion of patients had to reduce or suspend RBV in the G-CSF group, and this factor might have contributed to the smaller SVR rate in this group.

There are currently no guidelines on the use of G-CSF in IFN induced neutropenia. Therefore, no established common approach to its use is defined in both the immunocompetent and the transplanted patient. In this context we arbitrarily defined cut off values to begin G-CSF administration. In consideration of the immune depression of our patients we chose a high cut off level of 750 neutrophils/mm<sup>3</sup> for initiation of treatment in

order to prevent infections. We are aware that previous studies in immunocompetent patients did not show a correlation between the absolute number of neutrophils and infections<sup>[2,36,37]</sup>, but in immunodepressed oncological patients this correlation exists<sup>[8-10]</sup>; this induced us to choose a safer cut off value. Unfortunately ours was not a randomised study, because in our opinion it would be not ethical not to treat a severe neutropenia in a transplanted immunosuppressed patient. Nevertheless, in our study infections seemed to be independent of neutrophil count. Anyway, our study shows that G-CSF use in transplanted patients is safe and effective.

With regard to autoimmune diseases, our study suggests that G-CSF protected from *de novo* autoimmune hepatitis but not from other systemic autoimmune manifestations. This is not surprising, as G-CSF has been reported to have different effects in different autoimmune diseases<sup>[38-40]</sup>. This probably depends on the immune pathogenetic mechanisms underlying the different diseases. However, the apparently induced non hepatic autoimmune manifestations did not significantly impact on patients outcome while *de novo* hepatitis was severe and related to high patient and graft loss, suggesting that the onset of non hepatic autoimmune manifestations does not contraindicate G-CSF use in this population.

In conclusion, our study supports G-CSF administration in transplanted patients with HCV recurrence developing Peg-IFN induced neutropenia because it is effective in increasing the neutrophil count. G-CSF administration prolongs treatment duration in neutropenic patients leading to SVR rates which are comparable to that of non-neutropenic patients. A pre-emptive treatment with G-CSF could be advisable in patients with very low pre-treatment neutrophil counts. G-CSF seems to have a protective role against the occurrence of *de novo* autoimmune hepatitis, a recently defined pathogenetic entity related to a severe outcome. Prospective randomized studies are needed in order to evaluate the protective effect of prophylactic G-CSF administration to prevent this form of autoimmune hepatitis.

## COMMENTS

### Background

The treatment of liver transplanted patients with hepatitis C virus (HCV) recurrence is based on a combination of Pegylated Interferon (PegIFN) and Ribavirin, with disappointing results. A key factor for response to IFNs is adherence to treatment, which is challenging in immunodepressed patients in whom PegIFN dose reductions for neutropenia are often required. This study aims to evaluate the efficacy and the impact on virological response of granulocyte colony stimulating factors (G-CSF) administration in transplanted patients developing PegIFN related neutropenia.

### Research frontiers

There are no guidelines on the use of G-CSF for the treatment of IFN induced neutropenia. Moreover, the impact of G-CSF administration during antiviral therapy for chronic hepatitis C has not been determined yet. Nevertheless, the use of G-CSF is becoming a standard of care in this setting, especially in liver transplanted patients, and is recommended by several authors. A recent study by the authors' group showed that G-CSF administration has a protective effect on the development of *de novo* autoimmune hepatitis during antiviral therapy in transplanted patients.

### Innovations and breakthroughs

This is the first study conducted to investigate the efficacy of G-CSF in liver

transplanted patients with PegIFN induced neutropenia. It shows that G-CSF administration is effective at increasing the neutrophil count, prolonging treatment and leading to SVR rates comparable to those in non-neutropenic patients. Moreover, it confirms the observation that G-CSF has a protective role with regard to the occurrence of *de novo* autoimmune hepatitis.

### Applications

This study is of clinical interest to physicians treating recurrent HCV after liver transplantation. Based on the results, the use of G-CSF in liver transplanted patients with PegIFN induced neutropenia is advisable.

### Terminology

HCV recurrence: the reinfection of the graft by HCV post-liver transplantation is universal and is associated with a worse outcome. G-CSF is granulocyte colony stimulating factor hormone, which stimulates the bone marrow to produce granulocytes and stem cells

### Peer review

This work is an interesting pilot study describing the potential benefits of using G-CSF to treat IFN-induced neutropenia in recurrent HCV infection after liver transplantation.

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## Reasons of PEG failure to eliminate gastroesophageal reflux in mechanically ventilated patients

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**CONCLUSION:** Endoscopic grading of GEFV and the presence of severe reflux esophagitis are predisposing factors for failure of PEG to reduce GER in mechanically ventilated patients.

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**Key words:** Esophagitis; Gastroesophageal reflux; Percutaneous endoscopic gastrostomy

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### Abstract

**AIM:** To investigate factors predicting failure of percutaneous endoscopic gastrostomy (PEG) to eliminate gastroesophageal reflux (GER).

**METHODS:** Twenty-nine consecutive mechanically ventilated patients were investigated. Patients were evaluated for GER by pH-metry pre-PEG and on the 7th post-PEG day. Endoscopic and histologic evidence of reflux esophagitis was also carried out. A beneficial response to PEG was considered when pH-metry on the 7th post-PEG day showed that GER was below 4%.

**RESULTS:** Seventeen patients responded (RESP group) and 12 did not respond (N-RESP) to PEG. The mean age, sex, weight and APACHE II score were similar in both groups. GER (%) values were similar in both groups at baseline, but were significantly reduced in the RESP group compared with the N-RESP group on the 7th post-PEG day [2.5 (0.6-3.8) vs 8.1 (7.4-9.2,  $P < 0.001$ )]. Reflux esophagitis and the gastroesophageal flap valve (GEFV) grading differed significantly between the two groups ( $P = 0.031$  and  $P = 0.020$ , respectively). Histology revealed no significant differences between the two groups.

### INTRODUCTION

Gastroesophageal reflux (GER) is a common problem in critically ill patients and constitutes a major mechanism of esophageal mucosal injury or erosive esophagitis in mechanically ventilated patients<sup>[1,2]</sup>. Nasogastric tube (NGT) plays a significant role in the development of GER in these patients<sup>[3]</sup>. GER is considered to be a major risk for ventilator-associated pneumonia (VAP), which is associated with prolongation of Intensive Care Unit (ICU) stay and increased mortality<sup>[4,5]</sup>. The judicious use of NGT, the semi-recumbent position and the application of strategies to improve gastric emptying are the proposed measures for preventing GER and thus minimizing VAP<sup>[5-7]</sup>.

The relationship between percutaneous endoscopic gastrostomy (PEG) and subsequent development of GER is complex and not well understood. The reported effects of PEG on GER are contradictory. Some authors reported the development or worsening of GER after PEG<sup>[8,9]</sup>, while others claimed the opposite<sup>[10,11]</sup>. Johnson *et al*<sup>[11]</sup> reported that PEG tube placement decreases

GER by increasing the lower esophageal sphincter pressure (LES), and showed that the anterior apposition of the stomach by PEG has a similar effect to gastropexy and increases LES pressure. The latter was argued by Coben *et al*<sup>[12]</sup>, who found that gastrostomy tubes had no effect on basal LES pressure. Few studies, however, have evaluated the effect of PEG on GER in mechanically ventilated patients. In a prospective randomized trial, we have shown that PEG when combined with the semi-recumbent position and control of gastric residue, can decrease GER in the majority of these patients<sup>[13]</sup>. In this new prospective study we aimed to explore the factors that interfere with failure of PEG to reduce GER in critically ill, mechanically ventilated patients.

## MATERIALS AND METHODS

Patients eligible for this study were all mechanically ventilated, were tolerating nasogastric feeding *via* NGT and had prolonged ICU stay. Enteral feeding was performed at a continuous infusion rate of 80 to 100 mL/h. Exclusion criteria were unstable hemodynamic state, the administration of morphine, atropine, theophylline, barbiturates or cisapride, the presence of bloated intestine, and a past history of gastroesophageal reflux disease or hiatus hernia. The patients were divided into 2 groups based on whether GER decreased to less than 4% (responders, RESP group) or remained unchanged or worsened (non-responders, N-RESP group) after PEG placement. The institutional review board approved the study and informed consent was obtained from each patient's next of kin.

### Study design

Antacids, H<sub>2</sub> receptor blockers or proton pump inhibitors were withdrawn 7 d before the study; sucralfate (2 g twice daily *via* NGT) was provided thereafter for gastric mucosa protection. Patients were neither sedated nor paralyzed. Enteral feeding was stopped 6 h before and during the period of pH-metry, which was performed on a 24 h basis. Gastrostomy was performed by the pull through (Ponsky) technique the day after<sup>[14]</sup>. Monitoring of pH was repeated on the 7th post-PEG day in order to assess the effect of PEG on eliminating or reducing GER%.

On the second post-PEG day, patients were administered continuous drip PEG-feeding with a polymeric diet at 60 mL/h and an energy content of 1000 kcal/L (Fresenius, Bad Homburg, Germany). Patients were placed in a semi-recumbent position (30°) and the volume of the gastric nutrient residue was determined to obviate gastric stasis. This was realized by aspirating the gastric content with a syringe at 8-h intervals to verify if the nutrient volume exceeded 200 mL. If the volume did exceed 200 mL, feeding was withheld and if volume persisted, the nutrient was drained. Feeding was re-administered when the volume was decreased.

### Endoscopic evaluation

The presence or absence of hiatus hernia, reflux es-

ophagitis and the grading of gastroesophageal flap valve (GEFV) were examined and scored according to the criteria below by two endoscopists. Biopsy specimens were obtained from the lower 3 cm of the esophagus for histological examination.

**Reflux esophagitis:** Reflux esophagitis was graded according to the Los Angeles classification<sup>[15]</sup>.

**GEFV:** This was assessed by the grading system of Hill *et al*<sup>[16]</sup>, as follows: Grade I: Normal ridge of tissue closely approximated the shaft of the retroflexed scope. Grade II: The ridge is slightly less well defined and opens with respiration. Grade III: The ridge is barely present and the hiatus is patulous. Grade IV: There is no muscular ridge and the hiatus is wide open at all times. GEFV grades I and II were regarded as representing normal GEFV, while grades III and IV were regarded as representing abnormal GEFV.

**Esophageal pH-metry:** The presence of gastroesophageal reflux was demonstrated by using 24-h double sensor catheter system pH monitoring. Baseline and 7th d post-PEG pH-metry was carried out using the portable recorder Digitrapper Mk III (Synectics Medical AB, Stockholm, Sweden). Briefly, after ordinary NGT removal, the sensor probe was introduced *via* the nose into the stomach until acid pH was recorded with the distal sensor as previously described<sup>[13]</sup>. The probe was then slowly withdrawn until the distal sensor channel detected a sudden pH change from acid (< 4) to above 5. The electrode was then withdrawn another 5 cm. In this way, the distal and proximal sensors were located at 5 and 20 cm above the lower esophageal sphincter, respectively. The correct positioning of the electrode was verified at the end of pH-metry and before its withdrawal by a chest x-ray. The recording device measured pH values every 4 s and stored the mean of 20 values every 80 s. Gastroesophageal reflux was expressed as the percentage of time that the esophageal pH was less than 4 in the given 24-h period [GER (%)], with a value of > 4% considered abnormal<sup>[17]</sup>. Additionally, the number of reflux episodes per hour and the number of reflux episodes lasting longer than 5 min in 24 h were measured and recorded. The pH-metry from the lower esophageal sensor consistently recorded GER 15%-20% higher than that from the upper sensor. However, since the importance of reflux detection in the upper part of the esophagus is greater in relation to aspiration, only data from the upper sensor are presented.

### Histological evaluation

Biopsies were assessed by two experienced histopathologists and scored for surface ulceration (present or absent), basal cell hyperplasia (score 0-3), acute inflammatory infiltration of the epithelium (score 0-3) and the lamina propria (score 0-3) and presence of long papillae (score 0-3) to produce an overall histological score (ranging from 0 to 12). The interobserver variability was <

Table 1 Patients' demographic data

	RESP group (n = 17)	N-RESP group (n = 12)	P value
Age, years	55 (32-65)	65 (49.5-68)	NS
Sex (M/F)	(12/5)	(5/7)	NS
Weight, kg	75 (70-80)	77 (68.5-80)	NS
APACHE II	17 (15-20)	15.5 (13.5-22.5)	NS
Indication for PEG placement			
Cerebrovascular attack	9	5	
Neuromuscular disorder	4	2	
COPD-pneumonia	2	2	
Head trauma	2	3	
Days on NGT	29.5 (25-38)	45 (25-53)	NS
Weaning, days	10 (6.5-14)	13 (12-20)	NS
Outcome (survived/died)	(14/3)	(7/5)	NS

Parameters are expressed in median (range). APACHE: Acute physiology and chronic health evaluation; COPD: Chronic obstructive pulmonary disease.

Table 3 Endoscopic and histological data of the groups studied

	RESP group (n = 17)	N-RESP group (n = 12)	P value
Endoscopic esophagitis <sup>1</sup>	1 (1-2)	2 (1-3)	0.031 <sup>a</sup>
A	11	4	
B	6	4	
C	0	4	
D	0	0	
GEFV <sup>2</sup>	2 (1-3)	3 (3-4)	0.020 <sup>a</sup>
Grade I	8	1	
Grade II	2	1	
Grade III	5	6	
Grade IV	2	4	
Histology	1 (1-2)	2 (1-2.5)	NS
Grade 0	2	1	
Grade I	8	3	
Grade II	6	5	
Grade III	1	3	

<sup>1</sup>LA classification; <sup>2</sup>GEFV: Gastroesophageal flap valve (grading after Hill classification<sup>[16]</sup>). <sup>a</sup>P < 0.05.

5%. In case of disagreement, reevaluation was performed jointly by the two observers so that a consensus could be reached. Complete surface ulceration was scored as 12 and no other epithelial changes were assessed. Overall histological score of 0-2 corresponded to grade zero, 3-4 corresponded to grade I, 5-8 to grade II and 9-12 to grade III histological esophagitis.

### Statistical analysis

Data are reported as median and inter-quartile range (Q1-Q3). Comparisons among groups (responders, non-responders) were made using the Mann-Whitney test. Comparisons (number of reflux episodes) within groups were performed by the Wilcoxon's rank sum test. The Pearson's  $\chi^2$  test or Fisher's exact test when appropriate, were used to examine the association between qualitative variables. The Spearman rank correlation coefficient ( $r_s$ ) was calculated to describe the relationships between variables. A P-value less than 0.05 was considered significant.

Table 2 Comparison of esophageal manometry findings between the RESP and N-RESP groups

	RESP group (n = 17)	N-RESP group (n = 12)	P value
Gastroesophageal reflux			
GER (%)			
Baseline	9.4 (7.4-13.5)	11.7 (7.6-15)	NS
7th post-PEG day	2.5 (0.6-3.8)	8.1 (7.4-9.2)	< 0.001 <sup>b</sup>
No. of reflux episodes/h			
Baseline	8 (3-10)	2.9 (2-4.4)	0.020 <sup>a</sup>
7th post-PEG day	1.3 (0.5-2)	2.9 (2.3-3.4)	0.002 <sup>a</sup>
No. of reflux episodes > 5 min			
Baseline	7 (4-9)	3.5 (2-6)	0.045 <sup>a</sup>
7th post-PEG day	1 (0-2)	3 (1.5-5)	0.003 <sup>a</sup>

Parameters are expressed in median (range). <sup>a</sup>P < 0.05, <sup>b</sup>P < 0.001.

## RESULTS

### Patients' profiles

A cohort of 29 consecutive mechanically ventilated patients undergoing PEG was prospectively evaluated. Demographic data are listed in Table 1. Patients were divided into the RESP group (n = 17) and the N-RESP group (n = 12). The mean age, sex, weight and APACHE II score were similar in both groups. The reasons for PEG tube placement were as follows: cerebrovascular attack 48.2% (n = 14), neuromuscular disorder 13.7% (n = 4), COPD-pneumonia 20.6% (n = 6), and trauma 17.2% (n = 5). The median number of days of nasogastric tube placement before PEG was 29.5 in the RESP group *vs* 45 in the N-RESP group (NS, Table 1), which denotes a similar pre-PEG timing in the two groups.

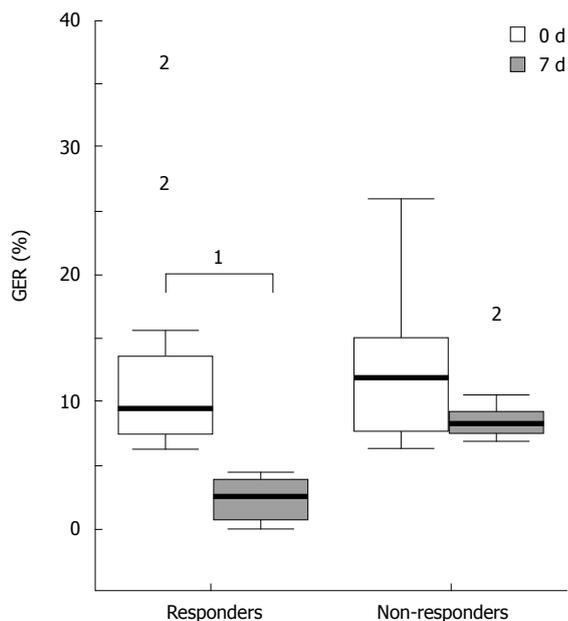
### Findings of pH monitoring

All variables from esophageal pH-metry are listed in Table 2. GER (%) values were similar in both groups at baseline, but were significantly reduced in the RESP group compared with the N-RESP group at the 7th post-PEG day (P < 0.001, Figure 1). The median (range) number of reflux episodes > 5 min and the number of reflux episodes/h decreased significantly at the 7th post-PEG day in the RESP group (P < 0.001) compared to baseline, but not in the N-RESP group (NS). Both variables were significantly higher at baseline in the RESP group compared with in the N-RESP group [7 (4-9) *vs* 3.5 (2-6), P = 0.045 and 8 (3-10) *vs* 2.9 (2-4.4), P = 0.020, respectively].

### Endoscopic findings

Reflux esophagitis assessed at PEG tube placement was observed in 100% of the study population (Table 3). There was a significant difference between the two groups: grade A:B:C:D = 11:6:0:0 in the RESP group (n = 17) *vs* 4:4:4:0 in the N-RESP group (n = 12), P = 0.031, respectively. The presence of reflux esophagitis correlated with GEFV grading ( $r_s = 0.465$ , P = 0.011). Hiatus hernia was not observed in any of the patients studied.

The GEFV appearance differed significantly between the two groups (P = 0.020, Table 3). Ten patients in the



**Figure 1** The median (interquartiles) variation in GER-% using two successive pH-metry readings performed in the responders (RESP) and non-responders (N-RESP) to PEG patients. <sup>1</sup>Denotes statistical significance; <sup>2</sup>Denotes outlier cases.

RESP group and 2 in the N-RESP group were evaluated as normal GEFV, while 7 and 10 patients, respectively, were evaluated as abnormal GEFV.

### Histologic findings

The histologic results are summarized in Table 3. The histology of esophageal biopsies was normal in 10.3% (2/17 and 1/12 in the RESP and N-RESP groups, respectively). There was no significant difference between the two groups: grade 0:1:2:3 = 2:8:6:1 in the RESP group ( $n = 17$ ) vs 1:3:5:3 in the N-RESP ( $n = 12$ ), respectively,  $P = \text{NS}$ . Histologic grading showed a good correlation with endoscopic grading ( $r_s = 0.705$ ,  $P = 0.000$ ).

Weaning from mechanical ventilation was achieved in 13/17 (76.4%) of responders within 10 (6.5-14) d, whereas 7/12 (58%) of non-responders required 13 (12-20) d (NS). Three patients in the RESP group (17.6%) and five in the N-RESP group (41.6%) died while in the ICU (NS). The deaths were unrelated to the PEG tube procedure.

## DISCUSSION

The results of this study confirm previous findings that PEG tube placement can eliminate GER in mechanically ventilated patients<sup>[13]</sup>. Our findings also provided evidence that the presence of severe reflux esophagitis and the gastro-esophageal flap valve grade are factors that predict failure of PEG to control GER in these patients.

The mechanisms that cause GER in mechanically ventilated patients differ from conscious patients<sup>[11]</sup>. Transient lower esophageal sphincter relaxation (TLESR) is believed to be the major mechanism of acid reflux in awake patients, with a defective basal LES pressure caused by hiatus hernia and straining associated with

increased abdominal pressure being contributing factors<sup>[18,19]</sup>. In contrast, the absent or very low basal LES pressure induced by mechanical ventilation, opiates, endotoxemia-related sepsis and nasogastric tube are the major causes of reflux in critically ill patients<sup>[1,2,7,20]</sup>. The latter is considered to be a key factor for both the development and the degree of GER<sup>[21]</sup>. In several studies, 48%-60% of ICU patients were found to have erosive esophageal lesions induced by GER, 3 or 5 d after NGT placement<sup>[2,22]</sup>. Furthermore, the degree of GER correlates with the duration of NGT. In an earlier study, we showed a positive correlation between the duration of nasogastric intubation and the degree of GER<sup>[13]</sup>. These findings were also validated in the current study, which showed 100% incidence of GER in patients with long-standing NGT.

Few data exist in the literature regarding the influence of PEG tube placement on GER in mechanically ventilated adult patients. We have reported the effectiveness of PEG in decreasing GER (%) by more than 60%, compared to baseline, at the 7th post-PEG day in 16 critically ill patients<sup>[13]</sup>. Our data are in agreement with previous findings and show a significant reduction in GER% at the 7th post-PEG day in 17/29 (58.6%) of the studied population. Similarly, both the number of reflux episodes/h and those lasting > 5 min were significantly reduced in the RESP group. However, this was not the case for the N-RESP group, although these variables were significantly increased at baseline in the former group of patients (Table 2). Overall, PEG tube placement improved (or at least did not deteriorate) both GER (%) and the two indices tested for gastroesophageal reflux. This is in agreement with previous findings which demonstrated that GER is not a contraindication to PEG<sup>[23]</sup>.

Reflux esophagitis occurs in approximately 25%-30% of critically ill patients undergoing endoscopy and GER is considered a major cause<sup>[1,7]</sup>. However, not all patients with gastroesophageal reflux have esophagitis. In this study, reflux esophagitis assessed by endoscopy, was present in 100% of the study population and showed a good correlation with GER ( $r_s = 0.465$ ,  $P = 0.011$ ). The increased incidence of reflux esophagitis and GER (%) in this study could be attributed to prolonged NGT placement [mean 29.5 (25-38) and 45 (25-53) d for the RESP and N-RESP groups, respectively] and to the removal of proton pump inhibitors 7 d prior to the study. It is well known that these agents bind with and inactivate the gastric parietal cell proton pump ( $\text{H}^+/\text{K}^+$ -ATPase) which, in turn, inhibits gastric acid production and raises gastric pH<sup>[24]</sup>.

Grading of GEFV differed significantly between the two groups ( $P = 0.020$ ). Approximately 83% of subjects with abnormal GEFV (grades III and IV) were in the N-RESP group vs 41% of the RESP group, suggesting that endoscopic grading of GEFV pre-PEG provides useful information for predicting failure of PEG to reduce GER.

Heikinen *et al*<sup>[25]</sup>, have demonstrated a poor valid-

ity between the severity of histologic evidence of esophagitis and significant GER. Our data are in keeping with this concept and showed no significant correlation between pre-PEG histological evaluation and the development of GER post-PEG. That is, evaluation of esophageal histology pre-PEG does not reliably predict the development of reflux after PEG placement.

Our study has some strengths and limitations. The strengths include the prospective design and the follow-up pH-metry on the 7th post-PEG day. In contrast, the study is limited due to the small sample size, so data should be extrapolated with caution.

In conclusion, the results of this study indicate that the endoscopic grading of GEFV and the presence of severe reflux esophagitis graded LA classification C or D are predisposing factors of failure of PEG to reduce GER in mechanically ventilated patients. We also provided evidence that gastroesophageal reflux is not a contraindication for PEG tube placement. The latter seems to eliminate or at least not exacerbate GER in these patients.

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## COMMENTS

### Background

Gastroesophageal reflux (GER) is a common problem in mechanically ventilated patients and contributes to the development of esophageal mucosal injury and even erosive esophagitis.

### Research frontiers

One of the main factors that have been implicated in the pathogenesis of GER in these patients is the placement and duration of a nasogastric tube (NGT). NGT may lead to GER through relaxation of the lower esophageal sphincter (LES) pressure. The authors have previously shown that percutaneous endoscopic gastrostomy (PEG) can eliminate GER by disengaging patients from the presence of NGT. In this study, the aforementioned findings are further confirmed and the authors identified factors, including the presence of severe reflux esophagitis and gastroesophageal flap valve grade that predict failure of PEG to eliminate GER.

### Innovations and breakthroughs

The presence of NGT and mechanical ventilation are factors predisposing to the development of GER. However, few data exist regarding the effect of PEG tube placement in the prevention of GER in mechanically ventilated adult patients. In the present study the authors identified factors which could predict PEG failure to reduce GER.

### Applications

By identifying the factors that predict failure of PEG to decrease GER, this study may represent a reference in deciding which patients are likely to benefit from PEG tube placement and thus protect them from the development of esophagitis and even ventilator-associated pneumonia (VAP).

### Terminology

Percutaneous endoscopic gastrostomy: Percutaneous endoscopic gastrostomy (PEG) is a procedure in which a feeding tube is passed through the abdominal wall directly into the stomach under endoscopic guidance, enabling nutrients, medication and fluids to be delivered directly into the stomach; Gastroesophageal reflux: GER is defined as the movement of stomach contents (solids or liquids) into the esophagus, and is caused mainly by dysfunction of the lower esophageal sphincter.

### Peer review

This is an interesting prospective study that provides valuable information re-

garding factors that predict failure of PEG to eliminate GER. The results are interesting and suggest that severe reflux esophagitis and the gastro-esophageal flap valve grade are the main contributing factors.

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## Manometric findings in patients with isolated distal gastroesophageal reflux

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### Abstract

**AIM:** To analyze manometric abnormalities in patients with isolated distal reflux and compare these findings in patients with erosive and non-erosive disease.

**METHODS:** Five hundred and fifty patients who presented to the outpatient clinic of Turkiye Yuksek Ihtisas Hospital with gastroesophageal reflux disease-like symptoms were enrolled. Each individual was evaluated with esophageal manometry, 24-h ambulatory pH monitoring, and upper gastrointestinal endoscopy. Manometric findings for the patients with isolated distal reflux were compared to findings in controls who were free of reflux disorders or hypersensitive esophagus. Findings for isolated distal reflux patients with and without erosive reflux disease were also compared.

**RESULTS:** Of the 550 subjects enrolled, 97 (17.6%, mean age 48 years) had isolated distal reflux and 100 had no abnormalities on ambulatory pH monitoring (control group, mean age 45 years). There were no significant differences between the isolated distal reflux group and control group with respect to age, body mass index, and esophageal body contraction amplitude (EBCA). Mean lower esophageal sphincter pressure was significantly higher in the control group ( $12.7 \pm 10.3$  mmHg vs  $9.6 \pm 7.4$  mmHg,  $P = 0.01$ ). Fifty-five (56.7%) of the 97 patients with isolated distal reflux had erosive reflux disease. There were no statistical differences between the erosive reflux disease and non-erosive reflux disease subgroups with respect to mean EBCA, lower esophageal sphincter pressure, or DeMeester score.

However, 13% of patients with gastroesophageal reflux disease had distal wave amplitudes  $\leq 30$  mmHg, whereas none of the patients with non-erosive reflux disease had distal wave amplitudes in this low category.

**CONCLUSION:** Patients with erosive and non-erosive disease present with similar manometric abnormalities. The only striking difference is the observation of very low EBCA exclusively in patients with erosive disease.

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**Key words:** Esophageal motility disorders; Isolated distal reflux; Gastroesophageal reflux disease; Manometry; Esophagitis

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### INTRODUCTION

Gastroesophageal reflux disease (GERD) is defined as the pathological retrograde movement of gastric contents into the esophagus. Patients with GERD are typically categorized into one of 3 groups: those without esophagitis [suffering from non-erosive reflux disease (NERD)]; those with esophagitis [suffering from erosive reflux disease (ERD)], and those with complicated forms of GERD<sup>[1]</sup>.

Non-erosive reflux disease is the most common presentation of GERD<sup>[2]</sup>. Peristaltic dysfunction of the esophagus is well documented in cases of GERD<sup>[3,4]</sup>. The main esophageal motility disorder in these patients is ineffective esophageal motility (IEM)<sup>[5]</sup>.

Gastroesophageal reflux can be classified as isolated proximal reflux (IPR), isolated distal reflux (IDR), or both proximal and distal reflux as determined by ambulatory pH monitoring. Whereas increased acid clearance time and IEM are strongly associated with IPR<sup>[6]</sup>, there are no data

that conclusively link motility disorders with IDR. Our aim in this study was to investigate manometric measurements in patients with IDR and compare the findings in individuals with and without erosive esophagitis.

## MATERIALS AND METHODS

Between January 2000 and July 2002, 550 patients with GERD-like symptoms were screened for the study. These individuals had typical GERD symptoms (acid regurgitation and heartburn) or extraesophageal symptoms (hoarseness, asthma-like clinical presentation, nocturnal cough, and nocturnal waking). They were evaluated at the outpatient clinic of Turkiye Yuksek Ihtisas Hospital's Gastroenterology Unit, which is a tertiary referral center. Demographic characteristics and body mass index (BMI) were recorded for each patient.

All subjects were referred to our motility unit for 24-h pH monitoring and manometric studies. Each was assigned to one of 5 groups according to the pH results: (1) those with IDR; (2) those with IPR; (3) those with both proximal and distal reflux; (4) those with hypersensitive esophagus; (5) those with normal findings.

All patients underwent gastrointestinal endoscopy. Individuals with GERD whose esophageal mucosa appeared normal on esophagogastroduodenoscopy were considered to have NERD. Those with varying degrees of esophagitis were considered to have ERD.

Patients were excluded if they had Barrett's esophagus, hiatal hernia, esophageal varices, connective tissue disease, primary esophagus disease, or had undergone endoscopic therapy. Informed consent was obtained from each participant and the study was approved by our hospital's ethics committee. All patients underwent esophagogastroduodenoscopy, esophageal manometry, and ambulatory 24-h pH monitoring as described below.

Esophagogastroduodenoscopy was performed after a 10-h fast using an Olympus GIF XQ endoscope (Tokyo, Japan). Local anesthesia was administered (1% xylocaine) and the esophagus, stomach and duodenum were evaluated. During endoscopy, special attention was paid to the distal esophageal mucosa. Patients with esophagitis were classified according to the Los Angeles classification<sup>[7]</sup>: Grade A, mucosal break < 5 mm in length; grade B, mucosal break > 5 mm; grade C, mucosal break continuous between > 2 mucosal folds; grade D, mucosal break > 75% of the esophageal circumference.

Esophageal manometry was carried out using a Medical Measurement Systems (MMS) unit (ver. 8.4i Beta) and an 8-channel Dent-sleeve catheter. Lower esophageal sphincter pressure (LESP) and esophageal body contraction amplitude (EBCA) were recorded. Based on these findings, patients were categorized as normal, hypotensive lower esophageal sphincter, or IEM<sup>[8]</sup>.

Ambulatory 24-h pH monitoring was performed using a Synetics Digrapper MHIII machine and double-channel, 15 cm antimony catheter. Findings were recorded and analyzed using Microsoft esophagram version 2.04. Based on the results, patients were categorized as normal, IDR, IPR, both proximal and distal reflux, or hypersensitive esophagus<sup>[9-12]</sup>. Hypersensitive esophagus

was defined if the symptom index for distal measurements (symptom index = number of symptoms at pH < 4 / total number of symptoms) was  $\geq 50\%$  while there was no measurable distal or proximal reflux.

Acid reflux was defined as a fall in esophageal pH below 4. All standard parameters (DeMeester score, percentage of time below pH 4, number of reflux episodes and number of long reflux episodes) were determined throughout the study period<sup>[13]</sup>.

A control group was established based on the combined results from the above battery of tests. Individuals who were free of abnormal esophageal conditions (distal or proximal esophageal reflux, or hypersensitive esophagus) comprised this group. Subjects were asked not to take antacids, H<sub>2</sub> blockers, prokinetic agents, or proton-pump inhibitors throughout the duration of the study. They were also directed not to consume acidic foods or foods containing bicarbonate during the study. During ambulatory pH monitoring, patients continued their normal daily routines. Throughout the 24 h of monitoring, individuals recorded their meal times, sleep periods and times of onset of heartburn complaints.

## Statistical analysis

All values are expressed as mean  $\pm$  SD. Comparisons between the study and control groups were made using the  $\chi^2$  test. The Student's *t* test was used to compare continuous variables between groups. *P*-values < 0.05 were considered significant.

## RESULTS

Of the 550 patients initially screened, 241 (43.8%) had combined proximal and distal esophageal acid reflux, 97 (17.6%) had IDR, 70 (12.7%) had IPR, 42 (7.6%) had hypersensitive esophagus, and 100 (18.2%) were free of these conditions. The latter 100 became the control group, with 42 women and 58 men of mean age  $44.9 \pm 12.8$  years. The 97 patients in the IDR group formed the study group, with 47 women and 50 men of mean age  $47.8 \pm 13.4$  years.

There were no significant differences between the IDR and control group means for age, BMI, and EBCA. However, mean LESP was significantly lower in the IDR group than in the control group ( $9.6 \pm 7.4$  mmHg *vs*  $12.7 \pm 10.3$  mmHg, *P* = 0.01) (Table 1).

Of the 97 patients with IDR, 42 (43.3%) had NERD and 55 (56.7%) had ERD. Of the 55 patients with ERD, 20 (36.4%) had grade A esophagitis, 18 (32.7%) had grade B esophagitis, 12 (21.8%) had grade C esophagitis, and 5 (9.1%) had Grade D esophagitis. In the ERD subgroup, mean age was  $45 \pm 13$  years, mean BMI was  $26.8 \pm 3.4$  kg/m<sup>2</sup>, mean DeMeester score was  $42.9 \pm 27.2$ , mean LESP was  $9.2 \pm 6.5$  mmHg, and mean EBCA was  $70.9 \pm 50.9$  mmHg (Table 2).

Patients in the NERD subgroup tended to be older than those in the ERD subgroup ( $51 \pm 13$  years *vs*  $45 \pm 13$  years, *P* = 0.03). There was no significant difference between the mean DeMeester scores for these 2 groups (Table 2). All 42 patients with NERD had EBCA > 30 mmHg,

**Table 1** Comparison of 24-h pH monitoring and esophageal manometry findings in the IDR group and control group

	IDR group <i>n</i> = 97	Control group <i>n</i> = 100	<i>P</i> -value
Age (yr)	47.8 ± 13.4	44.9 ± 12.8	0.134
Sex (F/M)	47/50	42/58	0.512
BMI (kg/m <sup>2</sup> )	26.6 ± 3.8	26.2 ± 3.8	0.437
EBCA (mmHg)	73.6 ± 44.4	77.6 ± 41.0	0.507
LESP (mmHg)	9.6 ± 7.4	12.7 ± 10.3	0.019
DeMeester score	42.6 ± 24.3	6.2 ± 4.8	0.000

IDR: Isolated distal reflux; BMI: Body mass index; EBCA: Esophageal body contraction amplitude; LESP: Lower esophageal sphincter pressure.

whereas 7 (12.7%) of the 55 patients with ERD had EBCA ≤ 30 mmHg. This difference was statistically significant (*P* = 0.01). In all cases where EBCA was less ≤ 30 mmHg, the patient had severe esophagitis (Grade C or D).

## DISCUSSION

Gastroesophageal reflux refers to retrograde passage of gastric contents into the esophagus. This movement of material is not considered pathologic until symptoms or mucosal damage occur, but at that stage the condition is termed GERD. The most important mechanisms and phenomena that protect the esophagus from gastric reflux are esophageal peristalsis, salivary pH, and gravity. Several well-characterized abnormalities of LESP and esophageal peristalsis are known to increase gastroesophageal reflux and acid-induced mucosal damage<sup>[14]</sup>.

The most common esophageal motor disorder in patients with GERD is IEM, and 20%-50% of patients with GERD are affected<sup>[15]</sup>. Ineffective esophageal motility is defined as esophageal contractions of amplitude < 30 mmHg and/or a 30% or higher rate of nontransmission of wet swallows to the distal esophagus<sup>[6,8]</sup>.

IEM is also the most prevalent motility abnormality in patients with IPR and GERD-associated respiratory symptoms<sup>[16]</sup>. However, the relationship between IDR and IEM is not clear. In our study, we focused specifically on manometric findings in patients with IDR.

We detected no statistical differences between our IDR group and control group with respect to age, BMI or EBCA. However, we did note significantly lower mean LESP for the IDR patients. This finding is in accordance with other studies that have documented motility abnormalities in patients with GERD<sup>[3,17]</sup>.

The presence of erosive disease in the esophagus is another factor that is thought to promote esophageal motility disorders in patients with GERD<sup>[18]</sup>. Impairment of esophageal body contraction, as manifested by reduced contraction amplitude and aperistalsis, is a frequent finding in patients with ERD. Twenty percent of individuals with moderate esophagitis and 50% of patients with severe esophagitis show aperistalsis and hypotensive contractions<sup>[19]</sup>.

Somani *et al*<sup>[17]</sup> analyzed manometric findings in 47 patients with GERD. They found that distal esophageal contraction amplitude was lower in cases of severe

**Table 2** Comparison of parameters for the ERD and NERD subgroups of IDR patients

	ERD ( <i>n</i> = 55)	NERD ( <i>n</i> = 42)	<i>P</i> -value
Age (yr)	45.2 ± 13.3	51.2 ± 13.0	0.032
Sex (F/M)	25/30	22/20	0.511
BMI (kg/m <sup>2</sup> )	26.8 ± 3.4	26.3 ± 4.2	0.485
EBCA (mmHg)	70.9 ± 50.9	77.1 ± 34.5	0.497
LESP (mmHg)	9.2 ± 6.5	10.2 ± 8.6	0.526
DeMeester score	42.9 ± 27.2	42.1 ± 20.1	0.876

ERD: Erosive reflux disease; NERD: Non-erosive reflux disease.

esophagitis than in cases of mild esophagitis (*P* = 0.001). Frazzoni *et al*<sup>[20]</sup> analyzed esophageal manometric findings in 88 patients with NERD, 76 with ERD, and 56 with complicated esophagitis. They found that mean EBCA was significantly lower in the complicated esophagitis and ERD groups than in the NERD group, but observed no significant difference between the 3 groups with respect to mean LESP. In contrast with these results, Lemme *et al*<sup>[21]</sup> assessed 70 patients with ERD and 40 patients with NERD using esophageal manometry and detected no statistical differences between these groups with respect to mean numbers of low amplitude, non-transmitted, and normal waves. The authors suggested that IEM alone is unlikely to be the major determinant of abnormal esophageal acid exposure, and that it is not a prerequisite for development of esophagitis. Similarly, Martinek *et al*<sup>[22]</sup> evaluated 111 patients with NERD, 77 patients with mild to moderate ERD, 33 patients with severe esophagitis, and 92 individuals with no evidence of gastroesophageal reflux using esophageal manometry and pH monitoring. They found no significant differences between NERD and ERD groups with respect to mean LESP or frequency of IEM. Similar proportions of patients in each group had low LESP and hiatus hernia. Martinek *et al*<sup>[22]</sup> suggested that a variety of other factors, including genetics, mucosal defense, and acid clearance, may influence patients' susceptibility to developing ERD. Ho *et al*<sup>[5]</sup> observed that patients with IEM were no more likely to have endoscopic evidence of esophagitis than individuals with normal manometry findings. They concluded that esophageal injury is not always associated with IEM.

All of the above-mentioned studies included patients with GERD, but IDR and IPR patients were not analyzed as separate groups in these studies. These 2 conditions may feature different manometric characteristics, and this might explain the contradictory results found in these studies. To our knowledge, no study to date has analyzed manometric data from patients with IDR alone. We focused solely on this patient group and detected no significant differences between the ERD and NERD subgroups with respect to mean EBCA or mean LESP. However, 12.7% of our patients with GERD exhibited EBCA ≤ 30 mmHg, whereas none of those with NERD had distal wave amplitudes in this category. In all cases where EBCA was ≤ 30 mmHg, the patient had severe esophagitis.

Various abnormalities of esophageal motor function

are observed in patients with GERD. Our data suggest that, among patients with IDR, those with NERD and those with ERD exhibit similar types and severity of esophageal motility disorders. The only striking difference between these 2 patient subgroups is that individuals with ERD have a significantly higher frequency of very low EBCA, and this is limited to patients with severe esophagitis.

## COMMENTS

### Background

Gastroesophageal reflux disease (GERD) is defined as the pathological retrograde movement of gastric contents into the esophagus. Various esophageal motility disturbances which may be important in reflux are observed in patients with GERD. The main esophageal motility disorder in these patients is ineffective esophageal motility (IEM). Increased acid clearance time and IEM have been shown to be strongly associated with isolated proximal reflux. However, there are no data that conclusively link motility disorders with isolated distal reflux.

### Research frontiers

In the present study, patients with symptoms of GERD were evaluated with esophageal manometry, 24-h ambulatory pH monitoring, and upper gastrointestinal endoscopy. The manometric findings of patients with isolated distal reflux were compared with those who did not have pathological reflux. Among patients with isolated distal reflux, the manometric findings of patients who had erosive disease and non-erosive disease were also compared. There were no significant differences between the isolated distal reflux group and control group with respect to age, body mass index, and esophageal body contraction amplitude. Mean lower esophageal sphincter pressure was significantly higher in the control group. There were no differences between the erosive reflux disease and non-erosive reflux disease subgroups with respect to mean esophageal body contraction amplitude (EBCA), lower esophageal sphincter pressure, or DeMeester score. However, IEM was observed only in patients with erosive reflux disease.

### Innovations and breakthroughs

In this study, the authors evaluated the manometric findings in a homogenous group of patients with isolated distal reflux. In this context, it is distinct from other related studies, since manometric findings have not been thoroughly analyzed in this special patient population. They also compared these findings in patients with erosive and non-erosive disease which has not been done previously.

### Applications

This study, the authors believe, provides more insight into the pathophysiology of reflux disease. The finding of very low EBCA being observed only in patients with erosive disease might be helpful in identifying these patients.

### Terminology

NERD: Patients with this condition exhibit typical reflux symptoms caused by reflux of gastric contents into the esophagus, but have no visible esophageal mucosal injury. ERD: Patients have visible esophageal mucosal injury on endoscopy. IPR: The upper esophageal sphincter localization was determined by manometry and proximal reflux was determined by the proximal probe localization and upper esophageal sphincter. If the proximal probe was localized in the upper esophageal sphincter or above it, a single acid reflux synchronously occurring with distal probe was accepted as pathologic acid reflux; if the probe was localized under the upper esophageal sphincter, acid contact time > 1% of total time was accepted as pathologic in proximal reflux. IDR: De Meester score > 14.72 and acid contact > 4.0% of total time below pH 4 were accepted as pathologic in distal reflux.

### Peer review

The study addresses an important question, is well written, clear, and is accompanied by legible and clear tables. In addition, the results are well presented and the limitations of the study appropriately addressed. Appropriate controls were chosen for the study.

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## Association between *Helicobacter pylori* seropositivity and digestive tract cancers

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seropositivity was determined by an enzyme linked immunosorbent assay method against *H pylori* IgG.

**RESULTS:** Presence of *H pylori* infection was significantly inversely associated with esophageal SCC [adjusted odds ratio (AOR): 0.315-0.472, all *P*-value < 0.05] but positively associated with gastric adenocarcinoma (both cardia and non-cardia) (AOR: 1.636-3.060, all *P*-value < 0.05) in comparison to the three control groups. Similar results were not found in cancers of the oral cavity and colon.

**CONCLUSION:** Our findings support the finding that *H pylori* seropositivity is inversely associated with esophageal SCC risk, but increases the risk of gastric cardia adenocarcinoma.

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**Key words:** *Helicobacter pylori* infection; Oral cancer; Esophageal squamous cell carcinoma; Gastric cardia adenocarcinoma; Colon cancer

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Wu IC, Wu DC, Yu FJ, Wang JY, Kuo CH, Yang SF, Wang CL, Wu MT. Association between *Helicobacter pylori* seropositivity and digestive tract cancers. *World J Gastroenterol* 2009; 15(43): 5465-5471 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5465.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5465>

### Abstract

**AIM:** To explore the role of *Helicobacter pylori* (*H pylori*) infection on the risk of digestive tract cancers.

**METHODS:** In total, 199 oral squamous-cell carcinoma (SCC), 317 esophageal SCC, 196 gastric cardia and non-cardia adenocarcinoma and 240 colon adenocarcinoma patients were recruited for serum tests of *H pylori* infection. Two hospital- and one community-based control groups were used for the comparisons. *H pylori*

### INTRODUCTION

It is widely accepted that chronic *Helicobacter pylori* (*H pylori*) infection, especially cytotoxin-associated gene A (CagA)-positive strains, causes non-cardiac gastric carcinoma via the intestinal metaplasia-carcinoma sequence<sup>[1,2]</sup>. However, there is debate in regard to the association between *H pylori* infection and gastric cardia adenocarcinoma. Most studies in Asian countries<sup>[3-5]</sup> have indicated an increased risk of cardia cancer in *H pylori*-infected subjects, while those in Western countries have reported a protective role

of *H pylori*<sup>[6]</sup> or a negative association<sup>[7-9]</sup> between these two.

Recently, cumulative evidence has indicated that *H pylori* infection plays a protective role in the development of reflux esophagitis<sup>[10,11]</sup> and esophageal adenocarcinoma (EA)<sup>[6,8,12,13]</sup>. In contrast, the relationship between *H pylori* infection and esophageal squamous cell carcinoma (SCC) is still inconclusive<sup>[8,14-17]</sup>. Very few studies have examined the role of *H pylori* infection on colon and oral cancers. Therefore, we aim to expand our esophageal SCC cases and to examine the relationship between *H pylori* infection and the risk of oral SCC, gastric cancer, especially gastric cardia adenocarcinoma and colon cancer, in a Taiwanese population.

## MATERIALS AND METHODS

### Selection of cases and controls

This was a hospital-based case-control study design. Three hundred and seventeen newly pathologically proven ESCC patients (301 males and 16 females) were recruited from two medical centers in the Kaohsiung metropolitan area in southern Taiwan, including the Kaohsiung Medical University Hospital (KMUH) and the Kaohsiung Veterans General Hospital (KVGH) between 2000 and 2007. The detailed information was described elsewhere<sup>[18]</sup>. Patients with newly pathologically proven oral SCC ( $n = 199$ ), gastric ( $n = 196$ ) and colon adenocarcinoma ( $n = 240$ ) were recruited from the KMUH in the same period.

The first hospital-based control group was recruited from the Department of Preventive Medicine in these two hospitals and matched with each esophageal SCC patient (case:control = 1:1-4) by gender, age (within a 4 year age difference) and hospitalization (within 4 wk after each case was identified). In this study, we randomly selected 1 out of 1-4 matched controls ( $n = 305$ , 289 males and 16 females) for each case to investigate the status of *H pylori* infection<sup>[19]</sup>. Twelve cases did not have suitable controls. In order to examine the different prevalence of *H pylori* infection from different areas or different sources of selection, we included another two comparison groups, defined as the second hospital-based control group and the community-based control group, in this study. The second hospital-based control group ( $n = 403$ , 374 males and 29 females) were originally used for the esophageal cancer study in National Taiwan University Hospital (NTUH) in Taipei<sup>[20]</sup>. They were cancer-free, had visited for a health check-up in NTUH, and had enough stored serum specimens for testing *H pylori* status during the same period of this study. The community-based control group ( $n = 395$ , 204 males and 191 females) consisted of healthy subjects living in Kaohsiung who voluntarily participated in one large multi-year study of childhood neoplasms at the same period of this study<sup>[14,21]</sup>. The second hospital-based and the community-based control groups were not matched by age and gender with our esophageal SCC patients.

All subjects provided blood samples before any treatment or blood transfusion for *H pylori* seropositivity. For those with positive *H pylori* antibodies, CagA status was further examined using an enzyme immunoassay. The institutional review boards at NTUH and KMUH reviewed and approved this study, and all participants signed written sheets of informed consent.

### Location of esophageal SCC and gastric adenocarcinoma

Lesions were classified with respect to their location in the upper, middle or lower third of the esophagus, described previously<sup>[22,23]</sup>. Gastric cardia adenocarcinomas were defined as a tumor centered within 3 cm distal to the gastroesophageal junction. There was no difficulty in distinguishing lower third esophageal cancer with cardia invasion from gastric cardia cancer extending to the lower esophagus, because SCC comprise more than 95% of esophageal cancers in Taiwan<sup>[24]</sup>. Cardia adenocarcinoma was classified as being of gastric origin, if Barrett's esophagus was not detected in the adjacent tissue.

### *H pylori* serology and CagA status

Non-heparinized whole blood was collected and serum was isolated and stored at  $-70^{\circ}\text{C}$  for subsequent analysis. A commercially available immunochromatographic screening test (Chembio *H pylori* STAT-PAK, Chembio Diagnostic System, Inc., Medford, NY, USA) utilizing a combination of *H pylori* antigen coated particles and anti-human IgG was used to qualitatively and selectively detect *H pylori* antibodies in serum<sup>[14]</sup>. According to the manufacturer's instructions, the sensitivity and specificity of STAT-PAK were 94% and 98%, respectively. Final readings were recorded after verification by at least two research technicians blinded to the status of cases and controls.

A commercial kit, Helori CTX IgG (ravo Diagnostika GmbH, Freiburg, Germany), was used to detect the IgG antibodies against serum CagA by enzyme immunoassay. The average optical density ( $A$ ) values of subject specimens, blank, negative and positive controls were calculated. The  $A$  of negative and positive controls must be always  $< 0.100$  and  $> 0.800$ , respectively, in each set of samples. The concentration of each sample was expressed as units obtained from the formula: sample  $A \times$  calibrator value (units)/calibrator  $A$ . Based on guidelines set forth by the manufacturer, we set negative and positive cutoff points for each concentration as  $< 5$  and  $\geq 5$  units, respectively. The research technicians were also blinded to the status of cases and controls.

### Statistical analysis

Multiple logistic regression models were used to examine the association between *H pylori* infection (seropositivity by STAT-PAK) and esophageal SCC risk using each of three control groups as a basis of comparison before and after adjusting for other covariates, including age (continuous), gender, educational levels ( $\leq$  primary school, junior high and high school, and  $>$  high school), cigarette

Table 1 Demographic characteristics of digestive tract cancer patients and controls *n* (%)

	Cancer				Control		
	Oral SCC	Esophageal SCC	Gastric adenocarcinoma	Colon adenocarcinoma	1st hospital-based control	2nd hospital-based control	Community-based control
<i>n</i>	199	317	196	240	305	403	395
Age mean ± SD (min, med, max)	50.6 ± 9.9 (26, 50, 78)	58.3 ± 11.6 (34, 58, 86)	63.2 ± 13.5 (22, 65, 91)	64.5 ± 12.7 (24, 66, 89)	57.1 ± 11.4 (34, 57, 82)	60.0 ± 10.2 (39, 60, 84)	40.2 ± 6.2 (30, 40, 58)
Sex							
Male	188 (90.5)	301 (95.0)	123 (62.8)	139 (57.9)	289 (94.8)	374 (92.8)	204 (51.6)
Female	11 (5.5)	16 (5.0)	73 (37.2)	101 (42.1)	16 (5.2)	29 (7.2)	191 (48.4)
Educational level							
≤ primary school	117 (58.8)	176 (55.5)	118 (60.2)	133 (55.4)	78 (25.6)	162 (40.2)	41 (10.4)
Junior high & high school	76 (38.2)	120 (37.9)	53 (27.0)	72 (30.0)	93 (30.5)	76 (18.9)	241 (61.0)
> high school	6 (3.0)	21 (6.6)	25 (12.8)	35 (14.6)	134 (43.9)	165 (40.9)	113 (28.6)
Cigarette smoking							
No	32 (16.1)	39 (12.3)	101 (51.5)	143 (59.6)	168 (55.1)	248 (61.5)	283 (71.6)
Yes	167 (83.9)	278 (87.7)	95 (48.5)	97 (40.4)	137 (44.9)	155 (38.5)	112 (28.4)
Alcohol consumption							
No	64 (32.2)	60 (18.9)	145 (74.0)	193 (80.4)	215 (70.5)	288 (71.5)	298 (75.4)
Yes	135 (67.8)	257 (81.1)	51 (26.0)	47 (19.6)	90 (29.5)	115 (28.5)	97 (24.6)
Betel quid chewing							
No	29 (14.6)	149 (47.0)	171 (87.2)	213 (88.8)	280 (91.8)	381 (94.5)	-
Yes	170 (85.4)	168 (53.0)	25 (12.8)	27 (11.2)	25 (8.2)	22 (5.5)	-

SCC: Squamous cell carcinoma.

Table 2 Serum status of *H pylori* and CagA in cancer patients and control *n* (%)

	Cancer				Control			
	Oral SCC	Esophageal SCC	Gastric cardia adenocarcinoma	Gastric non-cardia adenocarcinoma	Colon adenocarcinoma	1st hospital-based control	2nd hospital-based control	Community-based control
<i>n</i>	199	317	29	167	240	305	403	395
<i>H pylori</i>								
-	104 (52.3)	205 (64.7)	8 (27.6)	48 (28.7)	105 (43.8)	141 (46.2)	164 (40.7)	235 (59.5)
+	95 (47.7)	112 (35.3)	21 (72.4)	119 (71.3)	135 (56.3)	164 (53.8)	239 (59.3)	160 (40.5)
CagA <sup>1</sup>								
-	14 (14.7)	8 (7.3)	1 (4.8)	9 (7.6)	12 (8.9)	24 (14.6)	-	11 (6.9)
±	13 (13.7)	10 (9.2)	3 (14.3)	8 (6.8)	7 (5.2)	14 (8.5)	-	6 (3.8)
+	68 (71.6)	91 (83.5)	17 (81.0)	101 (85.6)	116 (85.9)	126 (76.8)	-	142 (89.0)

<sup>1</sup>CagA status was determined among the subjects with *H pylori* seropositivity (No CagA data was available in 3 esophageal SCC, 1 gastric non-cardia adenocarcinoma and 1 community-based control).

smoking, alcohol drinking and betel nut chewing. Information about betel nut chewing was not available in the community control, so it was not evaluated in the regression models when the community control was used as the control group. The data were analyzed using the SAS statistical package; all *P*-values were two-sided.

## RESULTS

Distribution of demographic variables and substance use for cancer cases and controls is summarized in Table 1. The majority of oral and esophageal SCC patients were male, and more than half of all cancer patients had an educational level of primary school or below. The ratio of women to men among the esophageal SCC was 3.5% (6/173) in KVGH, which is slightly lower, but not significantly different, than that in KMHU (6.9%, 10/144, *P*-value = 0.18). The mean age of the community con-

trol group (40.2 ± 6.2 years) was about 20 years younger than the other two control groups.

The *H pylori* seropositivity in esophageal SCC patients was 35.3%, which was consistently lower than those in the three controls (40.5%-59.3%) (Table 2). The seropositivity of *H pylori* among the ESCC patients was similar in those two hospitals: 36.1% (KMHU 53/147) and 34.7% (KVGH 59/170) (data not shown). In contrast, *H pylori* seropositivity in gastric adenocarcinoma patients (71.4%) was higher than that in all three control groups. *H pylori* seropositivity in gastric cardia and non-cardia adenocarcinoma patients was similar (Table 2). In addition, the prevalence of CagA-positive subjects among *H pylori*-infected cancer cases was 71.6%-85.9%, similar to that among *H pylori*-infected controls (76.8%-89.0%).

In multivariate analyses, there was still a significantly inverse association between *H pylori* seropositivity and esophageal SCC (AOR: 0.315-0.472, all *P*-values < 0.05).

Table 3 Association between *H pylori* infection and cancer risks

		Odds ratio (95% CI)	P-value	Adjusted odds ratio (95% CI) <sup>1</sup>	P-value
Oral SCC <i>vs</i>	1st hospital control	0.105 (0.787-1.553)	0.5640	0.961 (0.637-1.449)	0.8494
	2nd hospital control	0.627 (0.445-0.882)	0.0074	0.378 (0.190-0.751)	0.0055
	community control	1.342 (0.952-1.891)	0.0932	1.064 (0.622-1.820)	0.8211
Esophageal SCC <i>vs</i>	1st hospital control	0.470 (0.340-0.648)	< 0.0001	0.454 (0.290-0.710)	0.0005
	2nd hospital control	0.375 (0.277-0.508)	< 0.0001	0.315 (0.203-0.489)	< 0.0001
	community control	0.802 (0.591-1.089)	0.1581	0.472 (0.257-0.865)	0.0151
Gastric adenocarcinoma <i>vs</i>	1st hospital control	2.149 (1.465-3.152)	< 0.0001	2.015 (1.278-3.174)	0.0025
	2nd hospital control	1.715 (1.187-2.479)	0.0041	1.636 (1.071-2.501)	0.0228
	community control	3.672 (2.538-5.312)	< 0.0001	3.060 (1.650-5.674)	0.0004
Colon adenocarcinoma <i>vs</i>	1st hospital control	0.785 (0.549-1.123)	0.1857	0.595 (0.319-1.113)	0.1040
	2nd hospital control	0.882 (0.639-1.219)	0.4476	0.851 (0.581-1.245)	0.4055
	community control	1.888 (1.365-2.613)	0.0001	1.117 (0.612-2.039)	0.7177

<sup>1</sup>Compared to KMHU or NTUH controls: adjusting for age (continuous), gender (female *vs* male), educational levels ( $\leq$  primary school *vs* > high school; junior and high schools *vs* > high school), cigarette smoking (yes *vs* no), alcohol consumption (yes *vs* no) and betel nut chewing (yes *vs* no); Compared to community controls: Adjusting for the above factors except for betel nut chewing.

In the multivariate analyses of esophageal SCC and 1st hospital control, besides the significant protective effect of *H pylori*, we found that cigarette smoking (AOR = 2.608, 95% CI: 1.540-4.414), alcohol consumption (AOR = 5.078, 95% CI: 3.130-8.240), and betel nut chewing (AOR = 5.142, 95% CI: 2.916-9.065) are still the significant risk factors for esophageal SCC. However, we did not see any significant interaction between *H pylori* and any of those substances (tobacco, alcohol, and betel nut) in the multivariate analyses.

*H pylori* infection was positively related to gastric adenocarcinoma, including the cardia (AOR: 1.636-3.060; all *P*-values < 0.05), in comparison to all three control groups, after adjusting for other co-variables (Table 3). Oral SCC was found to have a significantly inverse association with *H pylori* infection (AOR = 0.378, 95% CI: 0.190-0.751), when compared with the 2nd hospital-based control group, but not the other two. *H pylori* infection was not significantly associated with colon cancer, compared with all three control groups.

## DISCUSSION

After expanding the case number of esophageal SCC patients, we still found an inverse association between *H pylori* seropositivity and esophageal SCC when compared to different control groups. Conflicting results were reported in the previous two prevalence studies about the relationship between the two in Sweden<sup>[8,17]</sup>. One nationwide case-control study conducted by Ye *et al*<sup>[8]</sup> used enzyme-linked immunosorbent assay (ELISA) to detect both serum *H pylori* and CagA antibodies in 85 esophageal SCC patients and 499 controls in Sweden. Initially, they did not find any significant association between *H pylori* seropositivity and esophageal SCC risk (AOR = 0.9, 95% CI: 0.5-1.6). Thus, the authors further categorized the study subjects based on both statuses of *H pylori* and CagA seropositivity. They found subjects with negative *H pylori* but positive CagA, to have a significant 3.0-fold risk of developing esophageal SCC, compared with those who were found to be negative for both<sup>[8]</sup>. But the insignificant find-

ing was noted in the group of both positive *H pylori* and CagA patients (AOR = 1.6, 95% CI: 0.8-3.3). That was a post-hoc statistical analysis, so the preliminary finding is a warranty for further confirmation. Siman *et al*<sup>[17]</sup> updated their nested case-control study and found neither *H pylori* (OR = 0.44, 95% CI: 0.15-1.2) nor CagA seropositivity (OR = 2.0, 95% CI: 0.24-infinity) was significantly associated with esophageal SCC risk (*n* = 37). Recently, two studies were conducted in two major endemic areas in China-Huaian City<sup>[15]</sup> and Linxian<sup>[16]</sup>. The population-based case-control study in Huaian (case number 107) showed those with positive findings for *H pylori* antibody to be at 3-fold risk of developing esophageal SCC (AOR = 3.19, 95% CI: 1.11-9.15)<sup>[15]</sup>. But, in the case-cohort study including 335 ESCC patients in Linxian, no association was found between esophageal SCC risk and positive finding for antibodies to either *H pylori* (Hazard ratio = 1.17, 95% CI: 0.88-1.57) or CagA (Hazard ratio = 1.08, 95% CI: 0.80-1.47)<sup>[16]</sup>. In these two areas, the predominant risk factors for ESCC were diet and nutrition, instead of cigarette smoking and alcohol consumption mostly found in Western countries and Taiwan. The Linxian research group suggested the difference in risk factors for esophageal SCC in different areas may affect the relationship between *H pylori* and esophageal SCC<sup>[16]</sup>.

In Taiwan, about 95% *H pylori* strain is CagA positive<sup>[25]</sup>. This study has shown that subjects with positive *H pylori* serology have > 85% CagA<sup>+</sup> strains in both esophageal SCC patients and controls, suggesting using IgG serology to measure *H pylori* infection can represent the CagA<sup>+</sup> strains in Taiwan. The relatively low seroprevalence of *H pylori* infection in the community control group may be due to younger age (average age = 40.2 years), compared to those in the two hospital-based control groups (average age = 57.1 and 60.0 years).

The exact mechanism by which *H pylori* infection affects the development of EA and esophageal SCC is still puzzling. The strongest risk factor of distal EA is Barrett's esophagus, a condition secondary to long-term gastroesophageal reflux disease, and *H pylori* colonization may protect against Barrett's esophagus<sup>[11,13,26]</sup>. Rich-

ter *et al*<sup>27</sup> also hypothesized that *H pylori* can prevent EA development through gastric atrophy and possibly by increased intragastric ammonia production. Ye *et al*<sup>8</sup> challenged this hypothesis by demonstrating an unaffected protective effect for EA after adjusting for gastric atrophy. However, they hypothesized that CagA<sup>+</sup> *H pylori* infection caused esophageal SCC through the pathway of gastric atrophy<sup>8</sup>. An atrophic stomach might enhance the overgrowth of bacteria and increase endogenous nitrosamines production, which causes esophageal SCC<sup>28</sup>. In contrast, another hypothesis is *H pylori*-induced gastric atrophy, which decreases intragastric acidity<sup>14</sup>, might protect the lower esophageal mucosa from repeated injury by acid exposure, and thus decrease the esophageal SCC risk in an Asian population<sup>29</sup>. Another mechanism concerning the protective effect of *H pylori* infection on esophageal cancer risk directly involves apoptosis<sup>30,31</sup>. Jones *et al*<sup>30</sup> found significantly increasing apoptosis in OE33 Barrett's-derived EA cells, but not in normal esophageal cells, after being treated with intact *H pylori* wild-type strains. Our *in vitro* study demonstrated similar apoptosis effect on esophageal SCC cells, but not in AGS cells, after being co-cultured with *H pylori*<sup>31</sup>. However, this proposed mechanism is still speculative and needs to be further verified.

The prevalence of *H pylori* infection in patients with cardia and non-cardia adenocarcinoma was about 70%, significantly higher than control subjects (about 56%), which was similar to the previous study done in China<sup>41</sup>. Fewer cardia cancer patients ( $n = 29$ ) were noted in our study, but the prevalence of *H pylori* and CagA seropositivity was similar to that in non-cardia cancer patients. Previous studies have linked *H pylori* colonization to carditis and intestinal metaplasia in the cardia, indicating a role distinct from Barrett's esophagus<sup>32,33</sup>. The subsites of gastric cancer were not well defined in some of the previous studies, but most researchers in Asian countries<sup>3-5,34</sup> supported at least a trend of positive association between *H pylori* seropositivity and proximal gastric cancer. On the contrary, observational studies in Western countries indicated no association<sup>7-9</sup> or inverse association<sup>6</sup> between *H pylori* seropositivity and cardia cancer. Further studies are needed to investigate the discrepancy of findings between Asian and Caucasian populations.

*H pylori* infection was not significantly associated with the risk of colon adenocarcinoma in this study. Hartwich *et al*<sup>35</sup> found a positive association between colon adenocarcinoma risk and *H pylori* seropositivity, especially CagA<sup>+</sup> strains. However, there are also reports indicating no association between *H pylori* seroprevalence and colorectal neoplasia<sup>36,37</sup>. In addition, a recent report from Taiwan did not find any association between *H pylori* infection by the <sup>13</sup>C-urea breath test and the risk of colon adenomatous polyp, which is the pre-cancer of colon adenocarcinoma<sup>38</sup>.

The effect of *H pylori* infection on oral SCC risk has not yet been determined. Our study found conflicting results when *H pylori* infection status in three comparison controls was compared to that in oral cancer patients.

Previous studies have shown that *H pylori* was detectable in the oral cavity by histopathologic diagnosis or polymerase chain reaction<sup>39,40</sup>, but this bacterium may be present as a transient organism because of the difficulty of surviving in the oral cavity<sup>41</sup>. Thus, further studies are necessary to elucidate the relationship between *H pylori* infection and oral cancer risk.

Several limitations were present in this study: (1) The control group is mainly for the comparison of esophageal SCC, but not for other digestive cancers; (2) This is a cross-sectional case-control study design; the causal relationship may be not clearly elucidated; (3) Like other epidemiological studies<sup>8,15-17</sup>, we only measured *H pylori* antibodies in human serum, which may not be representative for actual *H pylori* infection.

In summary, our study supports an inverse relationship between *H pylori* prevalence and esophageal SCC risk in Taiwan. In contrast, *H pylori* infection increases gastric adenocarcinoma risk, including the cardia and non-cardia. There is an inconsistent or negative association between *H pylori* infection and the risks of oral and colon cancers. The choice of the control groups in this study were mainly for the comparison of esophageal SCC, but not for other patient groups. Thus, the role of *H pylori* in digestive tract cancers, except esophageal SCC, in this study still requires further examination.

## COMMENTS

### Background

There is debate in regard to the association between *Helicobacter pylori* (*H pylori*) infection and gastric cardia adenocarcinoma. Recently, cumulative evidence has indicated that *H pylori* infection plays a protective role in the development of esophageal adenocarcinoma. In contrast, the relationship between *H pylori* infection and esophageal squamous cell carcinoma (SCC) is still inconclusive. Very few studies have examined the role of *H pylori* on colon and oral cancers.

### Research frontiers

This study aimed to explore the role of *H pylori* infection on the risk of digestive tract cancers, including cancers of the oral cavity, esophagus, stomach, and colon.

### Innovations and breakthroughs

*H pylori* seropositivity is inversely associated with esophageal SCC risk, but increases the risk of gastric non-cardia and cardia adenocarcinoma.

### Applications

Eradication of *H pylori* in all infected subjects should be cautious because *H pylori* might protect against other diseases, such as esophageal cancer.

### Terminology

STAT-PAK assay for *H pylori* is an immunochromatographic screening test to detect *H pylori* IgG antibodies in serum; Helori CTX IgG kit is an enzyme immunoassay to detect CagA (the virulent protein of *H pylori*) IgG antibodies in serum.

### Peer review

This is an important clinical observational study, indicating *H pylori* is not always a bad bug. Although *H pylori* can cause gastric non-cardia and cardia adenocarcinoma, it may protect against esophageal cancer.

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BRIEF ARTICLE

## Etiologic factors of gastric cardiac adenocarcinoma among men in Taiwan

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### Abstract

**AIM:** To elucidate etiologic associations between *Helicobacter pylori* (*H. pylori*), lifestyle, environmental factors and gastric cardiac adenocarcinoma (GCA) among men.

**METHODS:** A hospital-based case-control study was conducted in Taiwan from 2000 to 2009. All cases were newly confirmed as primary GCA. Five controls were selected matching with age, sex, and admission date to each case. Participants were informed

of potential risk factors with a structured questionnaire by trained interviewers during hospitalization and provided a blood sample for the determination of *H. pylori* infection. Odds ratio (OR) and 95% confidence interval (95% CI) were used to evaluate risk, and a multivariate conditional logistic regression model was performed.

**RESULTS:** All participants recruited for this study were men, consisting of 41 cases and 205 controls. Results of the univariate analysis showed that significant factors associated with the etiology of GCA included *H. pylori* (OR = 2.69, 95% CI = 1.30-5.53), cigarette smoking (OR = 2.28, 95% CI = 1.05-4.96), working or exercising after meals (OR = 3.26, 95% CI = 1.31-8.11), salted food (OR = 2.51, 95% CI = 1.08-6.11), fresh vegetables (OR = 0.28, 95% CI = 0.09-0.80), fruits (OR = 0.19, 95% CI = 0.04-0.89), and rice as principal food (OR = 0.53, 95% CI = 0.30-0.85). Multivariate conditional logistic regression models indicated that a significantly elevated risk of contracting GCA was associated with working or exercising after meals (OR = 3.18, 95% CI = 1.23-9.36) and *H. pylori* infection (OR = 2.93, 95% CI = 1.42-6.01). In contrast, the consumption of fresh vegetables (OR = 0.22, 95% CI = 0.06-0.83), fruits (OR = 0.28, 95% CI = 0.09-0.79) and rice as principal food (OR = 0.48, 95% CI = 0.24-0.93) remained as significant beneficial factor associated with GCA.

**CONCLUSION:** Working or exercising after meals and *H. pylori* infection increase the risk of GCA, but higher intakes of rice, fresh vegetables and fruits reduce the risk.

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**Key words:** Gastric cardiac adenocarcinoma; *Helicobacter pylori*; Diet; Obesity; Gastroesophageal reflux disease; Cigarette smoking; Family history

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## INTRODUCTION

The gastric cardiac adenocarcinoma (GCA) has attracted considerable attention recently as a consequence of the rapid increase in incidence, while the overall rate of gastric cancer has markedly declined in Western countries. Recent studies in China have also shown a statistically significant increasing trend of the disease in the last 16 years<sup>[1]</sup>. Another striking feature is the strong male predominance (the sex ratio being 6:1) among the patients<sup>[2]</sup>. However, no established risk factors can explain the male predominance. Previous studies evaluating the etiology of the GCA generally merged with data of both genders, which would distort the findings and dilute any associations. As the reasons for these variations of incidence remain uncertain, there emerged a critical need for epidemiological studies to identify risk factors in male as well as risk factors that might account for the rapid increases in incidence, although it is very rare.

Recent investigations suggest that obesity<sup>[3-5]</sup> and gastroesophageal reflux disease (GERD)<sup>[6]</sup> are important risk factors for GCA among the Western population<sup>[5]</sup>. A Swedish study found that GCA was related to GERD with an odds ratio of 2.3 (OR = 2.3, 95% CI: 1.2-4.3)<sup>[6]</sup>. Cigarette smoking is also a risk factor<sup>[7]</sup>, but with a lesser degree of strength for GCA<sup>[5]</sup>. Dietary factors are also thought to play an important role in the GCA etiology. Fresh vegetables and fruits have been inversely associated with GCA in several studies<sup>[7]</sup>. Diets high in cholesterol, animal protein and vitamin B12 increase the risk<sup>[8]</sup>, whereas several nutrients, particularly those found in plant food (fiber, ascorbic acid and carotenoids), are associated with a reduced risk of GCA<sup>[5,8]</sup>. Additionally, the use of supplemental calcium/Tums resulted in a significant risk of GCA in a US study<sup>[8]</sup>.

The other main candidate risk factor is the infection with *Helicobacter pylori* (*H pylori*). Evidences have shown that this infection increases the GCA risk in China and other East-Asian population<sup>[9-11]</sup>, but not in the Western population<sup>[12,13]</sup>. A US population-based case-control study even reported an inverse relationship between Cag<sup>+</sup>A strains (cytotoxin associated gene A positive strains) of *H pylori* and GCA<sup>[14]</sup>. The correlation between the GCA and cancer history in first-degree relatives suggests that inherited genetic susceptibility and shared environmental risk factors might contribute to cardiac carcinogenesis<sup>[15]</sup>. Great differences exist in lifestyles, diet habits and environment between Chinese and the Westerners. The features of Taiwanese lifestyle share many similarities with both the Chinese and the Westerners. Especially, some popular customs and traditions in Taiwanese are much alike to the Chinese. It would be interesting to know whether people in Taiwan share the risk factors of GCA similar to people in mainland China or in Western countries. We conducted the present study

to gain a better clue in the etiologic factors shared by lifestyles, diet, and other environmental exposures associated with GCA among men in Taiwan. We additionally investigated the association with *H pylori* infection.

## MATERIALS AND METHODS

### Study design

A hospital-based case-control study of matched pairs was derived from 2000 to 2009 in Kaohsiung City, Taiwan, to explore the risk factors of GCA associated with lifestyles, dietary habits, *H pylori* infection and other environmental exposure factors.

### Data sources

All subjects in our study were drawn from the Kaohsiung Medical University Chung-Ho Memorial Hospital, which has been accredited as a top-grade teaching hospital in southern Taiwan, and used by patients from all socioeconomic categories.

Patients were diagnosed with a primary histologically confirmed incident case of gastric cancer by pathologist as adenocarcinoma type. The pathologists were not aware of the clinical or endoscopic data. We used endoscopy to measure the distance between the gastroesophageal junction and the upper and the lower borders of the tumor. GCA was identified using an endoscope in a retroflexed position and defined as a tumor, and the majority of the tumors was within 5 mm of the squamocolumnar junction. Gastric cardiac cancers include small cancers located in the cardiac region of the stomach or large tumors located primarily in the cardiac region with slight involvement of the esophagocardiac junction<sup>[16]</sup>. Patients with non-cardia gastric cancer were excluded. Of the 52 cases of histologically confirmed primary GCA by pathologists and gastroenterologists, ten were women excluded in data analyses. One patient was too ill to participate. Eventually, 41 cases agreed to participate in the study.

For each case identified, 5 controls of matched with sex, age ( $\pm 3$  years), and admission date ( $\pm 2$  wk) were recruited from inpatients free of cancer in the Family Medicine Departments at the same hospital, including inpatients who came in for a general health check-up. And, subjects with stomach-related diseases were excluded. Overall, the majority of subjects in the control group were healthy, but some controls were patients with hepatitis B, diabetes mellitus, cataract, and/or benign prostate hypertrophy. Of 209 control subjects invited, 205 agreed to participate in the study.

With consent from subjects, trained professional interviewers performed face-to-face interviews at the hospital using a structured questionnaire. To minimize the variation between interviewers in handling the questionnaire, each interviewer interviewed cases and controls by pairs. We were unable to blind the interviewers to the status of cases or controls, but they were unaware of the study hypothesis and were trained to treat subjects with a similar manner. The structured questionnaire was designed to collect detailed information on sociodemo-

graphic characteristics, lifestyles such as cigarette smoking, alcohol drinking, areca quid chewing, and diet, the use of nutritional supplements and medication history, serologic assays, family history, and GERD symptoms.

Obesity was measured by body mass index (BMI), according to the height and weight measured at least 2 years prior to the interview. BMI was calculated as body weight in kilograms divided by the square of body height in meters ( $\text{kg}/\text{m}^2$ ). The habits of cigarette smoking, alcohol drinking, and areca quid chewing were quantified from information on the age at the initiating, quantity, frequency, and actual duration of consumption, and the cessation age. For analysis, a smoker was defined as someone who had smoked one cigarette or more per day for more than 1 year. Ex-smokers, ex-drinkers, and ex-chewers were defined as those who had quit the habit for more than 6 mo before being diagnosed with cancer or interviewed. Subjects were asked to report their usual consumption frequency of 15 food items more than 1 year before diagnosis or interviewed. This frequency of dietary history exposure was classified into 7 categories: (1) at least once or more per day, (2) three to six times per week, (3) less than three times per week, (4) at least once to three times per month, (5) less than once per month, (6) less than once per year, and (7) never. In addition to the food frequency questionnaire, we asked several questions about general dietary behaviors thought to be related to the digestive tract effect, such as principal food and whether with middle-intensity working or exercising at less than 30 min after meals.

Subjects were also asked to report whether they ever took any nutritional supplements at least 6 mo or longer, and, if so, the type and the frequency of tablets taken were queried. The use of medications history was also collected, such as aspirin, and non-steroidal anti-inflammatory drugs (NSAID).

A blood sample was obtained from subjects at the time of interview. Serum levels of IgG antibodies to *H pylori* and CagA were determined by antigen-specific ELISA. Subjects were first classified as *H pylori* positive or *H pylori* negative, and those who were *H pylori* positive were then classified as CagA positive or CagA negative. Finally, family history was defined as any first-degree relative self-reported by subjects as having been diagnosed with the gastric cancer or disease. To minimize unreliable information, we verified the reported information by asking the subject's family members for confirmation. GERD symptom was defined as someone presented with recurrent heartburn and regurgitation for more than 1 year before being diagnosed or interviewed. The frequency of GERD symptoms was also collected. A field supervisor inspected each subject's medical history and compared with the collected questionnaires, which were then transferred to coding sheets for analysis.

### Statistical analysis

All analyses were performed using STATA 7.0 statistical software. The analysis included  $\chi^2$  test for the similarity of demographic factors, and a multivariate technique de-

**Table 1** Demographic characteristics in GCA patients compared with matched hospital-based controls in a Taiwan case-control study, 2000-2009

Variables	Case	Control	OR <sup>2</sup>	95% CI
Age				
$\geq 60$ yr	28	132	1.03	0.57-1.96
$< 60$ yr	13	73	1.00	
Marital status				
Married	35	159	1.63	0.45-5.87
Other	6	46	1.00	
Ethnicity				
Others <sup>1</sup>	10	45	1.12	0.73-1.69
Chinese Taiwanese	31	160	1.00	
Years of schooling				
$\leq 9$ yr	27	115	2.96	0.65-13.69
$> 9$ yr	14	90	1.00	
Occupation				
Farmer	16	59	2.14	0.89-5.12
Business	6	22	1.92	0.73-5.06
Industry	9	52	1.36	0.41-4.38
White-collar	10	72	1.00	
Blood type				
Non- A type	32	150	1.03	0.50-2.11
A type	9	55	1.00	
Blood type				
Non-O type	19	114	0.83	0.32-1.96
O type	22	91	1.00	
Economics				
Poor	21	69	2.11	0.81-5.69
Not-poor	20	136	1.00	

<sup>1</sup>Others: Including Native, Chinese, and Hakka. GCA: Gastric cardiac adenocarcinoma; <sup>2</sup>OR: Odds ratio.

signed for matched case-control studies. Conditional logistic regression models were used to estimate odds ratio (OR) and 95% confidence interval (95% CI) for the associations between GCA and the covariates of interest. All analyses were performed controlling for age and education level to minimize the possible confounding effects. Sociodemographic characteristics, lifestyle and environmental exposure variables were categorized and compared between cases and controls to identify significant variables associated with GCA. We used the matched case-control data estimating in univariate and multivariate analyses of the relations among exposure factors, potential confounding variables, and cancer risk. The multivariate analysis was performed with no interaction terms to identify factors with the greatest impact on the fit of the model predicting the risk of GCA, taking into account other related exposure factors and covariates.

## RESULTS

### Demographic characteristics

To increase the study sensitivity of this male predominant disease, we confirmed 41 GCA male patients and enrolled 205 healthy male controls. Cases were older than controls, but not significant (average age:  $64.53 \pm 2.17$  years *vs*  $63.27 \pm 1.73$  years,  $P = 0.28$ ). BMI and other demographic characteristics were also comparable between these two groups (Table 1).

**Table 2** Association between suspected risk factors and risk of gastric cardiac adenocarcinoma in a Taiwan case-control study, 2000-2009

Variables	Case	Control	OR <sup>1</sup>	95% CI
BMI <sup>2</sup> (kg/m <sup>2</sup> )				
> 24	11	96	0.89	0.56-1.38
≤ 24	30	109	1.00	
Cigarette smoking				
Yes	29	110	2.28 <sup>a</sup>	1.05-4.96
No	12	95	1.00	
Alcohol consumption				
Yes	18	65	1.71	0.88-3.26
No	23	140	1.00	
Areca quid chewing				
Yes	9	20	2.75	0.86-8.77
No	32	185	1.00	
Daily exercise				
Yes	10	83	0.58	0.16-2.16
No	31	122	1.00	
Sleep with others in childhood				
Yes	37	165	2.10	0.44-10.24
No	4	40	1.00	
Working or exercising after meals				
Yes	27	74	3.26 <sup>a</sup>	1.31-8.11
No	14	131	1.00	
<i>H. pylori</i> serum				
Positive	30	118	2.69 <sup>a</sup>	1.30-5.53
Negative	11	87	1.00	
Cag <sup>+</sup> A <sup>3</sup>				
Positive	27	102	1.27	0.21-7.66
Negative	3	16	1.00	
GERD symptoms				
Yes	11	48	1.28	0.64-2.55
No	30	157	1.00	
Passive smoking (family)				
Yes	17	66	1.48	0.37-5.92
No	24	139	1.00	
Passive smoking (colleague)				
Yes	18	91	1.01	0.37-2.59
No	23	114	1.00	
Steaming hot dietary habits (/d)				
Yes	31	175	0.72	0.32-1.53
No	10	30	1.00	
Cold dietary habits (/d)				
Yes	12	62	0.94	0.32-2.75
No	29	143	1.00	
Water supply				
Non-tap-water	13	53	1.47	0.29-7.31
Tap-water	28	152	1.00	

<sup>1</sup>Odds ratio (OR): Adjusted by age and years of schooling; <sup>2</sup>BMI: Obesity: BMI more than 28 kg/m<sup>2</sup>; Overweight: BMI more than 24 and less than 28 kg/m<sup>2</sup>; Normal: BMI more than 18.5 and less than 24 kg/m<sup>2</sup>; Underweight: BMI less than 18.5 kg/m<sup>2</sup>; <sup>3</sup>Cag<sup>+</sup>A: Cytotoxin associated gene A positive strains. (Those who were *H. pylori* positive were classified by CagA strains). <sup>a</sup>*P* < 0.05.

### Suspected risk factors

Table 2 shows the relationship between lifestyle habits, environmental exposures and GCA controlling for age and years of schooling of participants. The estimated ORs were significantly elevated in persons with cigarette smoking (current smoking plus ex-smoking: OR = 2.28, 95% CI = 1.05-4.96), working or exercising after meals (OR = 3.26, 95% CI = 1.31-8.11) and *H. pylori* infection (OR = 2.69, 95% CI = 1.30-5.53). Cases were also more

**Table 3** Association between suspected dietary pattern and risk of gastric cardiac adenocarcinoma in a Taiwan case-control study, 2000-2009

Variables	Case	Control	OR <sup>1</sup>	95% CI
Nitrate food (times/wk)				
≥ 3	9	23	2.18	0.80-5.89
< 3	32	182	1.00	
Smoked food (times/wk)				
≥ 3	8	30	1.94	0.53-7.34
< 3	33	175	1.00	
Fermented beans (times/wk)				
≥ 3	11	24	2.87	0.82-9.77
< 3	30	181	1.00	
Salted food (times/wk)				
≥ 3	9	21	2.51 <sup>a</sup>	1.08-6.11
< 3	32	184	1.00	
Pickled vegetables (times/wk)				
≥ 3	8	34	1.20	0.41-3.36
< 3	33	171	1.00	
Biting food (times/wk)				
≥ 3	6	58	0.38	0.08-2.01
< 3	35	147	1.00	
Galic (times/wk)				
≥ 3	10	60	0.62	0.17-2.12
< 3	31	145	1.00	
Dairy products (times/wk)				
≥ 3	7	47	0.72	0.13-4.15
< 3	34	158	1.00	
Meat (servings/d)				
≥ 6	30	150	1.24	0.42-3.67
< 6	11	55	1.00	
Fresh vegetables (servings/d)				
≥ 2	25	184	0.28 <sup>a</sup>	0.09-0.80
< 2	16	21	1.00	
Fresh fruits (servings/d)				
≥ 2	15	169	0.19 <sup>a</sup>	0.04-0.89
< 2	26	36	1.00	
Stripped sweet potato (sun-dried)				
Yes	20	71	1.71	0.86-3.37
No	21	134	1.00	
Rice as principal food				
Yes	32	176	0.53 <sup>a</sup>	0.30-0.85
No	9	29	1.00	
Milk (/d)				
Yes	16	81	1.02	0.16-7.08
No	25	124	1.00	
Coffee preference (/d)				
Yes	13	62	1.20	0.55-2.53
No	28	143	1.00	
Tea (/d)				
Yes	18	110	0.69	0.36-1.29
No	23	95	1.00	

<sup>1</sup>Odds ratio (OR): Adjusted by age and years of schooling. <sup>a</sup>*P* < 0.05.

likely than controls to use alcohol and chew areca quit, but difference was not statistically significant compared with that for abstainers. And other environmental exposure factors were not significantly associated.

However, age-and-years of schooling-adjusted OR estimates revealed harmful associations for GCA with salted food (OR = 2.51, 95% CI = 1.08-6.11) (Table 3). In contrast, there were beneficial effects from the consumption of fresh vegetables (OR = 0.28, 95% CI = 0.09-0.80), fruits (OR = 0.19, 95% CI = 0.04-0.89), and rice as principal food (OR = 0.53, 95% CI = 0.30-0.85). We failed to

**Table 4 Association between nutritional supplement and risk of gastric cardiac adenocarcinoma in a Taiwan case-control study, 2000-2009**

Variables	Case	Control	OR <sup>1</sup>	95% CI
Vitamine				
Yes	6	42	0.63	0.18-1.95
No	35	163	1.00	
Vit A				
Yes	2	13	0.74	0.24-2.50
No	39	192	1.00	
Vit B				
Yes	2	18	0.49	0.07-3.46
No	39	187	1.00	
Vit C				
Yes	3	25	0.56	0.10-3.36
No	38	180	1.00	
Vit E				
Yes	5	23	0.99	0.20-4.81
No	36	182	1.00	
Vit B complex				
Yes	4	14	1.59	0.74-3.43
No	37	191	1.00	
Iron				
Yes	3	6	2.01	0.86-4.68
No	38	199	1.00	
Calcium tablet				
Yes	4	26	0.84	0.17-4.12
No	37	179	1.00	
Zinc tablet				
Yes	1	6	0.85	0.09-8.10
No	40	199	1.00	
Aspirin				
Yes	2	7	1.34	0.59-3.03
No	39	198	1.00	
NSAID				
Yes	4	14	1.29	0.47-3.71
No	37	191	1.00	

<sup>1</sup>Odds ratio (OR): Adjusted by age and years of schooling.

find significant associations between GCA and vitamin supplements and other supplements or medications such as iron, calcium tablet, zinc tablet, aspirin, and NSAID (Table 4).

The risk strength associated with family disease history is shown by adjusted OR in Table 5. To investigate the actual effects of family aggregation, we collected relevant information about patient's first-degree relatives. There were insignificant weak evidences to associate GCA risk with family anemia history and other forms of gastric disease as well as atrophy gastritis, gastric ulcer, lymphoma, gastric polypus, and gastric cancer. However, individuals with a family history of duodenal ulcer (OR = 0.82, 95% CI = 0.35-2.09) had a reduced nonsignificant risk for GCA.

All significant variables found in the univariate analyses were included in the multivariate conditional logistic regression analysis (Table 6). The significantly elevated OR for GCA were associated with working or exercising after meals (OR = 3.18, 95% CI = 1.23-9.36) and *H pylori* infection (OR = 2.93, 95% CI = 1.42-6.01). However, consumption of fresh vegetables (OR = 0.22, 95% CI = 0.06-0.83), fruits (OR = 0.28, 95% CI = 0.09-0.79), and rice as principal food (OR = 0.48, 95% CI = 0.24-0.93) remained as protective factors that reduced the risk of GCA.

**Table 5 Association between family history and risk of gastric cardiac adenocarcinoma in a Taiwan case-control study, 2000-2009**

Variables	Case	Control	OR <sup>1</sup>	95% CI
Anemia				
Yes	17	83	1.06	0.46-2.58
No	24	122	1.00	
Atrophy gastritis				
Yes	17	85	1.02	0.31-3.25
No	24	120	1.00	
Gastric ulcer				
Yes	15	73	1.23	0.39-3.79
No	26	132	1.00	
Duodenal ulcer				
Yes	16	88	0.82	0.35-2.09
No	25	117	1.00	
Lymphoma				
Yes	12	57	1.30	0.63-2.80
No	29	148	1.00	
Gastric polypus				
Yes	13	62	1.51	0.20-11.38
No	28	143	1.00	
Cancer				
Yes	10	24	2.54	0.83-7.75
No	31	181	1.00	
Sibling (Cancer)				
Yes	5	23	1.39	0.38-5.06
No	36	182	1.00	

<sup>1</sup>Odds ratio (OR): Adjusted by age and years of schooling.

**Table 6 Multivariate conditional logistic regression analysis for estimated OR and 95% CI for GCA patients compared with matched hospital-based controls in Taiwan, 2000-2009**

Variables	OR <sup>1</sup>	95% CI
<i>H pylori</i> infection	2.93 <sup>a</sup>	1.42-6.01
Cigarette smoking	2.26	0.35-15.20
Working or exercising after meals	3.18 <sup>a</sup>	1.23-9.36
Consumption of salted food	2.34	0.39-13.75
Consumption of fresh vegetables	0.22 <sup>a</sup>	0.06-0.83
Consumption of fresh fruits	0.28 <sup>a</sup>	0.09-0.79
Rice as principal food	0.48 <sup>a</sup>	0.24-0.93

<sup>1</sup>Odds ratio (OR): Adjusted by age and years of schooling. <sup>a</sup>*P* < 0.05.

## DISCUSSION

To our knowledge, this is the first study to explore simultaneously all aspects of factors that may associate with GCA, including *H pylori* infection, lifestyle, dietary and nutritional supplements taking history, and family history.

It took 10 years to identify 41 histologically confirmed primary GCA cases with useful data. But, this small sample size study has demonstrated important findings. The habit of working or exercising after meals was the strongest risk factors for GCA among men in Taiwan, which is consistent with the finding for Chinese population in Fujian Province<sup>[7]</sup>. One plausible biological explanation for this association is that working or exercising after meals may decrease blood supply in digestive tract and induce gastric ischemia to prolong the empty

time<sup>[17]</sup>. Besides, physical activity may increase the intra-abdominal pressure and wall tension in the digestive tract, and reduced gastric mucosal flow and ischemia<sup>[17]</sup>. And recent studies have indicated that the cardiac region differs from the rest of the stomach by remaining highly acidic after a meal<sup>[18]</sup>. Physical activity may thus increase the gastrointestinal transit time and also extend acid contact time between the gastric mucosa, cardiac region especially, and potential carcinogens in the stomach, eventually leading to an increased GCA risk.

Most of the studies from Western populations have found no significant association with *H pylori* in the GCA etiology<sup>[12,13]</sup>. Some studies even reported a reduced risk for the disease<sup>[14,19]</sup>. In contrast, most studies conducted in Asian population<sup>[9-11,20]</sup> have found elevated risks from *H pylori* infection. A meta-analysis of prospective studies, including only four studies, found no association between GCA and *H pylori*<sup>[21]</sup>. Of these, two studies from China indicated an elevated risk associated with infection while other two from Europe indicated a reduced risk. Clearly, our finding is consistent with observations in Asian studies, encouraging evidence of the harmful role of *H pylori* in the carcinogenic process for GCA. The divergent findings between European and Chinese leave a matter for future consideration.

However, we observed that CagA seropositivity played no significant role consistent with prospective studies from China<sup>[11]</sup> and Europe<sup>[10]</sup>. Of interest, CagA seropositivity was associated with a 6.2-fold greater risk for cancers in the upper one third of the stomach for Japanese (1.2-32.0)<sup>[12]</sup>. In contrast, CagA seropositivity (OR = 0.4, 95% CI = 0.2-0.8) was associated with a reduced GCA risk in the US<sup>[14]</sup>. The inconsistency of these findings may be due to the diversity of the studies in geography, and ethnicity among them because of important genetic components, host, and environmental heterogeneity.

Furthermore, the CagA antibodies measurement for each subject reduced our misclassification of *H pylori* status. This encourages the power of testing significance with such a small sample size in this study. Therefore, we suggest that *H pylori* measurement in early life is an important marker of GCA, and a future study to elucidate the transmission mechanism of *H pylori* infection for the GCA prevention relevancy is needed.

Salted food has been linked to stomach cancer risk<sup>[22-24]</sup>. We found that salted food was also associated with an increased risk for GCA. However, this risk disappeared in the multivariate statistical models. Salt is thought to induce an inflammatory process leading to the damage of the protective stomach mucosa. And, *H pylori* may interact with salt and enhances carcinogenesis after the gastric epithelium is damaged<sup>[24]</sup>. It may also increase cell susceptibility to carcinogenesis from salt intake<sup>[7]</sup>. Thus, we postulate that salt per se is not an independent carcinogen, as Cohen *et al*<sup>[25]</sup> argued, but a promoter of GCA.

Many studies have reported the protective effect from the intake of fresh vegetables and fruits against GCA<sup>[7,26,27]</sup>. We also showed a remarkably reduced risk of GCA for men consuming diet rich with fresh vegetables and fruits. In other words, the plant food was as-

sociated with a strong reduction in risk for GCA among men in Taiwan. Plant food is rich in fiber, ascorbic acid and carotenoids<sup>[5,8]</sup>. Several studies showed that dietary fiber was strongly related to a reduced GCA risk<sup>[18,27,28]</sup>. The protective mechanism of fiber is still unclear; it may play a cleansing role and could facilitate the removal of carcinogens at the epithelial surface<sup>[27]</sup> or promote the sloughing of damaged epithelial cells<sup>[8]</sup>. Ascorbic acid and carotenoids are both strong antioxidants in the process of gastric carcinogenesis. Nevertheless, our data not only confirmed the beneficial effect of fresh vegetables and fruits against GCA, but also suggested rice as the principal food is important against this disease.

This study showed that rice as principal food may well reduce the risk of GCA in Taiwan. Antioxidants and fiber effect in rice, with much lower protein composition than wheat and some other grains, may attribute to the anti-carcinogenicity<sup>[29-32]</sup>. The decreased protein content in meals may reduce gastric acid secretion<sup>[33]</sup>. Cardiac cancer in Western countries has been associated with normal or increased acid production<sup>[6]</sup>. Rice as principal food may lower acid secretion, thus reducing the GCA risk. According to our observation of the habit of working or exercising after meals or rice as principal food, similar to those of the West, we assume that chronic exposure to gastric acid plays a role in the aetiology of GCA. Although we failed to observe an association between GERD and the GCA risk, the result is well in agreement with other studies conducted in the US<sup>[31]</sup> and Taiwan<sup>[34,35]</sup>. GERD is less common in Asians than in Western population<sup>[34,36]</sup>. We, therefore, considered GERD as an indicator rather than a crucial cause of GCA.

Our results differed greatly from the Western studies<sup>[3,5]</sup> in the association with BMI. Similar to a recent North China study<sup>[37]</sup>, we found a negative association between obesity and GCA, although not significant. The reasons of the difference might be associated with the genetic background, cut-off points for BMI and lifestyle. Great differences exist in lifestyle and diet habits between Chinese and the Westerners. Asian populations historically eat low-fat diets<sup>[38]</sup> with more vegetables/fruits<sup>[39]</sup> and have a smaller BMI than Western populations<sup>[40]</sup>. This suggests that obesity is an indirect factor in the association with GCA for the men in Taiwan. Moreover, a recent China study has pointed out that the westernization in diet and eating habits is responsible for the higher risk of GCA in the urban area than in the rural area<sup>[41]</sup>.

Some studies found harmful effect from smoking for GCA<sup>[5,7,42,43]</sup>. The present study observed a significant risk associated with smoking, but it disappeared in the multivariate analysis. Thus, it may be attributed to the reinforcing factor but not an independent carcinogen for GCA. Besides, the sample size of GCA cases in this study might be not large enough to detect such a relationship; however, we still can not overlook its gravity.

A twin study assessing the contribution of hereditary and environmental factors has found that occupational exposures and viral infections make up the other 62% of the risk of developing stomach cancer. The statistical model used provided a perfect fit ( $P = 1.0$ ) in predicting

the involvement of major environmental factors plus minor genetic components for the risk of stomach cancer<sup>[44]</sup>. Our results support this notion, even though we have no direct witness to prove it. In our study, working or exercising after meals, vegetables, fruits and rice consumption and *H pylori* infection are significantly associated with GCA, but not in family history of gastric diseases with the familial (hereditary or nonhereditary) effects. This finding indirectly shows the etiological importance of environmental factors for GCA.

An important etiologic characteristic of GCA is the strong male predominance. This study did find 42 of 52 cases were men. Why did the strong male predominance exist among patients with GCA? As the family is the core unit of *H pylori* transmission<sup>[45]</sup> and childhood colonization has the same risks in males and females. The striking feature may be due to the fact that females work or exercise at the lower intensity than males, because of the Chinese social request of culture. Hence, males are at higher risk than females for GCA. Moreover, females have more frequent consumption of vegetables and fruits than males. Accordingly, we can find the fact of more protective effect in females than in males. Consequently, the strong male predominance will be continuously observed.

Our study is subject to some limitations. The major limitation is its relatively small sample size, as GCA remains a very rare disease not only worldwide but also in Taiwan<sup>[34]</sup>. But our stringent matching design has increased the effort in study precision and reduced bias<sup>[46]</sup>. Compared with published studies<sup>[3,19,21]</sup>, we are encouraged to have more GCA cases with no misclassification and no divergent effects. Another limitation found in this study is the potential for recall bias, a common limitation of case-control studies, as well as the recall bias of dietary and family history. It was not possible to adjust completely for biases such as the dietary recall bias. We did match, however, the time of hospital visits to avoid differences caused by seasonal dietary changes. Moreover, we used the structured questionnaire to collect categorized information to minimize errors in quantitative data caused by incorrect recall. Furthermore, family history data was only collected for first-degree relatives, such self-reported information is generally more reliable. And we did examine and reconfirm family histories by talking with other family members. But, it cannot still wholly excluded that some family histories may have been misclassified.

The advantage of this study was based on confirmed GCA cases validated by a panel of pathologists and gastroenterologists. The other strength in this study is our exact study design and statistical analysis. The high response rate (97.62% in cases and 98.09% in controls), absence of proxy interviews and the homogeneity of control group are the other important ones.

Despite the relatively low number of GCA cases, the main risk factors in Taiwan societies are well established. The epidemiological features of GCA in our study have in deed demonstrated that GCA is more closely governed by environmental factors. Concerning the etiology of GCA, unlike in the West, obesity, GERD and cigarette smoking may not be the independent contributing

factors for population in Taiwan. While lifestyle and dietary variables have an important role, infectious origin is still a major factor in the developing populations such as Taiwan. Our further analysis showed an interactive effect between working or exercising after meals and *H pylori* infection. The OR for GCA increased to 3.39 (95% CI 1.32-11.27), compared with individuals of neither working/exercising after meals and nor *H pylori* infection. In conclusion, this study suggests that healthy dietary habits to avoid the chronic exposure to higher and provocative gastric acidity and eradication of *H pylori* are important for the GCA prevention. Daily intake of fresh vegetables, fruits and rice as principal food should be encouraged for the beneficial effect to prevent GCA.

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## COMMENTS

### Background

The gastric cardiac adenocarcinoma (GCA) is a very rare disease. In recent decades, however, the incidence of the GCA has increased dramatically in many Western countries. An increasing trend in GCA is also observed in municipal regions but not in rural regions of China. Another striking feature is the strong male predominance among patients with GCA. Some of the reasons above are still unclear and require further epidemiological investigations.

### Research frontiers

The etiological factors associated with this rare disease remain unclear and require a comprehensive investigation in Taiwan. And no corresponding sex difference in the established risk factors for GCA can explain the male predominance. This is the first study which explores simultaneously all potentially factors associating with the disease among men, including *Helicobacter pylori* (*H pylori*) infection, lifestyle, diet and nutritional supplements intake history and family history of the disease in the Chinese population. And, this is the first study to explain the male predominance by sex differences in risk factor profiles of the disease.

### Innovations and breakthroughs

This study showed that working or exercising after meals, and *H pylori* infection may increase the risk of GCA. Moreover, the interactive effect between working or exercising after meals and *H pylori* infection augments the risk while rice as principal food and higher intakes of fresh vegetables and fruits may well reduce the risk.

### Applications

Avoiding laborious task after meals and diet control may help prevent GCA. Earlier detection and eradication of *H pylori* may refer to the clinical selective direction of prophylaxis and treatment of this disease.

### Peer review

This study evaluated retrospectively the risk factors of GCA among men. The results are interesting and suggest that working or exercising after meals and *H pylori* infection are important independent risk factors for GCA, but CagA strains of *H pylori* are not associated with this cancer. Furthermore, this study also demonstrates that rice as principal food and consumption of fresh vegetables and fruits may well reduce the risk of GCA.

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## Efficacy of surgical resection in management of isolated extrahepatic metastases of hepatocellular carcinoma

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patients with 1 or 2 isolated extrahepatic metastases and who concurrently exhibit good hepatic functional reserve and general performance status as well as successful treatment of intrahepatic HCC.

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**Key words:** Hepatocellular carcinoma; Hepatic resection; Extrahepatic metastases; Surgical resection; Outcome

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### Abstract

**AIM:** To clarify the benefit of surgical excision for patients with extrahepatic metastases of hepatocellular carcinoma (HCC).

**METHODS:** We retrospectively reviewed the medical records of 140 patients with pathologically proven extrahepatic metastases of HCC and evaluated the outcomes of those who had undergone surgical resection (SR) for extrahepatic metastatic lesions. Prognoses made on the basis of extrahepatic metastatic sites were also examined.

**RESULTS:** The survival rates of patients who underwent SR of extrahepatic metastases were significantly better than those of patients who did not receive SR. For the SR group, 1- and 3-year survival rates were 24% and 7%, respectively, while for the non-resection group, the survival rates were 8% and 0%, respectively ( $P < 0.0001$ ). Survival rates related to metastatic sites were also significantly superior after SR of extrahepatic metastases: median survivals were 32 mo with lung metastasis, 10 mo with bone metastasis, 6.1 mo with brain metastasis.

**CONCLUSION:** SR can provide survival benefits for

### INTRODUCTION

Hepatocellular carcinoma (HCC) is an aggressive malignant tumor that occurs throughout the world. It is one of the leading causes of cancer-related death among people in East Asian countries. Recent progress in diagnostic tools has led to an improved prognosis for HCC patients because of enhanced detection of early and small HCC and better identification of those patients who qualify for hepatic resection (HR)<sup>[1-5]</sup>. Moreover, treatment trends towards aggressive multimodal therapy in patients with intra-hepatic HCC have also improved long-term outcomes<sup>[6-9]</sup>. Nevertheless, extrahepatic metastasis of primary HCC is still considered terminal-stage cancer, and the prognosis for patients at this stage continues to be poor due to limited effective treatment. Despite recognition of this situation, there appears to be no consensus among the medical community regarding treatment strategies for HCC patients with extrahepatic metastasis. Therefore, to improve the overall long-term survival of patients with HCC, more active treatment of extrahepatic metastases is necessary. Several studies have recently demonstrated that surgical resection (SR) of extrahepatic metastases is able to provide benefits for selected patients with HCC<sup>[10-13]</sup>. However, the role

of SR in HCC patients with extrahepatic metastases required further clarification. In this study, we present our experience with patients with extrahepatic metastases of primary HCC and evaluate the benefits of surgical removal of extrahepatic lesions.

## MATERIALS AND METHODS

### Patients

A total of 2245 patients with primary HCC were treated at the Department of Surgery, Chang Gung Memorial Hospital-Linkou Medical Center in Taiwan, during the study period between June 1988 and June 2008. Among them, medical records of 151 patients with pathologically proven extrahepatic metastases from primary HCC were retrospectively reviewed. Metastasis of HCC was confirmed for all patients by histological examination of specimens derived from either biopsy or excision of extrahepatic tumor lesions. Of the 151 patients, 11 were lost to follow-up; thus, the remaining 140 patients, including 108 men and 32 women, were enrolled in the present study.

### Diagnosis and treatment of intra-hepatic HCC

Intrahepatic primary or recurrent HCC was diagnosed by dynamic liver computed tomography (CT) scans and hepatic angiography, which shows hyperattenuation in the arterial phase and hypoattenuation in the late phase indicating hypervascular tumor mass. Ultrasound-guided biopsy was performed only when considered necessary.  $\alpha$ -fetoprotein was also routinely checked as a parameter of diagnosis.

Treatment of intrahepatic HCC consisted of multimodal therapeutic procedures including hepatic resection (HR), radiofrequency ablation, percutaneous chemical reagent injection, transcatheter arterial chemoembolization (TACE), or a combination of these treatment modalities. Treatment methods were selected for hospitalized HCC patients based on the following principles. HR was considered a priority for patients with intrahepatic HCC whenever the tumor was determined to be resectable. An alternative approach, such as local ablation or TACE, was performed as indicated if patients had systemic multiple tumors, a difficult tumor location, unsuitable conditions for HR, or refused surgical treatment. Patients might also have received concurrent or sequential multimodal treatments if conditions warranted.

### Diagnosis and treatment of extrahepatic HCC

Detection of extrahepatic HCC and determination of tumor location was accomplished through X-ray, CT, magnetic resonance imaging (MRI), or bone scintigraphy as indicated and under two conditions: namely, recurrent and concurrent. Recurrent extrahepatic HCC was noted during post-operative follow-up after treatment for primary HCC. Concurrent extrahepatic HCC was observed at the initial diagnosis stage of intrahepatic HCC. In order to ensure the significance and validity of our research, only those patients with pathologically

**Table 1** Clinical characteristics of patients with extrahepatic metastases of hepatocellular carcinoma

Characteristics	n (%)
Age (yr)	55.8 ± 14.4
Male:Female	108:32
Hepatitis B virus surface antigen	66 (47)
Hepatitis C virus antibody	30 (21.4)
Previous hepatic resection	
Yes	66 (47)
Single hepatic resection	57
Multiple hepatic resection	7
Liver transplantation	2
No	74 (53)
Location of metastatic tumor	
Lung	25 (17.8)
Bone	47 (33.6)
Brain	28 (20)
Soft tissue	35 (25)
Heart	2 (1.4)

proven extrahepatic metastasis of HCC were included in our study.

Treatment of extrahepatic metastasis varied according to the individual clinical characteristics of each patient. SR was selected for patients whose extrahepatic HCC was solitary, isolated, and considered resectable, and for whom intrahepatic tumor recurrence was not observed after previous treatment or for whom the intrahepatic tumor was completely treated by either HR or other treatment modalities. Systemic chemotherapy was reserved for those patients deemed unsuitable for SR. Radiotherapy was performed to relieve patients' symptoms as indicated.

### Follow-up

All patients were closely followed up at regular intervals until death or until the writing of this article. The duration of follow-up after first diagnosis of HCC ranged from 1 to 138 mo (median, 16 mo). Follow-up after diagnosis of extrahepatic metastasis ranged from 0.8 to 96 mo (median, 6.1 mo).

### Statistical analysis

All data were analyzed using the statistical software Prism 5.0 (GraphPad Software, San Diego, CA, USA). Survival rates were calculated by the Kaplan-Meier method, and the log-rank test was used to compare survival rates. Patients who died from surgical complications were excluded from the survival curve. A value of  $P < 0.05$  was considered statistically significant.

## RESULTS

### Clinical features of patients with extrahepatic metastases from HCC

The clinical characteristics of patients at initial diagnosis of extrahepatic metastases are summarized in Table 1. The average age of these patients was 55.8 years (range, 13 to 81 years). Predominant clinical elements were male gender (77%, 108/140) and a positive test for the

**Table 2** Clinical characteristics of patients who underwent surgical resection of extrahepatic metastases of hepatocellular carcinoma

Characteristics	n (%)
Age (yr)	54.1 ± 14.9
Male:Female	64:22
Hepatitis B virus surface antigen	46 (53)
Hepatitis C virus antibody	22 (21.4)
Concurrent treated intrahepatic lesion	
Yes	59 (69)
No	27 (31)
Location of metastatic tumor	
Lung	11 (12.8)
Bone	24 (27.9)
Brain	25 (29.1)
Soft tissue	24 (27.9)
Heart	2 (2.3)

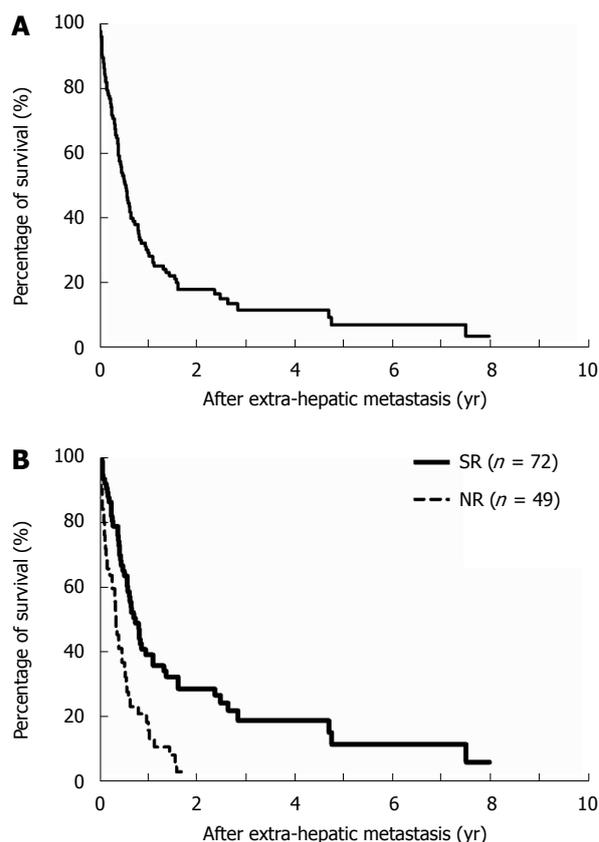
hepatitis B virus (47%, 66/140). These findings were similar to those of our previous reports on primary HCC<sup>[14,15]</sup>. Further, 66 patients (47%) had previously undergone HR for primary HCC. Among these patients, 7 had received multiple HRs for intrahepatic HCC and 2 had undergone liver transplantation. Extrahepatic metastases had occurred mainly at bone, brain, lung, and soft tissue locations. Patients categorized into the soft tissue group had extrahepatic metastases to intra-abdominal soft tissue (6 patients), intra-abdominal organs (4 patients), abdominal wall (16 patients), breast (1 patient), and skin plus subcutaneous tissue (8 patients).

SR of extrahepatic metastases of HCC was performed on 86 patients (64 male and 22 female; Table 2). Further, 59 out of 86 patients had intrahepatic tumors at the time of extrahepatic metastasis detection. Among these patients, 54 were also treated by TACE, local ablation, or a combination of both therapies for their intrahepatic lesion; thus, most patients received more than one course of treatment prior to resection of the extrahepatic lesion. Five patients underwent simultaneous HR for intrahepatic HCC and SR of extrahepatic metastases.

### Survival of patients

The median length of patient survival was 6.9 mo (range, 0.8 to 96 mo), and the overall 1-, 3-, and 5-year survival rates were 31%, 7%, and 4%, respectively, for all patients with extrahepatic metastases of HCC (Figure 1A). Long-term overall survival was significantly better in patients who underwent SR of extrahepatic metastases than in those who did not [non-resection (NR) patients]. The 1-year/3-year survival rates were 24%/7% for the SR group as compared with the 1-year/3-year survival rates of 8%/0% for the NR group (Figure 1B,  $P < 0.0001$ ).

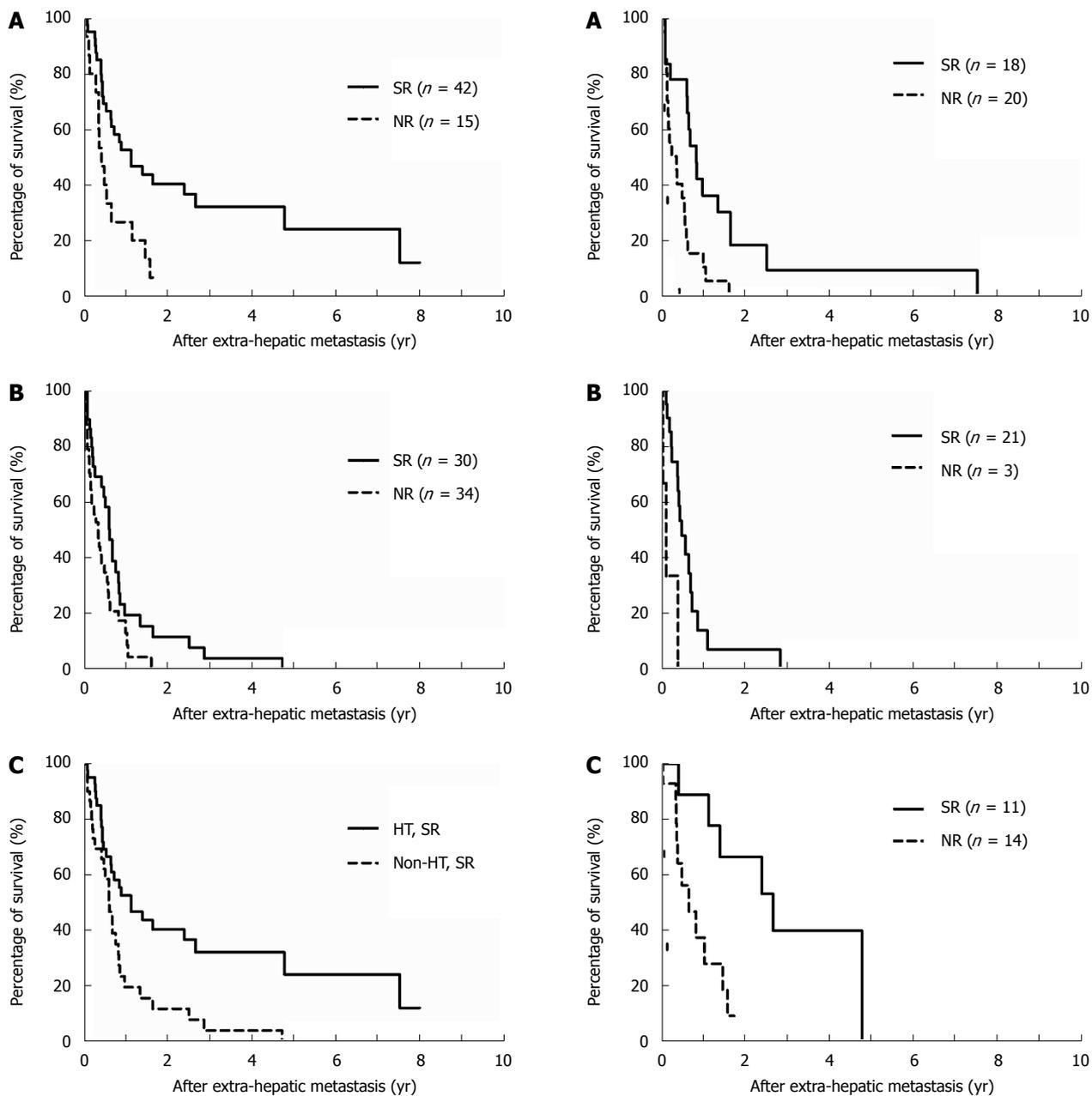
We further analyzed survival rates according to whether HR had been performed. Sixty-six patients had previously undergone HR (Table 1), including 5 patients who underwent simultaneous resection of intrahepatic and extrahepatic tumors at initial diagnosis and 61 patients who underwent resection of intrahepatic tumors followed by subsequent resection of extrahepatic tumor



**Figure 1** Survival rates. A: Overall cumulative survival curve of patients with extrahepatic metastases of HCC; B: Comparison of survival rates according to whether patients underwent surgical resection (SR) of extrahepatic metastases or not. NR: non-resection ( $P < 0.0001$ ).

recurrence. The outcome for patients who underwent SR was significantly better than that of the NR patients (Figure 2A and B), and the survival rate of SR patients in the HR group (1-year/3-year, 52.5%/32.1%) was considerably better than that of the non-HR group (1-year/3-year, 19.4%/3.9%,  $P = 0.0020$ ; Figure 2C).

The cumulative 1- and 3-year survival rates after extrahepatic metastases were 22.1%/4.1% for bony metastases, 11.8%/0% for brain metastases, 59.4%/20.8% for lung metastases, and 31.2%/27.3% for soft tissue metastases, respectively. The comparison of survival curves in relation to metastatic sites is depicted in Figure 3 and indicates that patients who received SR of extrahepatic HCC had a significantly better prognosis than patients in the NR group. The 1- and 3-year survival rates of the SR group *vs* the NR group by metastatic site were, respectively, 35.9%/9.0% (median survival, 10 mo) *vs* 10%/0% (median survival, 3.6 mo) for bony metastases (Figure 3A,  $P = 0.0022$ ); 13.6%/0% (median survival, 6.1 mo) *vs* 0%/0% (median survival, 1.7 mo) for brain metastases (Figure 3B,  $P = 0.0055$ ); 89%/40% (median survival, 32 mo) *vs* 37.5%/9.4% (median survival, 7.9 mo) for lung metastases (Figure 3C,  $P = 0.0063$ ); and 40.2%/40.2% (median survival, 10.1 mo) *vs* 16.7%/0% (median survival, 3.5 mo) for soft tissue metastases (Figure 3D,  $P = 0.0080$ ). Among those patients who had undergone SR, resection of lung metastases demonstrated

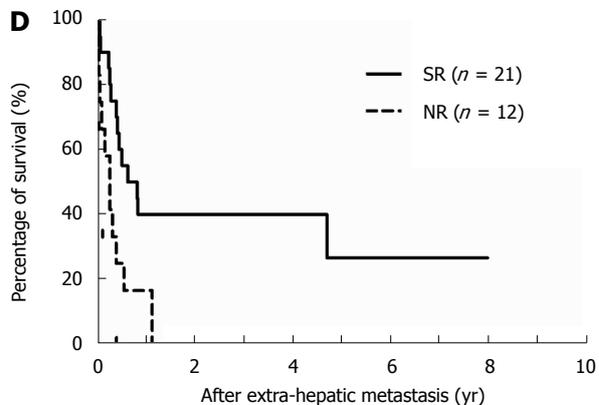


**Figure 2** Comparison of survival curves between surgical resection (SR) and non-resection (NR) groups for extrahepatic metastases in terms of patients (A) who had undergone previous hepatectomy for primary HCC ( $P = 0.0054$ ), and (B) who had not received hepatectomy ( $P = 0.0247$ ); (C) Among patients with surgical resection for extrahepatic metastases, the survival curve of the HT group was significantly better than that of the non-HT group. ( $P = 0.0020$ ).

most favorable outcomes when compared with the other metastatic sites (Figure 4,  $P < 0.05$ ).

**Patients with long-term survival**

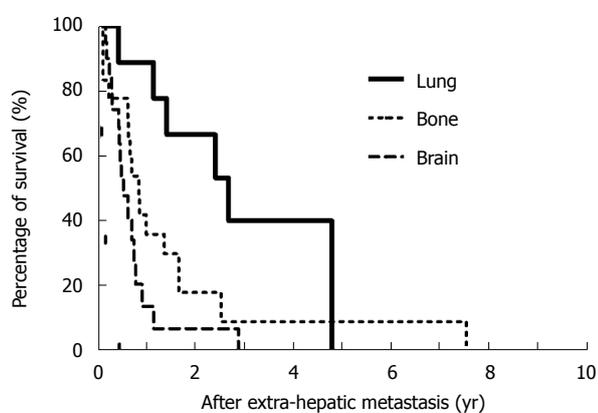
The clinical features of patients with favorable outcomes who survived more than 3 years after extrahepatic metastases diagnosis are listed in Table 3. Four patients had undergone HR and one had received TACE for their primary HCC. All patients had experienced intrahepatic recurrence and were treated by either TACE or local ablation after initial treatment. A solitary metastatic lesion at



**Figure 3** Comparison of survival curves between surgical resection and non-resection groups according to metastatic sites. A: Bone,  $P = 0.0022$ ; B: Brain,  $P = 0.0055$ ; C: Lung,  $P = 0.0063$ ; D: Soft tissue,  $P = 0.0080$ . The data show that surgical resection resulted in significantly better patient survival rates at all extrahepatic metastatic sites.

**Table 3** Clinical features of patients who underwent surgical resection for extrahepatic metastases from HCC with favorable outcomes

No. of patient	Age (yr)/ Sex	Treatments for intrahepatic HCC	Extrahepatic metastases		Follow-up after metastases (mo)	Outcome
			Duration to metastases (mo)	Location/Management		
1	44/Male	Hepatic resection: 1 time TACE: 1 time	7	Lung/wedge resection	40	Alive
2	36/Female	Hepatic resection: 1 time TACE: 1 time	16	T-spine/resection of tumor Lung/wedge resection	89	Dead
3	31/Female	Hepatic resection: 1 time TACE: 3 times	11	Transverse colon and peritoneal soft tissue/surgical resection	67	Alive
4	51/Male	Hepatic resection: 1 time TACE: 2 times PEI: 1 time RFA: 1 time	32	Peritoneal soft tissue/surgical resection	96	Alive
5	67/Male	TACE: 11 times PEI: 8 times	7	Abdominal wall/wide excision	57	Dead

**Figure 4** Among patients with surgical resection of extrahepatic metastases, the survival curves are compared according to metastatic sites. Patients who had undergone surgical resection of lung metastases benefit from better survival curves than patients with the other two metastatic sites. (Lung vs bone,  $P = 0.0418$ ; lung vs brain,  $P < 0.001$ ).

the 12th thoracic spine was noted in patient 2 (Table 3) 16 mo after hepatic resection, and another episode of isolated pulmonary metastasis was encountered in the subsequent 32 mo. Both events were successfully treated by SR, but the patient died 89 mo after initial detection of extrahepatic metastasis due to systemic metastases. Three patients were still living at the end of this study; follow-up of patient 4 has been ongoing for 96 mo after SR of his intra-abdominal metastatic lesion.

## DISCUSSION

HCC is an aggressive malignant neoplasm with dismal prognosis because of the high incidence of intrahepatic recurrence after initial treatment. However, recent trends towards aggressive treatment of intrahepatic recurrence with multimodal therapy have significantly improved the overall outcome of patients with HCC<sup>[6,9,16]</sup>. This trend, along with better treatment of intrahepatic HCC, may not only prolong a patient's survival but may also retard anticipated extrahepatic metastases. Because the prognosis of patients with extrahepatic metastases is considered very

poor and because there is limited information regarding treatment strategies for these patients, it is necessary to actively develop more aggressive treatment methods in order to further improve the long-term survival of HCC patients with extrahepatic metastases.

HCC is a hypervascular tumor and is believed to spread mainly through a hematogenous route, causing extrahepatic metastases. It has been reported that extrahepatic metastases occur in 13.5%-41.7% of HCC patients<sup>[17-20]</sup>; however, actual prevalence may be higher than that reported. Although all HCC patients are followed up at regular intervals for intrahepatic recurrence using a variety of diagnostic tools, not all patients receive complete examination for extrahepatic metastases. Moreover, the majority of patients with extrahepatic metastases experience no specific symptoms, and it is possible to overlook extrahepatic metastases upon examination. Therefore, increased detection of extrahepatic metastases suitable for aggressive treatment may provide great benefit to patients and improve overall survival of those with HCC.

Increasing evidence shows that SR of metastatic lesions with curative intent has become standard practice for the management of several malignancies. SR of isolated metastatic lesions from colorectal cancer, gastrointestinal stromal tumors, neuroendocrine cancers, renal-cell carcinoma and sarcoma is associated with favorable outcomes<sup>[21-24]</sup>. Furthermore, improvements in patient safety during complex surgeries have also lowered the threshold for more aggressive surgical intervention. Recently, several reports have shown that SR of HCC metastases prolonged survival in selected patients<sup>[10,11,13,25]</sup>. Our results appear to be comparable with those reports which indicated that patients who received SR of extrahepatic metastases had better outcomes than patients who did not receive SR. In addition, in our study, patients undergoing SR of lung metastases had better survival rates than patients with bony and brain metastases, which is comparable with previous reports that surgical resection of pulmonary metastasis from HCC has favorable outcome. Although the small number of patients involved in this study

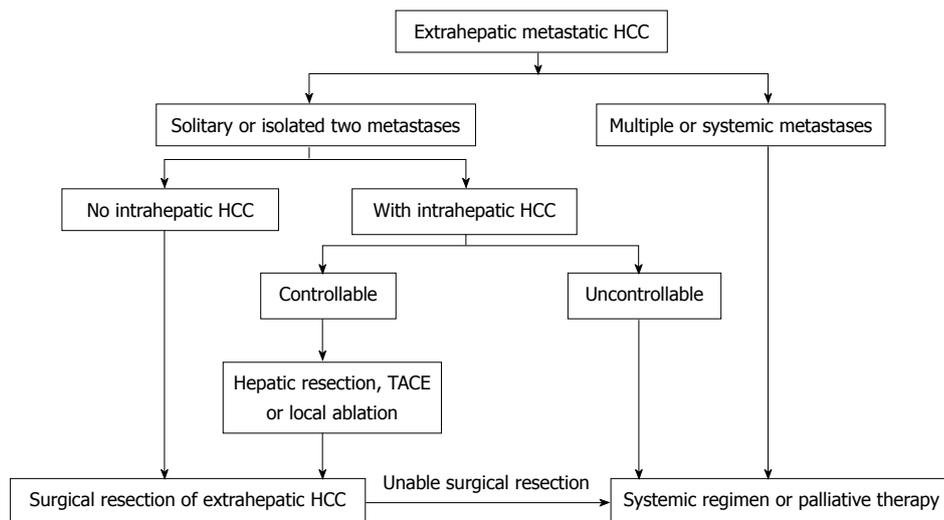


Figure 5 Suggested strategy for management of extrahepatic metastases from primary hepatocellular carcinoma.

might not reflect a real variation in outcomes due to metastatic location, we believe that our observations are valid. However, the difference in outcomes based on metastatic site may be related to the molecular biological pattern of HCC; therefore, further studies involving basic science and using a larger number of patients should be conducted to clarify the significance of our results.

Bony metastasis is one of the most frequent extrahepatic metastasis of HCC and occurs as multiple metastases in most patients<sup>[19,26,27]</sup>. In this study, the outcome of HCC patients with bony metastases was poor. Patients with metastases to bone had a median survival of 6.7 mo, which is similar to previous reports<sup>[28,29]</sup>. Data also indicate that the spine is the most common site of bony metastasis of HCC, and further studies suggest that surgical treatment for spinal metastatic HCC lesions usually improves quality of life instead of prolonging patient survival<sup>[30]</sup>. Nevertheless, prolongation of survival may still be achievable in selected patients, as observed in the current study. The longest survival of a patient who had undergone resection of metastasis at the left femoral shaft was 30 mo after the detection of extrahepatic metastasis.

Brain metastasis of HCC is relatively rare and is detected in less than 1% of HCC patients<sup>[20,31]</sup>. The overall prognosis of patients with brain metastases is extremely poor, and the most recent report showed that the median survival of these patients was only 6.8 wk after the detection of metastatic brain lesions<sup>[32]</sup>. Clinically, brain metastases are frequently associated with mass-producing hemorrhage, a condition requiring further surgical treatment. Although the surgical excision of brain metastases is considered a palliative treatment, several reports have also shown that a favorable outcome was achievable in patients with a single brain metastasis and good liver function and performance status<sup>[32,33]</sup>. Our study showed that patients who had undergone SR of brain metastases fared significantly better than NR

patients, with a median survival of 6.1 mo *vs* 1.7 mo. Thus, SR of metastatic brain lesions of HCC should be considered in specific patients not only to improve quality of life but also to prolong life expectancy.

Nonetheless, one might argue that patients who are not suitable for surgical resection are naturally in poorer condition than patients who underwent SR for extrahepatic metastasis. Indeed, although HCC with extrahepatic metastasis is considered the terminal-stage of TNM staging, the outcome of patients with systemic multiple metastases theoretically might be poorer than those of one or two isolated extrahepatic metastases. However, the accumulated data and this study have shown that SR of extrahepatic metastases can effectively provide real benefits for patients with HCC. Although there is no standard treatment for extrahepatic metastases of HCC, aggressive treatment in patients with HCC should be considered, not only in cases of intrahepatic HCC, but also in the event of extrahepatic metastases. Taking into consideration that there are no effective nonsurgical treatment modalities for extrahepatic metastases of HCC, surgical treatment may be the only modality available for curing selected patients. As our study has shown, patients who have undergone HR prior to SR for extrahepatic metastases had the best outcome, indicating that patients should be closely followed up at regular intervals after initial treatment of primary HCC to ensure early detection of extrahepatic metastases at a resectable stage.

In conclusion, we herein propose the following algorithm for surgical management of extrahepatic metastases from HCC (Figure 5). Surgical excision of extrahepatic metastases should be considered in patients with one or two isolated extrahepatic metastases if the patient has otherwise good performance status, good hepatic functional reserve, and well-controlled intrahepatic HCC. Although the retrospective nature of the current study and the small number of patients with resectable extrahepatic metastases makes it hard to compare outcome of patients undergoing SR for isolated

extrahepatic metastasis with those who do not receive SR, the marked differences we observed may prove helpful in the management of patients with HCC. Furthermore, to achieve better long-term outcomes of patients with HCC, as well as effective treatment of extrahepatic metastases, new promising treatments such as a novel systemic chemotherapy or targeting therapy should be developed.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is a common malignant tumor, and ranked within the top five of cancer-related deaths worldwide. Patients with extrahepatic metastases of primary HCC are clinically considered at terminal-stage cancer, and there is still no effective treatment as well as no consensus on treatment strategies for these patients. Recently, several reports have shown benefit of surgical resection of extrahepatic metastases from HCC. However, the role of surgical resection in the treatment of extrahepatic metastasis of primary HCC remains uncertain.

### Research frontiers

The concept of treating metastatic cancer has been changing recently. Patients with several types of metastatic cancers such as colon cancer, gastrointestinal stromal tumor, neuroendocrine cancer, sarcoma, renal cell carcinoma, *etc.* are considered to gain benefit from surgical resection of metastatic lesions. Therefore, it seems reasonable to attempt aggressive surgical resection for HCC patients with extrahepatic metastases.

### Innovations and breakthroughs

In the present study, the authors gathered their experience in the management of HCC, and further clarified the role of surgical resection in dealing with extrahepatic metastases from HCC. The results show that although the outcome of patients with extrahepatic metastases remains very poor, surgical resection could provide survival benefits for patients who had 1 or 2 isolated extrahepatic metastases under a prerequisite that patients has good performance status and good hepatic functional reserve, as well as successful treatment of intrahepatic HCC.

### Applications

According to results from the study, the authors have proposed an algorithm for the management of extrahepatic metastases. Since there is no effective treatment for extrahepatic metastases in this era, surgical resection might be an applicable option and provide better outcome for well selected patients.

### Peer review

The authors reviewed their experience in dealing with primary HCC with extrahepatic metastasis, and proposed a treatment strategy suggesting that surgical resection might provide benefit to patients.

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## Small invasive ductal carcinoma of the pancreas distinct from branch duct intraductal papillary mucinous neoplasm

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### Abstract

Endoscopic ultrasonography (EUS) is a highly sensitive diagnostic method for the detection of small pancreatic carcinomas. Recently, there have been some reports describing the utility of contrast-enhanced harmonic EUS (CEH-EUS) which uses sonographic contrast agent for differentiation of a pancreatic mass. This report describes a case of small adenocarcinoma of the pancreas distinct from branch duct intraductal papillary mucinous neoplasm (IPMN) in which investigation by EUS took place every 6 mo and diagnosis was made accurately by additional CEH-EUS during the follow-up of the branch duct IPMN. A 68-year-old female was admitted to our hospital because of a branch duct IPMN in the pancreatic body. She had been followed-up by EUS every 6 mo. However, after 2 years EUS

demonstrated a low echoic area distinct from the branch duct IPMN which was vaguely discernible by EUS, and accurate sizing and differential diagnosis were considered difficult on the EUS imaging. CH-EUS with Sonazoid revealed a hypovascular tumor and we suspected small pancreatic carcinoma. The histopathological diagnosis was adenocarcinoma (10 mm) in the pancreatic tail, distinct from the branch duct IPMN of the pancreatic body. EUS and CEH-EUS may play an important role in the correct diagnosis of small pancreatic tumors, including synchronous and metachronous occurrence of IPMN and ductal adenocarcinoma of the pancreas.

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**Key words:** Intraductal papillary mucinous adenoma; Small pancreatic cancer; Endoscopic ultrasonography; Contrast-enhanced harmonic endoscopic ultrasonography; Pancreatic cancer; Pancreas; Contrast enhanced endoscopic ultrasonography

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### INTRODUCTION

Endoscopic ultrasonography (EUS) is now commonly used worldwide and is more sensitive for the detection of small pancreatic lesions when compared with transabdominal ultrasonography (US) or contrast-enhanced computed tomography (CE-CT)<sup>[1,2]</sup>. Recently, there have been some reports describing the utility of contrast-enhanced EUS (CE-EUS) and contrast-enhanced harmonic EUS (CEH-EUS) with sonographic contrast agent for differentiation of pancreatic masses<sup>[3-7]</sup>.

Intraductal papillary mucinous neoplasm (IPMN)

of the pancreas is a unique clinicopathological entity characterized by cystic dilatation of the main or branch pancreatic duct, mucus production and intraductal papillary growth<sup>[8-11]</sup>. Metachronous occurrence of IPMN and ductal carcinoma of the pancreas has been recently reported<sup>[12-15]</sup>. These authors concluded that special attention should be paid to the development of ductal carcinoma of the pancreas during the follow-up of IPMN. However, the detection of ductal carcinoma at an early stage is still difficult, even under close surveillance by imaging examinations such as abdominal US and computed tomography (CT) during the follow-up of IPMN.

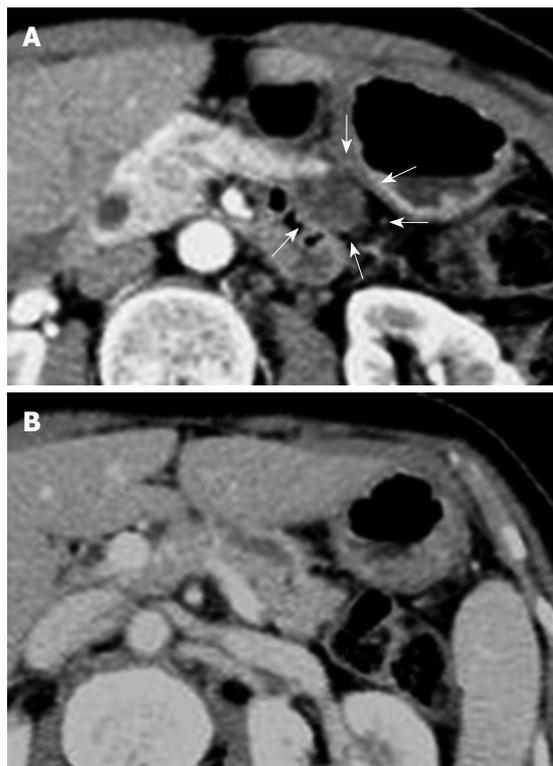
Here, we report a patient with small invasive ductal carcinoma of the pancreas that is distinct from branch duct IPMN. The lesion in this patient was detected by EUS and the diagnosis was established accurately by contrast-enhanced harmonic EUS during the follow-up of the branch duct IPMN.

## CASE REPORT

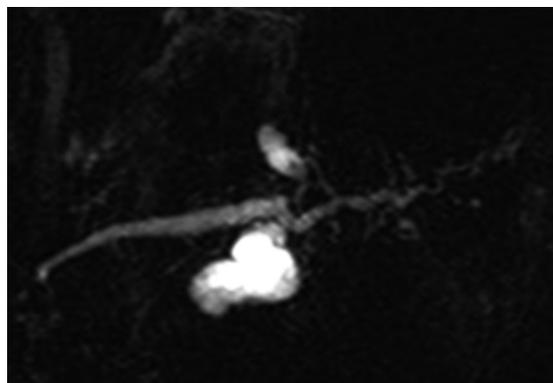
A 68-year-old female was admitted to our hospital because of a branch duct IPMN in the pancreatic body that was detected by contrast-enhanced computed tomography (CE-CT) (Figure 1). Magnetic resonance cholangiopancreatography (MRCP) (Figure 2) and EUS revealed a dilatation of the branch pancreatic ducts in the pancreatic body (22 mm × 9 mm, 8 mm × 5 mm); however, no nodules were found in the cyst and in any other parts of the pancreas. The patient was followed up by performing EUS every six months, as well as MRCP and CE-CT once a year. The size of the branch ducts was unchanged one year after the last MRCP (Figure 3). EUS performed at two years revealed a low echoic area that was distinct from the dilatation of the branch duct IPMN. This finding was vague and the area had unclear margins with a low echoic area, as assessed by EUS (Figure 4A). Thus, it was very difficult to determine the accurate size of the area and perform a differential diagnosis based on the EUS imaging. We performed CEH-EUS using an Olympus GF-UE260P apparatus (Olympus Medical Systems Co. Ltd., Tokyo, Japan) for endoscopy, Extended Pure Harmonic Detection (ExPHD) mode of ALOKA Prosound α-10 (ALOKA Co. Ltd., Tokyo, Japan) for image analysis and Sonazoid (15 μL/kg intravenously, Daiichi Sankyo, Tokyo, Japan; GE Healthcare, Milwaukee, Wisc., USA) as a contrast agent. CEH-EUS revealed the presence of a 10 mm hypovascular tumor, which led us to suspect a small carcinoma of the pancreas (Figure 4B). Pancreatoduodenectomy was performed. The histopathological diagnosis established was a moderately differentiated tubular adenocarcinoma in the 10 mm range at the maximum length of the pancreatic tail, which was distinct from the branch duct intraductal papillary mucinous adenoma of the pancreatic body (Figures 5 and 6).

## DISCUSSION

We report here the discovery of independent development of a ductal carcinoma of the pancreas during follow-up



**Figure 1** Contrast-enhanced computed tomography (CE-CT) scan of pancreas. A: A cystic lesion in pancreatic body (arrows); B: A dilatation of the main duct in the pancreatic tail. Solid masses were not observed.

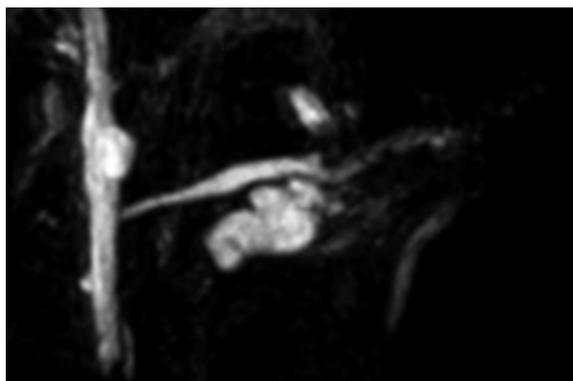


**Figure 2** Magnetic resonance cholangiopancreatography (MRCP) at initial examination showing dilatation of the branch duct.

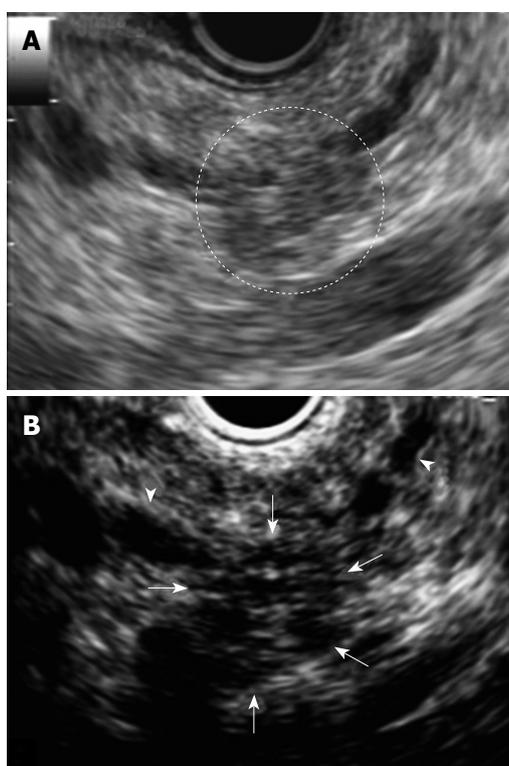
of an IPMN, similar to the metachronous occurrence of IPMN and ductal carcinoma of the pancreas which has been recently reported<sup>[13-15]</sup>.

Uehara *et al*<sup>[13]</sup> reported results from a follow-up study of 60 patients with branch duct IPMNs over an average period of 87 mo, and the follow-up was performed by US every three or six months. These authors detected ductal pancreatic carcinomas that were distinct from IPMN in 5 of 60 patients (8%). Four of the 5 ductal carcinomas detected during follow-up were resectable, but all of them were advanced cancer.

Tada *et al*<sup>[14]</sup> followed-up 197 cystic lesions of the pancreas, including 80 branch duct IPMNs and 117 non-IPMN cysts, for an average period of 3.8 years, and found 5 ductal carcinomas between 14 and 60 mo after



**Figure 3** The size of the branch ducts was unchanged one year after the last MRCP.

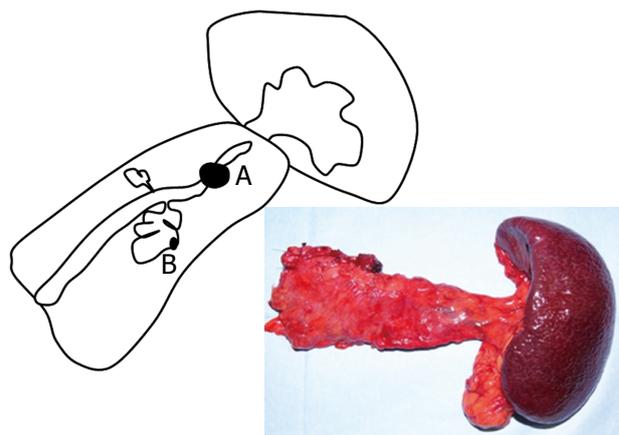


**Figure 4** Endoscopic ultrasonography (EUS) at two years later examination showing pancreatic tail. A: EUS showing low echoic lesion which is vague and the area had unclear margins in the pancreatic tail; B: Contrast-enhanced harmonic EUS showing a clear margin of 10 mm in diameter and hypovascular nodule compared with surrounding pancreatic tissue (arrows). MPD: Main pancreatic duct (arrowheads).

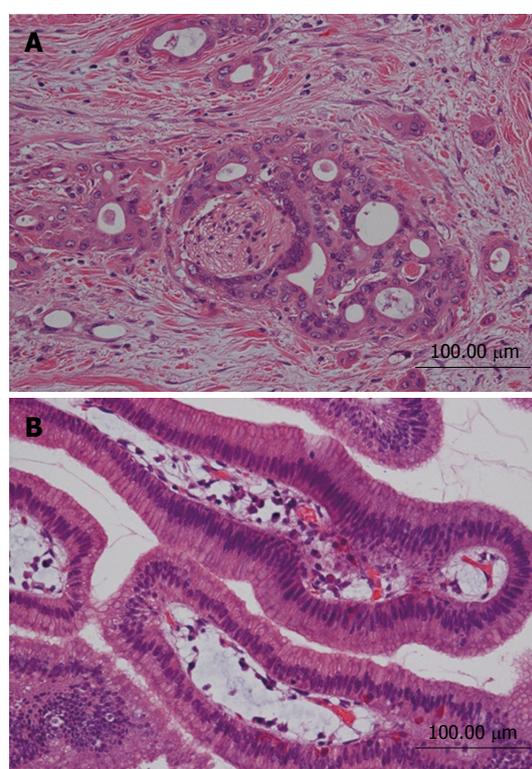
the diagnosis of the cystic lesions. In two patients, ductal carcinomas were < 2 cm in size, arising in apparently different sites from the cystic lesion. In one patient, the ductal carcinoma developed around the pre-existing cystic lesion. In the remaining two patients, the tumors were too large to evaluate any local relationship between carcinoma and cystic lesion.

These results indicate that the detection of ductal carcinoma at an early stage was still difficult even under close surveillance imaging examination such as US.

EUS is a highly sensitive diagnostic method for detection of pancreatic carcinoma, especially small



**Figure 5** Scheme showing the pancreatic body and tail by pancreatoduodenectomy. A: Intraductal papillary tumor of the pancreatic body; B: Invasive adenocarcinoma of the pancreatic tail.



**Figure 6** Histological appearance of two lesions (HE). A: Intraductal papillary mucinous adenoma; B: Moderately differentiated tubular adenocarcinoma.

pancreatic carcinomas. In the case reported here, the small pancreatic mass was not detected by CE-MDCT, US or MRCP. EUS was the only imaging technique that detected this mass. During the follow-up of branch duct IPMN, special attention should be paid to the occurrence of ductal carcinoma distinct from IPMN as detected by EUS. Recent reports have described the utility of contrast-enhanced EUS (CE-EUS) and CEH-EUS using sonographic contrast agent for the differentiation of pancreatic masses<sup>[3-7]</sup>. In the case reported here, CEH-EUS imaging using Sonazoid clearly distinguished the margin of the tumor and accurately measured the size of the lesion. Moreover, CH-EUS revealed that the mass was

hypovascular when compared with surrounding tissue, which suggested an adenocarcinoma of the pancreatic tail. Therefore, CEH-EUS should be used for the accurate diagnosis of small pancreatic tumors, including the synchronous and metachronous occurrence of IPMN and ductal adenocarcinoma of the pancreas.

Here, we reported a patient with a very small ductal carcinoma of the pancreas distinct from a branch duct IPMN. The performance of EUS every six months coupled with CEH-EUS imaging during the follow-up of the branch duct IPMN allowed the establishment of an accurate diagnosis of the disease.

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## Spontaneous liver rupture in a patient with peliosis hepatis: A case report

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### Abstract

Peliosis hepatis is a rare pathological entity and may cause fatal hepatic hemorrhage and liver failure. Here, we present a young male patient with aplastic anemia, who had received long-term treatment with oxymetholone. The patient suffered from sudden onset of intra-abdominal hemorrhage with profuse hemoperitoneum. The patient was treated successfully with a right hemihepatectomy and is in good health after 13 postoperative months. We suggest that peliosis hepatis be considered in patients with hepatic parenchymal hematoma, especially in patients under prolonged synthetic anabolic steroid medication. The possibility of a potentially life-threatening complication of massive intra-abdominal bleeding should also be considered.

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**Key words:** Peliosis hepatis; Spontaneous rupture; Liver

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### INTRODUCTION

Peliosis hepatis (PH) was first reported in the German literature in 1861 by Wagner, and named by Schoenlank in 1916<sup>[1]</sup>. It is a rare condition characterized by multiple small cystic blood-filled spaces from 1 mm to several centimeters in diameter in the hepatic parenchyma, resulting from the focal rupture of sinusoidal walls<sup>[2]</sup>. The etiology of PH is still unknown. Sinusoidal obstruction, hepatocellular necrosis, and direct injury to the sinusoidal barrier seem to be the most likely mechanism in adults<sup>[3]</sup>. PH is usually associated with chronic pathologic conditions, including cystic fibrosis, and human immunodeficiency virus (HIV) infection<sup>[4,5]</sup>. PH has also been reported after prolonged use of anabolic steroids (e.g. azathioprine<sup>[6]</sup>, 6-thioguanine, and 6-mercaptopurine<sup>[7]</sup>) and oral contraceptives<sup>[8]</sup>. PH varies from minimal asymptomatic lesions to larger massive lesions that may present with cholestasis, liver failure, portal hypertension, avascular mass lesion, or even spontaneous rupture<sup>[9]</sup>. Here, we report a case of a 20-year-old male patient with aplastic anemia who presented with hemoperitoneum. This patient had received long-term treatment with oxymetholone, and his imaging findings and extraphysiological changes were consistent with spontaneous hepatic rupture with PH. The patient was salvaged from a life-threatening hemorrhage by performing a right hemihepatectomy.

### CASE REPORT

The patient was a 20-year-old male who had been diagnosed with aplastic anemia 8 years previously and had received long-term anabolic steroid therapy. He presented with complaints of pain in the abdomen for one day and a seizure attack without any previous episode. The vital signs at presentation were blood pressure 84/44 mmHg, heart rate 140 beats/min, respiratory rate 24 breaths/min, and body temperature 36°C. The laboratory findings were white blood cell count  $5.1 \times 10^9/L$  (normal  $4 \times 10^9 - 10 \times 10^9/L$ ), hemoglobin 3.01 mg/dL (normal 13.1-17.5 g/L),

hematocrit 24.2% (normal 39%-52%), platelet count  $38 \times 10^9/L$  (normal  $140 \times 10^9 - 400 \times 10^9/L$ ), total bilirubin 1.287 mg/dL (normal  $< 1.3$  mg/dL), alkaline phosphatase 145 IU/L (normal  $< 220$  IU/L), alanine aminotransferase 495 IU/L (normal  $< 43$  IU/L), aspartate aminotransferase 332 IU/L (normal  $< 38$  IU/L), and prothrombin time 17.1 s (normal 11-15 s). Computed tomography (CT) of the brain was taken in the emergency department and revealed no specific abnormality, but the patient suffered from 3 more seizure attacks and became rapidly hypotensive and tachycardic, with abdomen distended and tense. The patient received a red blood cell (RBC) transfusion and fluid resuscitation. Abdominal and pelvic CT showed a laceration of the liver in the right lobe inferior portion and a moderate amount of hemorrhage (Figure 1). Suspecting spontaneous liver rupture with hemoperitoneum, the patient was transferred to the angiography unit for an emergency angioembolization of the right hepatic artery. The angiography showed multiple contrast filling small round lesions predominantly in the right hemiliver (Figure 2A). The right hepatic artery was selected, and gel-form embolization was attempted. On the venous phase angiogram, the peripheral portion of the right lobe of the liver was successfully embolized (Figure 2B). However, the patient continued to deteriorate, and hepatic and/or portal venous bleeding was suspected. An emergency exploratory laparotomy was performed.

Under general anesthesia, the patient was placed in the supine position. A bilateral subcostal incision was made and deepened to the peritoneum. On entering the peritoneal cavity, profuse hemoperitoneum was noted, and the liver was found to be lacerated on the right posterior sector. The Pringle maneuver was performed, but failed to lessen the bleeding. An urgent right hemihepatectomy was performed by the Kelly-clamp crushing method and the finger-fracture technique. After hepatectomy was done, the right upper quadrant was packed with a laparotomy gauze pad, and the peritoneal cavity was thoroughly examined. The remaining left hemiliver did not show gross abnormality nor did the other intraperitoneal organs. After the peritoneal cavity was irrigated with a warm saline solution, the packed gauze pad was removed and the cut surface was examined for further bleeding. Only minimal oozing was noted. Two closed suction drains were inserted at the hepatic cut surface, and the abdominal wall was closed layer by layer. A resected specimen showed extensive necrosis and hemorrhage and the cut surface of the liver parenchyma revealed small blood-filled cystic lesions (Figure 3A and B). The operation required 7 h, and 28 pints of leukoreduced RBC were transfused. The systolic blood pressure ranged from 90 mmHg to 130 mmHg throughout the operation.

Postoperatively, the patient was transferred to the intensive care unit and observed closely. When stable, the patient was admitted to the general ward on the 10th postoperative day, and oral intake was resumed. The drains were removed on the 20th postoperative day. The patient's vital signs were stable until the 17th postoperative day when body temperature began to rise abruptly



**Figure 1** CT of the abdomen showing laceration of the liver right lobe inferior portion (white arrow), with hyperdense fluid in the peritoneal cavity and liver parenchyma, suggesting acute blood collection in these areas (black arrows).

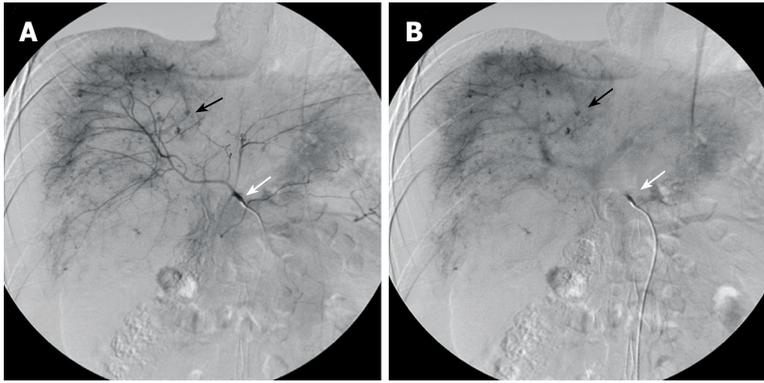
up to 38.5°C. Follow-up CT revealed an intraperitoneal abscess at the hepatectomy site. CT-guided percutaneous drainage was performed, and the patient's fever subsided with antibiotic therapy. The patient was discharged from hospital on the 46th postoperative day. The most recent follow-up was on the 13th postoperative month, and the patient was in good condition without significant complication from the surgery or PH.

The diagnosis of PH was determined from preoperative angiographic findings and histological features. Selective angiography of the celiac trunk showed hemorrhagic spots in the right hepatic parenchyma at the arterial phase with delayed washout at the portal venous phase. Microscopically, there were variable sized blood-filled cystic spaces, and hemorrhagic necroses were present adjacent to the peliotic spaces without lining endothelium (Figures 3 and 4).

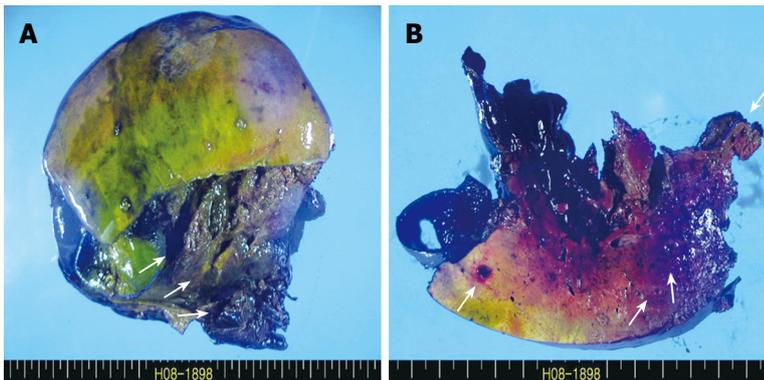
## DISCUSSION

Peliosis is an abnormality of the reticuloendothelial system and most commonly involves the liver. Occasionally, this condition may occur in the spleen and the bone marrow. Peliosis has mostly been reported in adult patients associated with chronic infections such as HIV<sup>[10]</sup> or the use of specific drugs<sup>[6,7,11]</sup>. In many cases, removal of the causative agent had caused regression of the disease<sup>[12]</sup>.

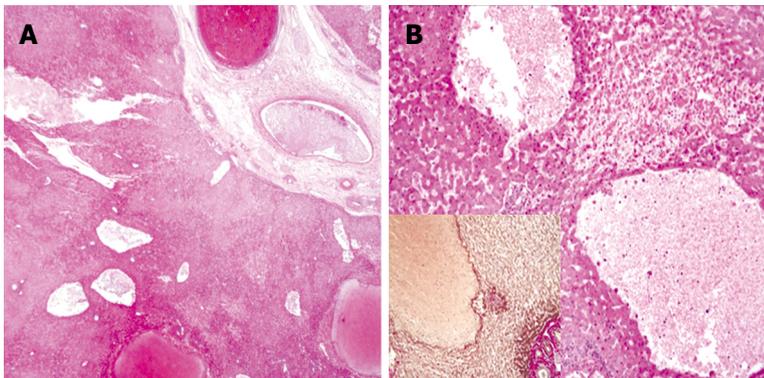
The exact pathogenesis of peliosis is unknown. PH is characterized by blood-filled cystic spaces from a few millimeters to 1 cm in diameter, but can reach up to 4 to 5 cm. Two morphological patterns of PH were described by Yanoff *et al*<sup>[13]</sup>. The parenchymal pattern is characterized by irregular blood-filled spaces, lined by fibrous tissue rather than the endothelium, which neither communicate with the central veins nor compress adjacent parenchymal cells. Instead, they associate with many areas of focal necrosis in the surrounding hepatic tissue. The phlebotatic pattern, the second morphological type, shows minimal hepatocyte necrosis. The regularly spherical, centrilobular blood-filled spaces are lined by endothelial cells and/or fibrotic tissue and freely communicate with hepatic sinusoids. They are an inte-



**Figure 2 Preoperative selective hepatic angiography.** A: Selective angiography of the celiac trunk (white arrow) shows hemorrhagic spots (black arrow) in hepatic parenchyma at the arterial phase. Note: Lesions are mainly distributed in the right hemiliver; B: Angiogram at the portal venous phase shows the lesions with delayed wash-out (black arrow).



**Figure 3 Gross findings.** A: Resected specimen shows extensive necrosis and hemorrhage (arrows); B: Cut surface of the liver parenchyma reveals small blood-filled cystic lesions (arrows).



**Figure 4 Microscopic findings.** A: Low magnification view shows variable-size, blood-filled cystic spaces (HE,  $\times 10$ ); B: High magnification view shows hemorrhagic necrosis in areas adjacent to peliotic spaces without lining endothelium (HE,  $\times 100$ ); Inset: immunohistochemical staining for reticulin ( $\times 100$ ).

gral part of the central vein, causing compression of the adjacent parenchymal cells, and they contain mural fibrin clots. However, Zak<sup>[1]</sup> considered these 2 morphological patterns to be one process, initiated by focal necrosis of the liver parenchyma, which transforms into an area of hemorrhage (parenchymal pattern). This pattern may progress to fibrous wall formation and endothelial lining around the hemorrhage (phlebotactic pattern), or it may heal by the resorption of blood. The phlebotactic pattern may heal by fibrin deposition, thrombosis, and sclerosis of vascular spaces. It has also been noted that a hyperplastic hepatocyte may be prolapsed into a terminal hepatic venule in anabolic steroid-induced peliosis. The theory that toxic substances may induce peliosis is supported by the finding of increased endothelial cell permeability with numerous RBCs in the space of Disse<sup>[14]</sup>. In the current case, the toxic substance that affected the sinusoidal wall was suspected to be the anabolic steroid oxymetholone, and the morphological characteristics

were consistent with the parenchymal pattern. Degott *et al*<sup>[15]</sup> also reported that peliosis could be secondary to fibrous thickening of the hepatic venule, possibly due to azathioprine in patients who had taken the drug long-term.

Eising *et al*<sup>[16]</sup> reported an increased tendency toward liver rupture following blunt trauma in patients with PH. In the present case, the patient had no trauma history, and the cause of the seizure attack was believed to be hemorrhagic shock induced by spontaneous liver rupture. In addition, the patient's usual platelet count was within the range of  $40 \times 10^9/L$  to  $70 \times 10^9/L$ , and the platelet count just prior to hepatic rupture was  $43 \times 10^9/L$ . To our knowledge, there has been no reported case of spontaneous liver rupture with this platelet level in the English literature. On the basis of these findings, we believe that the cause of spontaneous hepatic rupture in this patient was PH, and it was not the result of liver trauma or thrombocytopenia.

Interestingly, although systemic conditions were thought to be the cause of PH, the distribution of lesions within the liver was usually unilobar, favoring the right hemiliver. As far as we know, there has been no published report in the English literature on PH that mentions a preference in location. However, reported cases of liver rupture in PH patients are more common in the right lobe. Adam *et al*<sup>[17]</sup> and Toth *et al*<sup>[18]</sup> reported cases of PH rupture located in the left lobe of liver. But in other cases, the right lobe of the liver was the location of rupture<sup>[9,19-22]</sup>, including the current case. Although Saatci *et al*<sup>[23]</sup> reported a case of PH with multiple foci involving both right and left lobes by magnetic resonance imaging, the location of PH in most cases was unilobar, which may be explained by the distribution of hepatic blood flow. However further research is needed to verify the cause of differential distribution of PH rupture, as more case studies are reported. At the very least, it can be postulated that PH of the liver is a progressive disease that occurs in one lobe first, then extends to the whole liver, and hepatic rupture is distributed along with lesions.

PH is difficult to recognize, and the diagnosis often is missed or delayed because its appearance on radiological imaging is suggestive of a neoplasm or multiple abscesses. An ultrasound scan may show hypoechoic areas involving the liver with intraperitoneal fluid collection and a normal Doppler signal<sup>[24]</sup>. Occasionally, only subtle inhomogeneity is seen in the liver, which can also be seen in other diffuse liver parenchymal diseases. A contrast enhancement CT scan of the liver may show small lesions of a few millimeters to 1 to 4 cm in diameter. The lesions typically are hypodense in early arterial phase scans and enhanced in late venous phase. The CT appearance of PH can be difficult to differentiate from multiple abscesses, hemangiomas, and metastases. The diagnosis of PH on an angiography is made by visualizing multiple small accumulations of contrast material in the late arterial phase, which persists into the venous phase. This can be confused with irregular tumor vessels in focal nodular hyperplasia and adenomas. However, it is unclear if the angiography offers better diagnostic information than ultrasonography. In the current case, an emergency CT failed to show specific changes of PH other than that of hemoperitoneum caused by hepatorrhesis, and an angiogram was necessary to arrive at the diagnosis. A definitive diagnosis of PH was made from histological findings. A percutaneous needle biopsy can also be used to confirm the diagnosis. However, even when ultrasound-guided, the procedure has a high risk of a life-threatening hemorrhage<sup>[25]</sup>. Consequently, a laparotomy appears to be the more appropriate procedure when tissue confirmation is needed because it permits assessment of the macroscopic appearance of peliosis, and a liver biopsy can be performed with adequate hemostasis.

There is no specific treatment for PH. Hepatic artery embolization or partial hepatectomy has been reported<sup>[8,26]</sup>. Emergency liver transplantation is the ultimate

treatment in cases of imminent liver failure. The natural course of PH is not well known. Reports have described outcomes ranging from spontaneous resolution to hepatic failure or fatal intraperitoneal hemorrhage. Some reports have described the resolution of PH after withdrawal of causative drugs or after treatment of the associated conditions<sup>[12,26,27]</sup>. In other reports, patients presenting in fulminant hepatic failure eventually died. Fatal intraperitoneal hemorrhage has also been reported as another complication<sup>[9,28,29]</sup>. In summary, PH is an important disorder for consideration in the differential diagnosis of liver masses presenting acutely. PH is most often asymptomatic; in symptomatic patients, surgery should be reserved for cases when hemorrhage becomes life-threatening. Familiarity with imaging characteristics and awareness of this condition as a cause of hepatomegaly, hepatic failure, hepatic rupture, and hepatic hemorrhage can help in the early diagnosis of PH. Early removal of the known inciting agent may cause regression of this disease and prevent catastrophic complications. PH should be considered in all patients with known risk factors and presenting with typical morphological changes or hepatic mass, especially when the cause of the sudden onset of intraperitoneal hemorrhage is obscure. Although spontaneous regression of PH can occur in some patients, timely recognition and early treatment are essential to prevent life-threatening complications from the disease.

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LETTERS TO THE EDITOR

## Hepatitis B markers and vaccination-induced protection rate among Albanian pregnant women in Greece

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### Abstract

Hepatitis B has long been a serious public health problem both in Greece and in Albania. In the February 2009 issue of *World Journal of Gastroenterology*, Resuli *et al* presented the interesting epidemiological data concerning hepatitis B virus infection in Albania. The results of this study were discussed and several data from our similar research were provided.

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**Key words:** Hepatitis B; Vaccination; Pregnancy; Albania; Greece

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### TO THE EDITOR

In the February 2009 issue of *World Journal of Gastroenterology*,

Resuli *et al*<sup>[1]</sup> presented the interesting epidemiological data concerning hepatitis B virus (HBV) infection in Albania. The authors studied the positive hepatitis B surface antigen (HBsAg) and antibody to hepatitis B surface antigen (anti-HBs) among 3880 unvaccinated residents in rural and metropolitan areas of Albania. In the entire study population, the prevalence of HBsAg and anti-HBs was 9.5% and 28.7%, respectively, demonstrating that despite the estimated two-fold reduction of HBsAg prevalence in the general population, Albania remains a highly endemic country.

It is well known that vertical (mother-to-infant) transmission of HBV infection occurs mostly during the perinatal period and is responsible for the majority of the disease burden in endemic areas. The risk of vertical transmission generally depends on the level of maternal infectivity, particularly on the presence of hepatitis B e-antigen (HBeAg) and HBV DNA level<sup>[2]</sup>. In the study by Resuli *et al*<sup>[1]</sup>, 7.3% of the pregnant women (mean age 27.4 years) were chronically infected with HBV and 36.3% were positive for antiHBs. Despite the absence of serological data about the antiHBc status, it seems that more than 40% of the Albanian pregnant women studied have been exposed to HBV (taking into consideration a proportion of women with isolated anti-HBc seropositivity).

Hepatitis B has long been a serious public health problem in Greece also. Historically, Greece used to have the highest burden of HBV infection in the European Union. An early hepatitis B prevention program introduced in 1982, targeting mainly the high-risk groups, did not significantly impact the disease incidence and prevalence. More recent universal HBV mass vaccination programs launched in the past decade have proved to be the key measure in decreasing the disease burden, as well as demographic and socioeconomic changes, safer medical and nursing practices, and finally, screening of the blood donors have significantly declined the chronic HBV infection in our country<sup>[3]</sup>. However, a substantial intromission of refugees, especially descending from countries, endemic for HBV infection (mainly from Albania), is likely to have influenced this trend.

In a study conducted between September 2008 and June 2009 in the Maternal and Perinatal Hospital of Athens "Elena Venizelou", we evaluated the current prevalence of HBV serological markers in a multinational population of pregnant women, residing in Greece. A

total of 1333 pregnant women (mean age 28.5 years) who delivered at the Departments of Obstetric and Gynaecology of the Hospital were prospectively evaluated. HBsAg, HBeAg, antibody to hepatitis B e-antigen (anti-HBe), antibody to hepatitis B core antigen (anti-HBc) and anti-HBs were detected by commercially available routine enzyme immunoassays (Abbott Laboratories, Abbott Park, Illinois, US). All women of the study population were screened for HBsAg, anti-HBc and anti-HBs, whereas HBeAg and anti-HBe were evaluated only in those who were positive for HBsAg. The mean difference between two groups was evaluated by Student's *t*-test after the equality of variances was controlled with the Levene's statistic, while one-way analysis of variance (ANOVA) was used to assess the differences in continuous variables among more than two study groups.

More than half of the study population had Greek origin (756/1333, 56.7%), 30.6% were descendents from Albania (408/1333) and 12.7% (169/1333) from Eastern European countries (Russia, Romania, Bulgaria). Overall, 4.4% (58/1333) of the women were HBsAg (+) and the vast majority of them (45/58, 77.58%) were Albanians. Among the Albanian women, the prevalence of HBsAg was 11% followed by 2.4% among the women from Eastern European countries. The prevalence of HBsAg among the Greek women (1.2%) was very low and significantly lower than the mean value of the non-Greek population studied (7.4%,  $P < 0.001$ ). More than half (52%) of the Albanian women exhibited seropositive anti-HBc followed by Eastern European women (22.5%), whereas only 6.5% of the Greek women had serological markers compatible with previous HBV exposure. Moreover, serological markers of past HBV infection with spontaneous

recovery [antiHBc (+) and antiHBs (+)] were observed in 13.9% of the whole study population. Among the Greek women, only 3.6% exhibited serological markers of spontaneous recovery from HBV infection in contrast to 32.7% among the Albanian women and 14.8% among the Eastern European women ( $P < 0.001$  in all cases). Interestingly, only 33.8% of the Greek women and 40.3% of the Albanian women ( $P = 0.15$ ) exhibited vaccination-induced protection against HBV infection, characterised by the presence of positive isolated antiHBs.

Our data are in accordance with those presented by Resuli *et al*<sup>11</sup>, supporting that HBV infection is endemic among Albanian pregnant women. However, it seems that the awareness of Albanian immigrants, who live and work in Greece, about the control of HBV infection, is rising steadily, as supported by the comparable vaccination-induced protection rates among the Greek and Albanian pregnant women observed in our study. The relatively low vaccination-induced protection rates observed suggest that more intense surveillance and immunisation programs, targeting pregnant women, are necessary to avoid vertical transmission of HBV infection.

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## Meetings

### Events Calendar 2009

January 12-15, 2009  
Hyatt Regency San Francisco, San Francisco, CA  
Mouse Models of Cancer

January 21-24, 2009  
Westin San Diego Hotel, San Diego, CA  
Advances in Prostate Cancer Research

February 3-6, 2009  
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)  
Second AACR Conference  
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009  
Hyatt Regency Boston, Boston, MA  
Translation of the Cancer Genome

February 8-11, 2009  
Westin New Orleans Canal Place, New Orleans, LA  
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009  
Hong Kong Convention and Exhibition Centre, Hong Kong, China  
19th Conference of the APASL  
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009  
Orlando, Florida  
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009  
Vienna, Austria  
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease  
[www.easl.ch/vienna2009](http://www.easl.ch/vienna2009)

March 13-14, 2009  
Phoenix, Arizona  
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009  
Marriott Wardman Park Hotel  
Washington, DC  
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009  
Glasgow, Scotland  
British Society of Gastroenterology (BSG) Annual Meeting  
Email: [bsg@mailbox.ulcc.ac.uk](mailto:bsg@mailbox.ulcc.ac.uk)

April 8-9, 2009  
Silver Spring, Maryland  
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009  
Colorado Convention Center, Denver, CO  
AACR 100th Annual Meeting 2009

April 22-26, 2009  
Copenhagen, Denmark  
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)  
<http://www.easl.ch/>

May 17-20, 2009  
Denver, Colorado, USA  
Digestive Disease Week 2009

May 29-June 2, 2009  
Orange County Convention Center  
Orlando, Florida  
45th ASCO Annual Meeting  
[www.asco.org/annualmeeting](http://www.asco.org/annualmeeting)

May 30, 2009  
Chicago, Illinois  
Endpoints Workshop: NASH

May 30-June 4, 2009  
McCormick Place, Chicago, IL  
DDW 2009  
<http://www.ddw.org>

June 17-19, 2009  
North Bethesda, MD  
Accelerating Anticancer Agent Development

June 20-26, 2009  
Flims, Switzerland  
Methods in Clinical Cancer Research (Europe)

June 24-27 2009  
Barcelona, Spain  
ESMO Conference: 11th World Congress on Gastrointestinal Cancer  
[www.worldgicancer.com](http://www.worldgicancer.com)

June 25-28, 2009  
Beijing International Convention Center (BICC), Beijing, China  
World Conference on Interventional Oncology  
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009  
Snowmass, CO, United States  
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009  
Aspen, CO, United States  
Molecular Biology in Clinical Oncology

August 1-7, 2009  
Vail Marriott Mountain Resort, Vail, CO, United States  
Methods in Clinical Cancer Research

August 14-16, 2009  
Bell Harbor Conference Center, Seattle, Washington, United States  
Practical Solutions for Successful Management  
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009  
Beijing International Convention Center (BICC), Beijing, China  
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists (IASGO)  
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009  
Taipei, China  
Asian Pacific Digestive Week  
<http://www.apdwcongress.org/2009/index.shtml>

October 7-11, 2009  
Boston Park Plaza Hotel and Towers, Boston, MA, United States  
Frontiers in Basic Cancer Research

October 13-16, 2009  
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States  
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009  
Versailles, France  
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009  
Boston, MA, United States  
The Liver Meeting

November 15-19, 2009  
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States  
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009  
London, UK  
Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

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*Chinese journal article (list all authors and include the PMID where applicable)*

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

#### No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

#### Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

#### Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

#### No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

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#### Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

#### Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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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

### Statistical data

Write as mean  $\pm$  SD or mean  $\pm$  SE.

### Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as  $\chi^2$  (in Greek), related coefficient as *r* (in italics), degree of freedom as  $\nu$  (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

### Units

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose)  $6.4 \pm 2.1$  mmol/L; blood CEA mass concentration, *p* (CEA) =  $8.6 \pm 2.5$   $\mu$ g/L; CO<sub>2</sub> volume fraction, 50 mL/L CO<sub>2</sub>, not 5% CO<sub>2</sub>; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23243641.

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### Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

Restriction enzymes: *EcoRI*, *HindI*, *BamHI*, *Kho I*, *Kpn I*, etc.

Biology: *H. pylori*, *E coli*, etc.

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## Spontaneous bacterial peritonitis: A severe complication of liver cirrhosis

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### Abstract

This report presents a survey of current knowledge concerning one of the relatively frequent and severe complications of liver cirrhosis and associated ascites-spontaneous bacterial peritonitis. Epidemiology, aetiology, pathogenesis, clinical manifestation, diagnosis and present possibilities of treatment are discussed.

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**Key words:** Liver cirrhosis; Portal hypertension; Ascites; Spontaneous bacterial peritonitis

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### INTRODUCTION

Spontaneous bacterial peritonitis (SBP) is defined as an infection of initially sterile ascitic fluid (AF) without a detectable, surgically treatable source of infection<sup>[1]</sup>. It is a frequent and severe complication of cirrhotic ascites, first described in the middle of the 1960s<sup>[2]</sup>. Sometimes spontaneous infection of ascites is divided into three subgroups: (1) Spontaneous bacterial peritonitis is defined as a positive bacterial finding in ascites, together with increased polymorphonuclear leukocytes in ascites ( $> 250$  cells/mm<sup>3</sup>)<sup>[3]</sup>. Microorganisms responsible for SBP are isolated in 60%-70% of cases. (2) Culture-negative neutrocytic ascites (CNNA) - ascites is sterile, bacterial infection is not demonstrable by culturing, only an increased number of polymorphonuclear leukocytes above the limit of 250 cells/mm<sup>3</sup> is revealed. Of course, it is necessary to eliminate another cause of increased leukocytes in ascites, e.g. previous antibiotic therapy, hepatocellular carcinoma, peritoneal carcinomatosis or tuberculosis, pancreatitis or bleeding into ascitic fluid. If the sample of ascites contains blood, SBP diagnosis is made by finding more than one neutrophilic granulocyte per 250 erythrocytes. If left untreated, about one-third of cases can show, after some time, positive bacteriological findings. Both symptoms and mortality in patients with CNNA are similar to the course of disease in patients with diagnosed SBP; 33%-57% of these patients also show a positive blood culture, which provides evidence of a systemic bacterial infection. Infection is also confirmed by the fact that in patients with previous CNNA, there is a more frequent occurrence of SBP and *vice versa*<sup>[4]</sup>. (3) Monomicrobial non-neutrocytic bacterascites (or only bacterascites) has rarely been described. In this disorder, positive bacterial cultivation is presented without increased leukocytes. It is usually revealed in Child-Pugh class A patients. Recovery from bacterascites can be spontaneous (in 60%-80%), or it can develop into typical SBP. Bacterascites is often quite asymptomatic, and antibiotics should only be used if symptoms appear and cultivation finding is persistent.

As mentioned above, SBP and CNNA are identical, both from the clinical point of view and the therapeutic approach; therefore, the consensus conference of the

International Ascites Club<sup>[5]</sup> has recommended not to differentiate between these two entities; even in the case of CNNA, SBP is spoken about, and an increased number of neutrophils in ascites is sufficient for the diagnosis. A spontaneous infection complicating ascites may appear even in malignant ascites<sup>[6]</sup>, however, it is found most often in cirrhotic ascites.

Early, mostly retrospective, studies described SBP in about 8% of patients with ascites; later prospective trials revealed SBP in 10%-30% of patients with ascites admitted to hospital<sup>[5,7,8]</sup>. SBP is found in about 5% of non-selected outpatients<sup>[9]</sup>. Lethality is very high. Older studies reported 80%-100% lethality connected with SBP<sup>[10]</sup>, which was probably given partly by the generally worse therapeutic possibilities in cirrhotic patients and lack of availability of effective antibiotics, but better results - 20%-40% as reported in later studies<sup>[11]</sup> - are, to a certain extent, due to early diagnosis and treatment.

However, lethality has not decreased over recent years<sup>[12]</sup>. Even the long-term prognosis in these patients has been unfavourable. In 40%-70% of patients, SBP relapses within 1 year<sup>[11]</sup>. One-year survival after previous SBP patients is only 30%-40%, two-year survival is 20% and in patients with a Child-Pugh score > 10 survival is even lower<sup>[13]</sup>.

## AETIOLOGY

G<sup>-</sup> bacteria are found in 65% of positive culture results-*Escherichia coli* (*E. coli*) and *Klebsiellae* are the most common and second most common agents, respectively<sup>[14]</sup>. The remaining agents are represented by G<sup>+</sup> cocci<sup>[1,7]</sup>. There is a difference in bacteria responsible for SBP in hospitalised and non-hospitalised patients. G<sup>-</sup> bacteria prevail in the first group of patients, while G<sup>+</sup> bacteria are found in the latter<sup>[9]</sup>.

Experiments modelling hepatic cirrhosis and the accompanying SBP in a rat resulted in discovery of agents in AF that differed from those in humans. It was mainly *Enterococcus faecalis* that was isolated from AF in the rat affected by cirrhosis induced by tetrachloromethane and SBP was of polymicrobial origin in one half of cases<sup>[15]</sup>. None of the above findings is typical of human patients suffering from SBP.

## PATHOGENESIS

Bacteria participating in SBP come from the digestive tract. Extraintestinal bacteria such as those from the respiratory apparatus, urogenital tract or skin are much less frequent. Catheters and other equipment used during invasive procedures represent another possible source of infection. It is currently hypothesised that SBP follows an episode of bacteremia during which, due to the constant exchange of fluids between the peritoneal and intravascular space, AF gets infected<sup>[14]</sup>. The development of SBP thus depends on the antibacterial capacity of AF (the so-called opsonin activity) which is positively correlated to the content of the total protein in AF and the immunocompetence of the patient. The organism reacts to the infection of AF by activating neutrophilic

granulocytes which migrate into the peritoneal cavity and trigger a complex cytokine cascade; the fact being documented, for example, by increased concentrations of interleukin 6 and tumour necrosis factor  $\alpha$  in AF.

There are four key elements of SBP pathogenesis: (1) small intestinal bacterial overgrowth, (2) increased intestinal permeability, (3) bacterial translocation, and (4) immunosuppression. These key elements are not separate, but interlinked.

### **Small intestinal bacterial overgrowth (SIBO)**

Considering bacteria commonly found in the gastrointestinal tract, only some of them frequently participate in translocation. The most frequent bacteria are *E. coli*, *Proteus spp.*, *K. pneumoniae* and other *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *enterococci*, *streptococci* and *staphylococci* - i.e. organisms causing infections in immunocompromised individuals. Bacterial small intestinal overgrowth creates conditions favourable for translocation of the above-mentioned bacteria. Liver cirrhosis is one of the conditions accompanied by SIBO. The main reasons for SIBO in patients affected by liver cirrhosis can be summarised as reduced intestinal passage, abnormalities in bile secretion, hypochlorhydria, abnormalities in IgA production and malnutrition<sup>[16]</sup>. A total of 20%-60% cirrhotic patients are affected by bacterial overgrowth. SBP is more often diagnosed in alcoholics affected by liver cirrhosis and SIBO as compared to patients affected in the same way but without SIBO<sup>[17]</sup>. Some authors are doubtful about the importance of SIBO in the pathogenesis of SBP<sup>[18]</sup>; according to this study, SIBO is associated with the use of drugs decreasing secretion of HCl and this therapeutic approach can increase the risk of SBP. Bacterial translocation found in a healthy individual after ingestion of extremely high numbers of microorganisms<sup>[19]</sup> can serve as a distant parallel of the relationship between SIBO and bacterial translocation.

### **Increased intestinal permeability**

In severely ill patients, such as those with liver cirrhosis, small intestinal motility is impaired, resulting in bacterial overgrowth and the related translocation of microbes through dysfunctional mucosal barrier. The increased intestinal permeability and thus impaired function of the intestinal barrier are mainly due to portal hypertension. The consequences are dilated vessels in the intestinal mucosa, oedema of the lamina propria mucosae, fibromuscular proliferation, hypertrophy of the lamina muscularis mucosae and compromised integrity of the intestinal mucosa. Portal hypertension can play an important role, as can the toxic effects of alcohol, bile secretion disorders, malnutrition, decreased secretion of growth factors (insulin-like growth factor I), changes in the composition and flow of bile, or higher levels of nitric oxide. Increased intestinal permeability is likely to be proportional to the degree of portal hypertension, but independent of the severity and aetiology of liver disease<sup>[20,21]</sup>.

### **Bacterial translocation**

The term bacterial translocation was first used in 1979<sup>[22]</sup>.

Bacterial translocation is defined as either active or passive penetration of living microorganisms and their toxic products through the mucosal epithelial layer to the lamina propria mucosae. From there, microbes migrate to mesenteric lymph nodes and/or extraintestinal sites. Under normal circumstances, there are only small numbers of bacteria readily destroyed by the immune system in the lamina propria mucosae. Translocation is only possible if their numbers are high, up to  $10^8$  bacteria in 1 g of faeces<sup>[17]</sup>.

According to clinical significance, there are four degrees of bacterial translocation. Degree 0: Bacteria and/or their components penetrate the mucosa by various mechanisms: active intracellular penetration, diffusion, absorption, endocytosis or phagocytosis by macrophages; Degree 1: Bacteria and/or their components enter the mesenteric lymphatic system and penetrate it centripetally; Degree 2: Bacteria and/or their components are already detectable in the systemic circulation and certain organs. They may also pass directly into small intestinal venules and from there into the portal circulation. Some of the bacteria are probably even capable of intracellular passage through the muscularis propria into the peritoneal cavity; Degree 3: The organism is systemically overwhelmed by bacteria and/or their components, leading to a septic response.

Bacteria escaping both phagocytosis and destruction by the complement system may even get into the bloodstream. *Enterobacteria*, *staphylococci* and *enterococci* are capable of translocation, i.e. passing live across the intestinal epithelium to the mesenteric lymph nodes, blood and other organs, whereas most anaerobic organisms lack this ability. Bacterial translocation may be confirmed by positive culture from the mesenteric lymph nodes. The main mechanisms underlying the translocation are deficient local mucosal immune response, lower phagocytic activity of both macrophages and neutrophils, increased permeability of the intestinal barrier and the above-mentioned intestinal bacterial overgrowth<sup>[16,17]</sup>.

### **Immunosuppression**

Patients with liver cirrhosis suffer from decreased phagocytic activity of neutrophilic granulocytes and the mononuclear phagocytic system, deteriorated humoral immunity and decreased opsonin activity of AF<sup>[23]</sup>. Neutrophilic granulocytes of patients with hepatic cirrhosis show not only a decrease in the phagocytic activity, but also intracellular destruction of bacteria, deteriorated metabolic activities, frequent apoptosis and considerably reduced chemotaxis. For proper protection against bacteria, neutrophilic granulocytes have first to adhere to the vascular endothelium, then migrate to the endothelial cellular junctions, pass through by diapedesis and migrate further into the target tissue. Neutrophilic granulocytes of cirrhotic patients adhere to the vascular endothelium to a greater extent and their transendothelial migration is thus decreased<sup>[24]</sup>. Neutrophilic granulocytes show decreased chemotaxis probably due to the presence of inhibitors of chemotaxis in the blood plasma of the cirrhotic.

The opsonin activity of AF correlates with the concentration of immunoglobulins, complement, fibronectin and total protein in AF<sup>[5,25]</sup>. Patients with a reduced total protein content in AF are prone to the development of SBP.

## **CLINICAL MANIFESTATION**

SBP is particularly revealed in patients with more severe liver functional damage (Child-Pugh classification C), often after bleeding from the upper gastrointestinal tract due to portal hypertension and often recurs. The clinical picture is non-specific. In a lot of cases the infection is quite asymptomatic, common signs - subfebrile states, diffuse abdominal pain - are not very conspicuous. They are frequently manifested only by the occurrence or deepening of symptoms that accompany the course of liver cirrhosis - increased ascites and failure of diuretic therapy, deteriorated encephalopathy, vomiting, *etc.* Therefore, an active search for the ascites infection is necessary.

Diagnostic paracentesis with leukocyte investigation is recommended in all patients with ascites admitted to hospital as well as in cirrhotics (whether in hospital or not) with worsened ascites, who have presented with signs of abdominal or systemic infection (abdominal pain or tenderness, disturbed intestinal passage, fever, acidosis, peripheral leukocytosis) or with encephalopathy or worsened renal functions<sup>[5]</sup>. An active approach to the SBP diagnosis is extraordinarily important even from the prognostic point of view. If SBP is diagnosed at the first paracentesis carried out in all patients hospitalised with ascites, this infection has no influence on the patient's prognosis. However, if SBP appears over the course of studying these patients, the risk of lethality increases two-fold<sup>[26]</sup>. This can probably be explained by the damaged renal function under a longer-lasting untreated infection.

High lethality is not primarily associated with the severity of the infection, and patients do not die of sepsis. An infection only worsens the changes present in cirrhotic patients, especially blood supply and renal function<sup>[27]</sup>. Splanchnic vasodilatation occurring in cirrhotics is worsened due to endotoxemia; effective arterial blood volume decreases which can damage renal function and can cause hepatorenal syndrome. Patients who showed a higher level of urea and higher portal pressure at the moment of diagnosis of an infection are threatened with renal failure and the associated high lethality<sup>[28]</sup>.

## **DIAGNOSIS**

Diagnosis is relatively easy. SBP is diagnosed by revealing polymorphonuclear leukocytes in ascites  $> 250$  cells/mm<sup>3</sup> (less accurately at the increase of all leukocytes over 400/mm<sup>3</sup>). Reagent papers used for examining leukocytes in urine could be used for the immediate detection of leukocytes in ascites<sup>[29]</sup> although the sensitivity is not sufficient. A positive cultivation finding is not necessary for diagnosing SBP, it is usually revealed in about 30% of cases. Some trials have mentioned a higher bacteriological

detection if ascites is inoculated on the medium at the bedside as it is carried out for the investigation of blood culture - BactAlert test<sup>[30]</sup> - however, the advantage of this test has not been demonstrated by other studies<sup>[8,31]</sup>.

In the case of diagnostic doubts, the serum procalcitonin level (the limit of 0.75 ng/mL has 95% sensitivity, and 98% specificity) or the interleukin 6 level in ascites (the limit of 5.0 ng/mL has 100% sensitivity) may be determined<sup>[32]</sup>. Nowadays, examination of the ascites pH (for the diagnosis of SBP < 7.35) or arterial: ascites pH gradient (> 0.1) as recommended previously is no longer used.

## TREATMENT

Therapy should be initiated immediately after revealing increased leukocytes in ascites. Empiric antibiotic therapy, e.g. an intravenous third-generation cephalosporin, preferably cefotaxime in a single dose of 2 g is the recommended antibiotic drug. Mostly, it is sufficient to administer cefotaxime every 8 h as this regimen is as effective as its administration every 6 h<sup>[33]</sup>. Even a shorter length of application is possible, cefotaxime administered every 8 h for 5 d has the same effects as its 10 d application and from a practical and financial point of view, this regimen seems to be the most suitable. Two 3rd generation cephalosporins may alternatively be used-cefonicid (2 g every 12 h) or ceftriaxone 2 g/d<sup>[34]</sup>. In uncomplicated SBP (i.e. if diagnosed during preventive examination of ascites without clinical signs of infection) a combination of amoxicillin, clavulanic acid<sup>[35]</sup>, or ofloxacin 400 mg may be administered every 12 h for a period of 7-10 d<sup>[36]</sup>. Although more than 10 therapeutic studies have been published since 1985, clear proof of the efficacy of these antibiotics supported by evidence-based medicine is still missing, and treatment is based more on clinical experience<sup>[37]</sup>. Moreover, there are reports on increasing occurrence of enterobacteria resistant to the 3rd generation cephalosporins<sup>[38]</sup>, therefore, a large, well-designed randomised trial is necessary to ensure explicit demonstration of the effectiveness of each antibiotic drug.

As mentioned above, the high lethality is associated with damaged renal function due to impaired hypovolemia. Therefore, the efforts of expansion of intravascular volume could be contributory, and some studies have demonstrated that simultaneous application of albumin (as plasma expander) at a dose of 1.5 g/kg during the first 6 h and 1 g/kg on the 3rd d together with antibiotics decreased both the occurrence of renal damage and lethality<sup>[39]</sup>. This albumin replacement is recommended in patients with even clinical suspicion of SBP and serum creatinine > 1 mg/dL, blood urea nitrogen > 30 mg/dL, or total bilirubin > 4 mg/dL.

### Preventive treatment

Three groups of patients are at higher risk of SBP - patients with gastrointestinal bleeding, patients who have survived an episode of SBP and patients with low opsonic activity of ascites.

Preventive administration of antibiotics is recommended in the first two groups. Intravenous ceftriaxone

for 7 d or norfloxacin 400 mg twice a day for 7 d should be administered to prevent bacterial infections in patients with cirrhosis and gastrointestinal bleeding. Patients who have survived an episode of SBP should receive long-term prophylaxis with daily norfloxacin (400 mg once a day)<sup>[40]</sup> or trimethoprim/sulfamethoxazole. In these patients, liver transplantation should be considered as the lethality after passed SBP is higher than that after liver transplantation<sup>[5,13]</sup>. If transplantation is indicated, antibiotics should be applied until the operation. If transplantation is not indicated, according to literature, they should be applied for the rest of the patient's life. However, in clinical practice, an individual approach to each patient has been recommended.

Preventive application of antibiotics in the last group of patients at higher risk of infection - patients with the ascitic fluid protein < 15 g/L (i.e. with low opsonin activity) has not been generally recommended so far<sup>[41]</sup>. Long-term use of norfloxacin (or trimethoprim/sulfamethoxazole) can be justified in these patients if at least one of the following is present together with a low level of protein: serum creatinine > 1.2 mg/dL, blood urea nitrogen > 25 mg/dL, serum sodium < 130 mEq/L or Child-Pugh > 9 points with bilirubin > 3 mg/dL<sup>[42]</sup>.

Intraperitoneal re-infusion of concentrated ascites can effectively increase the protein level and opsonic activity of ascites<sup>[43]</sup>. On the other hand, it is not clear if just this procedure can reduce the risk of SBP in patients with low ascitic fluid protein.

Reports on possible influencing of bacterial translocation (and thus the development of SBP) using prokinetics<sup>[44,45]</sup> or probiotics<sup>[46]</sup> have appeared in the literature.

## CONCLUSION

Spontaneous infection of ascites is a very frequent and severe complication of ascites occurring in a high number of patients admitted to hospital with ascites. This infection must be actively searched for. Diagnostic paracentesis must be performed with examination of polymorphonuclear leukocytes in ascites in all patients suffering from ascites at the moment of their admission to hospital as well as in all cirrhotics with a sudden increase of ascites or worsening of their general condition.

If the number of polymorphonuclear leukocytes in ascites exceeds 250 cells/mm<sup>3</sup>, a positive bacteriological finding is not necessary, SBP is diagnosed and cefotaxime at the minimum dose of 2 g every 8 h for 5 d is administered. If patient has no clinical symptoms and SBP was diagnosed during preventive examination, ofloxacin 400 mg every 12 h for 7-10 d is an alternative. Although more studies are lacking, prevention of the development of simultaneous renal damage due to severe hypovolemia by administration of plasma expander (albumin at a dose of 1.5 g/kg during the first 6 h, and 1 g/kg on the third day) seems to decrease lethality.

Preventive application of antibiotics is indicated in two groups of patients with a high risk of developing SBP. Norfloxacin 400 mg twice a day is recommended

in patients with bleeding from the upper gastrointestinal tract and portal hypertension. The other group involves patients with previous SBP. As secondary prevention, norfloxacin 400 mg once a day is administered. Liver transplantation is recommended and antibiotics are applied until the surgery. Long-term administration (lifelong according to literature) is indicated in patients who are not indicated for transplantation.

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## Liver transplantation for hepatocellular carcinoma

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### Abstract

Hepatocellular carcinoma (HCC) is the commonest primary malignancy of the liver. It usually occurs in the setting of chronic liver disease and has a poor prognosis if untreated. Orthotopic liver transplantation (OLT) is a suitable therapeutic option for early, unresectable HCC particularly in the setting of chronic liver disease. Following on from disappointing initial results, the seminal study by Mazzaferro *et al* in 1996 established OLT as a viable treatment for HCC. In this study, the "Milan criteria" were applied achieving a 4-year survival rate similar to OLT for benign disease. Since then various groups have attempted to expand these criteria whilst maintaining long term survival rates. The technique of living donor liver transplantation has evolved over the past decade, particularly in Asia, and published outcome data is comparable to that of OLT. This article will review the evidence, indications, and the future direction of liver transplantation for liver cancer.

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**Key words:** Hepatocellular carcinoma; Selection criteria; Liver transplantation; Living donors

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### INTRODUCTION

Hepatocellular carcinoma (HCC) is the third most common cause of cancer death and the fifth most prevalent cancer in the world<sup>[1]</sup>. Cirrhosis itself is the major risk factor for the development of HCC<sup>[2-6]</sup>. Overall, 80%-90% of HCC develop in the context of liver cirrhosis<sup>[7]</sup>. Hepatitis B infection (HBV) particularly, can cause HCC in the absence of cirrhosis as a result of its direct carcinogenic effect<sup>[8]</sup>. Hepatitis B is the commonest cause of chronic liver disease in the East, whereas hepatitis C (HCV) and alcohol predominate in the West<sup>[9]</sup>. This has resulted in widespread geographical variation in HCC prevalence. In particular, in Asia and sub-Saharan Africa the incidence is far higher than in North America and Europe. In our Unit in London, 70% of patients with HCC on the background of liver disease have underlying chronic viral hepatitis; 40% have HCV, 30% HBV and a minority have a combined etiology including viral, steatohepatitis and/or alcoholic chronic liver disease.

The incidence of HCC is increasing in many parts of the world<sup>[10]</sup>. In the United States, for example, the rate of HCC has increased by 80% over the past 20 years<sup>[11]</sup>. This may be partly due to the fact that screening for HCC in patients with cirrhosis has become established practice. However, in our Unit over the past 5 years, only 55% of patients with HCC had their cancer detected as part of a screening program. Another likely reason for rising HCC incidence is the increasing burden of chronic viral hepatitis. In addition to HBV and HCV, both fatty liver disease and alcohol-related liver disease continue to rise. As a result, it is predicted that HCC prevalence will increase further over the coming decades.

Without specific treatment the prognosis is poor with median survival of early and advanced HCC being 6-9 mo and 1-2 mo, respectively<sup>[12]</sup>. Tumor resection in the context of cirrhosis and portal hypertension

leaves a residual cirrhotic liver which remains at risk of developing new lesions and a liver which is at risk of decompensation. Recurrence rates of 50%-70% have been reported after resection of HCC<sup>[13]</sup>. Many patients have advanced cirrhosis at presentation and are not eligible for resection. In addition, HCC is often multifocal and thus resection is not feasible.

## EVOLUTION

The initial liver transplants were performed by Starzl in 1963<sup>[14]</sup>. Four years elapsed before there was a long term survivor<sup>[15]</sup>. Over the subsequent decades advances in organ preservation and particularly prevention of organ rejection led to a steady rise in both transplant numbers and outcomes. With the widespread acceptance of OLT, transplantation for HCC was attempted. Unfortunately, the initial results of OLT for HCC showed disappointing short and long term survival rates and high levels of tumor recurrence<sup>[16]</sup>.

Given the huge demand for organs, transplantation needed to be limited to those with a good prognosis, and liver transplantation continued to flourish as the definitive treatment of end stage liver disease. A change in thinking arose from the following two observations. Firstly, finding small unexpected HCC in explanted livers did not affect the outcome of OLT<sup>[17]</sup>. Secondly, there was no significant difference in terms of overall survival between liver transplantation and resection in cirrhotic patients with hepatocellular carcinoma<sup>[18]</sup>. Furthermore, in small tumors, < 3 cm, whether uni- or bi-nodular, transplantation resulted in better survival than resection. This culminated in the development of the "Milan criteria" which has cemented OLT as an effective treatment for HCC.

## MILAN CRITERIA

The seminal study by Mazzaferro *et al*<sup>[19]</sup> in 1996 established OLT as a viable treatment for HCC. In this study in Milan, Italy, 48 patients with HCC and cirrhosis were followed and transplanted. Twenty eight patients had preoperative therapy, mainly transarterial chemoembolization (TACE). All patients fulfilled the following restrictive radiographic criteria: single lesion  $\leq$  5 cm, up to 3 separate lesions all less than 3 cm, no evidence of vascular invasion, no nodal or distant metastases. The aim of the criteria was to achieve a good prognosis in those who fulfilled them, avoiding a poor prognosis in those who exceeded them. The overall actuarial survival after 26 mo was 75% and recurrence-free survival was 83%. Patients who fulfilled these criteria on the basis of explant pathology had actuarial survival of 85% and recurrence-free survival of 92%. These rates are not dissimilar to the outcome of patients undergoing OLT without HCC. These criteria have become known as the "Milan criteria" and these results have been confirmed throughout the world.

## EXPANDED CRITERIA

Since the acceptance of OLT for the treatment of HCC,

excellent post-transplant survival has been reported in many centers. With increasing experience of OLT many centers have recognized that many explanted livers have had HCC beyond the size described in the "Milan criteria". These transplanted patients have been followed over time and many have had excellent 1- and 5-year survival. Also, advances in imaging have occurred in the past decade since these criteria were originally published. As a result several groups have attempted to broaden the restrictive limits. A group from the University of California at San Francisco (UCSF) have reported a 5-year survival of 75% using less restrictive criteria derived by retrospectively analyzing 2000 patients undergoing OLT<sup>[20]</sup>. This was achieved by using the following criteria: a maximum tumor size 6.5 cm or 2 lesions < 4.5 cm diameter with a total tumor diameter < 8 cm. The same group validated the 'San Francisco criteria' on 168 patients based on preoperative imaging assessments<sup>[21]</sup>. Various other groups have published expanded criteria with results not dissimilar to the original "Milan criteria"<sup>[22-28]</sup>. The same group in Milan has recently published retrospective data regarding outcome in 1112 patients exceeding the original Milan criteria<sup>[29]</sup>. In this study, a 71.2% 5-year overall survival could be achieved using the "up-to-7" criteria (HCC with 7 as the sum of the largest tumor (cm) and the number of tumors). It is clear that the larger the tumor size and number, the worse the outcome. Tumor understaging, by preoperative imaging of patients has been one of the major concerns for liberalizing the "Milan criteria"<sup>[30-32]</sup>. Also, there can be significant tumor progression during the waiting time to transplantation<sup>[33]</sup>. At the present time, the "Milan criteria" remains the only universally accepted and recurrently validated criteria.

## COMPARISON OF OLT TO OTHER TREATMENT STRATEGIES

Various treatment options are available for HCC including the surgical options as outlined above, and local ablative techniques such as radiofrequency ablation (RFA), transarterial chemoembolization (TACE), and percutaneous ethanol injection (PEI). Systemic chemotherapy has been unsuccessful in the studies thus far<sup>[34]</sup>. The local ablative treatments themselves are used as a palliative procedure or as a bridge to resection or transplantation. The only curative treatments are that of hepatic resection or transplantation.

The published observational studies to date suggest survival following OLT is at least as good as following resection in patients with adequate hepatic reserve<sup>[18]</sup>. Liver resection can leave residual liver which is of insufficient size to provide adequate function and as stated before, has the possibility of developing further lesions. In patients with well-compensated cirrhosis (Child Pugh A) and HCC, the decision whether to resect or transplant remains controversial. However, in the current realm of organ shortage and long waiting times, the decision to resect in this group appears attractive.

If tumor recurrence were to recur than salvage transplantation can be performed. This strategy has been retrospectively analyzed by different groups with conflicting outcome data. One retrospective study has shown no difference in either outcome or disease-free survival when comparing primary OLT to resection and salvage OLT<sup>[35]</sup>. In contrast, an observational series has shown primary OLT to have lower operative mortality, recurrence rates and survival rates<sup>[36]</sup>.

## REQUIREMENTS FOR LISTING

The United Network for Organ Sharing (UNOS) administers the allocation of organs for transplantation in the United States. UNOS provides a set of specific requirements<sup>[37]</sup> for listing patients with HCC: (1) Rough evaluation of the number and size of tumors and to rule out extra-hepatic spread by ultrasound, computed tomography (CT) or magnetic resonance imaging (MRI) plus CT of the chest; (2) Prelisting biopsy is not mandatory, however patients must have one of the following: (a) a biopsy confirming HCC; (b) a vascular blush corresponding to the area of suspected HCC; (c) an  $\alpha$  fetoprotein (AFP) > 200 mg/mL; (d) an arteriogram confirming a tumor; (e) a history of local ablative therapy (TACE, RFA, PEI); (3) Patients with chronic liver disease and a rising AFP > 500 mg/dL can also be listed even in the absence of discrete tumor on imaging studies; (4) The patient must not be a resection candidate; (5) Reimaging by CT or MRI every 3 mo is required to ensure continued eligibility for OLT.

## BRIDGING THERAPY

The “dropout” due to tumor progression whilst waiting for OLT is reported to be at least 20%<sup>[20]</sup>. This problem has furthered the use of local-regional adjuvant therapy (LAT) whilst awaiting transplantation such as TACE, RFA, and PEI. Unfortunately no randomized, controlled trials have been conducted to assess this approach<sup>[38]</sup>. As a result, there is no universal consensus as to the optimum bridging therapy prior to transplantation.

The ultimate aim of LAT is to provide complete tumor necrosis in an attempt to halt tumor progression. Analyses of explant specimens subjected to RFA and TACE have shown complete tumor necrosis rates of 47%-66% and 16%-27%, respectively<sup>[30,39,40]</sup>. A retrospective study looking at tumor necrosis in 61 patients did not find any particular modality of LAT to be superior<sup>[41]</sup>.

Retrospective analyses have shown that multi-modality local treatment is associated with a modest survival benefit<sup>[42,43]</sup>. As a result most units offer dual as well as single modality ablative treatments, in an attempt to reduce tumor progression and recurrence rates. Hepatic resection has also been used as a bridge to transplantation<sup>[44]</sup>. This is in contrast to the traditional view of resection and transplantation being opposing strategies. Unlike LAT which has been shown in studies to achieve only partial tumor necrosis, resection should

achieve the best tumor control. Resection also allows a thorough intra-operative assessment of liver status and tumor burden. A detailed histological analysis can be made of the resected specimen and can give invaluable information about the natural history of the tumor and the presence of microvascular invasion. Resection, however, is associated with increased risk and, as stated earlier, should only be attempted in well-compensated cirrhotic patients.

## PROGNOSTIC FACTORS

Conforming to the “Milan criteria”, in terms of tumor size and tumor burden, gives rise to 3-4-year recurrence-free survival rates of up to 92%<sup>[45-47]</sup>. Multivariate analysis has shown these to be the only independent variables predicting patient survival and tumor recurrence. Other biological factors such as tumor grading, microvascular invasion and microsatellites appear to play a role, but within the constraints of the size and number burden. These biological factors can only be accurately assessed post-transplantation or post-resection. The histological grade of the tumor can be assessed by lesional biopsy and several authors have recommended this approach<sup>[48-50]</sup>. Lesional biopsy does however carry the risk of tumor seeding which has been estimated at approximately 2%<sup>[51]</sup>. Furthermore, significant histological heterogeneity has been described in large tumors, which limits the utility of needle biopsy<sup>[52]</sup>.

A scoring system incorporating tumor differentiation in addition to tumor size and number has been validated in a 2007 French multicenter study<sup>[53]</sup>. In this study, tumor histology improved the Milan criteria in predicting post-transplant outcome. These results conflict with a more recent 100 patient study in which tumor differentiation did not predict HCC recurrence<sup>[54]</sup>.

## LIVING DONOR TRANSPLANTATION

Living donor liver transplantation (LDLT) has evolved over the past decade, mainly in response to the scarcity of donor livers. Deceased liver donation is particularly scarce in Asia, where organ donation rates are less than 5 donors per million population compared to 10-35 per million in Western countries<sup>[55]</sup>. This is also compounded by the fact that in most Asian countries HCC is the most common cancer and a frequent indication for OLT. LDLT, in particular right liver transplantation, has dramatically increased the number of potential donors. This can eliminate the problem of long waiting times and ‘dropout’ whilst waiting for an organ because of disease progression. Furthermore, as there is no direct ‘competition’ from other potential transplant recipients the restrictive criteria on tumor burden can be relaxed somewhat.

The results from LDLT appear to show good long term survival rates with retrospective studies showing comparable rates to OLT<sup>[56-58]</sup>. There are, however, published retrospective studies which show a higher rate of tumor recurrence than with conventional OLT<sup>[59-61]</sup>.

The reason for this is not entirely evident. It has been postulated that many candidates are not transplanted because of tumor progression whilst waiting for OLT; these patients, with more aggressive tumor biology, are then removed from the outcome analysis. In LDLT there is no protracted waiting time and hence the patients with aggressive tumors are transplanted.

LDLT has been performed, in some centers, in patients who have a tumor load beyond the Milan criteria with the consent of both the donor and recipient<sup>[62]</sup>. In addition, LDLT carries a risk to the donor during hepatectomy with morbidity and mortality rates of 14%-21% and 0.25%-1%, respectively<sup>[63]</sup>. This highlights the need for a universal consensus on the use of LDLT.

Akin to the published “expanded criteria” in OLT, several groups have attempted to determine an “expanded criteria” in LDLT. Using a scoring system including the measurement of “protein induced by vitamin K absence or antagonist- II” (PIVKA- II), a Japanese group have achieved a 5-year recurrence rate of only 4.9%<sup>[24,64]</sup>. PIVKA- II, also known as des-carboxyprothrombin, is an abnormal prothrombin protein found in the serum of patients with HCC and in patients with vitamin K deficiency or on warfarin therapy. It has been suggested as an alternative marker to AFP for the surveillance of HCC, but is not currently used as standard practice internationally. This study reported an 86.7% survival rate in patients with  $\leq 10$  tumors, all  $\leq 5$  cm (on pre-transplant imaging) and with PIVKA- II values  $\leq 400$  mAU/mL. Without the application of PIVKA- II, patients with  $\leq 10$  tumors, all  $\leq 5$  cm had similar 5-year recurrence rates as those conforming to the Milan criteria (7.3% vs 9.7%)<sup>[24,64]</sup>.

## CONCLUSION

Liver transplantation remains the definitive treatment for HCC complicating cirrhosis. Since the publication of the “Milan criteria”, OLT has progressed into a universally accepted treatment for HCC. The results of OLT for HCC continue to improve with time. The retrospective studies to date suggest outcome for OLT for HCC is only marginally worse than for end stage liver disease itself. Over the past decade LDLT has evolved but at present needs more stringent universal guidance on its usage. However, the results of LDLT for HCC seem slightly worse than for OLT but this may be a result of selection bias. The use of single or combined local ablative therapy in the period prior to OLT appears to be accepted. As operative techniques have improved, resection as a bridge to transplantation now appears a viable option in compensated cirrhotic patients. Resection also offers the ability to gain invaluable information about the tumor.

Undoubtedly further work will be published on expanded criteria for transplantation. The various studies to date have shown that acceptable long term outcomes are possible with OLT for HCC outside the “Milan criteria”. With time a new universal consensus will be required to standardize these ‘expanded’ criteria.

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## Rheumatic manifestations of inflammatory bowel disease

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### Abstract

This article reviews the literature concerning rheumatic manifestations of inflammatory bowel disease (IBD), including common immune-mediated pathways, frequency, clinical course and therapy. Musculoskeletal complications are frequent and well-recognized manifestations in IBD, and affect up to 33% of patients with IBD. The strong link between the bowel and the osteo-articular system is suggested by many clinical and experimental observations, notably in HLA-B27 transgenic rats. The autoimmune pathogenic mechanisms shared by IBD and spondyloarthropathies include genetic susceptibility to abnormal antigen presentation, aberrant recognition of self, the presence of autoantibodies against specific antigens shared by the colon and other extra-colonic tissues, and increased intestinal permeability. The response against microorganisms may have an important role through molecular mimicry and other mechanisms. Rheumatic manifestations of IBD have been divided into peripheral arthritis, and axial involvement, including sacroiliitis, with or without spondylitis, similar to idiopathic ankylosing spondylitis. Other periarticular features can occur, including enthesopathy, tendonitis, clubbing, periostitis, and granulomatous lesions of joints and bones.

Osteoporosis and osteomalacia secondary to IBD and iatrogenic complications can also occur. The management of the rheumatic manifestations of IBD consists of physical therapy in combination with local injection of corticosteroids and nonsteroidal anti-inflammatory drugs; caution is in order however, because of their possible harmful effects on intestinal integrity, permeability, and even on gut inflammation. Sulfasalazine, methotrexate, azathioprine, cyclosporine and leflunomide should be used for selected indications. In some cases, tumor necrosis factor- $\alpha$  blocking agents should be considered as first-line therapy.

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### INTRODUCTION

The association between arthritis and inflammatory bowel disease (IBD) was originally described in 1929<sup>[1]</sup>, but it was not until the 1950s when peripheral arthritis associated with IBD was distinguished from rheumatoid arthritis, and in the 1960s, the concept of spondyloarthropathy (SpA) was established<sup>[2-4]</sup>. Here, we present a review of all pertinent literature from Medline regarding rheumatic complications of IBD. We include original and review articles, as well as relevant case reports published from 1929 to 2009.

With time, clinical and experimental evidence of the close relationship between IBD and some rheumatic diseases, particularly seronegative SpAs, has grown. In fact, IBD is considered part of the concept of SpA<sup>[5]</sup>.

Some studies have shown that up to 70% of patients with SpA have inflammatory intestinal lesions, and up to 26% of patients with SpA who undergo ileocolonoscopy

have intestinal abnormalities compatible with Crohn's disease (CD). In fact, 6%-10% of patients with SpA develop IBD on follow-up; besides, studies with serial ileocolonoscopy have shown strong correlation between the presence of gut and joint inflammation in SpA<sup>[6-10]</sup>. Therefore, some authors have suggested that joint abnormalities are the initial manifestation of IBD, and after several years, these patients later may develop florid intestinal abnormalities. On the other hand, some studies that have evaluated extraintestinal manifestations of patients with IBD have shown that 36%-46% of patients have at least one extraintestinal manifestation, and rheumatic abnormalities are the most frequent (22%-33%)<sup>[11,12]</sup>. Besides, rheumatic manifestations are significantly more common in patients with disease confined to the colon. For instance, some patient series with ulcerative colitis (UC) have reported a prevalence of joint involvement of 62%<sup>[13]</sup>. Several studies have evaluated the prevalence of seronegative SpA in patients with IBD: 18%-45% of patients with IBD fulfill the criteria for SpA; 3%-9.9% fulfill diagnostic criteria for ankylosing spondylitis (AS); around 14% develop one or more clinical manifestations of SpA without fulfilling diagnostic criteria, and some of these patients (up to 24%) have asymptomatic sacroiliitis<sup>[14-18]</sup>.

## IMMUNOPATHOGENESIS OF GUT AND JOINT INFLAMMATION

### *Immunologic alterations shared by patients with IBD and SpA*

There have been interesting studies about the immunopathogenesis of IBD and SpA, which have shown alterations in key molecules that regulate the immune response in the gut of patients with SpA, and some of them are almost the same as those found in CD<sup>[19]</sup>. For instance E-cadherin, a transmembrane glycoprotein that mediates the intercellular adhesion of epithelial cells is expressed highly in the gut of patients with IBD<sup>[20]</sup> and in patients with SpA<sup>[21]</sup>, and subclinical acute and chronic gut inflammation, even in those without macroscopic lesions, which indicates that the alteration in the regulation of these proteins may be an early event in the development of gut inflammation. Besides, E-cadherin is a ligand of  $\alpha 4\beta 7$  integrin in intraepithelial T cells. Two studies have shown increased expression of this integrin in T cell cultures from the gut mucosa of patients with AS<sup>[22]</sup> and CD<sup>[23]</sup>. Increased expression of this integrin has been found in patients without histological signs of gut inflammation, which suggests that there are common abnormalities in patients with SpA and IBD that precede clinical inflammation.

Other alterations that are common in both groups of diseases are those described in CD4<sup>+</sup> T cells. Currently, four types of CD4<sup>+</sup> T cells have been described: Th1 cells are identified as interferon  $\gamma$  (IFN- $\gamma$ ) producers; Th2 cells produce primarily interleukin (IL)-4, IL-5, IL-10 and IL-13; Th17 cells produce mainly IL-17A and

they also produce IL-17F, IL-21, IL-22, granulocyte-macrophage colony-stimulator factor (GM-CSF), CCL-20, and potentially tumor necrosis factor (TNF) and IL-6. These cytokines have pro-inflammatory properties and they act on a broad range of cell types to induce the expression of several cytokines (TNF, IL-1 $\beta$ , IL-6, GM-CSF, G-CSF), chemokines (CXCL1, CXCL8, CXCL10), and metalloproteinases. On the other hand, regulatory T cells (Tregs) have a suppressor function; they influence the immune system by cell-to-cell contact and also *via* regulatory mechanisms that are still not fully elucidated. It is known that they are able to produce IL-10 and transforming growth factor (TGF)- $\beta$ , and it is interesting that Th17 and Treg developmental programs are reciprocally interconnected. Upon T-cell receptor stimulation, a naïve T cell can be driven to express Foxp3 and become a Treg cell in the presence of TGF- $\beta$ , but in the presence of TGF- $\beta$  plus IL-6 or IL-21, the Treg developmental pathway is abrogated, and instead, T cells develop into Th17 cells<sup>[24]</sup>. Initial studies have shown Th1 predominance in intestinal mucosa of patients with IBD and SpA, however, recent studies have suggested that, in both groups of patients, Th17 cells may have an important role in initiation and perpetuation of autoimmune inflammation. One study that involved 499 patients with CD and 216 with UC has shown increased IL-17F mRNA expression in intestinal biopsies of patients compared to controls<sup>[25]</sup>. A recent study has shown an increased proportion of Th17 cells in patients with SpA<sup>[26]</sup> and increased IL-17, IL-6, TGF- $\beta$  and IFN- $\gamma$  levels in synovial fluid of patients with SpA, when compared with patients with rheumatoid arthritis<sup>[27]</sup>. Also, some authors have suggested that dysfunction of Tregs participates in the immunopathogenesis of these diseases, and they have proposed their use as therapeutic agents in IBD<sup>[24,28]</sup>.

TNF- $\alpha$  is a pro-inflammatory cytokine that is produced mainly by macrophages and activated T cells. It is a key molecule in chronic inflammation of SpA and IBD. In the latter, the interaction between antigen-presenting cells (APCs) and intestinal bacterial flora contributes to the development of uncontrolled CD4<sup>+</sup> cell activation, which leads to the release of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-6, IL-12, IL-23 and IL-17. In SpA, models of transgenic rats and clinical observations have suggested that pathogenic microorganisms and their interaction with APCs also have a crucial role in the initiation and perpetuation of the altered immune response that leads to joint and enthesitis inflammation. Additionally, increased intestinal permeability has been found in patients with SpA and IBD. This fact could alter the innate immune response to bacterial antigens<sup>[29]</sup>. These discoveries have had important therapeutic implications for these groups of patients.

Other relevant molecules involved in the pathogenesis of both groups of diseases are the toll-like receptors (TLRs) that play an important role in the innate immune response against pathogenic microorganisms. Several studies have shown increased expression of TLR-4 and TLR-2 in APCs of patients with SpA<sup>[30,31]</sup> and in intestinal

biopsies of patients with UC and CD<sup>[32-34]</sup>. The alterations in function and regulation of these molecules may have an important role in the initiation and perpetuation of chronic inflammation in these diseases<sup>[35]</sup>. There are studies that have correlated several mutations and genetic variations of these receptors with susceptibility for these diseases. The results are controversial, probably due to the heterogeneity of the patients and the ethnic groups that have been included in the studies<sup>[36-39]</sup>.

#### **Other immunopathogenic abnormalities in IBD and SpA**

Abnormal responses to certain microorganisms have been described in patients with IBD or SpA; for instance, increased levels of the same serotypes of *Klebsiella pneumoniae* have been found more frequently in patients with IBD and SpA, compared with healthy controls and controls with celiac disease<sup>[40]</sup>. Besides, several studies have shown increased levels of antibodies against *Klebsiella* antigens, and against collagens type I, III, IV and V in patients with CD or AS. There are molecular similarities between *Klebsiella* nitrogenase and HLA-B27 genetic markers and between *Klebsiella* pullulanase and collagen fibers types I, III and IV. Therefore, several authors have proposed that there may be molecular mimicry between *Klebsiella* and human molecules that could participate in the initiation and perpetuation of these diseases<sup>[41]</sup>, i.e. anti-*Klebsiella* IgA cross-reacts with HLA-B27 antigen, and antibodies to enteric bacteria are able to lyse lymphocytes from HLA-B27 patients with AS.

The participation of the HLA-B27 molecule in the immunopathogenesis of SpA has been well known since 1973 when Brewerton and Schlosstein detected its high prevalence in patients with AS, psoriatic arthritis (PsA), reactive arthritis and anterior uveitis<sup>[42]</sup>. Its functional relevance was demonstrated elegantly in 1990 by Hammer *et al.*<sup>[43]</sup>; their group developed a transgenic rat with the human HLA-B27 molecule. These rats developed a multisystemic inflammatory disease that had several clinical and histopathological similarities to SpA and IBD<sup>[43]</sup>. An important finding was that these rats did not develop joint or gut inflammation when they were in a bacteria-free environment, which supports the theory of the participation of microorganisms in the pathogenesis of these diseases<sup>[44]</sup>. Some studies involving patients with IBD have shown an association of HLA-B27 molecule with the presence of sacroiliitis, spondylitis, enthesitis, peripheral arthritis, erythema nodosum, uveitis and oral ulcers<sup>[14]</sup>.

Other genes have been associated with the clinical presentation of several arthropathies associated with IBD<sup>[45]</sup>. The Oxford Group has found an association of type 1 arthropathy (pauci-articular, self limited, with relapses) with HLA-DRB1\*0103, HLA-B35 and HLA-B24, while type 2 arthropathy (polyarticular, progressive) is associated with HLA-B44 in British patients<sup>[46]</sup>. One study that evaluated Spanish patients has found an association of the mini-haplotype TNF a6B5 with the presence of joint manifestations in patients with UC<sup>[47]</sup>. Another study has found that a polymorphism of the gene CARD15 is associated with the presence of gut inflammation in

patients with SpA, and the same polymorphism is over-represented in patients with CD<sup>[48]</sup>.

On the other hand, there is evidence to suggest that, for patients with IBD, tobacco use and history of appendectomy are risk factors for developing extraintestinal manifestations, particularly seronegative SpA and dermatological manifestations such as erythema nodosum and pyoderma gangrenosum<sup>[49]</sup>.

In summary, the autoimmune pathogenic mechanisms shared by IBD and SpA include genetic susceptibility to abnormal antigen presentation, aberrant recognition of self, the presence of autoantibodies against specific antigens shared by the colon and other extra-colonic tissues, and increased intestinal permeability. The response against microorganisms may have an important role through molecular mimicry and other mechanisms.

## **CLINICAL PRESENTATION OF THE RHEUMATIC MANIFESTATIONS OF IBD**

The rheumatic manifestations of IBD have been divided into peripheral arthritis, and axial involvement, including sacroiliitis, with or without spondylitis, similar to the presentation of classic AS. There may be other periarticular manifestations such as enthesopathy, which is the involvement of tendinous insertions, tendonitis, periostitis, clubbing and granulomatous lesions in joints and bones. There may also be osteoporosis and osteomalacia secondary to IBD and to its treatment<sup>[50]</sup>.

#### **Peripheral arthritis**

The frequency of peripheral arthritis in IBD ranges from 17% to 20%, and it is more common in CD<sup>[51]</sup>. In a retrospective study that included 1459 patients with IBD, peripheral arthritis was present in 6% of patients with UC and in 10% of patients with CD<sup>[52]</sup>. Another study has shown arthritis in 10%, enthesitis in 7%, and a history of arthritis in 29% in a group of patients with IBD<sup>[53]</sup>.

The Oxford Group<sup>[52]</sup> has classified the peripheral arthritis associated with IBD (also called peripheral enteropathic arthropathy) without axial involvement, as pauci-articular or polyarticular arthropathy (Table 1). In the pauci-articular form, joint symptoms are usually acute and self-limited; the joint involvement is asymmetric and migratory, with participation of both large and small joints, and lower limbs are more affected. Many episodes last from 6 to 10 wk but relapses are frequent and they tend to coexist with IBD relapses; these patients have high frequencies of other extraintestinal manifestations such as erythema nodosum and uveitis. It is interesting that in 31% of these patients the arthropathy may appear even 3 years before the diagnosis of IBD is made. On the other hand, the polyarticular form tends to have a chronic course and it may be destructive; its course is independent of IBD exacerbations and the coexistence of other extraintestinal manifestations is rare, except for uveitis<sup>[50,54]</sup>. Enthesopathy, particularly affecting the Achilles tendon or plantar fascia insertion are common manifestations.

Table 1 Classification of peripheral arthropathy associated with IBD

Type 1 (Pauci-articular)	Type 2 (Polyarticular)
Less than five joints	Five or more joints
Asymmetric involvement	Persistent inflammation for months or even years
Lower limbs more affected	May be erosive
Self limited episodes that last < 10 wk	Affects both large and small joints
Usually concomitant IBD relapse	It can be symmetric or asymmetric
High frequency of other extraintestinal manifestations	Clinical course is independent of IBD activity
	Associated with uveitis

IBD: Inflammatory bowel disease.

Table 2 AS prevalence studies in patients with IBD

Author and reference number	n	Origin of patients	Diagnostic criteria	Prevalence (%)				
				AS	SpA	Sacroiliitis (Rx or MRI)	Inflammatory low back pain	Articular manifestations
de Vlam <i>et al</i> <sup>[53]</sup>	103	Referral center	ESSG	10	35	30	30	39
Scarpa <i>et al</i> <sup>[13]</sup>	79	Referral center	New York	25			18	43
Salvarani <i>et al</i> <sup>[17]</sup>	160	Open population	ESSG	2 (CD) 5.1 (UC)	18.1			32.5
Palm <i>et al</i> <sup>[58]</sup>	654	Open population	ESSG	6 (CD) 2.6 (UC) 3.7 Total	22	2	19	
Wordsworth <i>et al</i> <sup>[60]</sup>	42	Referral center	ESSG	25 (CD)		36		

Rx: Radiography; MRI: Magnetic resonance image; ESSG: European Spondyloarthropathy Study Group.

In most of the cases, intestinal symptoms precede or coexist with joint manifestations, but in some patients, arthritis precedes the gut manifestations, even by several years. In a prospective study that included 123 patients with SpA, 6% of the patients developed CD from 2 to 9 years after the first joint symptom. For UC, some studies have described a temporal relationship between arthritis and gut inflammation relapses<sup>[50]</sup>; there have been case reports of patients that have developed arthritis at the same time as ileal pouchitis after total proctocolectomy for UC activity. However, other studies have shown that colectomy has almost no effect on joint inflammation<sup>[50,55]</sup>. The most consistent finding is that colon involvement increases the susceptibility to peripheral arthritis.

There have been isolated case reports about patients that have presented with progressive, destructive monoarthritis with granulomatous synovial inflammation, and CD has been diagnosed when extension studies have been carried out as part of the diagnostic workup<sup>[56]</sup>.

### AS and other forms of axial involvement

Axial involvement is part of CD and UC, although it is more common in CD (5%-22%) than in UC (2%-6%), and in general, the prevalence is 10%-20% for sacroiliitis and 7%-12% for AS<sup>[51,57,58]</sup>. A study that included patients from a referral center has shown that 30% of the patients with IBD had inflammatory low back pain, 33% had abnormal Shober index, and 30% had unilateral or bilateral grade I or II sacroiliitis. In fact, 35% of the patients fulfilled the European Spondyloarthropathy Study Group criteria for SpA<sup>[53,59]</sup>, and 10% fulfilled the criteria for AS. Other studies from referral centers have shown similar results and higher prevalence than for population

studies, which probably reflects referral bias<sup>[13,17,53,58,60]</sup> (Table 2).

The clinical picture is virtually the same as the one for patients with AS without extra-articular manifestations. Axial symptoms usually precede gut symptoms; clinical course is totally independent of the intestinal manifestations and intestinal surgery does not alter the course of SpA. Ankylosing spondylitis associated with IBD can develop at any age, whereas idiopathic AS usually starts before 40 years of age. In idiopathic AS, males are more affected than females (ratio 2.5:1), while in AS associated with IBD, the male to female ratio is 1:1<sup>[61]</sup>.

### LABORATORY AND OTHER DIAGNOSTIC WORKUP

The most common laboratory abnormalities are: anemia due to chronic inflammation and intestinal bleeding, leukocytosis, thrombocytosis, elevated acute phase reactants such as C reactive protein, and erythrocyte sedimentation rate. Perinuclear anti-neutrophil cytoplasm antibodies are found in up to 60% of patients with UC and in some patients with CD, and they seem to be directed against lactoferrin autoantigens<sup>[62]</sup>. Antinuclear antibodies and rheumatoid factor are usually absent. Synovial fluid may be inflammatory but sterile.

Anti-*Saccharomyces cerevisiae* antibodies (ASCAs) were first described in patients with CD (both IgG and IgA isotypes). Several studies have confirmed their relevance as markers of CD. One multicenter study has shown that their presence in patients with IBD, who are negative for ANCA, has a positive predictive value of 94.2% for CD,

and it is associated with severe clinical manifestations of this disease<sup>[63]</sup>. Another recent study has shown that the prevalence of ASCAs in patients with CD is 80.6%, and they have a sensitivity of 81%, specificity of 78%, positive predictive value of 45.5%, and negative predictive value of 95% when used as a single marker in patients with IBD<sup>[64]</sup>. One study that evaluated 87 SpA patients positive for HLA-B27 has found increased levels of IgA but not IgG ASCAs, particularly in patients with AS; although patients with increased levels of the antibody had no gastrointestinal symptoms. It remains unclear if these antibodies are associated with the development of IBD in patients with SpA, but this finding supports the etiopathogenic association of AS with IBD<sup>[65]</sup>.

Sacroiliitis and spondylitis in IBD are associated with the presence of HLA-B27, although in lower frequencies than in AS (33% *vs* 71%). Besides, patients with AS without HLA-B27 have a higher risk of developing IBD than those with HLA-B27<sup>[53]</sup>. The Oxford study<sup>[52]</sup> has shown an association of pauci-articular arthritis with the presence of HLA-B27 (27% *vs* 7% in controls), HLA-B35 (32% *vs* 15%) and HLA-DRB1\*0103 (33% *vs* 3%), while the polyarticular form was associated with HLA-B44 (62% *vs* 30%)<sup>[45]</sup>. Another study has shown that the presence of the shared epitope is associated with synovitis in patients with IBD without sacroiliitis<sup>[50,66]</sup>.

Radiological studies of peripheral joints have shown changes of acute arthritis such as increased volume of soft tissue. Erosions are uncommon in patients with self-limited arthritis. Patients with persistent arthritis may develop joint erosion and, if the hips are affected, there may be also loss of joint space. Axial radiographs show typical AS changes, although some studies have suggested that asymmetric sacroiliitis frequency may be higher<sup>[67]</sup>.

## OTHER RHEUMATIC MANIFESTATIONS ASSOCIATED WITH IBD

Less frequently, IBD has been associated with Sjögren's syndrome<sup>[68]</sup>, rheumatoid arthritis<sup>[69]</sup>, inflammatory myopathy and Takayasu arteritis<sup>[70,71]</sup>, although these come from isolated case reports and no confirmed clinical or pathophysiological association can be made. There has been one study that has evaluated the prevalence of fibromyalgia and spread of musculoskeletal pain in patients with IBD, which has found equal frequencies to those in the general population<sup>[72]</sup>. In addition, it is advisable to evaluate IBD patients for osteoporosis if they have received high-dose steroids for long periods, and to implement prophylactic measures such as calcium and vitamin D supplements<sup>[73]</sup>.

## TREATMENT OF RHEUMATIC MANIFESTATIONS OF IBD

Treatment depends on the severity of the clinical picture. Patients with mild oligoarthritis usually respond to relative rest, physiotherapy and intra-articular steroid in-

jections<sup>[74]</sup>. Most of the patients respond to nonsteroidal anti-inflammatory drugs because they control the symptoms and joint and enthesitis inflammation, but they do not stop joint destruction, and they may have important side effects including exacerbation of IBD<sup>[75,76]</sup> and produce small intestine and colon ulcers<sup>[77]</sup>. Hence, they are recommended for patients with mild exacerbations, to control symptoms in arthritis flares, but their use must be limited to the minimal effective dose and time.

Sulfasalazine and 5-aminosalicylic acid are often recognized as useful for IBD, and are efficacious for moderate peripheral arthritis in SpA and IBD-associated arthritis, particularly in UC<sup>[78,79]</sup>. Their usefulness in CD is less clear. Some studies have shown promising results, others, no benefit at all, to control CD-associated arthritis<sup>[80-82]</sup>. These drugs have no effect in the progression of aggressive arthritis and their utility in axial involvement is marginal; also, they do not prevent the appearance of IBD in patients with SpA<sup>[83]</sup>. Their beneficial effect may be explained by their anti-inflammatory effects in the intestinal wall, by normalizing its permeability and preventing antigen entrance through an abnormal intestinal wall<sup>[84]</sup>.

Immunosuppression with methotrexate, azathioprine, 6-mercaptopurine, cyclosporine and leflunomide has been successful in some patients with peripheral arthritis and other extraintestinal manifestations, although there are no controlled studies that have demonstrated their efficacy<sup>[74,85-88]</sup>, and the evidence of their efficacy comes from uncontrolled studies or case reports.

The description of the pathogenic mechanisms of SpAs and IBD have relevant therapeutic implications for both groups of diseases. The initial observation that intestinal manifestations of patients with both SpA and IBD improve with TNF- $\alpha$  blocking agents has led to the use of these medications in patients with IBD, with favorable results, particularly with infliximab and adalimumab in CD<sup>[89]</sup>. Currently, infliximab is a first-line treatment for patients with active AS associated with IBD, and other TNF- $\alpha$  blocking agents such as certolizumab that have shown good results for patients with IBD, are promising<sup>[90,91]</sup>.

Also, considering the pathogenic relationship between gut and joint inflammation in SpA, there are other agents with potential benefits in both diseases. Some of these include IL-10, IL-11, IL-6, intercellular adhesion molecule 1, mitogen-activated protein kinase and integrin ( $\alpha$ 4 and  $\alpha$ 4 $\beta$ 7) blockade, as well as TLR modulation<sup>[92-95]</sup>.

Some groups have proposed the use of probiotics for the treatment of patients with IBD and arthritis or AS. Their use is based on modulation of intestinal flora by bacteria and their products. The objective is to treat patients with persistent arthralgia in the early phases of the disease, before chronic damage is established, to improve the quality of life and have a positive influence on the natural course of the disease. Some interesting studies have demonstrated that these compounds improve experimental colitis in murine models and in patients with IBD. One of these models has shown that the anti-

inflammatory effect depends on the probiotic DNA, in an IFN-mediated response induced by TLR9. Further studies should evaluate these and other mechanisms in animal models<sup>[96,97]</sup>.

## CONCLUSION

Rheumatic manifestations of IBD include peripheral arthritis, enthesitis and axial involvement, and they are present in up to 62% of patients with IBD. There is an important immunopathogenic relationship between gut and joint abnormalities in patients with IBD. TLR variants and abnormalities, altered function and balance of T-cell subpopulations and their production of pro-inflammatory cytokines, as well as integrin and E-cadherin dysfunction are partially responsible for such a relationship, and research in these areas will help to clarify the pathogenesis of these complex diseases. Medical treatment of rheumatic manifestations of IBD includes sulfasalazine and mesalamine, immunomodulators and TNF- $\alpha$  inhibitors. New treatments for IBD directed against pro-inflammatory cytokines and the regulation of the immune response may have long-term benefits for rheumatic manifestations.

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## Acoustic radiation force imaging sonoelastography for noninvasive staging of liver fibrosis

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### Abstract

**AIM:** To investigate the diagnostic accuracy of acoustic radiation force impulse (ARFI) imaging as a noninvasive method for the assessment of liver fibrosis in chronic hepatitis C (CHC) patients.

**METHODS:** We performed a prospective blind comparison of ARFI elastography, APRI index and FibroMax in a consecutive series of patients who underwent liver biopsy for CHC in University Hospital Bucharest. Histopathological staging of liver fibrosis according to the METAVIR scoring system served as the reference. A total of 74 patients underwent ARFI elastography, APRI index, FibroMax and successful liver biopsy.

**RESULTS:** The noninvasive tests had a good correlation with the liver biopsy results. The most powerful test in predicting fibrosis was ARFI elastography. The diagnostic accuracy of ARFI elastography, expressed

as area under receiver operating characteristic curve (AUROC) had a validity of 90.2% (95% CI AUROC = 0.831-0.972,  $P < 0.001$ ) for the diagnosis of significant fibrosis ( $F \geq 2$ ). ARFI sonoelastography predicted even better F3 or F4 fibrosis (AUROC = 0.993, 95% CI = 0.979-1).

**CONCLUSION:** ARFI elastography had very good accuracy for the assessment of liver fibrosis and was superior to other noninvasive methods (APRI Index, FibroMax) for staging liver fibrosis.

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**Key words:** Elasticity imaging techniques; Hepatitis C; Liver biopsy; Liver fibrosis

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### INTRODUCTION

The prognosis and clinical management of chronic liver diseases are highly dependent on the extent of liver fibrosis. This is particularly true in patients with chronic hepatitis C (CHC), which is the leading cause of cirrhosis in many countries. CHC progresses to cirrhosis through a sequence of morphological changes that reflect increasing fibrosis and architectural distortion<sup>[1]</sup>. Staging systems of varying complexity based on these morphological changes have been used in patient management as a guide to prognosis, and in clinical investigations as an estimate of natural history and response to therapy.

Liver biopsy is the current reference examination for the assessment of liver fibrosis. It is important to remember that a needle biopsy is merely a sample of the entire liver, and that scarring in chronic liver disease is typically irregularly distributed in the liver. Needle liver biopsy removes only about 1/50000th of the liver and carries substantial interpreter errors<sup>[2]</sup>. Liver biopsy is an invasive procedure with certain unavoidable risks and complications. Therefore, the development of noninvasive tests to assess hepatic inflammation and fibrosis has been an active area of research. Furthermore, as new antifibrotic therapies are studied, there is a growing need to develop noninvasive tests for fibrosis for use in clinical trials.

Several noninvasive methods have been proposed to stage liver fibrosis, including biochemical tests and imaging techniques. The biochemical tests consist of sophisticated indices and scores, or a large number of serological markers of liver fibrosis. However, the value of these diagnostic methods remains debated.

Among the imaging methods, transient elastography is a new technique that rapidly and noninvasively measures mean tissue stiffness. It is largely accepted that hepatic stiffness is related to the degree of fibrosis. Most clinical studies have been performed with FibroScan. Recently, acoustic radiation force impulse (ARFI) imaging sonoelastography has been proposed as an alternative method to assess liver elasticity. To the best of our knowledge, no study has compared ARFI sonoelastography and biochemical tests (APRI index, FibroMax) for the assessment of liver fibrosis.

The aim of our study was to evaluate the value of ARFI elastography for the diagnosis of liver fibrosis and to compare the accuracy of ARFI elastography, APRI index and FibroMax for staging liver fibrosis in patients who underwent liver biopsy for CHC.

## MATERIALS AND METHODS

### Study population

The study was a single-centered, prospective, blind comparison of ARFI sonoelastography, APRI index and FibroMax in 100 patients with CHC who underwent liver biopsy at the University Hospital Bucharest between January 2007 and June 2008. Patients with CHC were selected out of 215 patients who attended the University Hospital Bucharest for hepatic cytolysis.

Inclusion criteria were the presence of HCV RNA in serum without previous antiviral treatment and hepatic cytolysis [alanine aminotransferase (ALT) > 1.5 × normal]. Patients with other forms of chronic liver disease and those with ascites were excluded from the study.

Hepatitis C virus (HCV) infection was defined by the presence in the serum of anti-HCV antibodies using the third generation ELISA. HCV infection was confirmed by performing the COBAS TaqMan HCV test (Roche Molecular Systems, Inc., NJ, USA). This test is an *in vitro* amplification of the HCV nucleic acid, which

uses the High Pure System Viral nucleic acid kit (Roche Diagnostics Corp, IN, USA) for manual preparation and the COBAS TaqMan 48 analyzer (Roche Diagnostics Corp, IN, USA) for automatic amplification and detection. The RNA titer was expressed in IU/mL. The detection limit was > 15 IU/mL with a positive rate of 95%.

Patients who fulfilled the inclusion criteria were enrolled and underwent blood tests for APRI, FibroMax measurement, and ultrasound elastography on the day before the liver biopsy.

The study was approved by the local ethics committees and all individuals provided written informed consent prior to enrollment in the study.

### Biochemical evaluation of the liver

Liver function tests were performed prior to the liver biopsy. Blood samples were obtained under fasting conditions and routine liver function tests [ALT and aspartate transaminase (AST), total proteins, serum albumin and  $\gamma$  globulins,  $\gamma$ -glutamyl transferase (GGT), total bilirubin, alkaline phosphatase, international normalized ratio of prothrombin time] were performed. All were measured using Dade Behring reactants and the Dimension RXL analyzer (Dade Behring, FL, USA).

### APRI index

AST levels and platelet counts were measured with a Dimension RXL analyzer and a Hematology System analyzer (Beckman-Coulter, Inc., CA, USA). The APRI index was calculated as follows:  $\text{AST} / (\text{Upper Limit of Normal}) \times 100 / \text{platelet count} (10^9/\text{L})$ . The upper normal limit of AST was considered as 38 U/L.

### FibroMax

FibroMax (Biopredictive, France) is a new noninvasive panel of markers reported to predict advanced fibrosis. It contains five tests: FibroTest (to assess the degree of fibrosis); ActiTest (to assess the degree of activity, inflammation and necrosis); SteatoTest (to diagnose hepatic steatosis); NashTest (to diagnose nonalcoholic steatohepatitis); and AshTest (to diagnose severe alcoholic steatohepatitis). FibroMax combines the measurement of 10 indirect parameters adjusted for age, sex, weight and height:  $\alpha$ 2-macroglobulin, haptoglobin, apolipoprotein A1, total bilirubin, GGT, ALT, AST, fasting glucose, triglycerides and total cholesterol.

FibroMax serum samples were taken on the day before biopsy from patients in the fasting state. Biochemical parameters were measured using Dade Behring reactants and the Dimension RXL analyzer. Specific proteins,  $\alpha$ 2-macroglobulin, haptoglobin and apolipoprotein A1, were measured by immunonephelometry using the BN Pro-Spect Dade Behring system. Using FibroMax, fibrosis was staged on a scale of 0-4 with respect to the METAVIR fibrosis staging. For FibroTest, 0-0.21 fibrosis was staged as F0, 0.22-0.27 as F0-F1, 0.28-0.31 as F1, 0.32-0.48 as F1-F2, 0.49-0.58 as F2, 0.59-0.72 as F3, 0.73-0.74 as F3-F4, and 0.75-1 as F4.

### Ultrasound elastography

ARFI imaging is a new tissue strain imaging technology that utilizes sound waves to interrogate the mechanical stiffness properties of tissues. Virtual Touch tissue imaging and Virtual Touch tissue quantification (Siemens AG, Germany) are the first available applications to implement this technology. Unlike conventional B-mode sonography, which provides anatomical details based on differences in acoustic impedance, Virtual Touch imaging describes relative physical tissue stiffness properties. In complement, Virtual Touch™ tissue quantification provides accurate numerical measurements related to tissue stiffness at user-defined anatomical locations.

Conventional elastography, however, requires manual compression of the tissues in order to provide relative displacement between the mass and the surrounding structures and therefore an assessment of strain. This can be difficult to apply to abdominal structures because of the presence of the ribs and abdominal wall. ARFI technology quantifies stiffness without manual compression. Using the Virtual Touch application, the tissue is compressed by acoustic energy. Virtual Touch tissue quantification is a quantitative assessment of tissue stiffness, through measurement of shear wave speed. Shear waves are generated by displacement of tissue and attenuate approximately 10 000 times more rapidly than conventional ultrasound waves.

Prior to liver biopsy, the patients underwent liver ultrasonographic elastography, under fasting conditions, using ARFI imaging [Acuson S2000 (Siemens AG, Germany) with Virtual Touch tissue quantification software]. The system uses a standard ultrasonographic probe and offers elastography with a flexible metering box of 1 cm at variable depths. An acoustic push pulse transmitted by the transducer (3.5 MHz) toward the tissue induces an elastic shear wave that propagates through the tissue. The propagation of the shear wave is followed by detection pulses that are used to measure the velocity of shear wave propagation, which is directly related to tissue stiffness: speed increases with stiffness.

Liver stiffness was assessed by the same physician blinded to clinical and biological data. The measurements were performed on the right lobe of the liver through the intercostal spaces, with the patient lying in the decubitus dorsal position, with the right hand under the head and the head turned toward the left. The tip of the probe was covered with coupling gel and placed on the skin between the ribs at the level of the right lobe of the liver. The operator positioned the probe over the area of interest: segment eight of the right lobe, away from motion and portal/hepatic vessels, about 2 cm from the liver capsula, at a depth between 3.8 and 5.5 cm. When the target area had been located after optimizing the B-mode image, the operator pressed the button Virtual Touch tissue quantification, and asked the patient to stop breathing for a moment. Then, we observed the velocity of shear wave on screen. We used this protocol because we saw that, when the right lobe was scanned through the intercostal spaces with normal breathing, the variance of

measurement was low. A total of 12 valid measurements per patient were performed. In difficult patients, to obtain better access to the liver without excessive pushing or breath holding, the measurements were performed on patients lying in the left lateral decubitus position, or using a subcostal approach to the left lobe. The results of ARFI ultrasonographic elastography were expressed as liver propagation velocity (m/s). The success rate of liver elasticity measurements was calculated as the ratio between validated and total measurements. Results were expressed as the median value of the total measurements (12 measurements per patient) in m/s, with values ranging from 0.8 to 3.7 m/s. Only procedures with a success rate of at least 60%, and with the interquartile range of all validated measurements < 30% of the median value, were considered reliable.

### Histological assessment

Percutaneous liver biopsy was performed by senior operators using the Menghini technique with a 1.4-mm-diameter needle (Hepafix; B Braun Melsungen AG, Germany). After biopsy, the liver samples were fixed in formalin, paraffin embedded, and stained with hematoxylin-eosin and Masson's trichrome. All biopsy specimens were analyzed by an expert pathologist blinded to the biological and clinical data and to the results of ultrasound elastography. The length of each liver biopsy was established in millimeters and the number of portal tracts was counted.

Liver fibrosis and necroinflammatory activity were evaluated by the METAVIR scoring system. Fibrosis was staged on a four-point scale according to this score: F0 represented no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and a few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis. Necroinflammatory activity was also graded on a four point scale: A0, none; A1, mild; A2, moderate; A3, severe.

Steatosis was assessed according to the number of hepatocytes with degeneration, as follows: 0, none; 1, steatosis in 1%-10% of hepatocytes; 2, steatosis in 11%-33% of hepatocytes; 3, steatosis in 34%-66% and 4, 67%-100% of hepatocytes<sup>[3]</sup>.

### Statistical analysis

Results are presented as the mean  $\pm$  SD, counts and percentages. The results are illustrated as the median and 25th- to 75th-percentile values. The correlation between the noninvasive tests and liver biopsy was tested using the non-parametric Spearman's correlation coefficient. The overall validity was measured using area under the receiver operating characteristic curve (AUROC) with 95% CI. Cutoff values that defined prediction regions for each fibrosis stage were defined by a common optimization step that maximized the sum of the sensitivities in predicting the single stages. Finally, sensitivity, specificity, and positive (PPV) and negative predictive value (NPV) were calculated. The optimal cutoff was chosen at the highest left point on the curve. For all tests, significance was achieved at  $P < 0.05$ .

Table 1 Patient characteristics

Characteristics	Patients included (n = 74)
Sex (male/female)	32/42
Age (yr)	55.32 ± 9.6 (31-72)
BMI (kg/m <sup>2</sup> )	25.59 ± 4.03 (17.23-33.29)
AST (IU/L)	50.06 ± 32.36 (18-188)
ALT (IU/L)	67.06 ± 26.79 (20-147)
INR	1.12 ± 0.15 (0.9-1.74)
Bilirubin (mg/dL)	0.69 ± 0.32 (0.29-2.08)
Albumin (g/dL)	3.9 ± 0.55 (2.6-4.9)
Cholesterol (mg/dL)	192.36 ± 46.58 (120-311)
Triglycerides (mg/dL)	123.24 ± 73.33 (48-467)

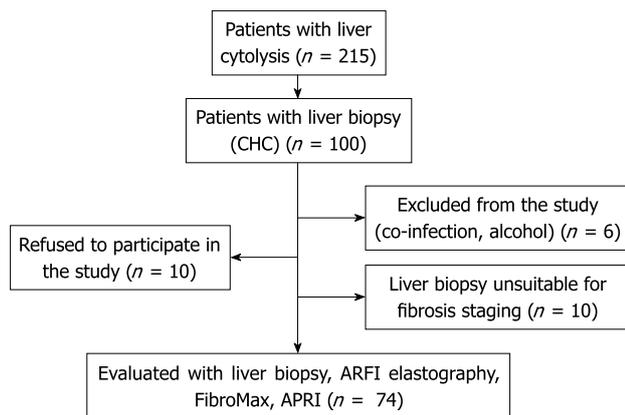


Figure 1 Diagram of patients with liver biopsy for CHC in the University Hospital Bucharest during the 18-mo study period.

## RESULTS

### Patient characteristics

A total of 100 patients underwent liver biopsy for CHC. The patients were selected out of 215 patients who attended the University Hospital Bucharest for hepatic cytolysis. Ten patients refused to participate and six were excluded: three had hepatitis B virus coinfection, and three had a current daily alcohol intake of at least 60 g/d. In 10 patients, liver biopsy was unsuitable for fibrosis staging with the length of sample < 15 mm.

Seventy-four patients underwent ARFI elastography, APRI index, FibroMax and successful liver biopsy (Figure 1). Demographic, clinical and biochemical characteristics of the patients included in the study are shown in Table 1.

### Histological characteristics

The median biopsy length was 20 mm (range: 15-25 mm); the number of portal tracts was 12 ± 4 (range: 8-16). Patient distribution according to METAVIR fibrosis stage and activity grade and steatosis are presented in Table 2.

### Relationship between liver elasticity and histological stages

Figure 2 shows the median value (95% CI) of liver elasticity compared with fibrosis stages according to METAVIR score. A high correlation of increasing liver stiffness with increasing stage of fibrosis was observed. The Spearman's correlation coefficient between the liver

Table 2 Patient distribution according to fibrosis stage, activity grade and steatosis

Fibrosis Stage	Activity Grade		Steatosis Grade		
	n (%)	n (%)	n (%)	n (%)	
0	1 (1.3)	0	1 (1.3)	0	24 (32.4)
1	9 (12.1)	1	32 (43.2)	1	23 (31.08)
2	25 (33.7)	2	29 (39.1)	2	12 (16.2)
3	19 (25.6)	3	12 (16.2)	3	11 (14.8)
4	20 (27.02)			4	4 (5.40)

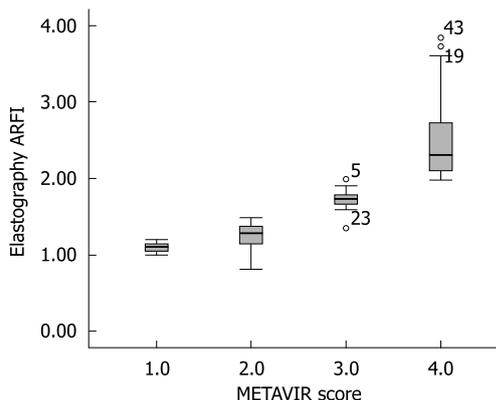


Figure 2 Box plot of METAVIR score and ARFI elastography. Top and bottom of boxes represent first and third quartiles, respectively. Length of box represents interquartile range within which 50% of values are located. Thick line through each box represents the median. Error bars mark minimum and maximum values (range). Small circles represent outliers. Skewed data for fibrosis stage 0/1 can be explained by the small number of patients in this group.

stiffness and the histological fibrosis stages was highly significant with a value of 0.919 ( $P < 0.001$ ). Steatosis is a frequent occurrence in CHC<sup>[4]</sup>. According to ANOVA test, no influence of the degree of activity or steatosis on elasticity values was observed in this study.

To determine the cutoffs that predicted each degree of fibrosis, we used the ROC curve method (Figure 3). ARFI elastography could predict fibrosis F2 or more, with a validity of 90.2% (95% CI AUROC = 0.831-0.972,  $P < 0.001$ ). The optimal cutoff point between F1 and F2 was 1.215. At this value, ARFI elastography had a sensitivity of 89.4% and a specificity of 100%.

ARFI sonoelastography predicted even better F3 or F4 fibrosis (AUROC = 99.3%, 95% CI = 0.979-1). The optimal cutoff between F3 and F4 was 1.54, with sensitivity and specificity of 97% and 100%, respectively. The specificity of 100% shows that all study patients who presented with a value of  $\geq 1.54$  upon sonoelastography had liver fibrosis F3 or more (confirmed by liver biopsy).

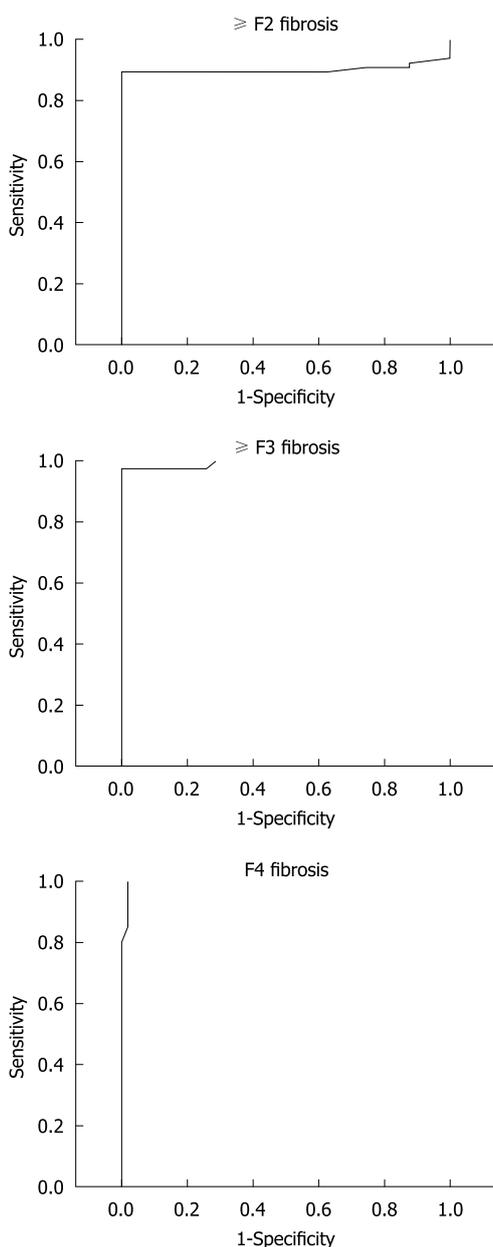
The same high validity was maintained as in predicting cirrhosis: (AUROC = 99.3%, 95% CI = 0.989-1).

The optimal cutoff in predicting cirrhosis was 1.94 with a sensitivity of 100% and a specificity of 98.1%. In other words, every patient in this study that had a sonoelastography result with a value < 1.94 did not have cirrhosis.

The most discriminative cutoff values of ARFI elastography are presented in Figure 4.

**Table 3 Most discriminative cutoff values of ARFI elastography**

	≥ F1	≥ F2	≥ F3	F4
Cutoff (m/s)	1.185	1.215	1.54	1.94
Sensitivity (95% CI)	89% (79-95)	100% (91-100)	97% (86-100)	100% (83-100)
Specificity (95% CI)	87% (47-99)	71% (53-85)	100% (90-100)	98% (90-99)
PPV (95% CI)	98% (91-99)	79% (65-89)	100% (90-100)	95% (76-100)
NPV (95% CI)	50% (23-76)	100% (86-100)	97% (85-100)	100% (93-100)
Positive likelihood ratio	7.1	3.5	3.5	54



**Figure 3 ROC curves: elastography predicting ≥ F2 fibrosis, ≥ F3 fibrosis and F4 fibrosis.**

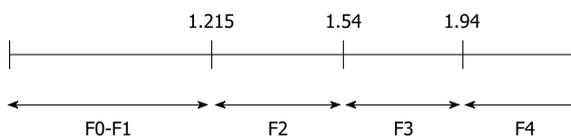
The corresponding sensitivity, specificity, PPV and NPV are shown in Table 3.

**Relationship between APRI index and FibroMax and histological stages**

A high correlation of increasing noninvasive measurements with increasing stage of fibrosis was observed. The

**Table 4 Noninvasive tests and their correlations with liver pathology**

	Noninvasive test	Correlation (Spearman coefficient)	95% CI	P
Liver biopsy	Elastography ARFI	0.919	0.872-0.949	< 0.001
	APRI index	0.712	0.572-0.811	< 0.001
	FibroMax	0.674	0.521-0.784	< 0.001



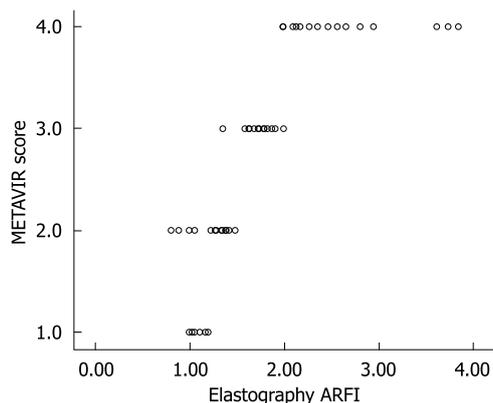
**Figure 4 Cutoff points for degrees of fibrosis.**

Spearman’s correlation coefficient between APRI index and FibroMax and the histological fibrosis stages was highly significant: 0.712 for APRI index and 0.674 for FibroMax. Table 4 shows the correlation between noninvasive tests and liver histology.

The correlation between two noninvasive tests should be better than with histological assessments for a correct prediction of liver fibrosis stage. The inter-test correlation coefficients, ARFI elastography/APRI index, and ARFI elastography/FibroMax were 0.712 and 0.718, respectively. All noninvasive tests used (Elastography ARFI, APRI index and FibroMax) were highly correlated with each other.

**DISCUSSION**

In chronic viral hepatitis, the knowledge of the stage of liver fibrosis is important for prognosis and for decisions about antiviral treatment<sup>[5]</sup>. Significant fibrosis (≥ F2) in these patients is an indicator for antiviral treatment, hence the great therapeutic value of a highly accurate diagnostic test. At present, liver biopsy is used as the reference standard for the assessment of liver fibrosis<sup>[2]</sup>. However, liver biopsy is becoming increasingly useless in the management of HCV-related chronic liver disease because of large sampling error, consistent inter-observer disagreement, unavoidable risks and complications, and the fact that it is a snapshot of a process that is everything but a static one. Replacement of liver biopsy in the assessment of chronic liver disease is the goal of any noninvasive technique. Therefore, a test with diagnostic performance for precise estimation



**Figure 5 Correlation between METAVIR and ARFI elastography.** Correlation between METAVIR and ARFI elastography showed that the overlap between stages F1 and F2 fibrosis limits the discriminatory value of the test in this context.

of the degree of liver fibrosis is of great therapeutic value. Previous studies have reported that transient elastography (FibroScan) can detect liver fibrosis and accurately predict significant fibrosis<sup>[5-11]</sup>. In the present study, we showed that APRI elastography should be taken into consideration as a noninvasive sonographical method for evaluation of liver fibrosis. A significant positive correlation between liver stiffness and the stages of fibrosis was noticed in patients with CHC. Significant AUROC for fibrosis F2 or more had a validity of 90.2% (95% CI AUROC = 0.831-0.972,  $P < 0.001$ ).

ARFI proved to be an even better predictor of F3 or F4 fibrosis (AUROC = 99.3%, 95% CI = 0.979-1). Early and rapid detection of significant fibrosis using this noninvasive procedure is essential since patients with significant fibrosis are at a high risk of developing complications, such as portal hypertension or hepatocellular carcinoma, and consequently need specific follow-up.

We considered ascites as one of the exclusion criteria from our study since ARFI, similar to Fibro-Scan, is performed in close contact with the liver. Nevertheless, ascites indicates the presence of cirrhosis, which makes noninvasive staging of fibrosis unnecessary<sup>[5]</sup>.

Liver elastometry with FibroScan is unsuccessful in patients with narrow intercostal spaces and in those with morbid obesity<sup>[7,8]</sup>. These are the major limitations of FibroScan measurements<sup>[12]</sup>. In the present study, patients with a body mass index (BMI) of up to 30 were enrolled, and ARFI elastography was performed successfully in all included patients. For the majority of the patients, the measurements were performed through a right intercostal space, and patients did not need to hold their breath during the examination. In difficult patients with narrow intercostal spaces, the examination was improved because of the subcostal approach to the left liver lobe. Elastography of the left liver lobe is especially helpful in obese patients. Rifai *et al*<sup>[13]</sup> have found recently that ARFI results of the right and left liver lobe were comparable ( $n = 26$ ,  $r = 0.44$ ,  $P < 0.03$ ). Furthermore, ARFI elastography is less time consuming than FibroScan. The new software allows one to do 12

measurements in under 1 min, as compared to FibroScan that does 10 measurements in under at least 4 min<sup>[9]</sup>.

The current study confirmed that, in CHC, no influence of steatosis or activity grade on correlation between elasticity values and fibrosis stages was observed. According to ANOVA test, the elasticity values were not affected by steatosis or activity grade. Similar results have been reported from studies analyzing transient elastography with FibroScan<sup>[7-9]</sup>.

Three noninvasive tests were performed. The tests had a good correlation with the liver biopsy results. The most powerful test in predicting fibrosis was ARFI elastography, with a non-parametric correlation coefficient of 0.919 (95% CI = 0.872-0.949,  $P < 0.001$ ). Other correlation coefficients were: 0.712 (95% CI = 0.572-0.811,  $P < 0.001$ ) for APRI index, and 0.674 (95% CI = 0.521-0.784,  $P < 0.001$ ).

In the present study, we found highly significant positive correlations between liver propagation velocity obtained with ARFI elastography and the METAVIR fibrosis stage. The correlation coefficients showed a linear relationship between ARFI elastography and liver biopsy fibrosis, but to determine the cutoffs that predicted each degree of fibrosis, we used the ROC curve method.

An AUC value of 0.902 (0.831-0.972) was obtained using ARFI elastography for the diagnosis of significant fibrosis ( $\geq F2$ ), with a cutoff value of 1.215 m/s. In comparison, three recent studies that analyzed the non-invasive assessment of liver fibrosis with FibroScan have revealed AUCs between 0.75 and 0.84 for the diagnosis of significant fibrosis ( $\geq F2$ )<sup>[7-9]</sup>. The ARFI sonoelastography predicted even better F3 or F4 fibrosis (AUROC = 99.3%, 95% CI = 0.979-1).

Considering the increased values of sensitivity and specificity, ARFI sonoelastography was shown to be a valuable investigative technique that could be of great help in the evaluation and follow-up of liver fibrosis.

At present, it is difficult to determine the real impact of ARFI elastometry in the early diagnosis of fibrosis in patients with CHC. Figure 5 clearly indicates an overlap observed between F0-F1 and F2. The increase in liver propagation velocity was more important between stages F2 (1.21 m/s) and F3 (1.54 m/s) than between stages F1 (1.18 m/s) and F2 (1.21 m/s). This is consistent with the fact that the increase in fibrous tissue is more important between stages F2 and F3 than between stages F1 and F2.

This limit of ARFI elastography (reduced discriminatory value between F1 and F2) was overcome by the fact that significant fibrosis ( $\geq F2$ ) is considered a hallmark of progressive liver disease. Studies have shown that antiviral treatment of patients with CHC prolongs life, improves quality of life, and is cost-effective<sup>[14,15]</sup>. However, treatment may be associated with severe side effects and the decision for treatment needs to be made on an individual basis. Patients with fibrosis stage F2 are at increased risk for developing cirrhosis with its complications (ascites, encephalopathy, or portal hypertension). Therefore, patients with fibrosis stage  $\geq F2$  have a stronger indication for treatment as compared with patients with no or mild fibrosis (F1)<sup>[16,17]</sup>.

Compared with fibrosis biomarkers, the disadvantage of liver elastometry is the absence of a large control group to assess the limit of normal values<sup>[14-18]</sup>. In studies using liver biopsy as a reference method, as in our study, the number of patients without fibrosis (F0) is very small.

Liver biopsy was considered the gold standard in our study because it is the only reference method available at present<sup>[1,19]</sup>. However, this technique is an invasive procedure that is known to have serious limitations. Various studies have shown that a single-needle liver biopsy can miss the diagnosis of cirrhosis in 20%-50% of patients<sup>[19]</sup>. To maximize the diagnostic yield of liver biopsy, we selected only the patients with a biopsy length > 15 mm and histological sections with at least eight portal tracts. The major advantage of ARFI liver elastography compared with liver biopsy is that it is painless, rapid, has no risk of complications, is very well tolerated and it is more representative of the entire liver parenchyma than is liver biopsy (1/50000 of the total liver mass)<sup>[2]</sup>.

To assess the performance of ARFI elastography, we have compared the liver propagation velocity with predictive blood tests (FibroMax) and APRI index in the same population. Our results showed that the most powerful test in predicting fibrosis was ARFI elastography. The high correlation between these three noninvasive tests suggests a sequential combination of them in algorithms used for staging liver fibrosis in patients with CHC.

In conclusion, our study showed that ARFI sonoelastography stands out on account of its very good correlation with liver biopsy (gold standard for evaluating liver fibrosis), good sensitivity and excellent specificity. It has proved to be a noninvasive imaging method, which is far superior to other methods investigated (APRI and FibroMax), for staging liver fibrosis. Further investigations on a larger number of patients are necessary to validate ARFI elastography as a noninvasive method of diagnosing liver fibrosis.

## COMMENTS

### Background

Liver biopsy is an invasive procedure with certain unavoidable risks and complications. Therefore, the development of noninvasive tests to assess hepatic inflammation and fibrosis has been an active area of research. Several noninvasive methods have been proposed to stage liver fibrosis, including biochemical tests and imaging techniques. The biochemical tests consist of sophisticated indices and scores, or a large number of serological markers of liver fibrosis. However, the value of these diagnostic methods remains debatable. Among the imaging methods, transient elastography, based on ultrasound, is a new technique that rapidly and noninvasively measures mean tissue stiffness.

### Research frontiers

Transient elastography is a noninvasive method with performance equivalent to that of serum markers for the diagnosis of significant fibrosis in patients with hepatitis C. Combining transient elastography with serum markers as first-line assessment could avoid liver biopsy in the majority of these patients. There is now a general consensus that liver fibrosis is potentially reversible. Follow-up biopsy is too insensitive for real-time monitoring of progression and regression of fibrosis. When considering anti-fibrotic therapy, it is important to recognize that fibrosis is a dynamic process. Clinical proof and monitoring of anti-fibrotic drug effects requires better noninvasive tests for fibrosis. Incorporation of noninvasive tests into large studies and therapeutic trials should be a priority in the next few years.

### Innovations and breakthroughs

Acoustic radiation force impulse (ARFI) imaging sonoelastography has been proposed as an alternative method to assess liver elasticity. No comparison of ARFI sonoelastography and biochemical tests (APRI and FibroMax) has been reported for the assessment of liver fibrosis.

### Applications

ARFI imaging could be applied to the noninvasive assessment of hepatic fibrosis initially, or after treatment. This study evaluated ARFI imaging in patients with chronic hepatitis C, but probably it could be used for other liver diseases that lead to fibrosis (we need further studies).

### Terminology

Elastography is a noninvasive method by which stiffness of soft tissue is evaluated.

### Peer review

The present study provides readers with a reliable, relatively inexpensive tool to measure liver fibrosis. It is a well designed, conducted and reported study.

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## Immunotherapy for nonalcoholic steatohepatitis using the multiple cytokine production modulator Y-40138

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### Abstract

**AIM:** To investigate the possible use of the multiple cytokine production modulator, Y-40138, as a novel immunotherapy in the rat nonalcoholic steatohepatitis (NASH) model.

**METHODS:** We allocated 6-wk-old male F344 rats to choline-supplemented, L-amino acid-defined (CSAA) diet (control group), CSAA diet + Y-40138 (control + Y-40138 group), choline-deficient, L-amino acid-defined (CDAA) diet (NASH group), or CDAA diet + Y-40138 (NASH + Y-40138 group). In each group, we measured the plasma alanine aminotransferase (ALT) levels, and the plasma and liver levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interferon- $\gamma$  (IFN- $\gamma$ ), and interleukin-10 (IL-10). Tissue specimens of phosphate buffered saline-perfused liver were subjected to hematoxylin and eosin staining, Azan staining, Sirius red staining, and immunohistochemical staining (for Kupffer cells and TNF- $\alpha$ ). We then extracted Kupffer cells from the collagenase-perfused livers using the Percoll gradient centrifugation method, and measured the TNF- $\alpha$  levels in the supernatant (*in vitro* TNF- $\alpha$  production by Kupffer cells) using an enzyme-linked immunosorbent assay kit.

**RESULTS:** In comparison to the NASH group, serum

ALT elevation was mild, production of serum and liver TNF- $\alpha$  and IFN- $\gamma$  was inhibited, and IL-10 production was increased in the NASH + Y-40138 group. Amelioration of liver histology was also noted in the NASH + Y-40138 group. Kupffer cell immunohistochemical staining revealed no differences between groups, whereas TNF- $\alpha$  immunohistochemical staining showed fewer stained cells in the NASH + Y-40138 group than in the NASH group. The TNF- $\alpha$  levels in the *in-vitro* Kupffer cell culture supernatant were lower in the NASH + Y-40138 group than in the NASH group.

**CONCLUSION:** Administration of Y-40138 to NASH model rats reduced hepatic inflammation and suppressed fibrosis. These results indicate that the multiple cytokine production modulator, Y-40138, is promising as a novel treatment for NASH.

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**Key words:** Nonalcoholic steatohepatitis; Y-40138; Tumor necrosis factor  $\alpha$ ; Interferon  $\gamma$ ; Interleukin-10; Kupffer cell; Innate immunity

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### INTRODUCTION

In recent years, nonalcoholic steatohepatitis (NASH) associated with obesity and diabetes mellitus has attracted a great deal of attention. Despite no history of alcohol intake, the histological findings of NASH are similar to

those of alcoholic hepatitis, and it is a progressive condition. A 2-component theory has been proposed for the etiology of NASH, the underlying condition being simple steatosis, with a first component of fatty degeneration as a result of insulin resistance, followed by a second component of aggravating factors such as inflammatory cytokines, including tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ), oxidative stress and endotoxins<sup>[1]</sup>. Kupffer cells, part of the innate immune system, produce various cytokines and are known to play a role in pathologies of the liver<sup>[2,3]</sup>. Abnormally functioning Kupffer cells are suspected to be involved in NASH<sup>[4]</sup>. Although the etiological mechanisms of NASH have not been fully elucidated, similar histopathological findings in NASH and alcoholic hepatitis suggest the existence of a common underlying mechanism. One possible mechanism is that increased endotoxin levels cause activation of Kupffer cells through surface Toll-like receptor-4 (TLR4), leading to increased expression of TNF- $\alpha$  and increased levels of reactive oxygen species (ROS)<sup>[5]</sup>. These induce inflammation and fibrosis, progressing to NASH.

It is known that the choline-deficient L-amino acid-defined (CDAA) fed rat model shows fatty accumulation and fibrosis, and after 16 wk the liver shows cirrhotic changes, and nodules appear as in hepatocellular carcinoma<sup>[6]</sup>. Although this model lacks obesity and insulin resistance, the 2 major characteristics of NASH in humans, the possible relation of Kupffer cells with inflammation, steatosis, and fibrosis may resemble human NASH<sup>[7]</sup>.

In this study, we administered Y-40138, which is a reciprocal cytokine production modulator, inhibiting production of TNF- $\alpha$  and interferon  $\gamma$  (IFN- $\gamma$ ), and promoting production of interleukin-10 (IL-10)<sup>[8,9]</sup>, to a CDAA-fed rat NASH model, to investigate its possible use as a novel immunotherapy for NASH.

## MATERIALS AND METHODS

### Animals and induction of NASH model

Male F344 rats (6-wk-old, weighing 180-200 g) were purchased from Japan SLC Inc. (Hamamatsu, Japan). The animals were housed in stainless steel mesh cages under controlled temperature ( $23 \pm 3^\circ\text{C}$ ) and relative humidity ( $50\% \pm 20\%$ ) conditions with 10 to 15 air changes per hour, and light illumination for 12 h a day. They were allowed access to tap water *ad libitum* throughout the duration of the study. CDAA and choline-supplemented L-amino acid-defined (CSAA) feeds were purchased from CLEA Japan Inc. (Tokyo, Japan). The details of both diets are described elsewhere<sup>[10]</sup>.

The study groups were fed a CDAA diet for 8 wk to produce NASH. After 8 wk, the control group rats (CSAA diet,  $n = 5$ ), control + Y-40138 group rats (CSAA + Y-40138 diet,  $n = 5$ ), NASH group rats (CDAA diet,  $n = 5$ ), and NASH + Y-40138 group rats (CDAA diet + Y-40138,  $n = 5$ ) were killed and bled from the portal vein. Plasma was obtained by centrifugation at 3000 g for 15 min, and stored at  $-20^\circ\text{C}$ . Liver tissues were excised after perfusion with phosphate buffered saline

*via* the portal vein. All procedures were approved by our Institutional Animal Care Committee, and conducted in accordance with the Nara Medical University Guidelines for the Care and Use of Laboratory Animals.

### Compounds

Y-40138 was obtained from Tanabe Mitsubishi Pharma Co. (Osaka, Japan), and dissolved in 0.5% hydroxypropyl methylcellulose solution. Y-40138 was orally administered to rats (10 mg/kg per day) for 8 wk.

### Plasma alanine aminotransferase (ALT)

The plasma ALT levels were measured in samples from rats of each group. The blood samples were centrifuged, and the plasma was collected and stored at  $-30^\circ\text{C}$  until used for ALT determination. The plasma ALT levels were measured using a 7170 Clinical Analyzer (Hitachi High-Technologies, Tokyo, Japan).

### Plasma TNF- $\alpha$ , IFN- $\gamma$ and IL-10

TNF- $\alpha$ , IFN- $\gamma$  and IL-10 concentrations were measured in plasma samples (50  $\mu\text{L}$ ) from rats from each group using an enzyme-linked immunosorbent assay (ELISA) kit (BioSource International, Camarillo, CA, USA).

### Liver TNF- $\alpha$ , IFN- $\gamma$ and IL-10

Liver tissue samples were frozen in liquid nitrogen, powdered, and 30 mg samples were homogenized in 1 mL of lysis buffer. TNF- $\alpha$ , IFN- $\gamma$ , IL-10 levels in the supernatant (50  $\mu\text{L}$ ) were measured using an ELISA kit (BioSource International).

### Histological examination

Liver tissue samples were fixed in 10% formalin, embedded in paraffin, and 5  $\mu\text{m}$  thick sections were cut from the paraffin blocks for staining with hematoxylin and eosin, Azan, and Sirius red. Histological grading and staging were performed using a modified scoring system based on the classifications of Matteoni *et al*<sup>[11]</sup> and Brunt *et al*<sup>[12]</sup>.

### Immunohistochemistry

Kupffer cell dynamics were analyzed by comparing cell counts in each group using immunohistochemical staining with anti-rat macrophage/dendritic cell monoclonal antibody (RM-4: Trans Genic Inc., Kobe, Japan), Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA, USA), and DAB peroxidase substrate solution (Vector Laboratories), with counterstaining by Hematoxylin Mayer. The stained areas were analyzed and compared using NIH image software (Version 1.61, U. S. National Institute of Health, Bethesda, MD, USA).

TNF- $\alpha$  immunohistochemical staining was performed on the paraffin sections using an anti-rat TNF- $\alpha$ /TNFSF1A antibody (R&D Systems, Minneapolis, MN, USA). We then used Vectastain ABC Elite Kit, and DAB peroxidase substrate solution, with counterstaining by Hematoxylin Mayer. The stained areas were analyzed using NIH image software.

### Isolation and culture of Kupffer cells

Kupffer cells were harvested from the liver using isolation buffers according to a previously described procedure<sup>[13,14]</sup>. The livers were perfused *in situ* with Ca<sup>2+</sup>-free minimum essential medium (Sigma, St. Louis, MI, USA), followed by 0.3% pronase (Roche Diagnostics Co., Indianapolis, IN, USA) and 0.05% collagenase type IV (Wako, Osaka, Japan) in Dulbecco's modified Eagle's medium/F-12 (Sigma) at a rate of 10 mL/min through the portal vein. The liver was carefully removed and minced using scissors. The minced livers were further incubated in a shaker water bath with 0.035% pronase and 62.5 U/mL DNase (Sigma) in Dulbecco's modified Eagle's medium/F-12 at 37°C for 20 min. The samples were then filtered through gauze mesh, and the parenchymal cells were removed using low-speed centrifugation. Percoll (GE Healthcare, Little Chalfont, UK) was used for density gradient centrifugation of the cells. Kupffer cells were collected from the layer between 1.04 and 1.06, and cultured in RPMI 1640 supplemented with 10% fetal bovine serum and SAG (50 µg/mL streptomycin, 50 µg/mL ampicillin, 0.3 g/L L-glutamine). The viability of the cultured Kupffer cells was determined using trypan blue exclusion. Kupffer cells were seeded at a density of 5 × 10<sup>5</sup> cells/mL on 12-well plastic plates with RPMI-1640 medium. For the lipopolysaccharide (LPS)-stimulated group, Kupffer cells were then exposed to 1 µg/mL of LPS (Sigma-Aldrich Corp.) and incubated for 4.5 h at 37°C in 50 mL/L CO<sub>2</sub>-humidified air. Cells not treated with LPS were used as a control. TNF-α levels in the supernatant were measured using an ELISA kit.

The purity of the isolated Kupffer cells was over 98%, as examined by the uptake of 1 µm latex beads<sup>[15]</sup>, and the viability was over 97% using the trypan blue dye exclusion test.

### Statistical analysis

All results are expressed as mean ± SD. Analyses were conducted using Statview software (version 5.0, SAS Institute, Cary, NC, USA). The statistical differences between groups were evaluated using the paired *t*-test. A *P*-value < 0.05 was considered statistically significant.

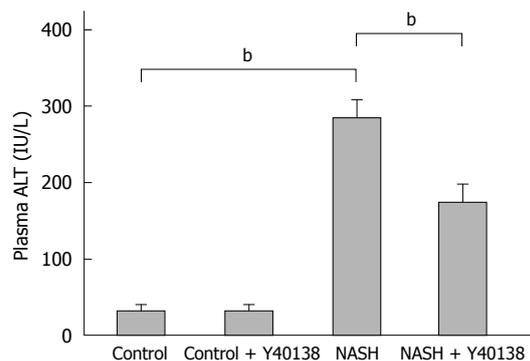
## RESULTS

### Plasma ALT levels

The mean plasma ALT level was 35.8 ± 4.7 IU/L in the control group, 38.3 ± 6.7 IU/L in the control + Y-40138 group, 279.0 ± 32.8 IU/L in the NASH group, and 171.3 ± 28.5 IU/L in the NASH + Y-40138 group. The NASH group showed a statistically significant elevation in the plasma ALT level in comparison to the control group (*P* < 0.01). The plasma ALT levels were significantly decreased in the NASH + Y-40138 group in comparison to the NASH group (*P* < 0.01) (Figure 1).

### Plasma TNF-α, IFN-γ and IL-10 levels

In comparison to the control group, the plasma TNF-α



**Figure 1** The plasma alanine aminotransferase (ALT) levels in the nonalcoholic steatohepatitis (NASH) group were significantly elevated in comparison to the control group. The plasma ALT levels in the NASH + Y-40138 group were significantly decreased in comparison to the NASH group (each group *n* = 5). <sup>b</sup>*P* < 0.01.

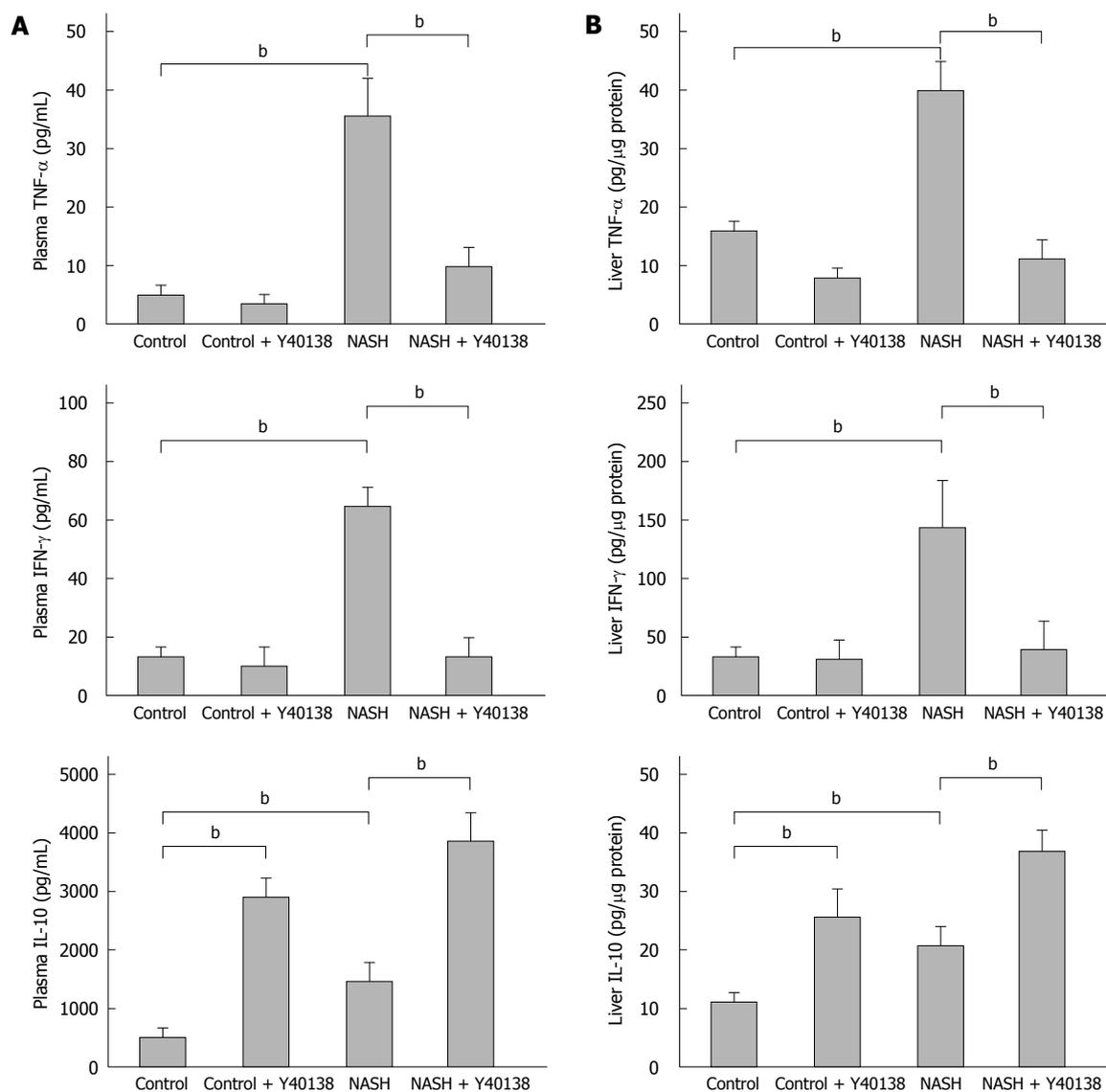
levels were significantly elevated in the NASH group (5.1 ± 1.4 pg/mL *vs* 35.2 ± 7.3 pg/mL, *P* < 0.01). The plasma TNF-α levels were significantly lower in the NASH + Y-40138 group (9.6 ± 3.6 pg/mL) than in the NASH group (*P* < 0.01). The plasma IFN-γ levels in the NASH group (62.4 ± 11.0 pg/mL, *P* < 0.01) were significantly elevated in comparison to the control group (12.3 ± 2.3 pg/mL). The plasma IFN-γ levels in the NASH + Y-40138 group (12.8 ± 6.3 pg/mL) were significantly decreased in comparison to the NASH group (*P* < 0.01). The plasma IL-10 levels in the control + Y-40138 group (2860 ± 350 pg/mL, *P* < 0.01) and the NASH group (1360 ± 380 pg/mL, *P* < 0.01) were significantly elevated in comparison to the control group (520 ± 96 pg/mL). The plasma IL-10 levels in the NASH + Y-40138 group (3820 ± 480 pg/mL) were significantly increased in comparison to the NASH group (*P* < 0.01) (Figure 2A).

### Liver TNF-α, IFN-γ and IL-10 levels

The liver TNF-α level in the NASH group was 39.1 ± 6.2 pg/µg protein, significantly higher than that in the control group (15.8 ± 3.6 pg/µg protein, *P* < 0.01). The liver TNF-α level in the NASH + Y-40138 group (12.4 ± 4.1 pg/µg protein) was significantly lower than that in the NASH group (*P* < 0.01). The liver IFN-γ level in the NASH group was 136 ± 39 pg/µg protein, significantly higher than that in the control group (36.3 ± 5.6 pg/µg protein, *P* < 0.01). The liver IFN-γ level in the NASH + Y-40138 group (45.6 ± 18.5 pg/µg protein) was significantly lower than that in the NASH group (*P* < 0.01). The liver IL-10 level in the control + Y-40138 group (24.7 ± 5.3 pg/µg protein) and the NASH group (19.3 ± 4.1 pg/µg protein) were significantly elevated in comparison to the control group (10.4 ± 2.5 pg/µg protein). The liver IL-10 level in the NASH + Y-40138 group (34.5 ± 4.3 pg/µg protein) was significantly higher than that in the NASH group (*P* < 0.01) (Figure 2B).

### Histological findings

In contrast to the control group, marked inflammation, fat deposition, and fibrosis were seen in the NASH



**Figure 2** Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interferon- $\gamma$  (IFN- $\gamma$ ), and interleukin-10 (IL-10) levels in the plasma and liver (ELISA method) in each group ( $n = 5$ ). A: The plasma TNF- $\alpha$  and IFN- $\gamma$  levels were significantly elevated in the NASH group, and the increase was attenuated in the NASH + Y-40138 group. The IL-10 levels were increased in the NASH group and further increased in the NASH + Y-40138 group rats. <sup>b</sup> $P < 0.01$ ; B: The liver TNF- $\alpha$ , IFN- $\gamma$  and IL-10 levels were significantly elevated in the livers of the NASH group rats. TNF- $\alpha$  and IFN- $\gamma$  levels were attenuated in the NASH + Y-40138 group. The IL-10 levels further increased in NASH + Y-40138 group rats. <sup>b</sup> $P < 0.01$ .

group, corresponding to Matteoni's type 4 and grade 3/ stage 3 of Brunt's NASH classification. The NASH + Y-40138 group findings corresponded to Matteoni's type 3 and Brunt's grade 2/ stage 2, and showed amelioration of inflammation and fibrosis in comparison to the NASH group (Figure 3).

#### Immunohistochemical findings

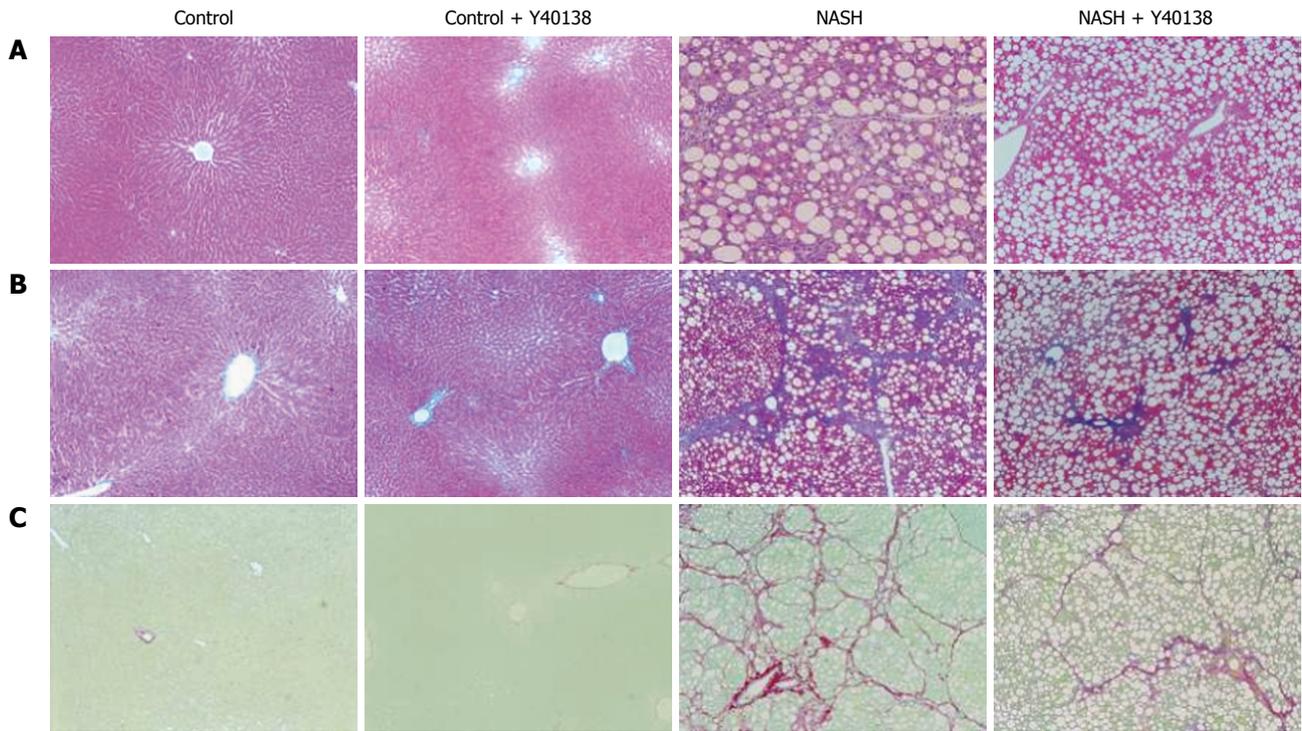
Kupffer cell immunohistochemical staining showed no significant difference in the number of stained cells per field between groups (Figure 4A and B). Moreover, quantitative analysis using NIH Image analysis software showed no significant difference between groups (Figure 4C).

The TNF- $\alpha$  immunoreactivity in the liver tissue samples from the NASH group rats was increased in comparison to the control group rats. Moreover, the

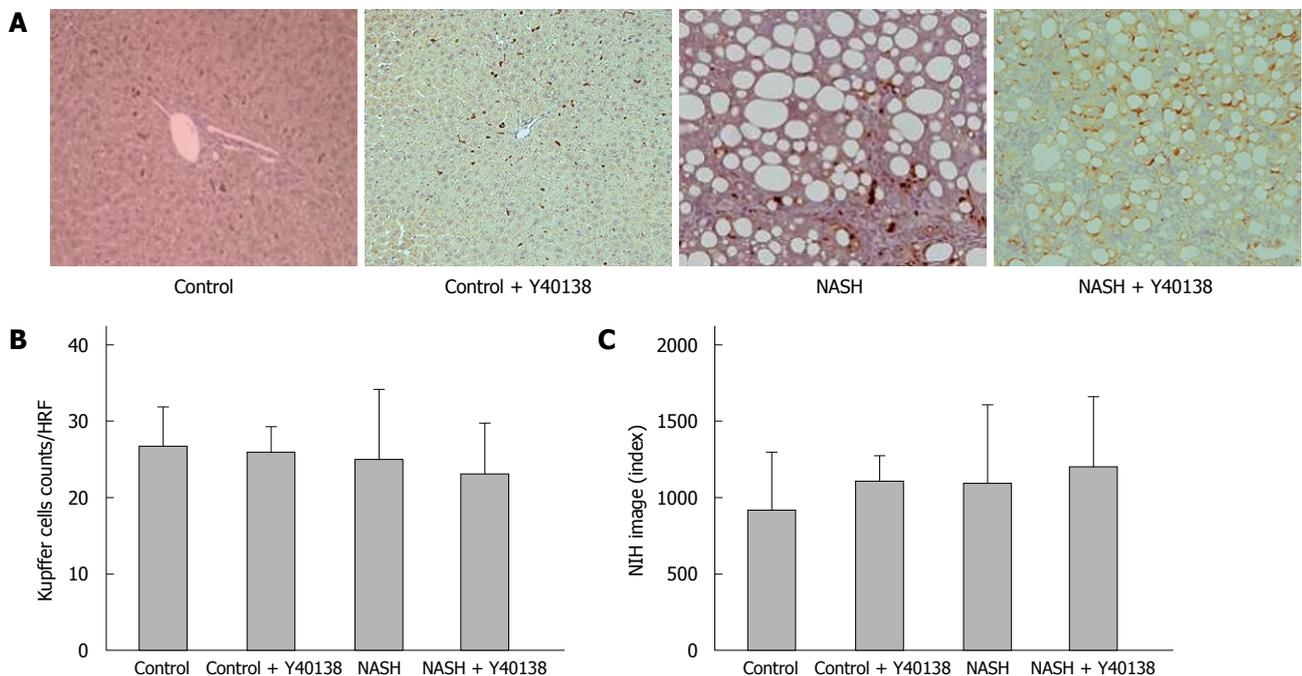
immunopositive areas in those animals were localized either to the periphery of fatty spots or to the non-parenchymal cells (Figure 5A). Semiquantitative analysis of TNF- $\alpha$  staining revealed significantly increased immunoreactivity in the livers of the NASH group rats ( $3250 \pm 390$ ) in comparison to the control group rats ( $260 \pm 80$ ) ( $P < 0.01$ ). Semiquantitative analysis of TNF- $\alpha$  staining revealed significantly decreased immunoreactivity in the livers of the NASH + Y-40138 group rats ( $820 \pm 350$ ) in comparison to the NASH group rats ( $P < 0.01$ ) (Figure 5B).

#### In-vitro TNF- $\alpha$ production by Kupffer cells

The viability of the cultured Kupffer cells was over 98% as determined by trypan blue exclusion. The basal TNF- $\alpha$  production by Kupffer cells isolated from the NASH group ( $26.6 \pm 3.4$  pg/mL) was equal to that



**Figure 3** Histological analysis of liver sections. A: Hematoxylin and eosin stain ( $\times 100$ ); B: Azan stain ( $\times 100$ ); C: Sirius red stain ( $\times 100$ ). Histological findings of liver tissue from the NASH group rats corresponded to Matteoni's type 4 and grade 3/stage 3 of Brunt's NASH classification. Sirius red staining revealed abundant collagen. NASH + Y-40138 group findings corresponded to Matteoni's type 3 and Brunt's grade 2/stage 2, and showed amelioration of inflammation and fibrosis in comparison to the NASH group ( $n = 5$ ).



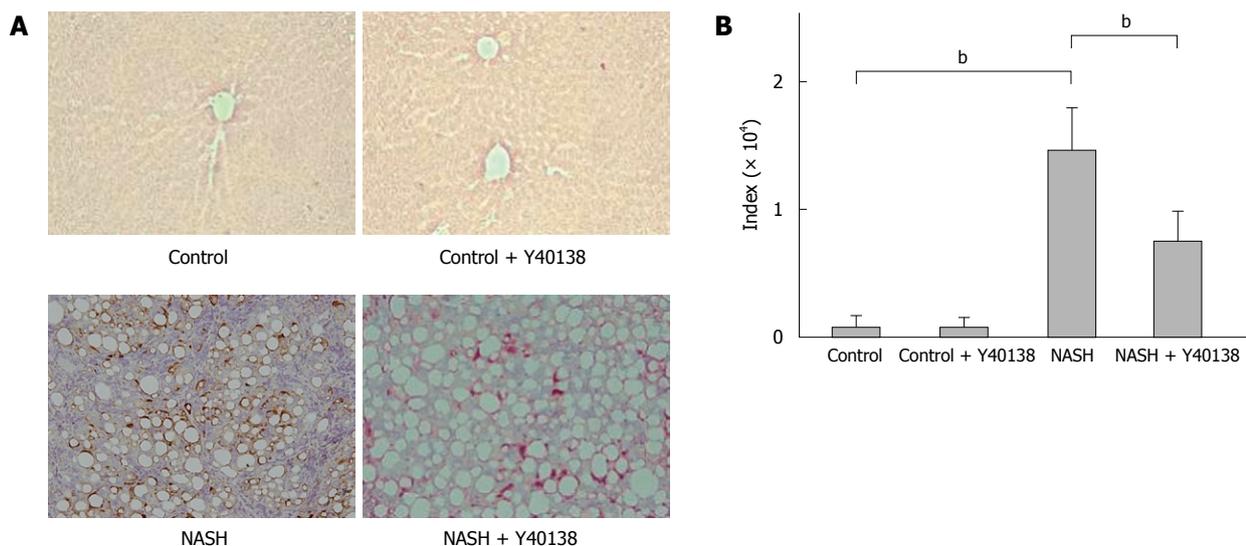
**Figure 4** Kupffer cell immunohistochemical staining, Kupffer cell number and semiquantification in the liver. A: Kupffer cell immunohistochemical staining ( $\times 200$ ). Brown-stained cells are positive; B: No significant differences were seen between groups in the stained cell counts per field; C: Quantitative analysis revealed no significant differences between groups ( $n = 5$ ). HRF: High rise filed.

from the control group ( $23.4 \pm 4.8$  pg/mL). After LPS stimulation, TNF- $\alpha$  production in the control group ( $48.9 \pm 7.5$  pg/mL) was significantly higher than the basal control level, and that in the NASH group ( $73.8 \pm 8.4$  pg/mL) was significantly higher than that in the control group ( $P < 0.01$ ). TNF- $\alpha$  production in the NASH + Y-40138

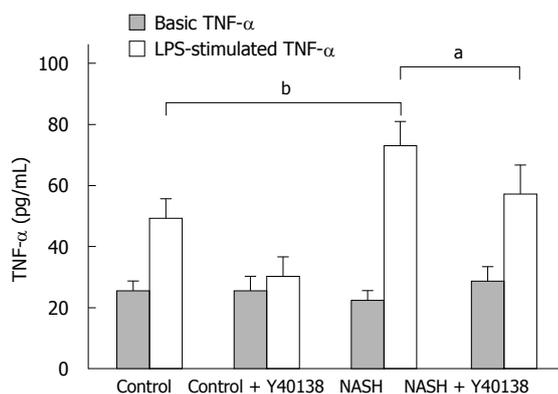
group ( $57.2 \pm 10.3$  pg/mL) was significantly lower than that in the NASH group ( $P < 0.05$ ) (Figure 6).

## DISCUSSION

Kupffer cells located in the hepatic sinusoids are important



**Figure 5** TNF- $\alpha$  immunohistochemical staining and semiquantification. A: TNF- $\alpha$  immunohistochemical staining ( $\times 200$ ). Brown-stained cells are positive; B: TNF- $\alpha$  immunoreactivities in the tissue samples from the NASH group were increased in comparison to the control group. The TNF- $\alpha$  immunoreactivities in the NASH + Y40138 group were decreased in comparison to the NASH group ( $n = 5$ ). <sup>b</sup> $P < 0.01$ .



**Figure 6** *In-vitro* TNF- $\alpha$  production by Kupffer cells. The basal TNF- $\alpha$  production by Kupffer cells isolated from the NASH group ( $26.6 \pm 3.4$  pg/mL) was equal to that in the control group ( $23.4 \pm 4.8$  pg/mL). After lipopolysaccharide (LPS) stimulation, TNF- $\alpha$  production in the control group ( $48.9 \pm 7.5$  pg/mL) was significantly higher than the basal control level, and was significantly higher in the NASH group ( $73.8 \pm 8.4$  pg/mL) than in the control group ( $n = 5$ ). <sup>b</sup> $P < 0.01$ . TNF- $\alpha$  production in the NASH + Y40138 group ( $57.2 \pm 10.3$  pg/mL) was significantly decreased in comparison to the NASH group ( $n = 5$ ). <sup>a</sup> $P < 0.05$ .

hepatic cells that possess phagocytic activity, antigen presenting activity, and produce inflammatory cytokines, making them responsible for the hepatic innate immunity. Inflammatory cytokines, in particular TNF- $\alpha$ , are reportedly involved in the etiology and progression of alcoholic hepatitis<sup>[16]</sup>. The histopathological findings in NASH are similar to those detected in alcoholic hepatitis, suggesting the existence of a common underlying mechanism. A possible mechanism is that increased endotoxin levels cause activation of Kupffer cells through TLR4, leading to increased expression of TNF- $\alpha$  and increased levels of ROS. These induce inflammation and fibrosis, progressing to NASH. The elevation in the hepatic tissue and plasma levels of TNF- $\alpha$  noticed in NASH<sup>[2]</sup> has been attributed to a combination of secretion by fat cells associated with obesity, and secretion by Kupffer cells activated by endotoxins<sup>[17]</sup>.

Once the trigger of TLR4 signal transmission has been initiated, Kupffer cells produce inflammatory cytokines including TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, as well as the anti-inflammatory cytokine IL-10<sup>[18]</sup>. Kupffer cells also induce the inflammatory response through production of transforming growth factor-1, matrix metalloproteinase, platelet-derived growth factor, and ROS<sup>[19]</sup>. NASH is characterized by fatty deposition in the hepatocytes, inflammatory cell infiltration, with consequent liver fibrosis<sup>[20]</sup>.

Methionine/choline deficient (MCD) and CDAA diets are widely used to produce NASH animal models<sup>[4]</sup>. Although these animals models lack obesity and insulin resistance, 2 of the major characteristics of NASH in humans, the possible relation of Kupffer cells with inflammation, steatosis, and fibrosis may resemble the human NASH<sup>[7]</sup>. We also demonstrated decreased Kupffer cell phagocytic activity in this model<sup>[4]</sup>. Increased sensitivity to LPS, the TLR4 ligand, occurs in MCD and CDAA-induced NASH, triggering the production of inflammatory cytokines<sup>[3]</sup>.

TNF- $\alpha$  is a cytokine widely involved in biodefence and immune mechanisms based on inflammation, and continued overproduction is harmful to the organisms, with potentially serious toxicity, resulting in various morbidities<sup>[21]</sup>. Anti-cytokine antibodies such as infliximab are now widely used clinically as pharmacotherapy for inflammatory diseases<sup>[19]</sup>.

In contrast, IL-10 is produced by macrophages and T-cells and has anti-inflammatory properties, inhibiting the production of inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8<sup>[22]</sup>. Therefore, IL-10 has shown efficacy in models of sepsis and rheumatoid arthritis<sup>[23]</sup> and clinically useful therapeutic effects have been reported for IL-10 in the treatment of steroid-resistant Crohn's disease<sup>[24]</sup>.

The chemical name of the new synthetic compound Y-40138 is N-[1-(4-[4-(pyrimidin-2-yl) piperazin-1-yl]

methylphenyl) cyclopropyl] acetamide/hydrochloride. Its molecular formula is  $C_{20}H_{25}N_5O/HCl$ , its molecular weight is 387.91 (free base: 351.45). Although the site of action of Y-40138 has not yet been elucidated, it is known to have reciprocal actions in inhibiting production of TNF- $\alpha$  and IFN- $\gamma$ , and increasing production of IL-10, as well as inhibiting production of chemokines and inflammatory cell chemotaxis, mechanisms not seen in any other agents<sup>[8,9]</sup>. Previous studies on Y-40138 have demonstrated improved survival rates in a D-galactosamine/LPS-induced mouse fulminant hepatic failure model<sup>[25,26]</sup> and a LPS-stimulated mouse sepsis model<sup>[27]</sup>, and reduced ALT levels in a concanavalin A-induced mouse hepatitis model<sup>[28]</sup>.

In this study using the rat NASH model, we detected increased plasma levels of ALT and TNF- $\alpha$ , and confirmed increased TNF- $\alpha$  levels in the liver using ELISA and immunohistochemical staining. These results indicate that TNF- $\alpha$  is involved in this NASH model, as in alcoholic hepatitis. Administration of Y-40138 to these same model rats reduced the plasma levels of ALT and TNF- $\alpha$ , and also decreased the TNF- $\alpha$  levels in the liver. We detected increased levels of IL-10 in the peripheral blood and liver. The TNF- $\alpha$  production by the isolated Kupffer cells was similar in the control and NASH rats. Kupffer cells from the control rats were sensitive to LPS stimulation. Furthermore, those from NASH were more sensitive to LPS stimulation, suggesting that continuous inflammation leads to up-regulation of the LPS-induced TNF- $\alpha$  production by the activated Kupffer cells. Administration of Y-40138 to the same model rats decreased LPS-induced TNF- $\alpha$  production *in vitro*. We presume that IL-10 restrains the production of TNF- $\alpha$  and IFN- $\gamma$  *via* inhibition of the activation of Kupffer cells through STAT3 (signal transducer and activator of transcription 3).

In conclusion, the multiple cytokine production modulator Y-40138 ameliorated hepatic inflammation and inhibited fibrotic changes in the liver. These results indicate that Y-40138 is promising as a novel treatment for NASH. Y-40138 may have a role in suppressing the progress of hepatitis and prolonging the lives of patients with liver damage such as NASH in which cytokines and/or chemokines are involved.

## ACKNOWLEDGMENTS

We gratefully acknowledge Tanabe Mitsubishi Pharma Co. for generously providing us with Y-40138 for our study.

## COMMENTS

### Background

Kupffer cells, part of the innate immune system, produce various cytokines and are known to play a role in pathologies of the liver. Abnormally functioning Kupffer cells are thought to be involved in nonalcoholic steatohepatitis (NASH). Although the etiological mechanisms of NASH have not been fully elucidated yet, observing similar histopathological findings in NASH and alcoholic hepatitis suggests the existence of a common underlying mechanism. In this study, the authors administered the multiple cytokine production modulator, Y-40138, to NASH model rats, to investigate its possible use as a novel immunotherapy for NASH.

## Research frontiers

Administration of Y-40138 to NASH model rats reduced hepatic inflammation and suppressed fibrosis. These results indicate that the multiple cytokine production modulator, Y-40138, is promising as a novel treatment for NASH. Therefore, Y-40138 may contribute to suppressing the progress of hepatitis and prolonging the life of patients with liver damage such as NASH.

## Applications

The present study is an experimental research using the rat NASH model, which was made by feeding a choline-deficient L-amino-acid-defined diet to rats, to examine their innate immunity. The authors believe that Y-40138 is a useful immunotherapy for NASH.

## Terminology

The chemical name of the new synthetic compound Y-40138 is N-[1-(4-[4-(pyrimidin-2-yl) piperazin-1-yl] methylphenyl) cyclopropyl] acetamide/hydrochloride. Although the mechanism of action of Y-40138 has not been fully elucidated yet, it is known to have reciprocal actions in inhibiting the production of tumour necrosis factor- $\alpha$  and interferon- $\gamma$ , and increasing the production of IL-10, as well as inhibiting the production of chemokines and inflammatory cell chemotaxis, and no other agents are known to have such mechanisms.

## Peer review

This is an interesting study on the use of a cytokine modulator in the treatment of an experimental NASH model. The paper is well written and the research is well designed and executed.

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## Macrophage migration inhibitory factor regulates proliferation of gastric cancer cells *via* the PI3K/Akt pathway

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### Abstract

**AIM:** To investigate the effects of macrophage migration inhibitory factor (MIF) on proliferation of human gastric cancer MGC-803 cells and expression of cyclin D1 and p27<sup>Kip1</sup> in them, and further determine whether the effects are related to the PI3K/Akt signal transduction pathway.

**METHODS:** Gastric cancer MGC-803 cells were cultured and then treated with 50 µg/L recombinant human MIF (rhMIF) with and without a PI3K inhibitor, LY294002 (25 µmol/L). MTT assay was used to detect the proliferation of MGC-803 cells. Cell cycle was detected by flow cytometry. Expression of cyclin D1 and p27<sup>Kip1</sup> mRNA was by reverse transcription-polymerase chain reaction. Protein expression of phosphorylated Akt (p-Akt), Akt, cyclin D1 and p27<sup>Kip1</sup> was examined by immunocytochemistry and Western blotting.

**RESULTS:** rhMIF significantly stimulated the proliferation of MGC-803 cells and cell cycle progression from G1 phase to S phase in a concentration- and time-dependent manner. After the MGC-803 cells were treated with rhMIF for 24 h, the expression of cyclin D1 was significantly up-regulated compared with the cells not treated with rhMIF at both mRNA and protein levels

( $0.97 \pm 0.02$  vs  $0.74 \pm 0.01$ ,  $P = 0.002$ ;  $0.98 \pm 0.05$  vs  $0.69 \pm 0.04$ ,  $P = 0.003$ ). The p27<sup>Kip1</sup> was down-regulated but only statistically significant at the protein level. rhMIF significantly increased the expression of p-Akt, which reached the peak at 30 min, but did not affect the expression of Akt. However, LY294002 inhibited all the effects of rhMIF.

**CONCLUSION:** Macrophage MIF increases the proliferation of gastric cancer cells, induces the expression of cyclin D1 at the transcriptional level and inhibits the expression of p27<sup>Kip1</sup> at the post-transcriptional level *via* the PI3K/Akt pathway.

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**Key words:** Macrophage migration inhibitory factor; Gastric cancer; Proliferation; Cell cycle; Cyclin D1; p27<sup>Kip1</sup>; PI3K/Akt

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Li GQ, Xie J, Lei XY, Zhang L. Macrophage migration inhibitory factor regulates proliferation of gastric cancer cells *via* the PI3K/Akt pathway. *World J Gastroenterol* 2009; 15(44): 5541-5548 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5541.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5541>

### INTRODUCTION

Gastric cancer is the most common gastroenterological malignancy worldwide, with its incidence rate being the fourth among all cancers<sup>[1,2]</sup>. There are approximately 934 000 new cases, and 700 000 patients die of gastric cancer each year. Gastric cancer is the second leading cause of cancer-related death worldwide<sup>[3-5]</sup>, and remains a disease of poor prognosis. The 5-year survival rate of gastric cancer patients is generally below 20%. The current major therapies for gastric cancer include surgery, chemotherapy combined with or without radiotherapy, but their efficacy is not satisfactory. It is, therefore,

necessary to explore and investigate the tumorigenesis of gastric cancer and its novel therapy targets<sup>[6]</sup>.

Macrophage migration inhibitory factor (MIF) is a multi-functional cytokine, which is associated with inflammation and tumorigenesis<sup>[7,8]</sup>. Recent studies have shown that MIF is related to the initiation and progression of gastric cancer, but its underlying mechanism is not very clear<sup>[9-11]</sup>.

It has been reported that the effect of MIF on cell proliferation is relevant to cell cycle factors<sup>[12]</sup>. Cyclin D1 and p27<sup>Kip1</sup> belong to the family of cyclin proteins and cyclin-dependent kinase inhibitors (CDKI), which regulates cell cycle progression from the G1 phase to the S phase<sup>[13,14]</sup>. Over-expression of cyclin D1 and aberrant expression of p27<sup>Kip1</sup> can lead to the loss of control of cell proliferation and differentiation, thus promoting tumorigenesis<sup>[15]</sup>.

The phosphoinositol-3-kinases (PI3K)/Akt pathway has been considered an important signaling pathway that is involved in the regulation of cell proliferation and differentiation<sup>[16]</sup>. It has been reported that NF- $\kappa$ B activates the transcription target, *via* PI3K/Akt signaling, to promote gastric tumorigenesis<sup>[17]</sup>. In a recent study with a gastric cancer cell line, AGS, Kim *et al*<sup>[18]</sup> reported that inhibition of the PI3K/Akt/PKB pathway could enhance the mitochondrial death pathway. Another recent study indicates that anti-cancer effects of celecoxib on gastric cancer cells are partly mediated by down-regulation of Akt signaling<sup>[19]</sup>.

The aims of the present study were to investigate the effects of recombinant human MIF (rhMIF) on cell proliferation of human gastric cancer MGC-803 cells and cell cycle. Activity of Akt and expression of cyclin D1 and p27<sup>Kip1</sup> were examined. Whether the PI3K/Akt pathway is involved in the effects of rhMIF was further investigated, using a PI3K/Akt inhibitor, LY294002.

## MATERIALS AND METHODS

### Chemicals

rhMIF and PI3K specific inhibitor, LY294002, were purchased from PROSPEC (Rehovot, Israel) and Cell Signaling (Danvers, MA, USA), respectively. Primers for GAPDH, cyclin D1 and p27 were produced by Shanghai Sangon Biological Engineering Technology & Service Company, Ltd. (Shanghai, China). Mouse anti-human  $\beta$ -actin primary antibody was bought from Beyotime Institute of Biotechnology (Shanghai, China). Rabbit anti-human cyclin D1 primary antibody was purchased from Epitomics (Burlingame, CA, USA), and rabbit anti-human p27, rabbit anti-human phosphorylated Akt (p-Akt) and Akt primary antibodies were bought from Santa Cruz Biotechnology (CA, USA). Goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Beyotime Institute of Biotechnology (Shanghai, China) and BioDev-Tech (BioDev, Beijing, China), respectively. Epics-XL II flow cytometer was purchased from Beckman Coulter (Beckman, Fullerton, CA, USA).

### Cell culture

Gastric cancer cell line, MGC-803, was provided by the

Institute of Cancer Research, Nanhua University (China), and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100  $\mu$ g/mL streptomycin and 100  $\mu$ /mL penicillin, and maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The cultured cells were then used in different experiments as described below.

### Cell proliferation assay (MTT assay)

Approximately  $2 \times 10^3$  cells/well were grown in 96-well microtiter plates and incubated overnight in 100  $\mu$ L of the culture medium. Cells were starved without FBS for 24 h at 70%-80% confluence and then treated with rhMIF at different concentrations (25, 50 and 100  $\mu$ g/L) with or without 1-h pretreatment with LY294002 (25  $\mu$ mol/L), for 12, 24 and 48 h, respectively. Cells without any treatment were used as controls. Twenty microliters of 5 mg/mL MTT (Sigma, St Louis, MO) labeling reagent was added to the designated well, and cells were incubated at 37°C for 4 h. The supernatant was removed, and then 150  $\mu$ L dimethyl sulfoxide (DMSO) was added to the designated well. After the plate was incubated at 37°C for 15 min, the absorbency was measured with a micro ELISA reader (Bio-Tek, Winooski, VT, USA) at a wavelength of 570 nm.

### Flow cytometry

Cells were harvested after treated with rhMIF, with or without 1-h pretreatment with LY294002 for 24 h as described earlier, and fixed with 75% cold alcohol at 4°C overnight. After washed with phosphate buffered saline (PBS), propidium iodide (PI) was added and cells were incubated at 4°C for 30 min. Cell cycle distribution was detected with an Epics-XL II flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA).

### Reverse transcriptase polymerase chain reaction (RT-PCR)

Cells were harvested after treated with rhMIF, with or without 1-h pretreatment with LY294002 for 24 h as described earlier. Total RNA was extracted from cells with a total RNA kit (BioDev, Beijing, China). One microgram of RNA was reversely transcribed to cDNA with thermoscript RT system reagent (MBI Fermentas, Glen Burnie, USA) according to its manufacturer's instructions. The primer sequences are as follows: cyclin D1, forward: 5'-CGTCCATGCGGAAGATC-3', reverse: 5'-CAGAGGGCAACGAAGGT-3' (406 bp); p27<sup>Kip1</sup>, forward: 5'-CGCAAGTGGAATTTTCGATT-3', reverse: 5'-ATGCGTGTCCCTCAGAGTTAGC-3' (215 bp); GAPDH, forward: 5'-CGGAGTCAACGGATTGTG-GTCGTAT-3', reverse: 5'-AGCCTTCTCCATGGTG-GTGAAGAC-3' (306 bp). PCR was performed according to the following conditions: denaturation at 94°C for 3 min, 40 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. Five microlitres of PCR product was used for agarose gel electrophoresis and analyzed using Alphamager 2200 (Imaging System, CA, USA).

### Western blotting

Whole cell lysates were prepared from cells with RIPA

lysis buffer containing protease inhibitors (Beyotime, Shanghai, China). Protein concentration was measured with a BCA protein assay kit (Beyotime, Shanghai, China). An aliquot of 40  $\mu\text{g}$  protein of each sample was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Then, the protein was transferred to a PVDF membrane (Millipore, Billerica, MA). Non-specific binding was blocked with 5% non-fat milk for 2 h at 37°C. The membrane was immunodetected with anti-cyclin D1 (1:500), anti-p27<sup>kip1</sup> (1:500), anti-p-Akt (1:500), anti-Akt (1:500), anti- $\beta$ -actin (1:1000) primary antibodies overnight at 4°C, and then with corresponding HRP-conjugated secondary antibodies (1:2000). Antigen-antibody complexes were visualized with a luminol reagent (Santa Cruz, CA, USA), and the results were analyzed with Alphamager 2200.  $\beta$ -actin was detected as the loading control.

### Immunocytochemistry

Cells were grown on slides at a concentration of  $1.0 \times 10^6$  cells/mL, treated with rhMIF (50  $\mu\text{g}/\text{L}$ ) with or without 1-h pretreatment with LY294002 (25  $\mu\text{mol}/\text{L}$ ) for 24 h at 70%-80% confluence. Cells without any treatment were used as controls. The treated and control cells were fixed with 75% cold alcohol at 4°C overnight and then subjected to immunostaining using the streptavidin-peroxidase technique. In brief, endogenous peroxidase was blocked with 3%  $\text{H}_2\text{O}_2$  in PBS, and then with a normal goat serum blocking solution for 20 min at room temperature. The slides were incubated with anti-p-Akt, anti-cyclin D1 and anti-p27<sup>kip1</sup> primary antibodies (1:200 diluted with 0.01 mol/L PBS), respectively, for 60 min at room temperature. Slides that were incubated with PBS instead of primary antibodies were used as negative controls. After washed with PBS, the slides were incubated with corresponding HRP-conjugated secondary antibodies for 30 min. The color of immunostaining was developed with a diaminobenzidine solution and counterstained with hematoxylin.

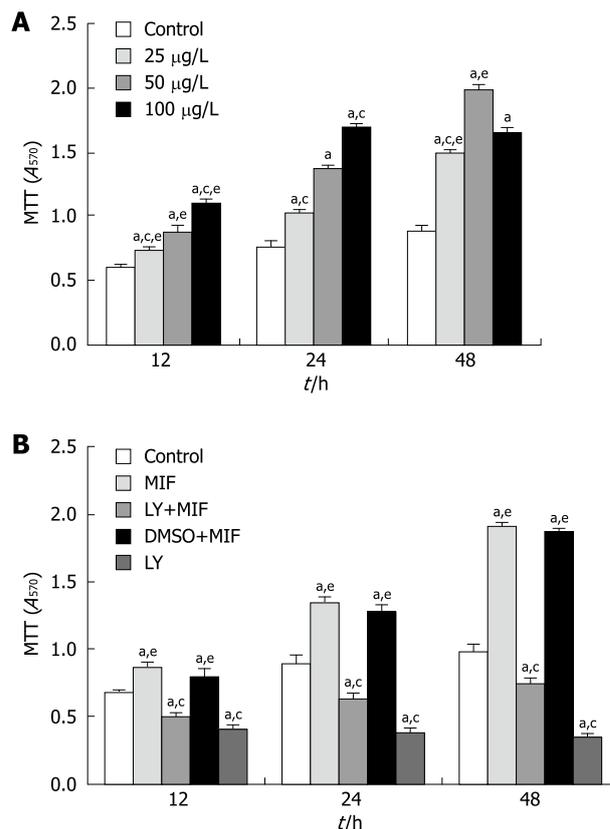
### Statistical analysis

Data were analyzed using SPSS 16.0 software. The results were expressed as mean  $\pm$  SD. Student *t*-test was used to determine the difference in numeric parameters between different groups.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Effects of rhMIF and LY294002 on the proliferation of MGC-803 cells

The MTT assay showed that treatment with rhMIF significantly increased the proliferation of MGC-803 cells in a dose- and time-dependent manner compared with the controls ( $P < 0.05$ ). The proliferation of MGC-803 cells reached its peak ( $1.823 \pm 0.04/0.647 \pm 0.02$ , 282%) when they were treated with 50  $\mu\text{g}/\text{L}$  rhMIF for 48 h (Figure 1A), which was significantly inhibited after 1-h pretreatment with 25  $\mu\text{mol}/\text{L}$  LY294002 ( $P < 0.05$ , Figure 1B). DMSO (the menstruum of LY294002, less than 0.1%) had little effect on the proliferation of MGC-803 cells.



**Figure 1** Effects of recombinant human migration inhibitory factor (rhMIF) and LY294002 on the proliferation of MGC-803 cells. A: MGC-803 cells were treated with 25, 50 and 100  $\mu\text{g}/\text{L}$  rhMIF for the indicated periods of time and cell proliferation was examined by MTT assay. <sup>a</sup> $P < 0.05$  vs control group; <sup>c</sup> $P < 0.05$  vs 50  $\mu\text{g}/\text{L}$  MIF group; <sup>e</sup> $P < 0.05$  vs 24 h group; B: With or without 1-h pretreatment with 25  $\mu\text{mol}/\text{L}$  LY294002, MGC-803 cells were exposed to 50  $\mu\text{g}/\text{L}$  rhMIF for the indicated periods of time, with a vehicle control of dimethyl sulfoxide (DMSO) (less than 0.1%) included. <sup>a</sup> $P < 0.05$  vs control group; <sup>c</sup> $P < 0.05$  vs MIF group; <sup>e</sup> $P < 0.05$  vs LY+MIF group. Data were represented as mean  $\pm$  SD of three independent experiments. DMSO (the menstruum of LY294002, less than 0.1%) had little impact on the rhMIF effect.

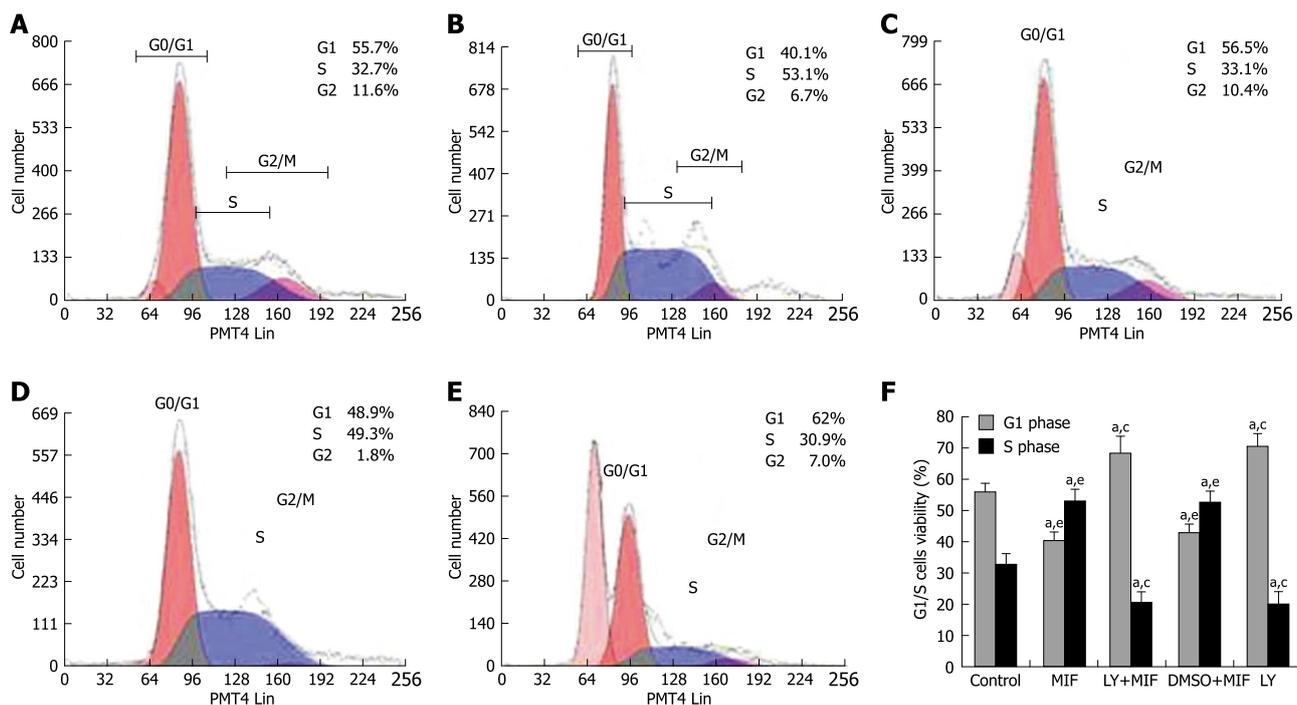
### Effects of rhMIF and LY294002 on the cell cycle of MGC-803 cells

The effect of rhMIF on cell cycle distribution is shown in Figure 2. rhMIF (50  $\mu\text{g}/\text{L}$ ) significantly induced the progression of MGC-803 cells from G1 phase to S phase ( $P < 0.05$ ), thus increasing the accumulation of cells in S phase. However, the cells treated with LY294002 were accumulated in G1 phase, namely G1 arrest, irrespective of rhMIF treatment ( $P < 0.05$ ). DMSO had little impact on the rhMIF effect (Figure 2).

### Effects of rhMIF and LY294002 on the mRNA and protein expression of cyclin D1 and p27<sup>kip1</sup> in MGC-803 cells

After treatment with 50  $\mu\text{g}/\text{L}$  rhMIF for 24 h, the mRNA level of cyclin D1 was increased significantly ( $P < 0.05$ ). However, 1-h pre-treatment with LY294002 reduced the mRNA expression irrespective of subsequent rhMIF treatment ( $P < 0.05$ , Figure 3A). DMSO had little impact on the rhMIF effect. Treatment with rhMIF or LY294002 had no effect on the mRNA level of p27<sup>kip1</sup> (Figure 3A).

Western blotting showed that after treatment with 50  $\mu\text{g}/\text{L}$  rhMIF for 24 h, the expression of cyclin D1



**Figure 2** Effects of rhMIF and LY294002 on cell cycle of MGC-803 cells. With or without 1-h pretreatment with 25  $\mu\text{mol/L}$  LY294002, MGC-803 cells were exposed to 50  $\mu\text{g/L}$  rhMIF for 24 h. A vehicle control of DMSO (less than 0.1%) was included. Cell lysates were prepared for the cell cycle detection. The cell cycle distribution was analyzed using an Eics-XL II flow cytometer. A: Control; B: MIF group; C: LY+MIF group; D: DMSO+MIF group; E: LY group; F: Quantification of G1/S viability(%) analysis of MGC-803 cells. Data were represented as mean  $\pm$  SD of three independent experiments. <sup>a</sup> $P < 0.05$  vs control group; <sup>b</sup> $P < 0.05$  vs MIF group; <sup>c</sup> $P < 0.05$  vs LY+MIF group. DMSO (the menstruum of LY294002, less than 0.1%) had little impact on the rhMIF effect.

protein was significantly up-regulated, but the expression of p27<sup>kip1</sup> protein was down-regulated ( $P < 0.05$ , Figure 3B). However, 1-h pretreatment with LY294002 prior to rhMIF treatment abrogated the effects of rhMIF on the expression of cyclin D1 and p27<sup>kip1</sup> proteins ( $P < 0.05$ ). Again, DMSO had little impact on the rhMIF effect (Figure 3B).

Immunocytochemistry demonstrated that the positive expression of cyclin D1 and p27<sup>kip1</sup>, in yellow or brown, was mostly located in the nuclei of MGC-803 cells, but rarely in the cytoplasm. rhMIF increased the expression of cyclin D1, but decreased the expression of p27<sup>kip1</sup> (Figure 4). However, 1-h pretreatment with LY294002 down-regulated the expression of cyclin D1 (Figure 4A), but up-regulated the expression of p27<sup>kip1</sup> (Figure 4B), which was consistent with the results obtained by Western blotting.

#### Effects of rhMIF on the expression of p-Akt and Akt in MGC-803 cells

Western-blotting showed that after treatment with different concentrations of rhMIF (25, 50, 100  $\mu\text{g/L}$ ) for 30 min, the expression of p-Akt in MGC-803 cells was up-regulated in a dose-dependent manner (Figure 5A,  $P < 0.05$ ). In addition, the cells treated with 50  $\mu\text{g/L}$  rhMIF for different periods of time (5, 15, 30 and 60 min) showed that the expression of activated Akt in MGC-803 cells started to increase at 5 min, reached its peak at 30 min, then decreased but remained at a level higher than that in the untreated control cells ( $P < 0.05$ , Figure 5B). Pretreatment with LY294002 prior to rhMIF treatment, however, inhibited the expression of p-Akt irrespective of

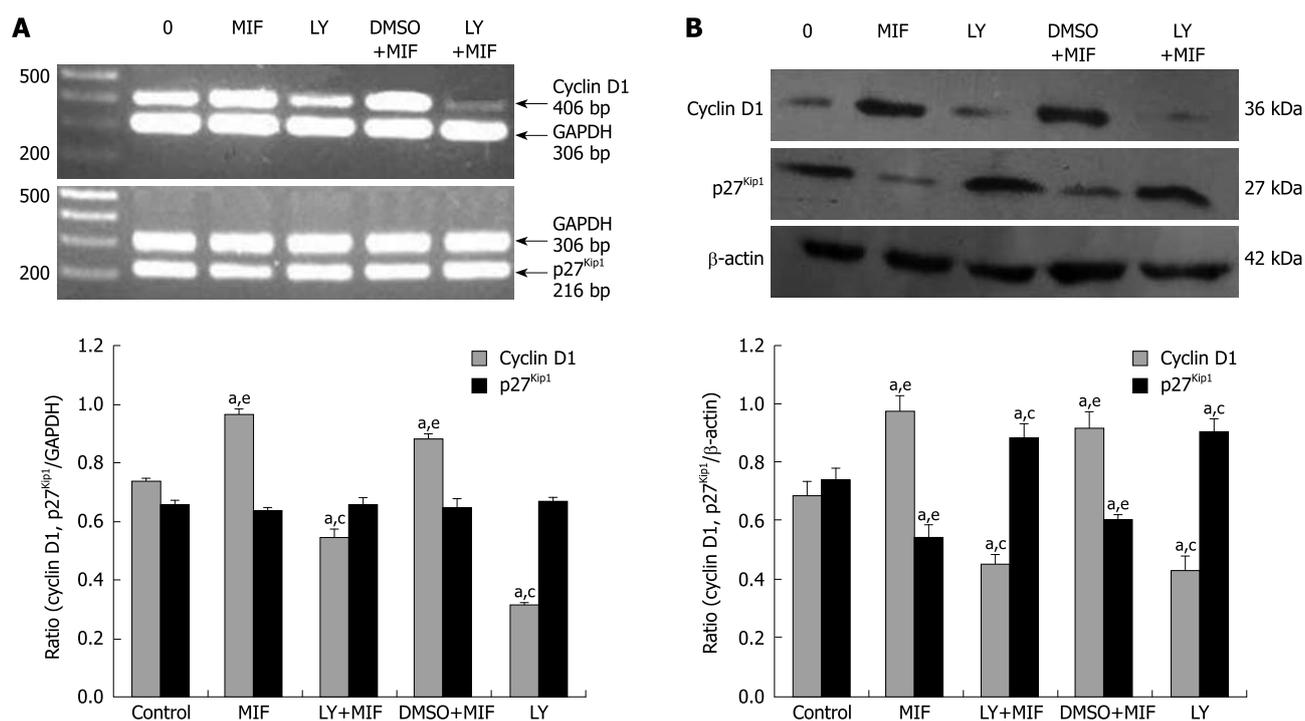
rhMIF treatment (Figure 5C). DMSO had little impact on the rhMIF effect. Both rhMIF and LY294002 had no effect on the expression of Akt (Figure 5A and C).

Immunocytochemistry showed that p-Akt was positively expressed in cytoplasm of the cells. rhMIF increased the expression of p-Akt. However, pre-treatment with LY294002 down-regulated the expression of p-Akt (Figure 5D).

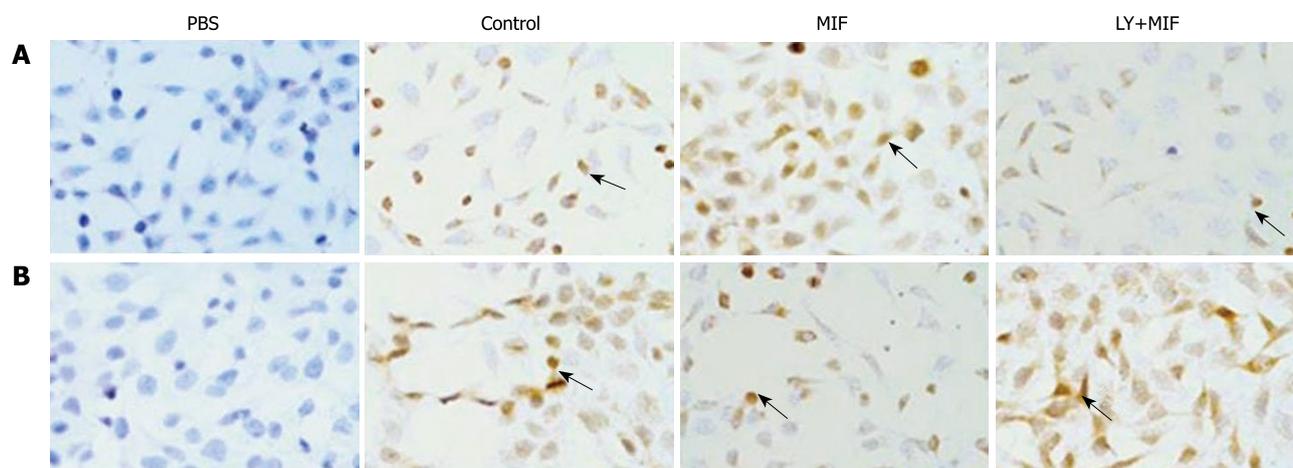
## DISCUSSION

MIF has multiple biological functions and plays an important role in inflammation as well as in carcinogenesis as a cytokine<sup>[20,21]</sup>. Increased expression of MIF has been reported in pre-cancerous, cancerous and metastatic tumors<sup>[10,22]</sup>. It has been shown that MIF-modulated cancer progress is associated with the ERK/MAPK, PI3K/Akt, and Rb-E2F pathways<sup>[22-25]</sup>. However, its detailed mechanism underlying inflammatory and malignant transformation as well as tumorigenesis needs to be further investigated.

In our study, rhMIF increased the MGC-803 cell proliferation in a dose- and time-dependent manner, and induced progression from G1 phase to S phase, leading to S phase arrest. It has been demonstrated that cyclin, cyclin-dependent kinase (CDK) and CDK inhibitor (CDKI) play a critical role in the orderly progression of cell cycle during the transition from G1 phase to S phase<sup>[26,27]</sup>. Since cyclin D1 is an important regulator of cell cycle progression, its increased expression contributes significantly to tumor development and malignant transformation<sup>[13,28]</sup>. p27<sup>kip1</sup>, an inhibitor of CDK, functions as



**Figure 3** Effects of rhMIF and LY294002 on the expression of cyclin D1 and p27<sup>kip1</sup> in MGC-803 cells. With or without 1-h pretreatment with 25  $\mu\text{mol/L}$  LY294002, MGC-803 cells were exposed to 50  $\mu\text{g/L}$  rhMIF for 24 h. A vehicle control of DMSO (less than 0.1%) was included. A: RT-PCR; B: Western blotting. The results were represented as mean  $\pm$  SD of three independent experiments. <sup>a</sup> $P < 0.05$  vs control group; <sup>c</sup> $P < 0.05$  vs MIF group; <sup>e</sup> $P < 0.05$  vs LY+MIF group. DMSO (the menstruum of LY294002, less than 0.1%) had little impact on the rhMIF effect.

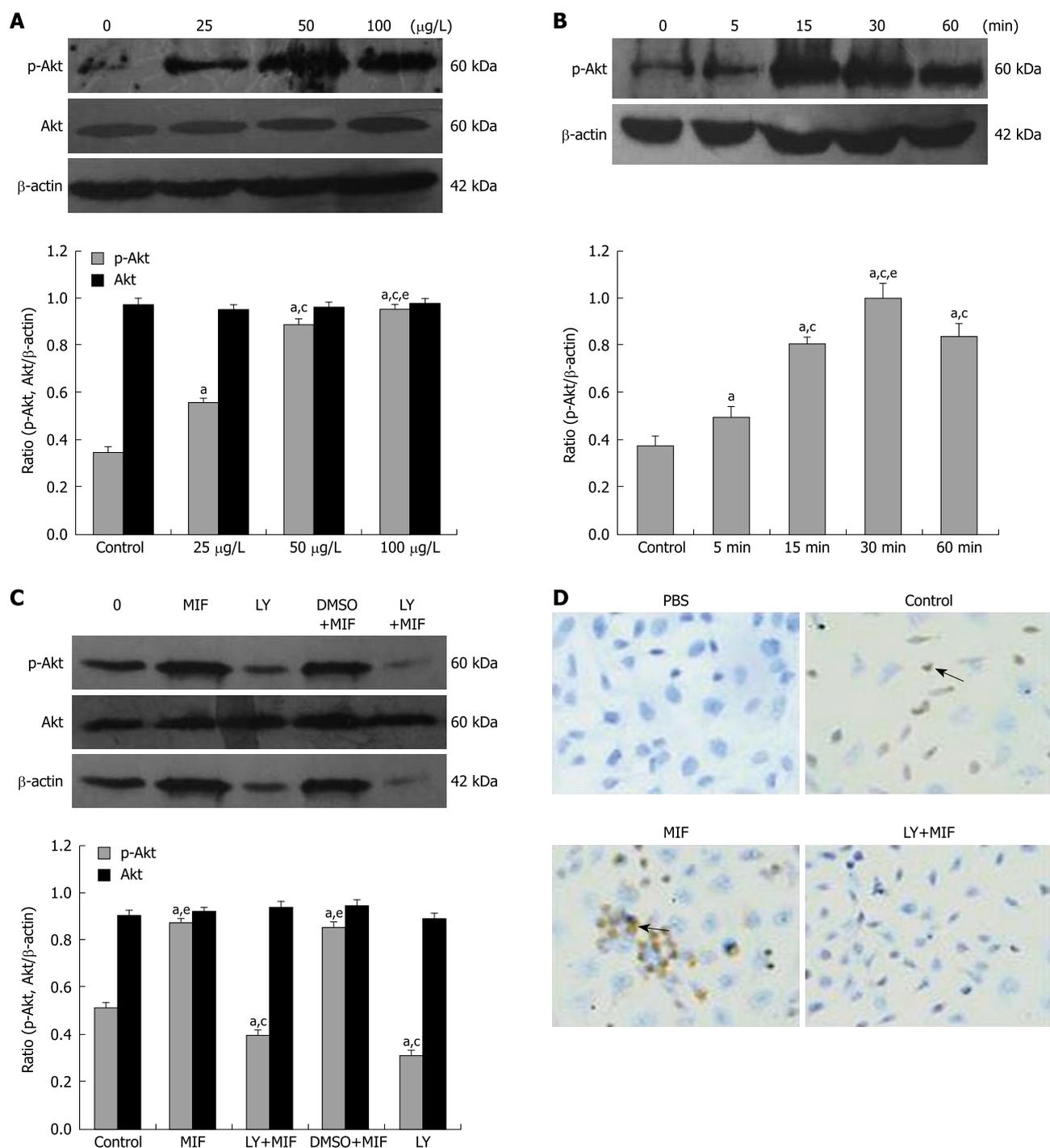


**Figure 4** Effects of rhMIF and LY294002 on the translocation of cyclin D1 (A) and p27<sup>kip1</sup> (B) in MGC-803 cells. With or without 1-h pretreatment with 25  $\mu\text{mol/L}$  LY294002, MGC-803 cells were exposed to 50  $\mu\text{g/L}$  rhMIF for 24 h. The arrow symbols represent immunocytochemical staining for nuclear and cytoplasmic expression of cyclin D1 (Original magnification,  $\times 200$ ) and p27<sup>kip1</sup> (Original magnification,  $\times 200$ ) in MGC-803. PBS: Phosphate buffered saline.

an integral brake of the cell cycle, thus also playing an important role in tumor suppression<sup>[29]</sup>. For example, deregulation of p27<sup>kip1</sup> has a profound effect on tumor progression in colorectal cancer and is suggested to be an accurate and independent prognostic marker<sup>[30]</sup>. Moreover, p27<sup>kip1</sup> may be a central target for growth regulation of the normal endometrium and for the pathogenesis of endometrial carcinoma<sup>[31]</sup>. In our study, rhMIF increased cyclin D1 mRNA expression, but had no effect on p27<sup>kip1</sup> mRNA expression. However, immunocytochemistry showed that rhMIF increased the expression of cyclin D1 protein and decreased the expres-

sion of p27<sup>kip1</sup> protein. These results were identified further by Western blotting. Therefore, our results indicate that rhMIF affects the cell cycle by inducing cyclin D1 and inhibiting p27<sup>kip1</sup>, thus promoting the proliferation of MGC-803 cells. In addition, rhMIF up-regulates the transcriptional cyclin D1, which is in contrast with the posttranscriptional protein reduction of p27<sup>kip1</sup> in cells. This phenomenon may be related to the degradation by its ubiquitin ligase<sup>[32,33]</sup>.

The PI3K/Akt pathway is crucial in tumorigenesis because the p-Akt can regulate the cell proliferation, apoptosis, angiogenesis and cell cycle by activating the



**Figure 5** Effects of rhMIF on the expression of p-Akt and Akt in MGC-803 cells. Cell lysates were isolated for Western blotting to detect the expression of p-Akt and Akt (A, B). A: MGC-803 cells were treated with 25, 50 and 100 µg/L rhMIF for 30 min. <sup>a</sup>*P* < 0.05 vs control group; <sup>c</sup>*P* < 0.05 vs 25 µg/L MIF group; <sup>e</sup>*P* < 0.05 vs 50 µg/L MIF group; B: MGC-803 cells were treated with 50 µg/L rhMIF at different time points (5, 15, 30 and 60 min). <sup>a</sup>*P* < 0.05 vs control group; <sup>c</sup>*P* < 0.05 vs 5 min group; <sup>e</sup>*P* < 0.05 vs 15 min and 60 min groups; C: With or without 1-h pretreatment with 25 µmol/L LY294002, MGC-803 cells were exposed to 50 µg/L rhMIF for 30 min with a vehicle control of DMSO (less than 0.1%) included, and then the expression of p-Akt and Akt was determined by Western blotting. <sup>a</sup>*P* < 0.05 vs control group; <sup>c</sup>*P* < 0.05 vs MIF group; <sup>e</sup>*P* < 0.05 vs LY+MIF group; D: The arrow symbols represent immunocytochemical staining for nuclear and cytoplasmic expression of p-Akt (original magnification, × 200) in MGC-803 cells. The results were represent as mean ± SD of three independent experiments. DMSO (the menstruum of LY294002, less than 0.1%) had little impact on the rhMIF effect.

downstream cell receptors or effectors<sup>[34-36]</sup>. Cinti *et al*<sup>[37]</sup> studied 50 cases of advanced gastric carcinoma using immunohistochemistry, showing a statistically significant direct correlation between p-Akt expression and depth of infiltration of the tumor, between the number of infiltrated lymph nodes and p34/cdc2 expression, and between prevalently nuclear p-Akt and cyclin D1 and cyclin E, but

an inverse correlation between nuclear pAkt and apoptotic index, and between cytoplasmic and nuclear pAkt and patient survival, suggesting that p-Akt may be considered an indicator of tumor progression and a prognostic factor for the survival of gastric cancer patients. Liang *et al*<sup>[38]</sup> reported that cellular prion protein up-regulates cyclin D1 at both mRNA and protein levels, thus promoting

cell proliferation and transition from G1 phase to S phase in gastric cancer GC7901 and AGS cells, at least partially by activating the PI3K/Akt pathway. Moreover, a PI3K specific inhibitor, LY294002, can significantly suppress the effects of cellular prion protein. In the present study, immunocytochemistry showed that rhMIF increased the p-Akt expression, activated p-Akt in a dose- and time-dependent manner, and induced the maximum Akt phosphorylation at 30 min. However, the total expression level of Akt remained unchanged, indicating that rhMIF affects specifically the activated Akt, namely the p-Akt. To further validate the involved Akt pathway in the effect of rhMIF on MGC-803 cells, the cells were pretreated with a PI3K specific inhibitor, LY294002, showing that LY294002 reversed or abolished all the effects induced by rhMIF, including the increased proliferation, cell cycle S arrest, up-regulation of cyclin D1 and p-Akt, down-regulation of p27<sup>Kip1</sup>, suggesting that rhMIF can induce the proliferation and cell cycle progression of MGC-803 cells *via* the PI3K/Akt pathway.

In conclusion, MIF increases the proliferation of gastric cancer cells, induces the expression of cyclin D1 at the transcriptional level and inhibits the expression of p27<sup>Kip1</sup> at the post-transcriptional level *via* the PI3K/Akt pathway. Further investigation is required to reveal whether macrophage MIF is a novel potential therapeutic target for the treatment of gastric cancer.

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## COMMENTS

### Background

Gastric cancer is one of the most common gastroenterological malignancies worldwide and remains a disease of poor prognosis. It is necessary to investigate the gastric tumorigenesis and potential new therapies. Macrophage migration inhibitory factor (MIF) is a multi-function cytokine involved in inflammation, auto-immune disease and development of several cancers. Recent studies have shown that over-expression of MIF is associated with tumorigenesis of gastric cancer, but the underlying mechanism is unclear.

### Research frontiers

MIF induces cell proliferation through many signal pathways, but the mechanism underlying the regulation of immunoreaction and tumorigenesis is complicated. The PI3K/Akt signaling pathway plays an important role in the regulation of cell growth, proliferation and differentiation. Recent studies have shown that the proliferation of cancer cells is suppressed by blocking the PI3K/Akt pathway. In this study, we investigated the effects of rhMIF on the proliferation of gastric cells and cell cycle progression, and whether the expression of cyclin D1 and p27<sup>Kip1</sup> and the regulation of PI3K/Akt pathway are involved in the effects of rhMIF.

### Innovations and breakthroughs

The study found that MIF induced the proliferation of MGC-803 cells and promoted the cell cycle progression from G1 phase to S phase in gastric cancer MGC-803 cells, and that rhMIF executed its effects by up-regulating cyclin D1 and down-regulating p27<sup>Kip1</sup> through the PI3K/Akt signal pathway.

### Applications

The study explored the mechanism by which MIF induces the proliferation of gastric cancer MGC-803 cells and promotes cell cycle, indicating that MIF may be the potential target for gastric cancer therapy.

### Terminology

MIF, a T-cell-derived cytokine, was identified and named by Bloom and David in

1966. PI3Ks is involved in the regulation of cell proliferation, differentiation and apoptosis. The signal pathway through PI3K and its down-stream protein PKB or Akt are closely related to the human tumorigenesis.

### Peer review

In this study, the authors investigated whether the PI3K/Akt pathway is involved in the effect of rhMIF on the proliferation of MGC-803 cells, using a PI3K/Akt inhibitor, LY294002. Overall, the study is interesting and significant, and the data are presented in a clear and logical manner.

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## Short-chain fatty acids act as antiinflammatory mediators by regulating prostaglandin E<sub>2</sub> and cytokines

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### Abstract

**AIM:** To investigate the effect of short-chain fatty acids (SCFAs) on production of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), cytokines and chemokines in human monocytes.

**METHODS:** Human neutrophils and monocytes were isolated from human whole blood by using 1-Step Polymorph and RosetteSep Human Monocyte Enrichment Cocktail, respectively. Human GPR41 and GPR43 mRNA expression was examined by quantitative real-time polymerase chain reaction. The calcium flux assay was used to examine the biological activities of SCFAs in human neutrophils and monocytes. The effect of

SCFAs on human monocytes and peripheral blood mononuclear cells (PBMC) was studied by measuring PGE<sub>2</sub>, cytokines and chemokines in the supernatant. The effect of SCFAs *in vivo* was examined by intraplantar injection into rat paws.

**RESULTS:** Human GPR43 is highly expressed in human neutrophils and monocytes. SCFAs induce robust calcium flux in human neutrophils, but not in human monocytes. In this study, we show that SCFAs can induce human monocyte release of PGE<sub>2</sub> and that this effect can be enhanced in the presence of lipopolysaccharide (LPS). In addition, we demonstrate that PGE<sub>2</sub> production induced by SCFA was inhibited by pertussis toxin, suggesting the involvement of a receptor-mediated mechanism. Furthermore, SCFAs can specifically inhibit constitutive monocyte chemotactic protein-1 (MCP-1) production and LPS-induced interleukin-10 (IL-10) production in human monocytes without affecting the secretion of other cytokines and chemokines examined. Similar activities were observed in human PBMC for the release of PGE<sub>2</sub>, MCP-1 and IL-10 after SCFA treatment. In addition, SCFAs inhibit LPS-induced production of tumor necrosis factor- $\alpha$  and interferon- $\gamma$  in human PBMC. Finally, we show that SCFAs and LPS can induce PGE<sub>2</sub> production *in vivo* by intraplantar injection into rat paws ( $P < 0.01$ ).

**CONCLUSION:** SCFAs can have distinct antiinflammatory activities due to their regulation of PGE<sub>2</sub>, cytokine and chemokine release from human immune cells.

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**Key words:** Short-chain fatty acids; GPR43; GPR41; Human monocytes; Prostaglandin E<sub>2</sub>; Chemokines; Cytokines

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## INTRODUCTION

The receptors for free fatty acids, including GPR40, GPR41, GPR42 and GPR43, constitute a subfamily of recently orphanized G-protein-coupled receptors (GPCRs) that are clustered on human chromosome 19q13.1<sup>[1]</sup>. They exhibit 30%-40% homology to one another and have diverse tissue distributions, yet all are activated by various free fatty acids, which function as intercellular lipid mediators through GPCRs<sup>[2,3]</sup>.

The free fatty acid receptors are functionally involved in both metabolism and the immune system. In contrast to GPR40 which is activated by medium- and long-chain fatty acids and abundantly expressed in the pancreatic islets<sup>[4-7]</sup>, GPR41 and GPR43 are functionally activated by short-chain fatty acids (SCFAs), including acetate, propionate and butyrate<sup>[8-10]</sup>. Although both receptors are activated by SCFAs, they have distinct tissue distribution profiles. GPR41 is preferentially expressed in adipose tissue, while GPR43 is highly expressed in hematopoietic tissues (such as spleen and bone marrow) and immune cells (particularly monocytes and neutrophils). Interestingly, the murine ortholog of GPR43 was initially identified as leukocyte-specific STAT-induced GPCR (LSSIG). It was reported that expression of both murine LSSIG and human GPR43 is induced during the differentiation of leukocyte progenitor cells to monocytes or neutrophils, suggesting their involvement in leukocyte function and host defense<sup>[11]</sup>.

SCFAs have long been known to modulate the immune response. Acetate, propionate and butyrate represent the most often described SCFAs that are capable of immune activation. SCFAs affect neutrophil function and migration<sup>[12-15]</sup>, and inhibit tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) or interleukin-1 $\beta$  (IL-1 $\beta$ )-induced vascular cell adhesion molecule-1 (and intercellular adhesion molecule-1) surface expression<sup>[16]</sup>. In addition, SCFAs reduce adherence of monocytes or lymphocytes to cytokine-stimulated human umbilical vein endothelial cells<sup>[17]</sup>, and inhibit interferon- $\gamma$  (IFN- $\gamma$ ) signaling<sup>[18,19]</sup>, a possible regulator of inflammation in inflammatory bowel disease (IBD). Although the molecular mechanisms of the effects of SCFAs remain to be elucidated, evidence in the literature indicates that SCFA-induced biological effects involve G-protein-mediated signaling<sup>[12,20]</sup>. GPR43, which is highly expressed in immune cells, could be the potential missing GPCR activated by SCFAs. GPR41 is also expressed in immune cells, but at lower levels compared to GPR43 and thus may play a lesser role<sup>[8-10]</sup>. As there are no well-validated specific drugs that can be used as tools to investigate the receptor, the emerging biology of these receptors is dependent on expression profiling. It is

believed that GPR43 is likely the main receptor contributing to the effect of SCFAs in immune cells. In human neutrophils, it was reported that SCFAs induced calcium flux and chemotaxis possibly through GPR43<sup>[9]</sup>. Nonetheless, the precise role of GPR43 in the effect of SCFAs in neutrophils needs to be established with the generation of a knockout model and specific small molecules. In addition, GPR43 is also highly expressed in monocytes and peripheral blood mononuclear cells (PBMC)<sup>[9]</sup>, and the effects of SCFAs in these important immune cells are of high interest, but are largely unknown.

SCFAs are produced by microbial anaerobic fermentation in the hindgut in millimolar (mmol/L) concentrations; therefore, the physiological site of GPR43 activation may be in the gut. Within the human colon, concentrations of acetate, propionate and butyrate were reported to be in the range of 20-43 mmol/L, 6-13 mmol/L and 6-15 mmol/L, respectively<sup>[21]</sup>. GPR43 has been reported to be expressed in human, mouse and rat colons<sup>[22-24]</sup>. Induction of anti-inflammatory activities by SCFAs in the colon may contribute to the bacterial evasion of the immune system<sup>[25]</sup>. GPR43-mediated activation may provide a link between the intestinal bacteria and the immune response in physiological and pathophysiological conditions.

We report the identification of new functional roles of SCFAs in production of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), cytokines and chemokines in human monocytes and in PBMC. We speculate that the effect of SCFAs is most likely through activation of GPR43, a potential therapeutic target for the development of an innovative treatment for colitis.

## MATERIALS AND METHODS

### *Isolation of human neutrophils, monocytes and PBMCs*

Human neutrophils were isolated from human whole blood by using 1-Step Polymorph (Accurate Chemical & Scientific Corp., Westbury, NY) according to the manufacturer's instructions. Human monocytes were isolated by RosetteSep Human Monocyte Enrichment Cocktail (StemCell Technologies, London, UK). Human PBMC were prepared by the Ficoll-Hypaque centrifugation method.

For the calcium flux assay, both human neutrophils and monocytes were treated with formate, acetate and propionate (Sigma, St. Louis, MO, USA) for 5-10 min. For measurement of PGE<sub>2</sub>, cytokines and chemokines, human monocytes and PBMC cells were incubated with formate, acetate, propionate and butyrate (Sigma, St. Louis, MO, USA) overnight.

### *Quantitative real-time polymerase chain reaction (PCR) analysis*

TaqMan primers and probes were designed with Primer Express software and purchased from ABI (Applied Biosystems, Foster City, CA, USA). The sequences of the human primers and probes for GPR41 and GPR43 are shown in Table 1. Quantitative real-time PCR was carried out with an ABI Prism 7900HT Sequence Detection System. The PCR reactions were prepared

**Table 1** Real-time polymerase chain reaction primers and probes designed for the detection and quantification of human GPR41 and GPR43

Gene	RefSeq ID	Forward primer	Reverse primer	Probe
GPR41	NM_005304	GCTTTGGGCCCTACAACGT	CCATGCCGGGCTTTC	TCCCATGTCGTGGGCTATACTGCG
GPR43	NM_005306	GGCTTCCCCGTGCAGTAC	ACCAGAGCTGCAATCACTCCAT	AGCTCTCCCCCGGCCTCTG

using the components from the iScript Custom One-Step RT-PCR Kit with ROX and assembled according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). The final concentrations of the primers and probe in the PCR reactions were 200 nmol/L and 100 nmol/L, respectively. The fluorogenic probes were labeled with 6-carboxyfluorescein as the reporter and 6-carboxy-4,7,2,7'-tetramethylrhodamine as a quencher. Each 10  $\mu$ L PCR reaction contained 10 ng of total RNA. The RT-PCR reactions were performed in triplicate in a 384-well plate according to the following protocol: one cycle for 10 min at 50°C, followed by one 5 min cycle at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. A eukaryotic 18S rRNA endogenous control probe/primer set (ABI) was used as an internal control for RNA quality.

The PCR data were quantified, based on a 12-point standard curve generated using 4-fold serial dilutions of a cDNA containing the gene of interest. The 4-fold dilutions began at 20000 fg. This procedure provides an absolute quantification of the amounts of GPR41 and GPR43 mRNA in a given sample.

### Calcium flux assay

Intracellular Ca<sup>2+</sup> assays were carried out as previously described with modifications<sup>[26]</sup>. Freshly isolated human neutrophils were resuspended at  $1 \times 10^6$  cells/mL in cell culture medium (RPMI 1640 with 2 mmol/L GlutaMAX and 5% fetal bovine serum) and incubated at 37°C, 50 mL/L CO<sub>2</sub>, for 1 h. Cells were spun and resuspended at  $1 \times 10^6$  cells/mL in a 1:1 mixture of cell culture medium and Calcium-4 no wash dye (Molecular Devices, Sunnyvale, CA, USA) containing a final concentration of 5 mmol/L probenecid (Sigma). The cells were seeded (50000/well) into poly-D-lysine-coated 384-well, black-wall, clear bottom microtiter plates (Becton Dickinson). Cells were sedimented in plates by spinning down briefly at low speed with no brake then incubated at 37°C, 50 mL/L CO<sub>2</sub>, for 1 h before assaying on a FLIPR-384 (Molecular Devices). Freshly isolated human monocytes were resuspended at  $1 \times 10^9$  cells/L in a 1:1 mixture of cell culture medium and Calcium-3 no wash dye containing a final concentration of 2.5 mmol/L probenecid. The cells were seeded (50000/well) into poly-D-lysine coated 384-well plates. Cells were dye loaded for 45 min at 37°C, 50 mL/L CO<sub>2</sub>, before assaying on a FLIPR-384.

Formate, acetate, propionate and butyrate (Sigma) were prepared in Hank's balanced salt solution, 25 mmol/L Hepes, and 0.1% bovine serum albumin. The maximum change in fluorescence over baseline was used to determine agonist response. The dose-response curves and EC<sub>50</sub> values were obtained by nonlinear regression (Prism 4; Graph Pad Software, San Diego, CA, USA).

### Measurement of PGE<sub>2</sub>, cytokines and chemokines

PGE<sub>2</sub> was measured by ELISA kit from Assay Designs (Ann Arbor, MI, USA). Cytokines and chemokines were measured by MesoScale ELISA (MesoScale Discovery, Gaithersburg, MD, USA) according to the manufacturer's instructions. To generate the conditioned media,  $1 \times 10^6$  cells per mL were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1 mmol/L L-glutamine, 10 mmol/L Hepes, 50000 U/L penicillin and 50  $\mu$ g/mL streptomycin; and seeded in 24-well plates for stimulation with SCFAs and/or incubation with selective inhibitors (Cayman Chemical, Ann Arbor, MI, USA), including MLnFP (methyl  $\alpha$ -linolenyl fluorophosphonate), a phospholipase inhibitor; 1400W, a selective iNOS inhibitor; SC-560, a selective cyclooxygenase-1 (COX-1) inhibitor; CAY10404, a selective COX-2 inhibitor; PTX, pertussis toxin. The tested concentrations of each inhibitor (except PTX) was chosen to be about 100 times of its IC<sub>50</sub> as reported in the data sheet provided by the manufacturer.

### Intraplantar injection of SCFAs into rat paws

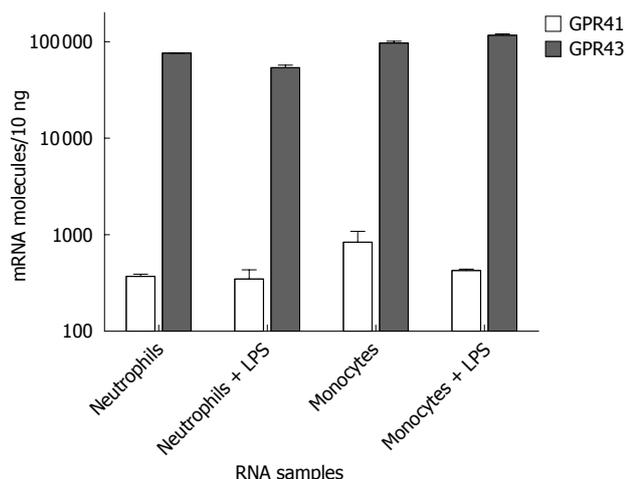
Sprague-Dawley male rats from Charles River Laboratories, 4 per group and approximately 200 g body weight, were anesthetized with isoflurane gas and the subplantar space of the paw injected with 0.1 mL of 100 mmol/L solutions of the SCFAs, either formate, acetate, propionate or butyrate prepared fresh in 0.9% saline. Lipopolysaccharide (LPS), *Escherichia coli* 0127:B8 (Sigma) was also injected at 3  $\mu$ g in saline either alone or in combination with 0.1 mL of 200 mmol/L sodium butyrate. Rats in the normal group were not injected. At 3 h post-injection, the rats were humanely euthanized and a uniform punch biopsy of the injected site was taken from each rat. The punch biopsies were immediately placed in PMSF (phenylmethanesulphonyl fluoride) buffer containing 10 g/L of indomethacin and frozen at -20°C. The tissues were homogenized in this collection buffer and assayed for PGE<sub>2</sub>.

All statistical analysis was performed by Mann-Whitney *U* test using GraphPad Instat version 3.06 for Windows XP (GraphPad Software, San Diego, CA, USA). All studies in animals were performed in accordance with the regulations specified by the National Institutes of Health Principles of Laboratory Animal Care (1985 revised version) and the Schering-Plough Research Institute Animal Care and Use Committee.

## RESULTS

### GPR43 is highly expressed in human neutrophils and monocytes

Both GPR43 and GPR41 are activated by SCFAs and

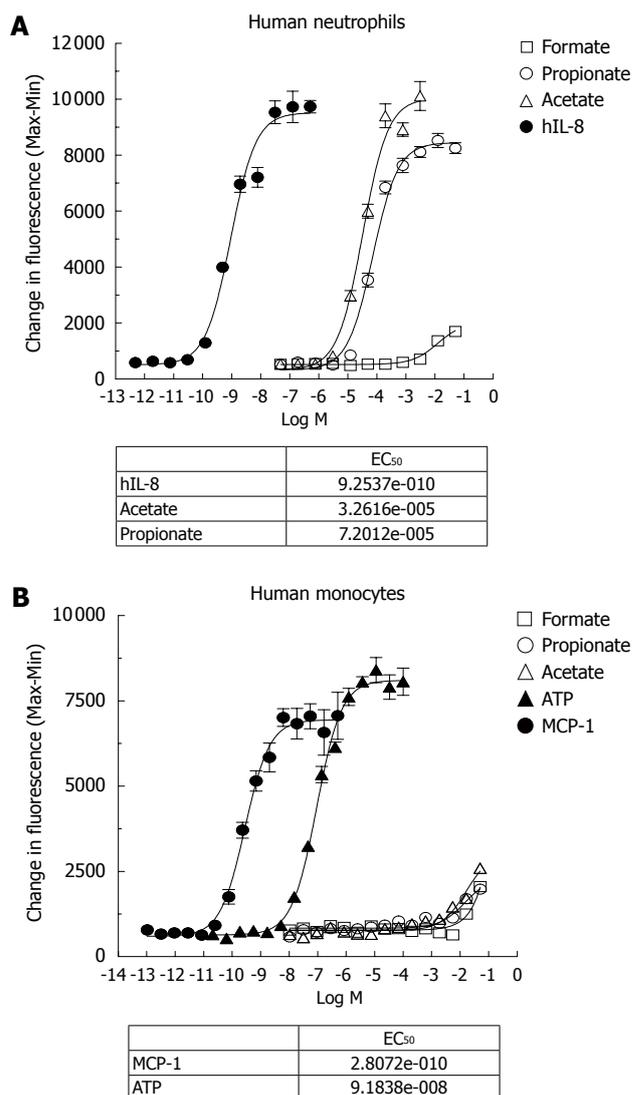


**Figure 1 GPR43 is highly expressed in human neutrophils and monocytes.** Human neutrophils and monocytes were isolated from human whole blood as described in Materials and Methods. Isolated human neutrophils or monocytes were stimulated with 100 ng/mL of lipopolysaccharide (LPS) for 3 h. RNAs were isolated and evaluated by Taqman analysis for absolute quantities of GPR43 and GPR41 mRNA molecules.

reported to be expressed in immune cells. To examine the role of GPR43 and GPR41 in human immune cells, we initially quantified their expression levels in human neutrophils and monocytes by Taqman analysis. Human neutrophils and monocytes were each isolated from human donors to 95% purity. Some of them were stimulated with LPS. RNAs were isolated and analyzed for GPR43 and GPR41 expression by Taqman. Figure 1 shows that GPR43 is expressed in both human neutrophils and monocytes at a much higher level than GPR41. It also appears that LPS stimulation did not affect their expression levels.

### SCFAs induce robust calcium flux in human neutrophils, but not in human monocytes

To investigate the biological activities of SCFAs, both purified human neutrophils and monocytes were exposed to various concentrations of SCFAs (formate, acetate and propionate) in a calcium flux assay. Formate was used as a negative control for the SCFAs. In addition, IL-8 was included as a positive control for neutrophil activation, while monocyte chemoattractant protein-1 (MCP-1) and ATP were used as the positive controls for monocyte activation. Since GPR41 couples to  $G_{i/o}$  only, SCFAs should not cause a calcium flux through this receptor, which was confirmed in a recombinant cell line expressing GPR41 (data not shown). Indeed, the agonist potency profile of the calcium response in human neutrophils (Figure 2A) was consistent with the GPR43 receptor response that has been described<sup>[9]</sup>. From 8 human donors, acetate had an average  $EC_{50}$  of  $58.25 \pm 12.44 \mu\text{mol/L}$  ( $n = 8$ ), and propionate had an  $EC_{50}$  of  $200.6 \pm 42.42 \mu\text{mol/L}$  ( $n = 8$ ). Formate was inactive and IL-8 had an  $EC_{50}$  about 1-2 nmol/L in human neutrophils. Acetate and propionate achieved the same maximal activation response as IL-8 in human neutrophils using this calcium flux assay.

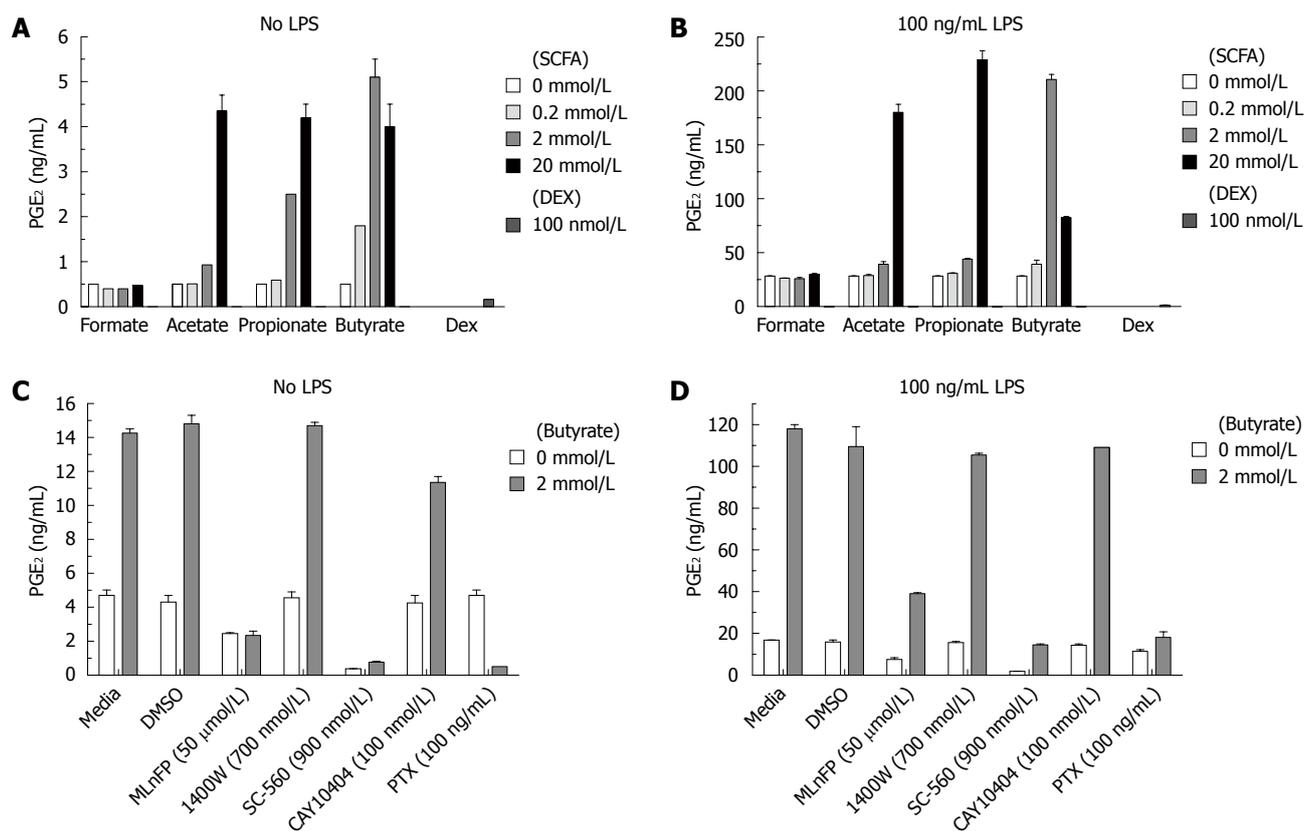


**Figure 2 Short-chain fatty acids (SCFAs) induce robust calcium flux in human neutrophils, but not in human monocytes.** Human neutrophils (A) and monocytes (B) were isolated and exposed to the indicated concentrations of formate, acetate and propionate for a calcium flux assay as described in Materials and Methods. Interleukin-8 (IL-8) is included as a positive control for human neutrophils, and monocyte chemoattractant protein-1 (MCP-1) and ATP for human monocytes. The dose-response curves and  $EC_{50}$  values were obtained by nonlinear regression using Graph Pad Prism 4 software.

On the other hand, Figure 2B shows that acetate and propionate, up to 100 mmol/L, did not induce significant calcium flux in human monocytes, while MCP-1 and ATP were able to induce robust calcium flux in the human monocyte preparation. It appears that the response of GPR43 to SCFAs for calcium flux is dependent either on cell type or on the state of differentiation of the cells. Indeed, it has been reported that propionic acid induced calcium mobilization in human neutrophils, but not in monocytes, platelets and lymphocytes<sup>[12]</sup>.

### SCFAs strongly induce PGE<sub>2</sub> production in human monocytes and the effect is receptor-mediated

To further explore the biological effects of SCFAs, human monocytes were isolated and incubated overnight with various concentrations of SCFAs in the presence



**Figure 3 SCFAs strongly induce PGE<sub>2</sub> production in human monocytes and the effect is receptor-mediated.** Freshly isolated human monocytes ( $1 \times 10^6$  cells/L) were incubated with the indicated concentrations of SCFAs or 100 nmol/L dexamethasone (Dex), and then cultured for 22 h without (A) or with (B) 100 ng/mL LPS. In a separate experiment (C, D), human monocytes were incubated with the indicated concentrations (in the parentheses) of inhibitors (described below) for 30 min, then with or without 2 mmol/L butyrate for another 30 min, and finally cultured for 22 h without (C) or with (D) 100 ng/mL LPS. The media and DMSO samples were used as controls with no inhibitors. All culture supernatants were assayed for PGE<sub>2</sub> by ELISA. MLnFP (methyl  $\alpha$ -linolenyl fluorophosphonate): Phospholipase inhibitor; 1400W: Selective iNOS inhibitor; SC-560: Selective cyclooxygenase-1 (COX-1) inhibitor; CAY10404: Selective COX-2 inhibitor; PTX: Pertussis toxin. (Cayman Chemical, Ann Arbor, MI). The tested concentrations of each inhibitor (except PTX) were chosen to be about 100 times its IC<sub>50</sub> as reported in the data sheet provided by the manufacturer.

or absence of LPS (100 ng/mL) as a proinflammatory stimulus. The supernatants were assayed for lipid mediators such as PGE<sub>2</sub>, proinflammatory cytokines and chemokines by either ELISA or Meso Scale Multi-Spot Discovery Technology. Dexamethasone was included in the experiment as a positive control for the induction of inhibition. Figure 3A shows that SCFAs alone strongly enhanced PGE<sub>2</sub> production in human monocytes across a panel of 10 human donors in a concentration-dependent manner. Furthermore, SCFAs synergistically enhanced the PGE<sub>2</sub> production induced by LPS in human monocytes (10 human donors), as shown in Figure 3B. The rank order of potency was butyrate > propionate > acetate, while formate was inactive. Dexamethasone at 100 nmol/L potently inhibited basal PGE<sub>2</sub> and LPS-induced PGE<sub>2</sub> production (Figure 3A and B). Similar results were obtained from 10 human donors. The culture supernatants were also assayed for PGI<sub>2</sub>, leukotriene B<sub>4</sub> (LTB<sub>4</sub>) and thromboxane B<sub>2</sub> (TXB<sub>2</sub>). SCFAs had no significant effect on these lipid mediators (data not shown), suggesting its effect on PGE<sub>2</sub> production is specific.

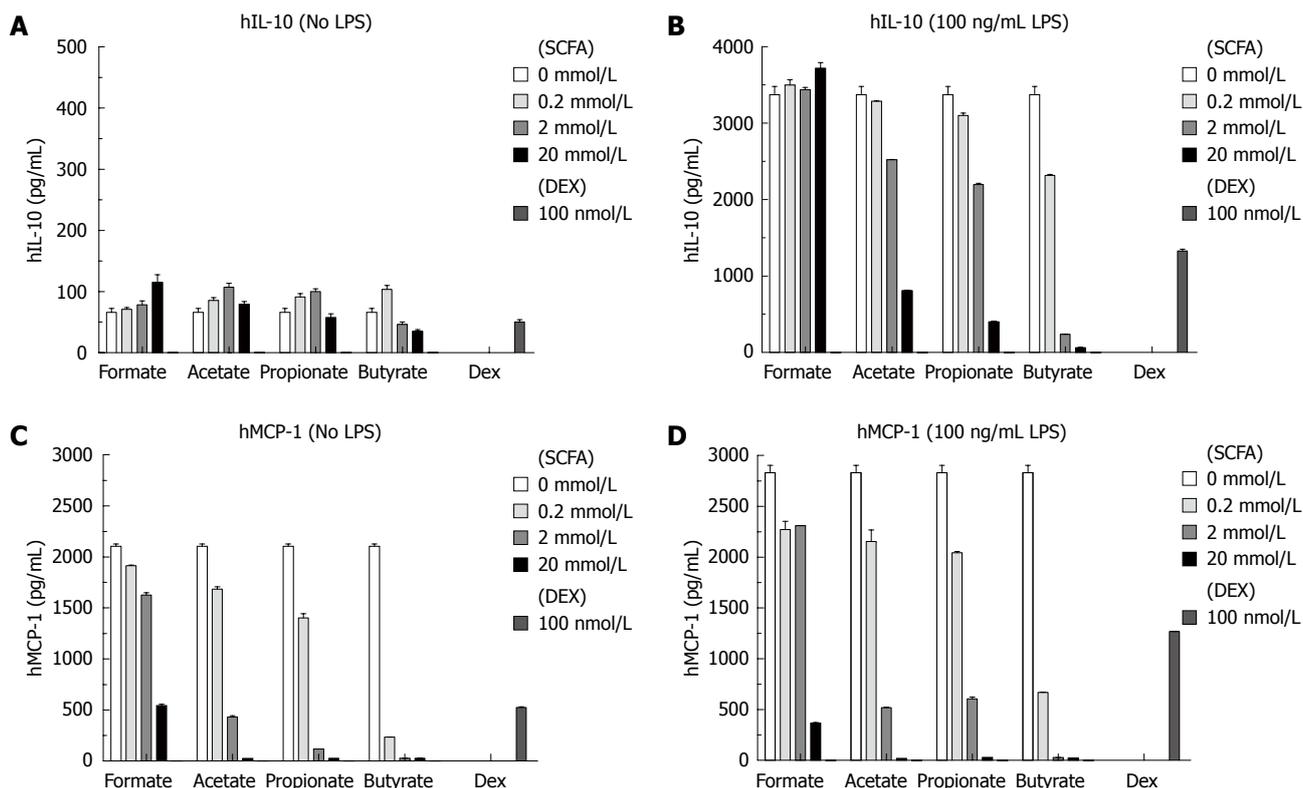
To examine possible mechanisms of PGE<sub>2</sub> production induced by SCFAs, human monocytes were preincubated with several inhibitors and then added to culture with butyrate and/or LPS. Figure 3C and D show that the

PGE<sub>2</sub> production induced by butyrate is inhibited by pretreatment with PTX, COX-1 and phospholipase inhibitors either in the absence or presence of LPS. In contrast, COX-2 and iNOS inhibitors had no effect on PGE<sub>2</sub> production induced by butyrate. The sensitivity to PTX suggests PGE<sub>2</sub> production induced by butyrate is receptor-mediated.

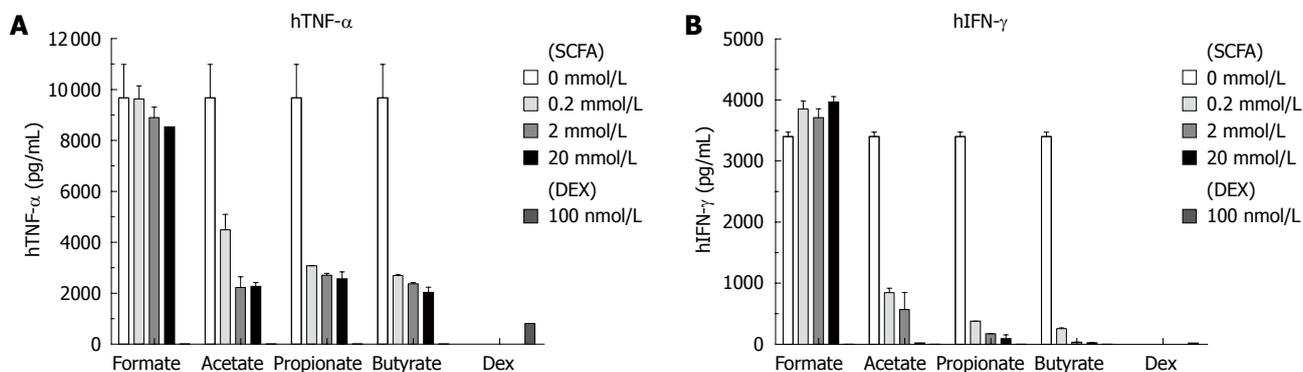
### SCFAs specifically inhibit LPS-induced IL-10 production and constitutive MCP-1 expression in human monocytes

The same culture supernatants described above were also analyzed for 8 proinflammatory cytokines including IL-1 $\beta$ , IL-2, IL-6, IL-8, IL-10, IL-12p70, IFN- $\gamma$ , TNF- $\alpha$  (Meso Scale Multi-Spot Discovery Technology). In the absence of LPS, SCFAs had no effect on the level of any of the 8 cytokines including IL-10, as shown in Figure 4A. In contrast, SCFAs specifically inhibited IL-10 production stimulated with LPS (Figure 4B), but had no effect on the other 7 cytokines with LPS stimulation (including LPS-induced production of IL-1 $\beta$  and TNF- $\alpha$ ). The effect of dexamethasone on the cytokine profile is consistent with what has been previously described; briefly, it inhibited LPS-induced production of IL-1 $\beta$ , IL-10 and TNF- $\alpha$ .

It is consistently observed that isolated human monocytes produce high levels of MCP-1 in the culture



**Figure 4** SCFAs specifically inhibit LPS-induced IL-10 production and constitutive MCP-1 expression in human monocytes. The culture supernatants as in Figure 3A and B were analyzed for 8 proinflammatory cytokines (including IL-1 $\beta$ , IL-2, IL-6, IL-8, IL-10, IL-12p70, IFN- $\gamma$ , TNF- $\alpha$ ) or 9 proinflammatory chemokines (including MCP-1, MCP-4, eotaxin, eotaxin-3, IL-8, IFN- $\gamma$  inducible protein-10, macrophage inflammatory protein-1 $\beta$ , macrophage-derived chemokine, thymus and activation-regulated chemokine) by Meso Scale Multi-Spot Discovery Technology. Only the data for IL-10 (A, B) and MCP-1 (C, D) are shown here. Similar results were obtained from 10 human donors.

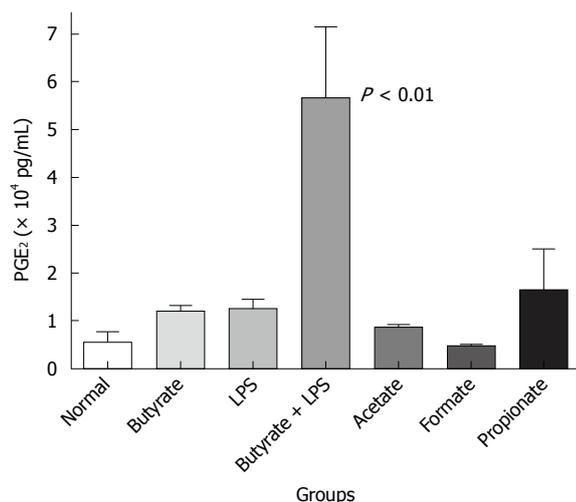


**Figure 5** SCFAs inhibit LPS-induced production of TNF- $\alpha$  and IFN- $\gamma$  in human peripheral blood mononuclear cells (PBMC) in a dose-dependent manner. Human PBMC isolated by Ficoll-Hypaque centrifugation procedure ( $1 \times 10^6$  cells/mL) were incubated with the indicated concentrations of short-chain fatty acids (SCFA) or 100 nmol/L dexamethasone (Dex), and then cultured for 22 h with or without 100 ng/mL LPS. Culture supernatants were analyzed for 8 proinflammatory cytokines including IL-1 $\beta$ , IL-2, IL-6, IL-8, IL-10, IL-12p70, IFN- $\gamma$ , TNF- $\alpha$  by Meso Scale Multi-Spot Discovery Technology. The data for TNF- $\alpha$  (A) and IFN- $\gamma$  (B) with LPS stimulation are shown here.

supernatants without stimulation. Figure 4C and D show that SCFAs inhibited this constitutive MCP-1 production in a concentration-dependent manner either in the absence or presence of LPS. SCFAs did not inhibit the constitutive high levels of eotaxin-3, macrophage-derived chemokine and macrophage inflammatory protein-3 $\beta$  in the cultures, suggesting that the effect of SCFAs on MCP-1 production is specific. In this experiment, 20 mmol/L formate showed some inhibition, which is likely the result of a nonspecific effect.

**SCFAs inhibit LPS-induced production of TNF- $\alpha$  and IFN- $\gamma$  in human PBMCs in a concentration-dependent manner**

To further confirm the effect of SCFAs, human PBMC were isolated by Ficoll-Hypaque centrifugation procedure and incubated with SCFAs and/or LPS. The culture supernatants were analyzed as before for PGE<sub>2</sub>, cytokines and chemokines. The effects of SCFAs on PGE<sub>2</sub>, MCP-1 and IL-10 production were similar to that observed in the isolated human monocytes. However, as shown in



**Figure 6 SCFAs induce PGE<sub>2</sub> production by intraplantar injection into rat paws.** The paw of male Sprague-Dawley rats was injected intraplantarly with 0.1 mL of 100 mmol/L formate, acetate, propionate or butyrate. Three micrograms of LPS in saline was also injected alone or in combination with 0.1 mL of 200 mmol/L butyrate. There were 4 rats per group. The normal group was not injected. Three hours post injection, rat paws were punched and fluids were collected in phenylmethanesulphonyl fluoride buffer with indomethacin. All punch fluids were assayed for PGE<sub>2</sub> by ELISA.  $P < 0.01$  for butyrate + LPS group vs normal group, Mann-Whitney  $U$  test.

Figure 5, SCFAs inhibited LPS-induced production of TNF- $\alpha$  and IFN- $\gamma$  in human PBMC in a concentration-dependent manner, an effect not observed in isolated human monocytes where IFN- $\gamma$  production was low and not stimulated by LPS addition. In the human PBMC experiment, dexamethasone inhibited LPS-induced production of IL-1 $\beta$ , IL-10, IFN- $\gamma$  and TNF- $\alpha$ .

#### SCFAs induce PGE<sub>2</sub> production by intraplantar injection into rat paws

To demonstrate the effect of SCFAs *in vivo*, SCFAs and LPS alone or in combination were injected by the intraplantar route into a rat paw. This was done to initiate an inflammatory response and determine what effect the butyrate would have on lipid mediator and cytokine induction. In the representative experiment shown in Figure 6, acetate, propionate and butyrate alone could induce PGE<sub>2</sub> production in rat paw. Formate was inactive and the level of PGE<sub>2</sub> was equivalent to that extracted from a normal (not injected) paw. In addition, suboptimal LPS also induced PGE<sub>2</sub> production and the combination of LPS and butyrate strongly enhanced PGE<sub>2</sub> production in a synergistic manner ( $P < 0.01$ ). These *in vivo* data, although preliminary in nature, support the effects of SCFAs on PGE<sub>2</sub> production observed in human monocytes and PBMC. Cytokine analysis was performed on the tissue homogenates but no clear pattern emerged that was similar to that obtained in the human monocytes and PBMC *in vitro* experiments. Sodium butyrate is extremely labile with a very high diffusion rate. Maintenance of a sufficient concentration in the paw subcutaneous space was a significant problem in these experiments.

## DISCUSSION

In this study, we have shown that SCFAs can induce either robust PGE<sub>2</sub> production alone or in synergy with LPS in human monocytes and PBMC. Since LPS stimulation did not affect the mRNA level of GPR43 and GPR41, the synergy is likely from 2 independent signaling pathways. This effect on PGE<sub>2</sub> production is specific, since other lipid mediators including PGI<sub>2</sub>, LTB<sub>4</sub> and TXB<sub>2</sub>, were not affected by SCFAs. Furthermore, this effect is receptor-mediated since it is sensitive to PTX.

Interestingly, we showed that SCFAs induced robust calcium flux in human neutrophils, but not in human monocytes. Our finding is consistent with the previous report that propionic acid induced calcium mobilization in human neutrophils, but not in monocytes, platelets and lymphocytes<sup>[12]</sup>. It is likely that SCFAs activate neutrophils and monocytes for the immune response through different signaling pathways that would be consistent with the different temporal roles of these cells in an inflammatory response. SCFAs induce calcium flux and chemotaxis in neutrophils which arrive early in an inflammatory response, while they regulate production of PGE<sub>2</sub>, cytokines and chemokines in monocytes which are also present in the inflammatory site but mature into macrophages during extravasation and remain at the site of inflammation until resolution.

This *in vitro* effect of SCFAs on human monocytes may have relevance to human diseases that target the intestinal tract. Among various prostanoids, PGE<sub>2</sub>, in particular, seems to play a critical role in inflammatory bowel disease (IBD) *via* the EP4 receptor, one of the four PGE<sub>2</sub> receptor subtypes (EP1-4)<sup>[27-31]</sup>. Among 8 prostanoid receptor-deficient mice, only EP4-deficient mice developed severe colitis with 3% dextran sodium sulphate (DSS) treatment. It is suggested that EP4 maintains intestinal homeostasis by maintaining mucosal integrity and downregulating the immune response<sup>[32]</sup>. Therefore, it is possible that SCFAs induce production of PGE<sub>2</sub> and play a protective role *via* the GPR43-expressing cells of the colon. Furthermore, we demonstrate that PGE<sub>2</sub> production induced by SCFAs was inhibited by a COX-1 inhibitor, but not a COX-2 inhibitor. This is consistent with the notion that in DSS-induced colitis, the significant reduction in PGE<sub>2</sub> resulted from decreased expression of COX-1, but not COX-2<sup>[33]</sup>.

In human monocytes, SCFAs specifically inhibited constitutive MCP-1 production and LPS-induced IL-10 production out of the total 16 cytokines and chemokines examined. The activities of SCFAs in our human monocyte cultures were all confirmed in human PBMC, which contain both monocytes and lymphocytes. We made the additional observation in these PBMC cultures that SCFAs would inhibit LPS-induced TNF- $\alpha$  and IFN- $\gamma$  production.

This unique effect of SCFAs on the production of cytokines and chemokines in monocytes and PBMC is relevant to the design of new therapies for colitis. Intestinal inflammation present in inflammatory bowel disease

is driven by the production of cytokines, chemokines and growth factors that draw in immune cells to the mucosa. It has been shown that MCP-1, IL-10, IFN- $\gamma$  and TNF- $\alpha$  were significantly upregulated in experimental colitis models<sup>[34,35]</sup>. Furthermore, the increased production of SCFAs from dietary fiber supplementation or probiotics administration inhibited the production of proinflammatory mediators and recovered damaged colonic mucosa in colitic animals<sup>[36,37]</sup>. These experiments show that SCFAs can inhibit multiple inflammatory mediators which may control intestinal inflammation. Clinical trial evidence for this use of SCFAs is still not widely available or accepted as a mainstream therapy.

We tried to design a model system *in vivo* to duplicate our *in vitro* findings. We present the results of a model system using the intraplantar space in the rat paw, a space from which there was limited diffusion of the injected SCFAs. We had previously tried injecting SCFAs into the pleural cavity and into the peritoneal cavity to induce an inflammatory response with and without suboptimal inflammatory stimuli such as LPS. However, SCFAs are extremely labile with a very high diffusion rate and require high concentrations to achieve their effect *in vivo*. Maintenance of a sufficient concentration of SCFAs at a local site or tissue space (such as the colon) has been a major challenge to obtain clear cytokine profiles and *in vivo* efficacy. Therefore, better pharmacological tools, such as GPR43 specific small molecules<sup>[38]</sup>, are needed to confirm our *in vitro* findings and further elucidate their biology. We were able to utilize the intraplantar space of the paw to illustrate the ability of SCFAs to synergize with LPS and induce mediator production but there was significant variability in this response.

The present findings of antiinflammatory activities of SCFAs in immune cells expand the database on these fatty acids as lipid mediators and may help explain the known beneficial effects of SCFAs in the colon and on colitis. Future studies are needed in knockout mice and with specific small molecules (both agonists and antagonists) to prove the exact molecular target for these effects of SCFAs.

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## COMMENTS

### Background

Short-chain fatty acids (SCFAs) are produced by bacterial fermentation of dietary fiber in the hindgut in millimolar concentrations. They have long been known to modulate the immune response and have a beneficial effect in the colon and on colitis.

### Research frontiers

The molecular mechanism for the effect of SCFAs in immune cells of the gastrointestinal tract has not been characterized. Recently, a free fatty acid receptor GPR43 was found to be highly expressed in both neutrophils and

monocytes and identified to be activated by SCFAs. In neutrophils, SCFAs induce calcium flux and chemotaxis. However, the effect of SCFAs in monocytes is of high interest, but is largely unknown.

### Innovations and breakthroughs

This study showed that SCFAs can induce specific and robust prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production either alone or in synergy with lipopolysaccharide in human monocytes and peripheral blood mononuclear cells (PBMC). Furthermore, SCFAs can specifically regulate production of monocyte chemotactic protein-1, interleukin-10, tumor necrosis factor- $\alpha$  and interferon- $\gamma$  in human monocytes and/or PBMC. Finally, we showed that SCFAs can induce PGE<sub>2</sub> production *in vivo* by intraplantar injection into rat paws.

### Applications

The present findings of antiinflammatory activities of SCFAs in immune cells expand the database on these fatty acids and may help explain the known beneficial effects of SCFAs in the colon and on colitis. Future studies are needed in knockout mice and with specific small molecules to prove the exact molecular target for these effects of SCFAs. The authors speculate that the action of SCFAs is most likely through activation of the free fatty acid receptor GPR43, a potential therapeutic target for the development of an innovative treatment of colitis.

### Peer review

This is a study in an important area of inflammatory activities of SCFAs and its effects on the related cytokines and chemokines. In the present paper, the authors examined the inflammatory activities of SCFAs and the production of the related receptors and analyzed the mechanisms. The experiments were well organized.

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BRIEF ARTICLE

## Analysis of *monocyte chemotactic protein-1* gene polymorphism in patients with spontaneous bacterial peritonitis

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### Abstract

**AIM:** To investigate a genetic polymorphism of the *monocyte chemotactic protein-1* (MCP-1) gene in patients with spontaneous bacterial peritonitis (SBP).

**METHODS:** MCP-1 genotyping was performed in 23 patients with SBP and 83 cirrhotic control patients with non-infected ascites.

**RESULTS:** The frequency of carriers of the G-allele was lower in SBP patients but this difference did not reach statistical significance. However, in the subgroup of patients with alcoholic cirrhosis ( $n = 80$ ), carriers of the G-allele were significantly less frequent in SBP-patients (38.1%) than in cirrhotic controls (67.8%,  $P = 0.021$ ).

**CONCLUSION:** In patients with alcoholic liver cirrhosis, the -2518 MCP-1 genotype AA is a risk factor for the development of SBP.

**Key words:** Monocyte chemotactic protein-1; Chemokines; Spontaneous bacterial peritonitis; Polymorphism; Liver cirrhosis

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### INTRODUCTION

Spontaneous bacterial peritonitis (SBP) is a common and potentially life-threatening complication in patients with cirrhosis. It is a prototypical infective disease in cirrhotic patients characterized by peritoneal neutrophil infiltration, which also serves as a diagnostic criterion for SBP (e.g. an ascites neutrophil count greater than  $250/\text{mm}^3$ )<sup>[1]</sup>.

Factors influencing the development of SBP in patients with liver cirrhosis are poorly understood. Previous studies have indicated that peritoneal macrophages of cirrhotic patients might contribute to the control of SBP or influence its associated pathology in human cirrhosis by producing high quantities of angiogenic peptides and nitric oxide<sup>[2-5]</sup>. Accordingly, elevated concentrations of proinflammatory cytokines are found in ascites of patients<sup>[6,7]</sup>.

Therefore, it is of particular interest to identify mechanisms underlying the recruitment and activation of macrophages/monocytes that infiltrate the ascites of cirrhotic patients. Chemotactic cytokines are known to be critical mediators of inflammatory cell trafficking into sites of injury and are crucial for the modulation of tissue injury, inflammation, and repair.

Monocyte chemotactic protein-1 (MCP-1) is one of the most potent chemokines for monocytes/macrophages and activated lymphocytes during infections<sup>[8]</sup>. In addition, several studies have shown that neutrophil infiltration is affected either directly or indirectly *via* MCP-1<sup>[9,10]</sup>.

Several lines of evidence indicate that MCP-1 might play a role in the recruitment and maintenance of the inflammatory infiltrate during liver injury. MCP-1 secretion is up-regulated during chronic hepatitis and correlates with the severity of hepatic inflammation<sup>[11-13]</sup>.

Furthermore, a previous study showed elevated MCP-1 levels in ascites of cirrhotic patients as compared to controls<sup>[14]</sup>. Moreover, during SBP, even higher MCP-1 levels were measured in ascites suggesting that this potent chemokine plays a pathophysiological role during the development and course of SBP.

Recently, a mutation in the distal regulatory region of the MCP-1 gene at position -2518 relatively to the transcription start site (based on the published sequence, Gene Bank accession number D26087) was identified (A-2518G)<sup>[15]</sup>. *In vitro* stimulated monocytes from individuals carrying a G-allele at -2518 produced more MCP-1 than cells from A/A homozygous subjects<sup>[15]</sup>. Several further studies have confirmed the functional effect of this single nucleotide polymorphism (SNP)<sup>[13,16-18]</sup>.

The prevalence of high MCP-1 producing genotypes has been shown to be associated with increased susceptibility and severity of several diseases, including bronchial asthma<sup>[19]</sup>, premature kidney graft failure<sup>[20]</sup>, Crohn's disease<sup>[21]</sup>, cutaneous vasculitis<sup>[22]</sup>, and tuberculosis<sup>[16]</sup>. Furthermore, this genetic polymorphism has been shown to be a risk factor for the progression of chronic viral hepatitis C infection<sup>[13]</sup>.

The aim of this study was to analyze the association of this functional promoter SNP of the MCP-1 gene with SBP.

## MATERIALS AND METHODS

### Patients and controls

We studied 106 patients with liver cirrhosis and ascites. Clinical charts of patients diagnosed with chronic liver disease at the medical department of the University of Regensburg during the period 2001-2007 were studied retrospectively. Based on the availability of DNA samples in an established tissue bank, patients were included in the study. Liver carcinoma, other neoplasms or HIV-infection were exclusion criteria. Clinical charts revealed diagnosis of SBP in 23 cases, while no SBP had ever occurred in 83 patients. In the majority of patients, chronic alcohol abuse was the cause of the underlying liver disease (Table 1). In the other patients, chronic hepatitis B or C infection and hemochromatosis were the cause of cirrhosis. Patients were considered to have alcohol related cirrhosis if alcohol intake had been in excess of 100 g/d for more than 10 years and if tests for viral, metabolic, and immune etiologies were negative. Patient characteristics are summarized in Table 1.

As an additional control group, 118 heavy drinkers without evidence of liver damage (90 male, 28 female; mean age: 42.5 ± 9.1 years) were analyzed. These unrelated individuals of German descent were recruited from an in-patient abstention program at the Department of Psychiatry of the University of Regensburg. Each

**Table 1** Characteristics of cirrhotic patients with SBP and cirrhotic control patients with ascites (mean ± SD)

	Control cirrhotic patients (n = 83)	Cirrhotic patients with SBP (n = 23)
Age (yr)	55.8 ± 10.5	58.7 ± 8.6
Male gender (%)	75.9	78.3
Albumin (g/dL)	3.0 ± 0.6	3.0 ± 0.6
Prothrombin activity (%)	63.8 ± 22.1	54.9 ± 20.8
Bilirubin (mg/dL)	4.9 ± 6.3	4.9 ± 5.9
Chronic alcohol abuse as etiology of cirrhosis (%)	71.1	91.3

SBP: Spontaneous bacterial peritonitis.

subject met the criteria of alcohol dependence according to ICD-10 alcohol dependence according to DSM-IV (American Psychiatric Association, 1994) and ICD-10 (World Health Organization, 1992).

At the day of admission, several serum parameters were analyzed, including transaminases (alanine aminotransferase, aspartate aminotransferase,  $\gamma$  glutamyltransferase), and anti hepatitis B virus (HCV)- and hepatitis B virus (HBV)-antibodies. Previous and current drinking habits, family history of non-alcoholic liver diseases, and potential previous complications of advanced liver damages, such as ascites or esophageal varices, were examined. Furthermore, within 14 d after admission a clinical and ultrasound examination of the abdomen was performed. Only alcohol-dependent subjects with an alcohol consumption of at least 100 g/d for more than 5 years were included. Furthermore, clinical or sonomorphological indications for advanced liver damage and serological evidence of HBV or HCV infection were exclusion criteria.

Patients and controls were Caucasians and their geographical origin was Southern Germany. Informed consent was obtained from all patients and the study was approved by the local ethics committee.

### DNA isolation and MCP-1 genotyping

DNA isolation and MCP-1 genotyping were performed as described previously<sup>[13,23]</sup>. Briefly, genomic DNA specimens were prepared from 200  $\mu$ L blood using the QIAamp blood kit, following the manufacturer's instructions (Qiagen, Hilden, Germany). The G to A polymorphisms at position -2518 of the MCP-1 gene was analyzed by performing PCR and restriction fragment length polymorphism analysis. PCR was performed under standard conditions (35 cycles, annealing temperature: 55°C) in a total reaction volume of 50  $\mu$ L containing 2  $\mu$ L of diluted genomic DNA, using the following pair of primers: forward: 5'-CCGAGATGTTCCCAGCACAG-3' and reverse: 5'-CTGCTTTGCTTGTGCCTCTT-3'. PCR products were digested by *Pvu*II, and the resulting fragments were separated by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining. With the -2518 A polymorphic base, the recognition sequence 5'-CAG/CTG-3' is modified to 5'-CAG/CTA-3', which is not cut by *Pvu*II.

Table 2 Frequencies of -2518 MCP-1 genotypes *n* (%)

-2518 MCP-1 genotype	Cirrhotic patients with ascites ( <i>n</i> = 106)	Patients with alcoholic cirrhosis and ascites ( <i>n</i> = 80)	Heavy drinkers w/o cirrhosis ( <i>n</i> = 118)
A/A	45 (42.5)	32 (40.0)	64 (54.2)
A/G	51 (48.1)	39 (48.8)	47 (39.8)
G/G	10 (9.4)	9 (11.3)	7 (5.9)

### Statistical analysis

Results are expressed as mean  $\pm$  SD (range) or, when indicated, as absolute number and percent. Genotype frequencies are reported with their group percentages. Contingency table analysis and two-sided Fisher's exact tests were used for comparison of qualitative variables.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Characteristics of the study population

The clinical and epidemiological characteristics of SBP patients and cirrhotic control patients are summarized in Table 1. There was no statistical difference between patients with SBP and non-infected cirrhotic patients with ascites with respect to age, distribution of sexes, and serum laboratory findings, such as albumin, bilirubin, or prothrombin activity. However, the percentage of patients with chronic excessive alcohol abuse as the cause of liver cirrhosis was significantly higher in the SBP group (91.3% vs 71.1% in the cirrhosis group,  $P = 0.046$ ), which agreed with previous studies that showed greater susceptibility to infections, especially SBP, in alcohol-induced liver cirrhosis<sup>[24]</sup>.

### Frequency of the -2518 MCP-1 polymorphism in patients and controls

The frequencies of the different MCP-1 genotypes in our cohort of patients with liver cirrhosis and ascites are summarized in Table 2. In addition, genotype distribution in the subgroup of patients with chronic excessive alcohol consumption as an underlying cause of liver disease and cirrhosis is depicted. Furthermore, MCP-1 genotypes were analyzed in heavy drinkers without evidence of liver damage.

The frequency of individual genotypes in the group of heavy drinkers without cirrhosis was similar to those previously reported in other Caucasian control populations<sup>[19,20,22,25]</sup>. Genotype distribution did not differ significantly between the whole group of cirrhotic patients with ascites and the subgroup of patients with alcohol related cirrhosis. However, irrespective of the underlying cause of liver disease, the genotype AA was less frequent in cirrhotic patients with and without ascites (40.0% and 42.5%, respectively) as compared to heavy drinkers without evidence of liver damage (54.2%) although these differences did not reach statistical significance ( $P = 0.084$  and  $P = 0.060$ , respectively).

Most previous *in vitro* and epidemiological studies reported functional and disease-related differences between

Table 3 Frequencies of carriers of the G-allele of the -2518 MCP-1 promoter polymorphism in patients with SBP and cirrhotic control patients with ascites *n* (%)

-2518 MCP-1 genotype	Whole cohort ( <i>n</i> = 106)		Alcoholic cirrhosis ( <i>n</i> = 80)	
	No SBP ( <i>n</i> = 83)	SBP ( <i>n</i> = 23)	No SBP ( <i>n</i> = 59)	SBP ( <i>n</i> = 21)
A/G or G/G	52 (62.7)	9 (39.1)	40 (67.8)	8 (38.1)
A/A	31 (37.3)	14 (60.9)	19 (32.2)	13 (61.9)

-2518 MCP-1 genotypes AA and non-AA (i.e. carriers of the G-allele: genotypes AG and GG); therefore, we continued this differentiation throughout the following analysis and focused on the comparison of patients with no G-allele (AA-homozygotes) and carriers of the G-allele (genotypes AG and GG).

### Frequency of the -2518 MCP-1 polymorphism in patients with and without SBP

In our cohort of 106 patients with ascites, SBP has been diagnosed in 23 individuals (21.7%), while in 83 cirrhotic patients (78.3%), despite mostly long lasting episodes of ascites, no SBP had occurred. The frequency of carriers of the G-allele was lower in the SBP group but this difference did not reach statistical significance [9/23 (39.1%) vs 52/83 (62.7%),  $P = 0.057$ , Table 3]. Also, in the subgroup of patients with alcoholic cirrhosis (*n* = 80), carriers of the G-allele were less frequent in SBP-patients compared to patients without SBP, and in this case, the difference reached statistical significance [8/21 (38.1%) vs 40/59 (67.8%),  $P = 0.021$ , Table 3].

## DISCUSSION

The aim of this study was to analyze the association of the functional MCP-1 promoter polymorphism (A-2518G) with SBP. Interestingly, in patients with alcoholic liver cirrhosis, the genotype AA was significantly more frequent in SBP-patients than in cirrhotic control patients with ascites only. Previously, this MCP-1 genotype has been shown to be associated with reduced MCP-1 release of monocytes *in vitro* and MCP-1 tissue levels *in vivo*, respectively<sup>[13,16]</sup>.

Furthermore, in a previous study<sup>[14]</sup>, MCP-1 levels in ascites were significantly higher when compared with their levels in serum, suggesting a chemotactic gradient towards the peritoneal cavity, even in the absence of infection. This chemotactic gradient could be operative in the chemotaxis of monocytes/macrophages. Antigenic stimuli drive these cells to synthesize further proinflammatory cytokines and chemokines, and thus might also modify the systemic response to the infection. Furthermore, the chemotactic MCP-1 gradient might directly or indirectly favor leukocyte migration towards the peritoneal cavity, even in the absence of infection. We could speculate that in patients with SBP, higher MCP-1 ascites fluid levels are implicated in the infiltration of the peritoneal cavity. Furthermore, in several experimental models of acute peritonitis, high concentrations of MCP-1 have

been shown to have beneficial effects by recruiting macrophages and neutrophils to the site of infection<sup>[26-28]</sup>. Taken together these data indicate that, in patients with alcoholic liver cirrhosis, the -2518 MCP-1 genotype AA is a risk factor for the development of SBP, possibly *via* reduced MCP-1 ascites levels as compared to patients carrying the G-allele.

Interestingly, only in the subgroup of patients with chronic excessive alcohol abuse as the underlying cause of liver cirrhosis was the association between MCP-1 genotype and SBP found. In a previous study, patients with alcohol induced cirrhosis and patients with other hepatopathies showed similar MCP-1 serum and ascitic fluid levels<sup>[14]</sup>.

Notably, carriers of the G-allele of the MCP-1 polymorphism were more frequent in patients with alcohol induced cirrhosis than in heavy drinkers without evidence of liver damage. This difference did not reach statistical significance, but taken together with our previous finding that carrying the -2518 MCP-1 G-allele is a risk factor for disease progression in patients with chronic hepatitis C infection<sup>[13]</sup>, these data indicate that this functional SNP also affects fibrosis progression in patients with alcoholic liver disease. Thus, the same SNP appears to affect two related pathophysiological mechanisms (namely development of cirrhosis and SBP) but to different extents, depending on the underlying liver disease. Inflammatory and immune responses are altered in cirrhotic patients in general<sup>[29,30]</sup>; however, it has to be considered that there are differences between the underlying liver diseases leading to liver cirrhosis. This might be one explanation why it is more difficult to identify an association with the functional MCP-1 polymorphism and SBP in patients with liver disease other than alcoholic liver disease. Furthermore, in patients with alcoholic cirrhosis, the mean endotoxin concentration was significantly higher than in patients with non-alcoholic cirrhosis<sup>[31]</sup>, and bacterial translocation and endotoxin concentration have been shown to play an important role in the pathophysiology of SBP development<sup>[32]</sup>. In addition, it has been shown that LPS downregulates the specific MCP-1 receptor, CCR2, and abolishes macrophage infiltration in an animal model of acute infection<sup>[33]</sup>. Therefore, we speculate that higher LPS levels in patients with alcoholic liver disease are the reason why an association between the -2518 MCP-1 polymorphism and SBP was found only in the subgroup of patients with this underlying liver disease.

Finally, as in most genetic association studies, it has to be considered that the SNP investigated is in linkage disequilibrium to a different disease-associated genetic variation. Theoretically, this potential genetic variation might particularly affect the pathophysiology of alcoholic but not viral or other liver diseases. However, the high levels of MCP-1 in ascites in patients suffering from SBP and the functional relevance of the -2518 MCP-1 polymorphism demonstrated in several studies, argue against this hypothesis.

In summary, this is one of the first genetic association studies in patients with SBP. Our data indicate that the -2518 MCP-1 genotype AA is a risk factor for the

development of SBP in patients with alcoholic cirrhosis. Of course, our results have to be confirmed in a larger cohort of patients with independent and prospective studies. However, it is intriguing to speculate that genotyping might be helpful to identify cirrhotic patients with a higher risk of developing this life threatening complication and to apply prophylactic treatment specifically to this subset of patients.

## COMMENTS

### Background

Spontaneous bacterial peritonitis (SBP) is a potentially life threatening complication in patients with liver cirrhosis and ascites, caused by peritoneal neutrophil infiltration. However, factors leading to enhanced neutrophil infiltration of the peritoneal cavity with consecutive SBP are poorly characterized.

### Research frontiers

Monocyte-chemotactic protein-1 (MCP-1) is an important chemokine for several proinflammatory cells, and there exists a functional single nucleotide polymorphism (SNP) of the MCP-1 promoter (A-2518G) leading to higher MCP-1 expression in carriers of the G-allele. In the present study, the frequency of this MCP-1 SNP was analyzed in patients with SBP and liver cirrhosis.

### Innovations and breakthroughs

Accumulating evidence suggests that genotypes leading to increased MCP-1 production contribute to susceptibility and severity of diseases such as asthma, kidney graft failure, Crohn's disease, cutaneous vasculitis, tuberculosis, and chronic hepatitis C infection. This is the first genetic association study in patients with SBP to show that the -2518 MCP-1 genotype AA is a risk factor to development of SBP in patients with alcoholic cirrhosis.

### Applications

Genotyping of the -2518 MCP-1 SNP in cirrhotic patients might be beneficial to identify patients with increased risk for the development of SBP. Thus, early antibiotic treatment of such patients could help to decrease the mortality of this severe complication in liver cirrhosis.

### Terminology

SBP is an infection of the ascites in patients with liver cirrhosis, diagnosed by a neutrophil count greater than 250/mm<sup>3</sup> in the ascitic fluid. MCP-1 is one of the most potent chemokines for the recruitment of inflammatory cell-types during infection. For example, MCP-1 is upregulated in chronic hepatitis.

### Peer review

This retrospective study identified an association of a genetic polymorphism of the chemokine MCP-1 with increased risk to develop SBP in patients with alcohol-related liver cirrhosis. This is one of the first genetic association studies in patients with SBP and the results might be of high clinical relevance.

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## Endoscopic ultrasound and magnetic resonance imaging for re-staging rectal cancer after radiotherapy

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T0-T2 (44% vs 33%,  $P > 0.05$ ) and N0 disease (87% vs 52%,  $P = 0.013$ ). However, MRI was more accurate than EUS in T and N staging for patients with more advanced disease after radiotherapy, though these differences did not reach statistical significance.

**CONCLUSION:** EUS and MRI are accurate imaging techniques for staging rectal cancer. However, after neoadjuvant RT-CT, the role of both methods in the assessment of residual rectal tumors remains uncertain.

**Key words:** Endoscopic ultrasound; Magnetic resonance imaging; Rectal cancer; Neoadjuvant chemoradiation therapy; Diagnostic accuracy

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### Abstract

**AIM:** To compare the sensitivity and specificity of two imaging techniques, endoscopic ultrasound (EUS) and magnetic resonance imaging (MRI), in patients with rectal cancer after neoadjuvant chemoradiation therapy. And we compared EUS and MRI data with histological findings from surgical specimens.

**METHODS:** Thirty-nine consecutive patients (51.3% Male; mean age:  $68.2 \pm 8.9$  years) with histologically confirmed distal rectal cancer were examined for staging. All patients underwent EUS and MRI imaging before and after neoadjuvant chemoradiation therapy.

**RESULTS:** After neoadjuvant chemoradiation, EUS and MRI correctly classified 46% (18/39) and 44% (17/39) of patients, respectively, in line with their histological T stage ( $P > 0.05$ ). These proportions were higher for both techniques when nodal involvement was considered: 69% (27/39) and 62% (24/39). When patients were sorted into T and N subgroups, the diagnostic accuracy of EUS was better than MRI for patients with

### INTRODUCTION

Endoscopic ultrasound (EUS) is the most accurate imaging technique for evaluating local invasion of rectal cancer and perirectal lymph nodes. Its overall accuracy for T staging before radiation therapy (RT) ranges from 73% to 94%, and from 70% to 80% for N staging<sup>[1]</sup>. The findings of rectal EUS serve to decide the type of treatment: surgery or preoperative chemoradiation (RT-CT)<sup>[2]</sup>. The response to neoadjuvant therapy must also be assessed accurately in patients with rectal cancer.

The benefits of this treatment have been reported in different studies, reporting lower local recurrence rates in patients with locally advanced rectal tumors. Rectal EUS helps select a group with advanced locoregional disease (stage T3 or T4) in whom this preoperative treatment offers most benefit<sup>[3-5]</sup>.

The accuracy of EUS in staging rectal cancer ranges from 80% to 95% compared to 65%-75% for computed

tomography (CT) and 75%-85% for magnetic resonance imaging (MRI)<sup>[6-9]</sup>. There is still debate about the role of cross-sectional imaging to restage rectal cancer after RT-CT, and data are scanty on accuracy, sensitivity, and specificity rates, suggesting that these modalities are not efficient enough for restaging.

The aim of this prospective study was to evaluate the diagnostic accuracy of two imaging techniques, EUS and MRI, in patients with rectal cancer, before and after neoadjuvant RT-CT. We compared the EUS and MRI data with histological findings on surgical specimens.

## MATERIALS AND METHODS

Over a 2-year period (January 2007 to January 2009), 50 patients with histologically confirmed distal rectal cancer were referred to our Unit for rectal endosonography to stage the disease. Out of these, thirty-nine consecutive cases (51.3% Male; mean age:  $68.2 \pm 8.9$  years) were enrolled (drop-out from the study: 11 patients).

Both examinations were done, in random order, by two experienced endosonographers (> 300 rectal examinations/year) and an expert radiologist, before and after RT. The second examiner was blind to the first one's conclusions. Patients were included in the study if they had rectal cancer shown by either EUS or MRI, and were scheduled for neoadjuvant RT.

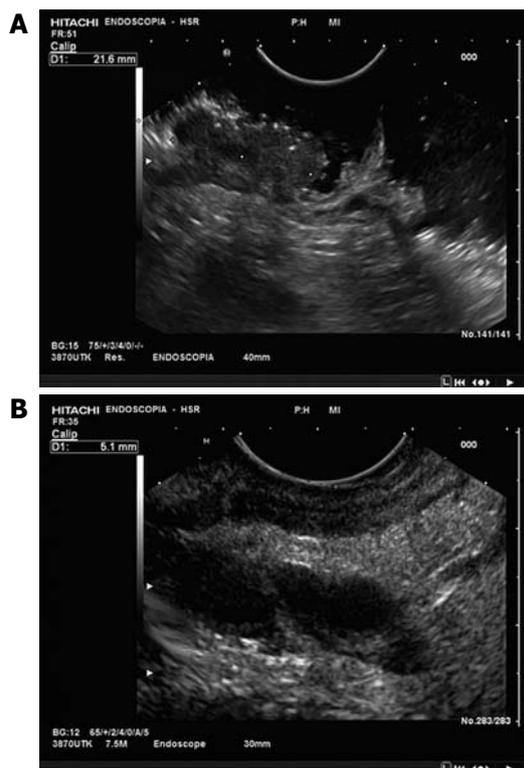
Usually, 30-40 d after termination of the RT protocol, patients were re-assessed by either EUS or MRI, followed by surgical excision during the same week. All patients gave informed consent.

Subjects with a history of rectal surgery, recurrent rectal cancer, or severe systemic illness were excluded from the study. Ten patients had neoplastic sub-stenosis of the lumen; therefore, it was not possible to evaluate their iliac lymph nodes by EUS.

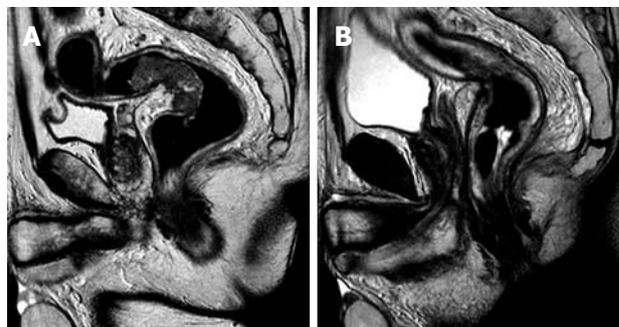
These two subgroups were formed to see whether the diagnostic accuracy of EUS or MRI was related to the tumor T stage after RT.

**EUS protocol:** Rectal EUS was performed using an oblique-forward viewing echo-endoscope (Pentax: FG-G36UX, FG-38UX, EG-3630U). All examinations were done with the patient lying on the left side under conscious sedation (midazolam i.v.). Patients were prepared with colonic lavage before EUS. The echo-endoscope was inserted and advanced beyond the lesion, under direct vision, to the rectosigmoid junction, high enough to detect iliac vessels on the EUS picture. Tumors were targeted to determine the depth of infiltration into or through the rectal wall. Frequencies commonly employed ranged from 7.5 to 10 MHz (Figure 1).

**MRI protocol:** All MRI examinations were performed on a 1.5T MRI scanner (Gyrosan Intera, Philips, The Netherlands). Iron contrast medium rectal distension and IM hypotonizing medication were performed. MRI examinations were performed before and after iv contrast media infusion (0.2 mL/kg). Multiplanar TSE T2 weighted sequences (TR: 4450, TE: 120, matrix:  $512 \times 344$ , NSA: 6, FOV: 240) were performed in order to local



**Figure 1 Rectal EUS.** A: Before neoadjuvant chemoradiation treatment: evidence of perirectal fat tissue invasion, with fat specculation; B: After neoadjuvant chemoradiation treatment: reduction of tumor size and perirectal fat invasion.



**Figure 2 Rectal MRI.** A: Before neoadjuvant chemoradiation treatment: evidence of perirectal fat tissue invasion, with fat specculation, and enlarged mesorectal lymph nodes; B: After neoadjuvant chemoradiation treatment: reduction of tumor size, perirectal fat invasion, enlarged lymph nodes; non-homogeneous mesorectal fat tissue.

stage rectal cancer; axial TSE T2 weighted sequences (TR: 3950, TE: 80, matrix:  $528 \times 372$ , NSA: 3, FOV: 370) were performed to analyze the presence of pathological lymph nodes (N). Axial FFE T1 weighted sequences (TR: 196, TE: 4.6, matrix:  $320 \times 216$ , NSA: 4, FOV: 250) with and without Fat Saturation, before and after contrast medium and sagittal FFE T1 weighted sequences (TR: 196, TE: 4.6, matrix:  $320 \times 216$ , NSA: 4, FOV: 250) with Fat Saturation, after contrast medium, were also performed. The MRI protocol used in our hospital is very similar to that commonly reported in the literature. Our Institute can be considered a medium-high flow hospital for rectal cancer staging; in fact, we performed at least 50 rectal contrast enhanced MRI in 1 year (Figure 2).

**Table 1** Post-treatment stage: imaging diagnosis *vs* surgical specimens

Stage	EUS	MRI
T0N0	6	4
T0N1	0	0
T0N2	0	0
T1N0	1	0
T1N1	0	0
T1N2	0	0
T2N0	0	0
T2N1	0	1
T2N2	0	0
T3N0	2	2
T3N1	4	6
T3N2	0	0
T4N0	0	0
T4N1	0	0
T4N2	0	0

**Neoadjuvant chemoradiation treatment:** Chemoradiotherapy consisted of administration of Oxaliplatin 100 mg/m<sup>2</sup> every 2 wk for three courses plus continuous infusion of 5-FU 200 mg/m<sup>2</sup>/die for six consecutive weeks. Concomitant hyperfractionated radiotherapy at a total dose of 45 Gy (1.25 Gy twice daily for 5 d every week with a four-field box technique, with 6-18 MeV photons) was started the same day as the second course of Oxaliplatin. EUS and pelvic MRI were repeated just before surgery to establish the clinical response.

**Surgical treatment:** Laparoscopic or laparotomic nerve sparing surgical resection (either low anterior or abdomino-perineal resection) with total mesorectal excision (TME) was scheduled 6-8 wk after completion of neoadjuvant treatment. Criteria for sphincter preservation were acceptable sphincter function, assessed with preoperative rectal manometry; and absence of direct invasion of the sphincter apparatus. These criteria remained unchanged throughout the treatment period. Creation of temporary loop ileostomy or colostomy was performed in selected cases as judged necessary by the surgeon.

### Rectal cancer staging

Rectal tumors were staged using the tumor-node-metastasis system (TNM)<sup>[10]</sup>.

### Statistical analysis

Pre-operative EUS and MRI findings were compared to assess the concordance between the two methods.  $\kappa$ -statistics were used to check how well EUS and MRI classified subjects in the T and N stage groups. The degree of agreement was quantified by weighted  $\kappa$ , which assumes the categories (T1, T2, *etc.*) are ordered and accounts for how far apart EUS and MRI are in classifying them.

EUS and MRI were repeated after RT, and the findings were compared with the T and N stages established on the basis of histological examination of surgical specimens. The proportions of concordant results between each method and histology were compared using the  $\chi^2$

**Table 2** Diagnostic accuracy (T and N-stage)

	All T	T0-T2	T3-T4	All N	N0	N 1-2
<i>n</i>	39	18	21	39	23	16
MRI	44%	33%	52%	62%	52%	75%
EUS	46%	44%	48%	69%	87%	44%

MRI: Magnetic resonance imaging; EUS: Endoscopic ultrasound.

test (2 × 2 table). Patients were considered all together and sorted into two subgroups according to their histological T stage: the first group had T0-T2 stage cancer and the second T3-T4.

## RESULTS

After neoadjuvant chemoradiation therapy, histological examination of surgical specimens was done for all patients, with the following results: 9 T0; 1 T1; 8 T2; 21 T3. Nodal involvement was N0 in 23; N1 in 14; N2 in 2. There were 18 patients with T0-T2 disease, 21 T3 (no T4), 23 without nodal metastasis (N0 disease) and 16 with N1-N2 disease. EUS and MRI correctly classified patients in line with their histological T stage in 46% (18/39) and 44% (17/39) (NS) of cases, respectively. These proportions were higher for both techniques when nodal involvement was considered: staging was correct in 69% (27/39) and 62% (24/39). Interestingly, when patients were sorted into T and N subgroups, the diagnostic accuracy of EUS was better than MRI for patients with T0-T2 (44% *vs* 33%, NS) and N0 disease (87% *vs* 52%,  $P = 0.013$ ). However, MRI was more accurate for patients with more advanced disease (both T and N) after RT, though these differences did not reach statistical significance (Tables 1 and 2).

## DISCUSSION

The prognosis for rectal cancer strongly correlates with the histopathological stage at diagnosis. Many imaging techniques are available, but each one differs in accuracy and applicability<sup>[11-13]</sup>. Accurate staging is important for planning surgery and deciding on adjuvant treatment.

ERUS (endorectal ultrasound) and MRI are considered to be the most accurate modalities for determining local tumor stage. Two recent meta-analyses have compared EUS, CT and MRI for rectal cancer staging. Bipat *et al*<sup>[14]</sup> found that ERUS was the most accurate modality when compared with CT and MRI for the evaluation of the T stage in rectal cancer. For lymph node involvement, the results of ERUS, CT and MRI were comparable. However, the T-staging system does not discriminate between T3 tumors with close or involved circumferential resection clearance. In this meta-analysis, the distance of the tumor from the rectal fascia or the anticipated circumferential resection clearance was not evaluated. Lahaye *et al*<sup>[15]</sup> conducted another meta-analysis regarding the accuracy of preoperative imaging for predicting the two most important risk factors that they recognized for local recurrence in rectal cancer; the circumferential resection

clearance and the lymph node status. Major progress has been made in the preoperative staging in rectal tumors by MRI and several authors have indicated that a tumor-free circumferential clearance of more than 1 mm can be predicted using this method. For nodal status, ERUS was slightly, but not significantly, better than MRI.

The introduction of trans-rectal EUS has made it easier to see the pattern of the layers of the rectal wall, improving treatment allocation by establishing the depth of tumor invasion more accurately<sup>[16-18]</sup>. EUS is the most accurate tool for evaluating local invasion of rectal cancer and perirectal lymph nodes. Its overall accuracy before radiation ranges from 73% to 94% in T staging, and from 70% to 80% in N staging. Comparative studies found EUS very accurate in staging rectal cancer from 80% to 95%, compared to 65% to 75% for CT and 75% to 85% for MRI<sup>[6-9]</sup>.

CT and MRI have proved disappointing for detecting small neoplastic lesions. MRI is not significantly superior to CT because of the limited resolution of conventional MRI techniques. It has, however, been reported useful in determining the status of the circumferential resection margin (meso-rectum), which is important for assessing the risk of local recurrence<sup>[19]</sup>.

Recent data suggest that EUS staging of rectal cancer after RT-CT is inaccurate<sup>[1]</sup>, while MRI seems to be cost-effective in selecting appropriate patients for neoadjuvant therapy and its use is justified for local staging. After RT-CT, EUS and MRI might be useful to demonstrate tumor shrinkage and down-staging in responsive tumors, which might occasionally disappear completely<sup>[17,18,20-23]</sup>. Increasing reflectivity and signal changes indicate fibrosis, but unless significant tumor bulk remains, neither modality seems to be able to exclude the persistence of tumor cells within fibrosis<sup>[24-28]</sup>.

Finally, EUS-FNA (endoscopic ultrasound-guided fine-needle aspiration) was proposed for N staging of rectal cancer following neoadjuvant chemoradiation<sup>[29]</sup>.

This study compared rectal EUS and MRI for staging rectal cancer before and, in particular, after neoadjuvant RT-CT. As reported in other studies, at the first staging examination after the diagnosis of rectal cancer, EUS and MRI offer similar accuracies. However, if we consider the degree of agreement between the two procedures in rectal cancer, EUS (without lymph node biopsy) and MRI give concordant results in T and N staging only in 64% and 54% of patients. Moreover, they only give concordant results for both T and N stages in one third of patients. The strength of agreement between EUS and MRI in staging advanced rectal cancer was very poor, implying that each method has limitations.

After RT, both EUS and MRI offered poor diagnostic performance in the assessment of T and N stages compared to the "gold standard", i.e. histological examination of surgical specimens. In the present study, compared to MRI, EUS offered significantly superior diagnostic accuracy in assessing nodal involvement after RT, giving much better results in patients with N0 disease. A higher proportion of false-positive results of MRI in patients without



**Figure 3** Rectal EUS and elastosonography before neoadjuvant chemoradiation treatment. Elastography showing hard tissue inside the lesion.

nodal metastasis are probably due to the effects of RT on perirectal tissues (edema, inflammation, and fibrosis), which renders MRI unreliable for excluding nodal disease. Both methods were weak in restaging the persistence and degree of tumor invasion of the rectal wall, and this might be mainly due to microscopic persistence (under-staging) in the wall or to inflammatory and scar changes in perirectal fat (over-staging). These might explain the lower sensitivity and accuracy of both methods in restaging after RT.

The recent development of elastosonography, a new real-time EUS modality that gives a fairly qualitative image of tissue elasticity, might improve the accuracy and sensitivity of EUS in this setting. In a preliminary report, we showed that adding elastosonography to real-time EUS boosted the accuracy in T staging of the disease<sup>[30]</sup>. The ability of elastosonography to distinguish tissues with different levels of elasticity means it can detect inflammatory (soft) tissues and tumor (hard) separately, particularly when the real-time modality does not exclude the suspicion of perirectal invasion (Figure 3).

Therefore, in the modern era of GI-oncology it seems that patients benefit mostly from interdisciplinary approaches. In considering the value of a diagnostic technique, based on its ability to influence the therapeutic choices by allowing a better selection of patients, we believe that in this situation these two modalities are complementary.

In conclusion, EUS and MRI are both accurate for staging rectal cancer. In particular, in pre-therapy staging, EUS is a good modality for T staging while MRI obtains other information, including the clearance. However, after RT-CT, neither method is reliable for establishing the T stage. EUS seems significantly better than MRI for assessing the N stage, but until significant improvements in both methods are achieved, their use in this setting should be considered only in controlled trials.

## COMMENTS

### Background

Endoscopic ultrasound (EUS) and magnetic resonance imaging (MRI) are both accurate for staging rectal cancer. However, after radiation therapy-computed tomography (RT-CT), neither method is reliable for establishing the T stage. EUS

seems significantly better than MRI for assessing the N stage but until significant improvements in both methods are achieved, their use in this setting should be considered only in controlled trials.

### Innovations and breakthroughs

This study compared rectal EUS and MRI for staging rectal cancer after neoadjuvant RT-CT.

### Peer review

This study compared rectal EUS and MRI for staging rectal cancer after neoadjuvant RT-CT and provides a good basis for selecting the best surgical or medical strategy in patients with rectal cancer.

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BRIEF ARTICLE

## Routine modified D2 lymphadenectomy performance in pT1-T2N0 gastric cancer

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### Abstract

**AIM:** To evaluate routine modified D2 lymphadenectomy in gastric cancer, based on immunohistochemically detected skip micrometastases in level II lymph nodes.

**METHODS:** Among 95 gastric cancer patients who were routinely submitted to curative modified D2 lymphadenectomy, from January 2004 to December 2008, 32 were classified as pN0. All level I lymph nodes of these 32 patients were submitted to immunohistochemistry for micrometastases detection. Patients in whom micrometastases were detected in the level I lymph node stations ( $n = 4$ ) were excluded from further analysis. The level II lymph nodes of the remaining 28 patients were studied immunohistochemically for micrometastases detection and constitute the material of the present study.

**RESULTS:** Skip micrometastases in the level II lymph nodes were detected in 14% (4 out of 28) of the patients. The incidence was further increased to 17% (4 out of

24) in the subgroup of T1-2 gastric cancer patients. All micrometastases were detected in the No. 7 lymph node station. Thus, the disease was upstaged from stage I A to I B in one patient and from stage I B to II in three patients.

**CONCLUSION:** In gastric cancer, true R0 resection may not be achieved without modified D2 lymphadenectomy. Until D2+/D3 lymphadenectomy becomes standard, modified D2 lymphadenectomy should be performed routinely.

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**Key words:** Gastric cancer; D2 gastrectomy; D2 lymphadenectomy; Micrometastases; Skip metastases; Skip micrometastases

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### INTRODUCTION

The lymphatic stream from a gastric tumor is wide and complicated, thus the exact pattern of lymphatic drainage remains obscure and poorly understood. However, histologically confirmed metastatic infiltration of perigastric and extragastric lymph nodes has been defined as the strongest independent dismal prognostic factor for both early<sup>[1]</sup> and large<sup>[2]</sup> gastric cancer patients.

Micrometastases and/or isolated tumor cells have been reported as immunohistochemically detectable in 10% of early gastric cancer patients<sup>[3]</sup>, in 52.6% of T2N0 patients<sup>[4]</sup>, and in 21%<sup>[5]</sup> to 49%<sup>[6]</sup> of all node-negative gastric cancer patients.

Skip metastases are defined as the detection of metastatically infiltrated extragastric lymph nodes (level II), in the absence of perigastric lymph node (level I) involve-

ment<sup>[7]</sup>. Particularly in the subgroup of level I lymph node negative patients, the incidence of histologically detected metastases in the level II lymph nodes (skip metastases) ranges between 2.8% in cases of early<sup>[7]</sup> and 5%<sup>[8]</sup> to 17.4%<sup>[9]</sup> in all other gastric cancers. Moreover, even in patients with histologically classified level I lymph node negative early gastric cancer, micrometastatically infiltrated level II lymph nodes are immunohistochemically detected in 10% of them<sup>[10]</sup>.

Most authors<sup>[11-13]</sup> agree that, except for early gastric cancer patients<sup>[3]</sup>, patients with immunohistochemically detected micrometastases have significantly worse 5-year survival rates compared to patients with undetectable micrometastases. However, the incidence, clinical implications and clinical significance of skip micrometastases in level II extragastric lymph nodes in patients with gastric cancer have not been properly studied.

The aim of the present study was to evaluate retrospectively the necessity for routine modified D2 lymphadenectomy in all gastric cancer patients, (as a prerequisite for R0 resection for locoregional control of the disease), based on the immunohistochemical detection of micrometastases in level II lymph node stations in patients who had been classified histologically and immunohistochemically as level I lymph node negative and histologically as level II lymph node negative.

## MATERIALS AND METHODS

Between January 2004 and December 2008, 207 patients with a preoperative diagnosis of gastric adenocarcinoma were subjected to surgery with curative intent, in our department. None of them had undergone preoperative chemotherapy or radiotherapy. In 95 patients, a modified D2 lymphadenectomy was offered as the standard surgical procedure. Postoperatively, the standard histological examination by hematoxylin and eosin (HE) staining disclosed metastatic infiltration of at least one lymph node in the level I or II lymph node stations in 63 of these patients. Thirty-two patients were classified as pN0, since standard histology did not disclose any evidence of metastatic infiltration of level I and level II peri- and extragastric lymph node stations. All level I lymph nodes of these 32 patients were submitted to immunohistochemistry for micrometastases detection. Patients in whom micrometastases were detected in the level I lymph node stations ( $n = 4$ ) were excluded from further analysis.

The level II lymph nodes of the remaining 28 patients were studied immunohistochemically for micrometastases detection and constituted the material of the present study.

### **Surgical technique**

The proximal resection margin of the stomach was calculated according to the location of the primary tumor. At least a 6-cm tumor-free (based on the frozen section result) proximal resection margin from the most proximal macroscopic border<sup>[14]</sup> was achieved in all cases. The dissection of the regional lymph nodes was based on the

Japanese Classification of Gastric Carcinoma<sup>[15]</sup>. Thus, for D1 lymphadenectomy, the appropriate (depending on the location of the primary tumor) nos. 1-6 lymph node stations were included in the gastrectomy specimen, whereas in the modified D2 lymphadenectomy, the nos. 7, 8a, 9, 11p, 11d and 12a lymph node stations, were routinely dissected. The level II lymph node stations were recognizable as they had been sent separately to the Pathology Department with special indices demonstrating their exact location. Dissection of the No. 10 lymph node station, splenectomy or distal pancreatectomy was not performed in any of the patients. For staging of the tumors, the TNM classification system according to the AJCC Staging Manual, 6th edition, was used<sup>[16]</sup>.

### **Histopathology and immunohistochemistry**

Primary tumors and lymph nodes were fixed in formalin and embedded in paraffin. The presence or absence of lymph node metastasis was examined routinely by HE staining by using a representative cut section through the largest diameter of the lymph nodes.

One additional section of 4- $\mu$ m thickness from each node was prepared for immunohistochemical staining with a monoclonal anti-cytokeratin (CK) antibody cocktail (AE1/AE3; Dako, Glostrup, Denmark) that reacts with a broad spectrum of human CKs, to detect micrometastases and/or clusters of isolated tumor cells. Briefly, for AE1/AE3 immunostaining, paraffin-embedded sections were deparaffinized in xylene and rehydrated through graduated ethanol to water. Endogenous peroxidase activity was blocked by incubation for 30 min with a solution of 1% hydrogen peroxide, and antigen retrieval was performed by autoclaving sections in 0.01 mol/L citrate buffer, pH 6.0 for 20 min at 800 W. A monoclonal mouse anti-human CK antibody (clone AE1/AE3) was applied at a dilution of 1:50. The Dako Real Envision kit was then used. Diaminobenzidine was used as a chromogen. Lymphoid tissue was used as an internal negative control, while additional sections from the primary tumors were used as positive controls.

Based on the 6th TNM classification<sup>[16]</sup>, micrometastasis (N1mi) was defined as metastatic focus  $> 0.2$  mm but  $\leq 2$  mm, and cluster of tumor cells [N0 (+)] was defined as cluster  $< 0.2$  mm according to previously accepted conventions.

## RESULTS

Pathologic review did not detect patients with previously missed evidence of lymph node metastasis on conventional HE staining.

In four patients, micrometastases were detected in the level I lymph node stations. These patients were excluded from further analysis.

The remaining 28 patients were 16 men with a median age of 72.5 years (IR 69-75) and 12 women with a median age of 66.5 years (IR 58-69.5) (Table 1). Skip micrometastases in the level II lymph node stations were immunohistochemically detected in four patients ( $n = 4$ ). All micrometastases were detected in the No. 7 lymph node station.

Table 1 Characteristics of the study population

Parameter	n
Sex	16
Males	12
Females	0
Age (yr) (median + IR)	70.5 (63.5-74)
Tumor location	
Upper third	0
Middle third	6
Lower third	22
Histological type (WHO classification)	
Enteric type	19
Diffuse type	5
Mixed type	4
Differentiation	
High	4
Moderate	19
Low	5
T	
T1	4
T2	20
T3	4

The profiles of these patients are presented in Table 2. There were three female and one male patients, with T1 ( $n = 1$ ), T2a ( $n = 2$ ) and T2b ( $n = 1$ ) tumors, located in the lower third ( $n = 2$ ) or middle third ( $n = 2$ ) of the stomach. Thus, following micrometastases detection, the disease was upstaged from stage I A to I B in one patient and from stage I B to II in three patients.

Based on the above, the overall incidence of micrometastases detection was 25% (8 out of 32 patients), while skip micrometastases in the level II lymph nodes were detected in 14% (4 out of 28) of gastric cancer patients, who had been classified histologically and immunohistochemically as level I lymph node negative. Furthermore, the incidence of skip micrometastases was increased to 17% (4 out of 24) in the subgroup of T1-2 gastric cancer patients.

## DISCUSSION

The present study disclosed that skip micrometastases in the level II lymph node stations were detected in 14% (4 out of 28) of the patients, who had been classified histologically and immunohistochemically as level I negative. This incidence was further increased to 17% (4 out of 24 patients) in the subgroup of T1-2 gastric cancer tumors.

Despite AJCC/UICC guidelines, which require the pathological examination of at least 15 lymph nodes for accurate gastric cancer staging<sup>[16]</sup>, only 29% of gastric cancer patients had more than 15 lymph nodes retrieved<sup>[17]</sup>. However, D2 lymphadenectomy clearly offers the mean number of the required lymph nodes for pathological examination, independently to the pathologist<sup>[18]</sup>.

Although D2 lymphadenectomy is recommended by the Japanese Surgical Society as the surgical option for gastric cancer treatment<sup>[19]</sup>, its performance has not gained popularity worldwide, since prospective randomized studies<sup>[20-24]</sup> and meta-analysis<sup>[25]</sup> have revealed significantly higher postoperative morbidity and mortality

rates and no 5- and 11-year survival benefit compared to D1 lymphadenectomy.

D2 lymphadenectomy increases the long-term survival of gastric cancer patients with lymph node metastases, however, it has been proposed as unnecessary for patients without lymph node metastases<sup>[9]</sup>. On the other hand, D2 lymphadenectomy improves survival even in node-negative early gastric cancer patients, probably due to the resection of the coexisting micrometastases<sup>[26]</sup>.

Thus, the favorable overall survival rates which were published following the Japanese-type, compared to the Western-type gastric cancer surgery, indicate that, with more extended lymph node dissections, more R0 resections are achieved<sup>[27]</sup>. This probably leads to locoregional control of the disease, better outcome and increased survival<sup>[28]</sup>.

Three methods have been used for the identification of micrometastasis, serial sectioning, immunohistochemical staining, and reverse-transcriptase polymerase chain reaction (RT-PCR). Serial sectioning constitutes a histological method, which can detect lymph node metastasis previously missed by the conventional technique, but it may still fail to identify isolated tumor deposits<sup>[13]</sup>. RT-PCR has been reported as highly sensitive<sup>[29]</sup>, but it is compromised by false-positive results caused by biological contamination<sup>[30]</sup>. Positive RT-PCR results indicate the presence of tumor DNA, however, they may not indicate the presence of viable tumor cells<sup>[31]</sup>. Thus, immunohistochemistry with human anti-CK antibodies represents the most accurate method for micrometastasis detection<sup>[32]</sup> and the most frequently applied technique in research<sup>[3]</sup>. One limitation of the method is CK expression by some dendritic cells in the lymph nodes<sup>[33]</sup>.

Based on the results of studies in colorectal<sup>[34]</sup> and non-small cell lung cancer<sup>[35]</sup>, it has been proposed that N1(-)/N2(+) patients represent a subgroup of pN2 disease with more favorable prognosis<sup>[35]</sup>. However, the clinical significance of skip metastases in gastric cancer patients remains controversial. The controversies are related mainly to the small number of patients enrolled in skip metastasis studies<sup>[26]</sup>, the probably different prognosis of patients with histologically *vs* micrometastatically detected skip metastases<sup>[32]</sup>, and the concern that patients with histologically detected skip metastases may represent cases of overlooked histological metastasis or micrometastasis in level I lymph nodes, thus being misclassified as patients with skip metastasis<sup>[36]</sup>.

Saito *et al*<sup>[36]</sup> have reported 5-year survival rates of 70.2%, 62.0% and 31.2% in patients with skip metastases, metastases in level I lymph nodes and metastases in level II lymph nodes, respectively. The prognosis of patients with metastases in the level II lymph nodes was significantly worse than that of the patients with either skip metastases or metastases in the level I lymph nodes. The authors have indicated that the clinicopathological characteristics and the prognosis of patients with skip metastases were similar to patients with level I lymph node metastases, but not to the patients with level II lymph node metastases. On the other hand, Li *et al*<sup>[9]</sup> have concluded that the cumulative survival rate is not statistically different between gastric cancer patients with solitary skip lymph node metastases, compared to

Table 2 Profiles of the patients with skip micrometastases

Pt	Sex	Age (yr)	No. of LN retrieved	(+) LN station	Location of the tumor	T size (mm)	T stage	Histological type	Lymphatic invasion	Vessel invasion
1	F	62	51	7	L, post	4	T2b	Mixed	+	-
2	F	56	16	7	M, less	30	T1	Diffuse	-	-
3	M	54	38	7	M, less	17	T2a	Enteric	-	-
4	F	71	29	7	L, post	26	T2a	Diffuse	-	-

Pt: Patient; LN: Lymph node; T: Tumor; L: Lower third; M: Middle third.

patients with solitary level I lymph node metastases. Moreover, Park *et al.*<sup>[26]</sup> have reported that, in patients with positive nodes extending into the level II lymph nodes, the survival curves did not show significant differences between skip(+) and skip(-) groups of patients, which further supports the theory that the number but not the level of lymph node metastases has prognostic significance.

The result of the present study, that all skip micrometastases were detected in the left gastric artery lymph node station, probably indicates that the clinical application of the sentinel node biopsy technique in selected cases might be useful, and lead to selective lymphadenectomy. Although the method has been reported as highly accurate (< 10% false-negative results) in breast cancer surgery<sup>[37]</sup>, similar findings have not been confirmed in gastric cancer surgery, since 20%-36% of positive lymph nodes were located outside of the sentinel lymph node basin<sup>[38]</sup>. Thus, the method is recommended currently to be used in conjunction with D2 lymphadenectomy<sup>[39]</sup>.

It has been suggested that the most likely route for para-aortic lymph node metastases is from the left gastric artery nodes, passing by the celiac artery<sup>[40]</sup>. Other common sites of skip metastasis are the 8a and 9 lymph nodes (around the celiac artery). Thus, these lymph nodes should always be evaluated, regardless of the mode of operation, even in the case of minimally invasive surgery. Moreover, Yanagita *et al.*<sup>[41]</sup> have investigated the clinical significance of morphological distribution of metastatic foci (metastasis, micrometastasis or isolated tumor cells) in sentinel lymph nodes with gastric cancer, and have concluded that, in patients with non-marginal sinus type sentinel node metastasis, attention should be paid to the possibility of non-sentinel node or even pN2 metastases. Thus, if the sentinel node cannot be identified in the perigastric lymph nodes, those around the celiac artery lymph nodes should be explored to reduce the likelihood of false-negative results in sentinel node mapping<sup>[7]</sup>.

In conclusion, the present study addressed the fact that, in up to 17% of T1-2 gastric cancer patients, true R0 resection may not be achieved without modified D2 lymphadenectomy. Thus, until D2+ or even D3 lymphadenectomy becomes the standard surgical option, modified D2 lymphadenectomy should be considered as the surgical option of choice in gastric cancer patients.

## COMMENTS

### Background

R0 resection constitutes the prerequisite for the locoregional control of gastric

cancer. However, the extent of the oncologically required lymphadenectomy (D1 or D2) remains a matter of debate and contradictory results have been published.

### Research frontiers

True R0 resection is characterized by complete resection of all viable tumor cells. As histology detects metastases > 2 mm, immunohistochemistry can detect micrometastases between 0.2 and 2 mm or even isolated tumor cells.

### Innovations and breakthroughs

Immunohistochemistry with human anti-cytokeratin antibodies showed clearly that, in level II extragastric lymph nodes, viable tumor deposits (skip micrometastases) were present in 17% of the gastric cancer patients who had been classified previously as node-negative by conventional histology.

### Applications

By understanding that, without modified D2 lymphadenectomy, true R0 resection may not be achieved, the present study indicates the usefulness of routine modified D2 lymphadenectomy in all gastric cancer patients.

### Terminology

Modified D2 lymphadenectomy offering the mean number of the required lymph nodes for pathological examination, contributes to accurate staging of the disease, probably defying the subgroups of patients who may benefit from adjuvant therapy.

### Peer review

The authors suggest that D2 lymphadenectomy might be necessary for complete resection of T1-2 N0 gastric cancer, which highlights clearly that skip metastases may be an overlooked problem with standard resection performed routinely in many countries.

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## Comparison of a modified anoscope and the purse-string anoscope in stapled haemorrhoidopexy

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surgeon's satisfaction increased with use of the modified anoscope, and fewer haemostatic sutures were required if the surgeon waited longer before and after firing the stapler.

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### Abstract

**AIM:** To compare the results of the anoscope of the PPH kit and a modified anoscope during stapled haemorrhoidopexy.

**METHODS:** The hospital records of 37 patients who underwent stapled haemorrhoidopexy between 2001 and 2006 were reviewed. The purse-string suture anoscope in the PPH kit was used on 15 patients (Group 1), and the modified anoscope was used on 22 patients (Group 2). Demographic characteristics of the patients, operation time, surgeon's performance, analgesic requirement, and complications were compared.

**RESULTS:** Operation time was significantly longer in Group 1 ( $42.0 \pm 8.4$  min vs  $27.7 \pm 8.0$  min,  $P = 0.039$ ). The surgeons reported their operative performance as significantly better in Group 2 (the results of the assessments were poor in ten, medium in four and good in one in Group 1, while good in all patients in Group 2,  $P < 0.001$ ). The need for haemostatic sutures was significantly higher in Group 1 (six cases) and was needed in two cases in Group 2 ( $P = 0.034$ ).

**CONCLUSION:** Operation time decreased and the

### INTRODUCTION

Stapled haemorrhoidopexy is not only a safe method that can be compared with conventional haemorrhoidectomy procedures, but also has advantages such as reduced postoperative pain and early return to work<sup>[1,2]</sup>. Moreover, some studies suggest the applicability of stapled haemorrhoidopexy as a day case procedure<sup>[3]</sup>. However, some complications were noted after stapled haemorrhoidopexy, such as rectal perforation, intra-abdominal haemorrhage, recto-vaginal fistula, and perirectal haematoma. These complications are not observed after conventional haemorrhoidectomy. In addition, although stapled haemorrhoidopexy is the most recent surgical method for the treatment of haemorrhoidal disease, the number of patients that developed pelvic sepsis after this surgical technique has reached the same number as after conventional haemorrhoidectomy<sup>[4-8]</sup>. It was suggested that these serious complications could be due to technical errors and could be avoided by eliminating the bite of the suture in deep layers of the rectum<sup>[9]</sup>.

The ideal purse-string suture must enable removal of a ring of the desired thickness from the most appropriate part of the rectum. One major obstacle to the surgeon during the operation is blockage of vision due

to internal haemorrhoids. If these internal haemorrhoids can be eliminated, then optimal purse-string suture can be performed<sup>[10]</sup>. Some studies suggest that after modification of the anoscope of the PPH stapler kit (Ethicon Endo-Surgery, Cincinnati, OH, USA), purse-string suture applications have been performed with relative ease<sup>[10-12]</sup>.

We first performed the stapled haemorrhoidopexy procedure in our clinic in 2001. We noticed the need for a modification of the anoscope of the PPH kit due to difficulties during the placement of the purse-string suture<sup>[10,11]</sup>. Subsequently, we tried several modifications of the anoscope and using the modified anoscope, purse-string sutures were performed with ease, and stapled haemorrhoidopexy was completed effectively.

In this study, the surgical findings and early postoperative results of patients in whom the purse-string suture anoscope of the PPH kit was used were compared with those of patients in whom the modified anoscope was used during stapled haemorrhoidopexy.

## MATERIALS AND METHODS

Hospital and follow-up records of patients with grade III and IV haemorrhoidal disease who underwent stapled haemorrhoidopexy in our clinic between 2001 and 2006 were reviewed retrospectively. Gender, degree of haemorrhoidal disease, preoperative treatment, operation time, performance of the surgeon, analgesic requirement, haemostatic sutures, histopathological examination of the excised ring, postoperative complications, and hospital stay were evaluated. Patients with associated proctological diseases such as anal abscess, anal fissure and fistula were excluded from the study. The patients were informed about the details of the procedure and written informed consent was obtained. The patients were grouped as: (1) the patients on whom the purse-string suture anoscope in the PPH kit was used (PA group), and (2) the patients on whom the modified anoscope was used (MA group). The modified anoscope was sterilized by ethylene oxide before use in each patient. The purse-string suture technique which has been described previously, was used in the MA group<sup>[10,11]</sup>. Stapled haemorrhoidopexy was completed using the stapler in the PPH (Ethicon Endo-Surgery, Cincinnati, OH, USA) kit in both groups. In the PA group, the surgeon waited for 30 s with the stapler closed after firing the stapler to ensure haemostasis. In the MA group, the surgeon waited for a total of 3 min, 90 s before and 90 s after firing the stapler. In the MA group, some of the operations were performed by inexperienced surgeons under the supervision of an experienced surgeon, whereas in the PA group an experienced surgeon performed the operations. The procedures were performed by six general surgeons, three of which were experienced. Diclofenac sodium 75 mg, IM was administered on demand after surgery, and pethidine HCl 50 mg, IM was given if diclofenac was insufficient. Patient age, gender, degree of haemorrhoidal disease, previous medical or surgical treatments, previous blood transfusions, histo-

pathological evaluation of the excised ring, the performance of the surgeons, postoperative analgesic requirement, early (during the first 30 d) or late (after the first 30 d) complications in both groups were investigated, and the differences between the groups were analyzed statistically.

In the first few postoperative hours, the surgeons were asked two questions in order to evaluate the procedure: Was the anoscope simple to use during the manipulation, and how would you evaluate your performance during the procedure? The surgeons who performed the operations were asked to evaluate their performances as good, moderate, or poor. The surgeons who performed operations in both groups evaluated their performances by making comparisons with their earlier experiences, however, the surgeons who performed operations only in Group 2 made their decisions without comparisons.

## Statistical analysis

SPSS 10.0 was used for statistical analysis. Considering numerical values, Student's *t* test was used for parametric measurements, and Mann Whitney *U* test for non-parametric measurements. Considering categorical values, Pearson's Chi-Square test was applied for parametric measurements and Fisher's exact correct test for non-parametric measurements. A *P* value less than 0.05 was considered statistically significant.

## RESULTS

The records of 37 patients, who underwent stapled haemorrhoidopexy for the treatment of haemorrhoidal disease were investigated. Two types of anosscopes were used to aid purse-string suture application: the purse-string anoscope in the PPH kit was used on 15 patients (40.5) between September 2001 and March 2004, and the modified anoscope was used on 22 patients (59.5) between December 2003 and May 2006.

Mean age of the patients was  $47.9 \pm 15.2$  years (range, 27-78 years), and  $49.0 \pm 11.9$  years (range, 30-70 years) for the PA group and the MA group, respectively. There were 11 male and four female patients in the PA group and 17 male and five female patients in the MA group. There were no statistically significant differences between the two groups with respect to age ( $P = 0.802$ ) and gender ( $P = 0.541$ ). Mean duration of complaints was  $94.8 \pm 83.5$  mo (range, 6 mo-20 years) for the PA group, and  $112.1 \pm 98.5$  mo (range, 6 mo-30 years) for the MA group. Mean postoperative follow up time was  $46.1 \pm 8.7$  mo (range, 36-66 mo) in the PA group and  $21.9 \pm 9.9$  mo (range, 10-39 mo) in the MA group.

When the disease stage was considered, nine patients had third degree, and six patients had fourth degree haemorrhoidal disease in the PA group, whereas; 17 patients had third degree, and five patients had fourth degree haemorrhoidal disease in the MA group. There were no statistically significant differences between the two groups with respect to stage of the disease ( $P = 0.222$ ).

**Table 1** General characteristics of the patients, operative findings, and the differences between the two groups *n* (%)

	Group 1 (PA)	Group 2 (MA)	<i>P</i> value
Number of patients	15 (40.5)	22 (59.5)	
Gender			
Male	11 (73.3)	17 (77.3)	0.541
The degree of haemorrhoidal disease			0.222
III	9 (60.0)	17 (77.3)	
IV	6 (40.0)	5 (22.7)	
Preoperative treatment			0.564
Medical	14 (93.3)	17 (77.3)	
Blood transfusion	-	1 (4.5)	
Surgery	-	1 (4.5)	
Not required	1 (6.7)	3 (13.6)	
Haemostatic suture			0.034
Required	6 (40)	2 (9.1)	
Early complication			0.683
Bleeding	5 (33.3)	7 (31.8)	
Urinary retention	1 (6.7)	1 (4.5)	
Anal prolapse	-	1 (4.5)	
Late complication			0.418
Anal fissure	1 (6.7)	-	
External pile	-	1 (4.5)	
Thrombosis of haemorrhoids	-	1 (4.5)	
Histopathology of the donut			0.933
Normal	13 (86.7)	19 (86.4)	
Smooth muscle	1 (6.7)	2 (9.1)	
Adenoma	1 (6.7)	1 (4.5)	
Evaluation of the performance of the surgeon			< 0.001
Poor	10 (66.7)	-	
Medium	4 (26.7)	-	
Good	1 (6.7)	22 (100)	

When previous treatment modalities (medical, blood transfusion, surgery) were considered, there were no statistically significant differences between the two groups ( $P = 0.564$ ). General characteristics of the patients, intraoperative findings, and the differences between the two groups are shown in Table 1.

Mean duration of surgery was  $42.0 \pm 8.4$  min (range, 30-60 min) for the PA group, and  $27.7 \pm 8.0$  min (range, 18-45 min) for the MA group, and the difference was statistically significant ( $P = 0.039$ ) (Table 2). Additional haemostatic suture application after removal of the stapler was required in six cases (40.0) in the PA group and in two cases (9.1) in the MA group; and the difference was statistically significant ( $P = 0.034$ ). When the need for analgesics was considered, mean diclofenac sodium usage was  $1.9 \pm 1.5$  times (range, 0-6 times) in the PA group, and  $1.5 \pm 1.1$  times (range, 0-4 times) in the MA group. Mean pethidine HCL administration was  $0.8 \pm 1.1$  times (range, 0-3 times) in the PA group, and  $0.3 \pm 0.6$  times (range, 0-2 times) in the MA group. These differences between the groups were not statistically significant ( $P = 0.284$ , and  $P = 0.070$ , for diclofenac and pethidine, respectively) (Table 2). Urinary retention developed as an early complication in one patient in each group, and was relieved by urinary catheterization. Anal prolapse developed in one patient due to straining 8 h after the operation. Digital rectal examination revealed that the staple line was intact. Prolapsed mucosa was

**Table 2** Operation time, need for analgesics, and length of hospital stay of the patients, and the differences between the two groups (mean  $\pm$  SD)

	Group 1 (PA)	Group 2 (MA)	<i>P</i> value
Operation time (min)	$42.0 \pm 8.4$	$27.7 \pm 8.0$	0.039
Needs for analgesics (average dose)			
Diclofenac sodium	$1.9 \pm 1.5$	$1.5 \pm 1.1$	0.284
Pethidine HCL	$0.8 \pm 1.1$	$0.3 \pm 0.6$	0.070
Hospital stay (d)	$1.7 \pm 1.1$	$1.5 \pm 0.7$	0.449

reduced, and gauze sponges were applied to avoid recurrence. Gauze sponges were removed 12 h later and the patient was warned about straining. The patient was free of complaints 24 h later. Anal prolapse did not recur and the patient had normal bowel movements. Bleeding occurred in five patients (33.3) in the PA group and in seven patients (31.8) in the MA group. Bleeding was minor in all cases and no additional treatment was required. Bleeding ceased spontaneously within 24 h in five patients in each group, and after 48 h in two patients in the MA group. No delayed haemorrhage occurred. When early complications were considered, there were no statistically significant differences between the two groups ( $P = 0.685$ ). The mean hospitalization period was  $1.7 \pm 1.1$  d (range, 1-3 d) in the PA group, and  $1.5 \pm 0.7$  d (range, 1-3 d) in the MA group, and the difference was not statistically significant ( $P = 0.449$ ) (Table 2). The surgeons ranked their performance as poor ( $n = 10$ ), moderate ( $n = 4$ ), and good ( $n = 1$ ) in the PA group, and as good in all 22 operations in the MA group. The difference was statistically significant ( $P < 0.001$ ). An anal fissure developed 6 mo after surgery in one patient in the PA group, and treatment with lateral internal sphincterotomy was unsuccessful. Thrombosed external haemorrhoids developed 1 mo after surgery in one patient in the MA group, and excision of the piles was performed under local anaesthesia. A skin tag excision was performed in one patient in the MA group 7 mo postoperatively. When late complications in the two groups were considered, the difference was not statistically significant ( $P = 0.418$ ). Histopathological evaluation of the donuts showed that the specimens from 13 patients in the PA group included only mucosa and submucosa. Additionally, a mucosal adenoma was reported in one patient, and muscularis propria in another patient in this group. In the MA group, 19 donuts included only mucosa and submucosa, two donuts also included muscularis propria, and one included a mucosal adenoma. There were no statistically significant differences between the two groups ( $P = 0.933$ ).

## DISCUSSION

Achieving a perfect purse-string may be difficult in stapled haemorrhoidopexy, even after meticulous application of the technique<sup>[1]</sup>. The surgeon starts by placing the purse-string suture at a distance of 3-4 cm above the dentate line

and just deep enough to take the mucosa and submucosa, however, if internal haemorrhoids obscure the surgical field and if the tip of the needle causes bleeding while passing through the mucosa, further bites of the suture may not pass at the same distance and at the same depth<sup>[10,13,14]</sup>. This may result in an uneven purse-string suture which is closer to the dentate line, goes through deeper tissues, or which skips the mucosa in some parts. Consequently, some parts of the stapler line may be closer to the dentate line, or some parts of the donut may be thicker, or it may not be intact<sup>[4,5,14]</sup>. A stapler line close to the dentate line may cause acute or persistent pain, or bring about a risk of incontinence<sup>[15,16]</sup>. It has been reported that patients with a stapler line uniformly 22 mm above the dentate line needed fewer postoperative narcotic analgesics and returned to work earlier<sup>[1]</sup>. In the case of an incomplete donut or a donut with thin parts, this may increase the risk of bleeding during the early postoperative period or recurrence in the late period<sup>[17]</sup>.

Various modifications of the purse-string anoscope have been used for easier application of the suture and a new anoscope has been designed<sup>[10-12,18,19]</sup>. Yamamoto *et al*<sup>[12]</sup> indicated that they have been using their modified anoscope over the last 2 years. We have been using our modified anoscope since 2003.

This is the first study to compare the conventional anoscope, and a modified anoscope used during stapled haemorrhoidopexy. Although this study is limited by its retrospective nature, the two groups of patients were statistically comparable in terms of age, gender, stage of haemorrhoidal disease and previous treatment modalities.

Median operation time for stapled haemorrhoidopexy has been reported to be between 15 and 38 min (range, 5-150 min)<sup>[13,20-23]</sup>. Median operation time for the PA group in our study was higher than that reported in the literature; however, the range in operation time was similar. When the MA group is considered, both median operation time and median range were in accordance with the literature. There may be a number of explanations for the difference in operation time between the two groups in the present study. First of all, the PA group covered a time period where the surgeons who applied this method were less experienced. In addition, the purse-string suture had to be repeated several times in some patients in this group, and the number of patients who required haemostatic sutures was higher in this group. We propose that all of these factors prolonged the operation time. One explanation for the shorter operation time in the MA group may be that the application of the purse-string suture with the modified anoscope was much easier. It can also be said that the surgeons performed the operation in a shorter time, as they had gained more experience with the procedure. However, it should be emphasized that inexperienced surgeons performed some of the operations under the supervision of an experienced surgeon in this group. Moreover, the purse-string suture was possible on the first attempt in most patients, and haemostatic sutures were required in a smaller percentage of patients in this group (9.1% in the

MA group, and 40% in the PA group). The necessity for haemostatic sutures was reported to be 84% in a study in which the time elapsed before and after firing the stapler was not noted<sup>[21]</sup>. Nahas *et al*<sup>[22]</sup> reported that they waited 30 s after firing the stapler and the necessity for haemostatic sutures was 20%. Recalbuto *et al*<sup>[20]</sup>, in their study in which one min elapsed before and after firing the stapler, did not refer to the rate of haemostatic sutures, but reported the rate of bleeding as 6%. The PA group consisted of the initial patients who underwent haemorrhoidopexy surgery. Initially we paused for 30 s after firing the stapler for haemostasis. After the team gained some experience we increased the time before and after firing to 180 s considering that more efficient haemostasis could be achieved by longer compression on the vessels at the surgical site. When the two groups in the present study were compared with respect to the application of haemostatic sutures, it was observed that the number of patients who required additional sutures was significantly less in the MA group than in the PA group. This was an extra finding, and was not due to the features of the videoanoscope. A 3 min pause after closing the stapler may be tedious, but it is better than trying to apply additional haemostatic sutures if bleeding occurs after the stapler is removed. The haemostatic efficacy of keeping the stapler closed needs to be investigated by prospective controlled studies.

The rate of bleeding reportedly ranges between 5% and 85%<sup>[9,13,17,22]</sup>. The rates of bleeding in the PA group and in the MA group in the present study were 33.3% and 31.8%, respectively. Bleeding was minor in all cases and stopped in most patients within 48 h without any need for additional interventions. Urine retention rates observed in the PA and MA groups were in accordance with the literature<sup>[21]</sup>. We could not find a similar complication in the literature to the anal prolapse that we observed in one patient in the MA group. It did not seem to be due to anastomotic dehiscence because the stapler line was intact. The reasons for anal prolapse as an early postoperative complication may be due to excessive venous congestion and edema of internal haemorrhoids. Improvement of the clinical picture after reduction of the haemorrhoids confirmed this hypothesis.

The need for analgesics in each group was consistent with that reported in the literature<sup>[1,25]</sup>. None of the patients suffered from persistent pain. Hospital stay, reported in the literature as 0-2 d was similar to that in the present study<sup>[20]</sup>. When the two groups were compared in terms of late complications, there were no significant differences, also consistent with the literature<sup>[17,20,21,24]</sup>. Results of the histopathological examination of the specimens obtained in this study were similar to those reported in the literature, and no significant differences between the two groups in this study were noted<sup>[15]</sup>.

Satisfaction of the surgeons regarding technical performance during surgery was significantly higher in the MA group than in the PA group. As this was a retrospective study, it was difficult for the surgeons to remember their initial operations and commented so.

The decisions made by the surgeons were based on a comparison of the two anoscopes as they performed surgery in both groups, and they described their operative performances as better with the modified anoscope. In the MA group, the surgeons who performed the procedure for the first time did not have the chance to compare the anoscopes, but they too described their operative performance as good or sufficient. As all the surgeons noted that they would prefer to use the modified anoscope in future operations, we have been using only the modified anoscope for 4 years. These results need to be supported by prospective randomized studies.

In conclusion, with the help of our modified anoscope, the purse-string suture is easy to perform, the operation is completed in a shorter time, and the surgeons were more satisfied with their operative performances. Additionally, by waiting a total of 3 min, 90 s before and after firing the stapler, fewer haemostatic sutures were required. Using the modified anoscope, optimum application of the purse-string suture is achieved during stapled haemorrhoidopexy.

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## COMMENTS

### Background

Modification of the purse-string anoscope was required to achieve an easier purse-string suture during stapled haemorrhoidopexy, because the purse-string anoscope could not prevent protrusion of large haemorrhoids.

### Research frontiers

Stapled haemorrhoidopexy is not only a safe method that can be compared with conventional haemorrhoidectomy procedures, but also has advantages such as reduced postoperative pain and early return to work. However, some complications were noted after stapled haemorrhoidopexy. It was suggested that these serious complications could be due to technical errors and could be avoided by eliminating the bite of the suture in deep layers of the rectum. This study compared the surgical findings and early postoperative results of patients in whom the purse-string suture anoscope of the PPH kit was used, with patients in whom the modified anoscope was used during stapled haemorrhoidopexy.

### Innovations and breakthroughs

Recent reports have highlighted the importance of purse-string suture during stapled haemorrhoidopexy. Various modifications of the purse-string anoscope have been used for easier application of the suture. We have been using our modified anoscope since 2003. This is the first study to compare the conventional anoscope with a modified anoscope during stapled haemorrhoidopexy.

### Applications

Using the modified anoscope, optimum application of the purse string can be performed during stapled haemorrhoidopexy, and it may prevent serious complications of the procedure.

### Terminology

Stapled haemorrhoidopexy is an alternative operative procedure for haemorrhoidal disease. It depends on the removal and the anastomosis of the mucosa above the haemorrhoidal tissue using the PPH kit or a circular stapler. Purse-string suture placement is an important part of the procedure. Anoscopes help to perform the purse string suture placement.

## Peer review

The authors have compared modified anoscope for the application of the purse string to the standard purse string suture anoscope in the PPH kit. Although the technique using the modified anoscope is well described, this is the first paper comparing the 2 techniques. The manuscript is well written and flows well.

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## Genetic and epigenetic characteristics of gastric cancers with JC virus T-antigen

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### Abstract

**AIM:** To clarify the significance of JC virus (JCV) T-antigen (T-Ag) expression in human gastric cancer.

**METHODS:** We investigated the relationship between T-Ag detected by immunohistochemistry and Epstein-Barr virus (EBV) infection, microsatellite instability (MSI), and genetic and epigenetic alterations in gastric cancers. Mutations in the *p53*, *β-catenin*, *KRAS*, *BRAF*, *PIK3CA* genes were analyzed by polymerase chain reaction (PCR)-single strand conformation polymorphism and DNA sequencing. Allelic losses were determined by PCR at 7 microsatellite loci. Aberrant DNA methylation was analyzed by MethyLight assay.

**RESULTS:** JCV T-Ag protein expression was found in 49% of 90 gastric cancer tissues. T-Ag positivity was not correlated with clinicopathological characteristics. T-Ag expression was detected in a similar percentage of EBV positive cancers (4 of 9, 44%) and EBV negative

cancers (35 of 73, 48%). T-Ag expression was detected in a significantly lower percentage of MSI-H cancers (14%) than in non MSI-H cancers (55%,  $P = 0.005$ ). T-Ag expression was detected in a significantly higher percentage of cancers with nuclear/cytoplasmic localization of  $\beta$ -catenin (15 of 21, 71%) than in cancers without (42%,  $P = 0.018$ ). *p53* mutations were detected in a significantly lower percentage of T-Ag positive cancers (32%) than in T-Ag negative cancers (57%,  $P = 0.018$ ). T-Ag positive gastric cancers showed a significant increase in the allelic losses and aberrant methylation compared with T-Ag negative gastric cancers ( $P = 0.008$  and  $P = 0.003$ ).

**CONCLUSION:** The results suggest that JCV T-Ag is involved in gastric carcinogenesis through multiple mechanisms of genetic and epigenetic alterations.

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**Key words:** JC virus; T-antigen; Epstein-Barr virus; Microsatellite instability; Gastric cancer

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### INTRODUCTION

Viruses have been proposed to play an etiologic role in human cancers. JC virus (JCV) is a polyomavirus that ubiquitously infects humans worldwide, and more than 80% of the adult population carries antibodies against the virus<sup>[1]</sup>. JCV has been implicated in various types of human cancers<sup>[2-9]</sup>. It has been reported that JCV

sequences are frequently present throughout the normal human gastrointestinal tract and in colorectal and gastric cancers<sup>[2,9]</sup>.

JCV encodes a transforming gene, T-antigen (T-Ag), which is believed to mediate the oncogenic potential of the virus. T-Ag protein expression was specifically present in the nuclei of colon cancer cells, but not in any adjacent normal colonic epithelium<sup>[8]</sup>. Previous studies have identified multiple pathways including p53, pRb, IRS, NF1/NF2 and  $\beta$ -catenin, which may be dysregulated by T-Ag<sup>[4,10,11]</sup>. T-Ag can bind and inactivate p53, pRb, and the spindle assembly checkpoint protein Bub1, resulting in disruption of chromosomal integrity and cell cycle checkpoints<sup>[12,13]</sup>. T-Ag can also bind and stabilize  $\beta$ -catenin<sup>[4,11]</sup>. T-Ag protein expression, rather than the simple presence of JCV DNA sequences, has been significantly associated with chromosomal instability (CIN) and the methylator phenotype in colorectal cancers<sup>[8]</sup>. The possible involvement in aberrant methylation is thought to provide a secondary link to microsatellite instability (MSI) in colorectal cancers<sup>[8]</sup>. Therefore, it has been suggested that JCV is involved in colorectal cancers through multiple mechanisms of genetic and epigenetic alterations<sup>[8]</sup>. Bhattacharyya *et al*<sup>[14]</sup> recently reported the interplay between  $\beta$ -catenin and Rac1 that is initiated by T-Ag and results in stabilization of  $\beta$ -catenin and its presence in cell membrane ruffles. T-Ag and  $\beta$ -catenin synergistically activate Rac1, an event that can trigger several oncogenic factors<sup>[14]</sup>.

The association between T-Ag expression and aberrant promoter methylation in colorectal cancers suggests that this viral oncogene induces the methylator phenotype<sup>[8]</sup>. Genomic methylation is a host defense mechanism that silences the transcription of transposons and retroviruses that have accumulated in the mammalian genome<sup>[8,15,16]</sup>. Methylation of host cell genes is not unique to JCV and occurs with other oncogenic viruses. It has been suggested that the key protein triggering methylation events for the polyomavirus SV40 is T-Ag, which mediates cellular transformation of cultured epithelial cells by regulating the activities of key *de novo* DNA methyltransferases, such as DNMT3b<sup>[8,17]</sup>.

A significant correlation has been found between Epstein-Barr virus (EBV) and methylation of multiple genes in gastric cancers<sup>[18,19]</sup>. A mutually negative association between EBV and MSI has been reported in gastric cancers<sup>[20]</sup>. T-Ag protein expression was found in 9 (39%) of 23 gastric cancers, whereas no expression was observed in any of the non-cancer tissues<sup>[9]</sup>. In contrast to colorectal cancers<sup>[8]</sup>, however, little is known about the significance of T-Ag expression in gastric cancers. In the current study, we investigated the relationship between T-Ag protein expression and EBV infection, MSI, and genetic and epigenetic alterations in gastric cancers.

## MATERIALS AND METHODS

### Tissue samples

A total of 90 paired specimens of gastric adenocarcinoma and adjacent noncarcinoma tissue were obtained from Japanese patients who had undergone surgical treatment.

Informed consent was obtained from each patient. The tumor-node-metastasis (TNM) system of the American Joint Committee on Cancer and the International Union against Cancer was used for the pathologic diagnosis and classification of variables. Clinicopathological characteristics were as follows: age ( $69 \pm 10$  years), gender (59 male and 31 female), Lauren histology (44 intestinal and 46 diffuse), and pTNM stages (stage I, 23; stage II, 16; stage III, 30; stage IV, 21).

### Immunohistochemistry and in situ hybridization

Immunohistochemistry using a mouse monoclonal antibody against SV40 large T-Ag (clone PAB416, 1:100 dilution; Oncogene Research Products, San Diego, CA, USA), which cross-reacts with T-Ag of JCV, was performed as described previously<sup>[8]</sup>. Immunohistochemistry with an anti-human  $\beta$ -catenin monoclonal antibody (BD Transduction Laboratories, San Jose, CA, USA) was done as described previously<sup>[21]</sup>. Cancer cases were categorized into the following 4 groups corresponding to immunostaining patterns of  $\beta$ -catenin as described previously<sup>[21]</sup>: membranous, membranous staining pattern similar to that in normal epithelium; weak, no staining or weaker staining than normal epithelium; cytoplasmic, diffuse staining in the cytoplasm as well as at the cell membrane; accumulated, strong staining in the nucleus and cytoplasm. EBV infection was analyzed by *in situ* hybridization for *EBER-1*<sup>[20]</sup>.

### MSI analysis

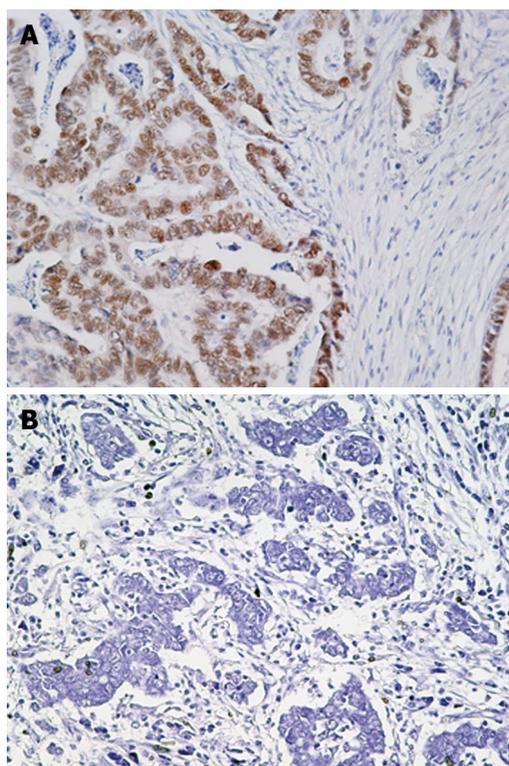
MSI was analyzed by polymerase chain reaction (PCR) using the mononucleotide (BAT26 and BAT25) and dinucleotide markers (D2S123, D5S346, and D17S250) proposed by the NCI workshop<sup>[22]</sup>. Based on the number of markers showing instability per cancer, cancers were divided into 3 groups; those with 2 or more of the 5 markers displaying instability (MSI-H), those with 1 of 5 markers displaying instability (MSI-low; MSI-L), and those with no instability (microsatellite stable; MSS).

### Mutation analysis

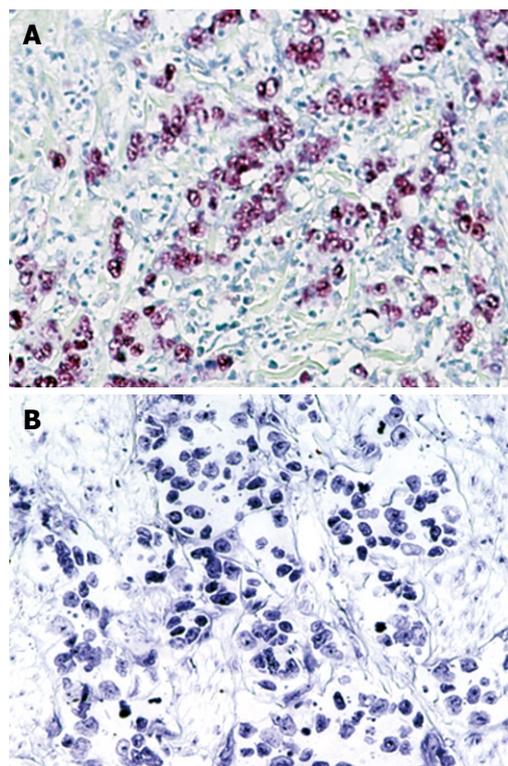
Mutations in exons 2-9 of *p53*, exon 3 of  *$\beta$ -catenin*, codons 12 and 13 of *KRAS*, codon 600 of *BRAF*, exons 9 and 20 of *PIK3CA* genes were analyzed by PCR-single strand conformation polymorphism and DNA sequencing as described previously<sup>[23-25]</sup>. Interstitial deletions spanning exon 3 of  *$\beta$ -catenin* was analyzed as described previously<sup>[24,25]</sup>.

### LOH analysis

LOH was analyzed as described previously<sup>[26]</sup>. Seven sets of microsatellite loci that are linked to tumor suppressor genes were used to identify significant allelic losses in gastric cancers. DNA was amplified by PCR at microsatellite loci linked to the *APC* locus on 5q21 (D5S505), possible tumor suppressor/senescence gene locus on 10p15 (D10S501 and D10S602), *p53* locus on 17p13 (TP53), *BRC1* locus on 17q21 (D17S855), and *DCC* locus on 18q21 (D18S58 and D18S61)<sup>[26]</sup>. Assessment of LOH was assigned when a tumor allele showed at least a 50% reduction in the relative intensity



**Figure 1** Immunohistochemistry for JCV T-Ag in gastric cancer tissues. A: Gastric adenocarcinoma positive for JCV T-Ag; B: Gastric adenocarcinoma negative for JCV T-Ag. Original magnification,  $\times 200$ .



**Figure 2** *In situ* hybridization for *EBER-1* in gastric cancer tissues. A: Gastric adenocarcinoma positive for *EBER-1*; B: Gastric adenocarcinoma negative for *EBER-1*. Original magnification,  $\times 200$ .

of 1 allele in cancer tissue compared with the matched non-cancer DNA as described previously<sup>[26]</sup>.

#### Quantitative DNA methylation analysis by real-time PCR (MethylLight assay)

Sodium bisulfite treatment of genomic DNA and MethylLight assay were performed as described previously<sup>[25,27-30]</sup>. We analyzed six promoters: calcium channel, voltage dependent, T type  $\alpha$ -1G subunit (*CACNA1G*), cellular retinoic acid binding protein 1 (*CRABP1*), neurogenin 1 (*NEUROG1*), *CDKN2A* (*p16*), *RUNX3*, and *SOCS1*. Primers, probes, and percentage of methylated reference (PMR, i.e. degree of methylation) were described previously<sup>[29,30]</sup>. The cutoff value of 4 (except for 6 in *CRABP1*) was based on previously validated data<sup>[28]</sup>.

#### Statistical analysis

The results were assessed for associations with clinicopathological parameters, using the following statistical tests: Student's *t* test for age, the Mann-Whitney test for depth of invasion, lymph node metastasis, and pTNM stage, and the chi-square test or Fisher's exact test for the remaining parameters.  $P < 0.05$  was considered significant. A  $P$  value between 0.05 and 0.10 was considered as a trend toward an association.

## RESULTS

### T-Ag protein expression in gastric cancers

T-Ag protein expression was found in 44 (48.9%) of 90 gastric cancers, whereas no expression was observed in

adjacent normal gastric epithelial cells or stromal cells (Figure 1 and data not shown). T-Ag positivity was not correlated with clinicopathological characteristics (data not shown).

### EBV infection

*EBER-1* expression was found in 9 (11.0%) of 82 gastric cancers, whereas no expression was observed in any of the non-cancer tissues (Figure 2). EBV infection was detected more frequently in diffuse type (7 of 44, 16%) than intestinal type (2 of 38, 5%), although this did not reach statistical significance. T-Ag expression was detected in a similar percentage of EBV positive cancers (4 of 9, 44%) and EBV negative cancers (35 of 73, 48%). The results for T-Ag protein expression and EBV infection were summarized in a Figure 3.

### MSI status

Following the NCI criteria, a total of 90 gastric cancers was classified as follows: 14 (16%) MSI-H, 10 (11%) MSI-L, and 66 (73%) MSS (Figure 4). T-Ag expression was detected in a significantly lower percentage of MSI-H cancers (14%) than in non MSI-H cancers (55%,  $P = 0.005$ ). None of the MSI-H tumors were EBV positive.

### Alterations of $\beta$ -catenin

Figure 5 shows representative results of immunohistochemistry for  $\beta$ -catenin in gastric cancer tissues. In normal gastric epithelium cells,  $\beta$ -catenin was moderately stained in a membranous distribution (Figure 5A), whereas fibroblasts, endothelial cells, and smooth muscle

Table 1 The exact mutations and their frequencies in gastric cancer tissues

p53 44% (40/90)	<i>n</i>		<i>n</i>		<i>n</i>	KRAS 6% (5/90)	<i>n</i>	BRAF 0% (0/90)	PIK3CA 4% (4/90)	<i>n</i>
G524A (R175H)	3	G469T (V157F)	2	G733A (G245S)	2	G35A (G12D)	3		A3140G (H1047R)	3
C742T (R248W)	2	G743A (R248Q)	2	C817T (R273C)	2	G35T (G12V)	2		G1624A (E542K)	1
G818A (R273H)	2	C844T (R282W)	2	C200G (P67R)	1					
G313T (G105C)	1	G422A (C141Y)	1	G461T (G154V)	1					
G473A (R158H)	1	C489G (Y163X)	1	G524T (R175L)	1					
C574T (Q192X)	1	A578G (H193R)	1	G586T (R196X)	1					
G587C (R196P)	1	A614G (Y205C)	1	C637T (R213X)	1					
G638A (R213Q)	1	G646A (V216M)	1	A659G (Y220C)	1					
G725T (C242F)	1	G731T (G244V)	1	G733T (G245V)	1					
C742G (R248G)	1	C817A (R273S)	1	T823G (C275G)	1					
C847T (R283C)	1									

Nucleotide substitution (amino acid change) is shown.

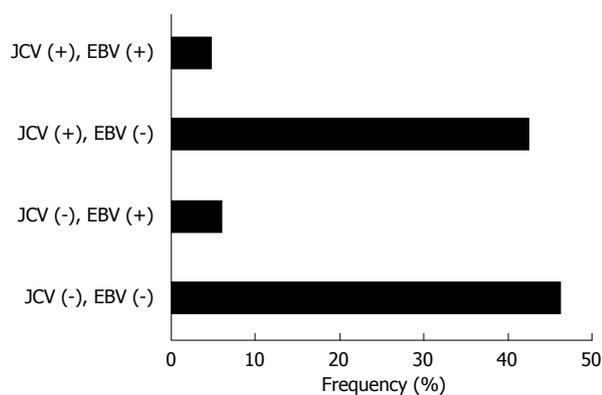


Figure 3 Classification of 90 gastric cancer tissues based on T-Ag protein expression and EBV infection.

cells were negative. Membranous, weak, cytoplasmic, and accumulated pattern was observed in 36 (40%), 33 (37%), 11 (12%), and 10 (11%), respectively. Neither point mutations nor interstitial deletions in exon 3 were detected (data not shown). T-Ag expression was detected in a significantly higher percentage of cancers with nuclear/cytoplasmic localization of  $\beta$ -catenin (15 of 21, 71%) than in cancers without (42%,  $P = 0.018$ ).

#### Mutations of the p53, KRAS, BRAF, PIK3CA genes in gastric cancer tissues

*p53* mutations were detected in 40 (44%) of 90 gastric cancer tissues. *p53* mutations were detected in a significantly lower percentage of T-Ag positive cancers (32%) than in T-Ag negative cancers (57%,  $P = 0.018$ ). *p53* mutations were detected in a significantly lower percentage of MSI-H cancers (14%) than in MSI-L cancers (60%,  $P = 0.028$ ) or in MSS cancers (48%,  $P = 0.021$ ). *KRAS* mutations were detected in only 5 (6%) of 90 gastric cancers. *BRAF* mutations were not detected. *PIK3CA* mutations were detected in 4 (4%) of 90 cancers. The exact mutations and their frequencies were described in a Table 1.

#### LOH analysis

Overall, 62 (69%) of 90 cancers had at least 1 LOH, with most frequent chromosomal losses observed on 17p (43%), followed by 18q (33%), 10p (27%), 5q (24%), and 17q (12%) (Figure 6A and B). Of 62 cancers with LOH, 4

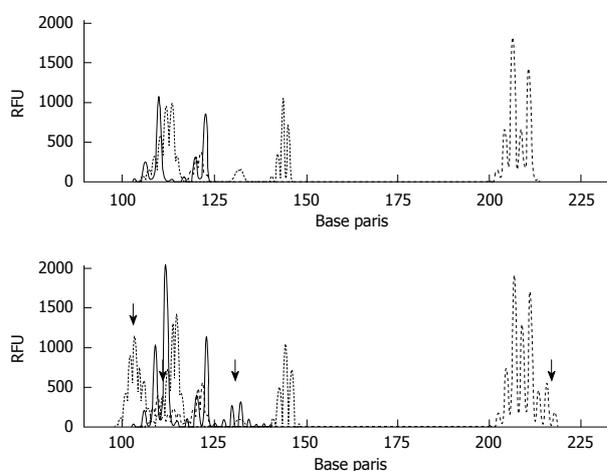


Figure 4 MSI analysis in gastric cancer tissues. MSI was analyzed by PCR using the mononucleotide (BAT26 and BAT25) and dinucleotide markers (D2S123, D5S346, and D17S250). Results of matched normal (upper panel) and tumor (bottom panel) samples (MSI-H) are shown. The arrows mean instability positive marker. RFU: Relative fluorescent units.

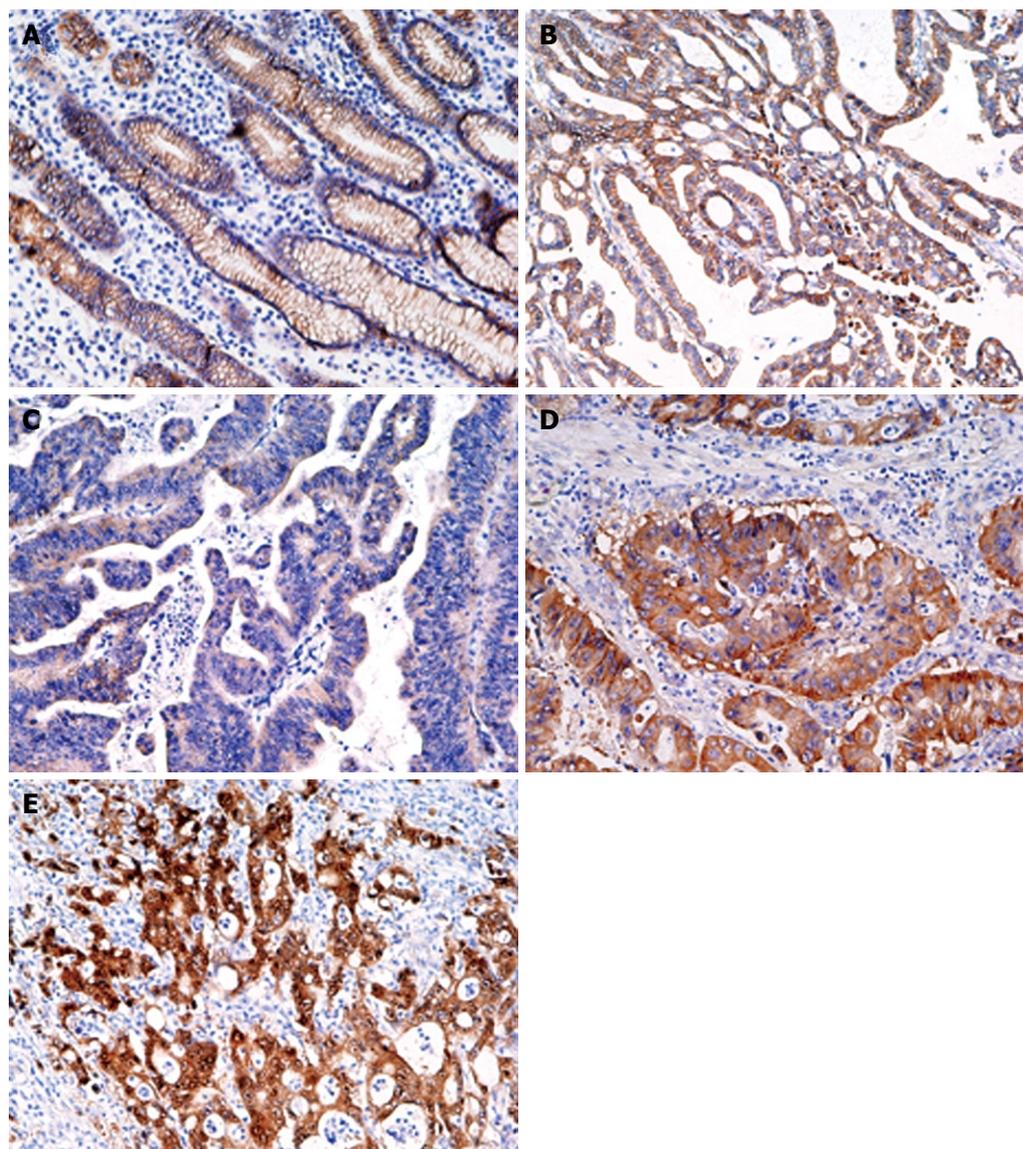
(6%) were also MSI-H. The data were expressed in terms of an allelic loss index (ALI), which reflects the number of chromosomal losses per the number of chromosomal loci showing heterozygosity but not MSI in each cancer specimen. T-Ag positive gastric cancers showed a significant increase in the ALI compared with T-Ag negative gastric cancers ( $0.41 \pm 0.12$  vs  $0.21 \pm 0.09$ ,  $P = 0.008$ ).

#### Methylation analysis

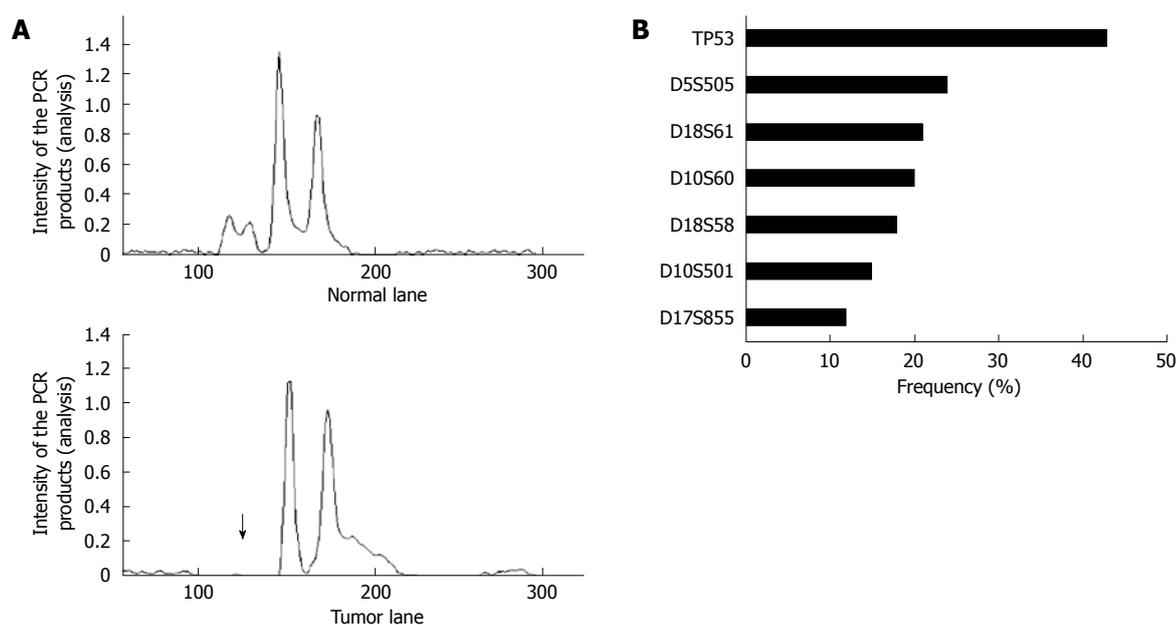
The results were summarized based on each individual marker (Figure 7A and B). The data were expressed in terms of a methylation index (MI), which reflects the number of abnormally methylated promoters per cancer specimen within the subsets of cancers. T-Ag positive gastric cancers showed a significant increase in the MI compared with T-Ag negative gastric cancers ( $3.01 \pm 0.92$  vs  $1.51 \pm 0.71$ ,  $P = 0.003$ ).

## DISCUSSION

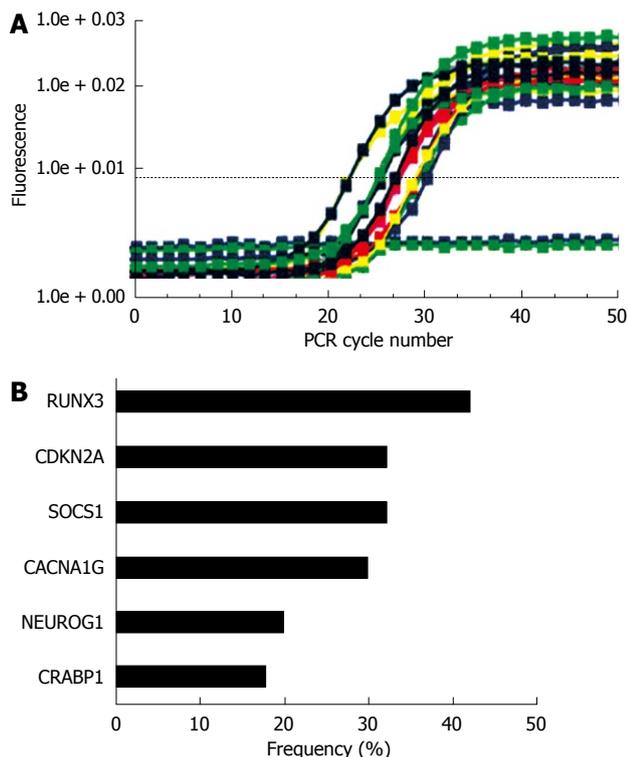
In the current study, T-Ag protein expression was positive in 44 (49%) of 90 gastric cancer tissues and it was observed specifically in gastric cancer cells but not in



**Figure 5** Immunohistochemistry for  $\beta$ -catenin in gastric normal (A) and cancer (B-E) tissues. A: Moderate membrane staining; B: Membrane staining pattern similar to that seen in normal epithelium; C: Weak staining; D: Diffuse staining in the cytoplasm and membrane; E: Strong staining of the nucleus and cytoplasm (Original magnification,  $\times 200$ ).



**Figure 6** LOH analysis in gastric cancer tissues. A: A representative example of a tumor with allelic loss in 5q21 (D5S505) is shown. The upper and bottom panels show the intensity plots of both the normal and tumor lane, respectively, demonstrating reduced relative intensity of allele one in the tumor sample (arrow) compared to the corresponding normal sample; B: LOH frequencies based on each individual marker.



**Figure 7** MethyLight analysis in gastric cancer tissues. A: Results of the *CRABP1* gene are shown. Bisulfite-converted DNA was used for quantitative methylation-specific PCR. The amount of hypermethylated DNA was determined by reading the midpoint of the linear portion of the S-shaped real-time curves, called the Ct point or threshold cycle. The Ct refers to the number of cycles it takes a sample to reach a specific fluorescence threshold; B: DNA methylation frequencies based on each individual marker.

the adjacent normal gastric epithelial cells or stromal cells. Previous study has also shown that T-Ag protein expression was found in 9 (39%) of 23 gastric cancers, whereas no expression was observed in any of the non-cancer tissues<sup>[9]</sup>. These results suggest an active role for this oncogenic protein in gastric carcinogenesis<sup>[9]</sup>.

The frequencies of EBV infection (11%) and MSI-H (16%) in this study were almost consistent with those reported in previous studies<sup>[20,31,32]</sup>. None of the MSI-H cancers were EBV positive. A mutually negative association between EBV and MSI has been reported in gastric cancers<sup>[20]</sup>. T-Ag was detected in a significantly lower percentage of MSI-H cancers (14%) than in non MSI-H cancers (55%). In contrast, T-Ag expression was present in similar frequencies in MSI-H and (8/15, 53%) and MSS/MSI-L (35/85, 41%) colorectal cancers<sup>[8]</sup>. From the view point of MSI, these results suggest a differential role of T-Ag in gastric and colorectal carcinogenesis.

T-Ag expression was detected in a significantly higher percentage of cancers with nuclear/cytoplasmic localization of  $\beta$ -catenin than in cancers without. T-Ag interacts with  $\beta$ -catenin, leading to its stabilization and resultant dysregulation of the WNT-signaling pathway in gastrointestinal cancers<sup>[4]</sup>. Neither point mutations nor interstitial deletions in exon 3 were detected. Although not analyzed in this study, the frequency of *APC* mutations is relatively low in gastric cancers. Therefore, T-Ag may play an important role in the stabilization of  $\beta$ -catenin in gastric cancers.

*p53* mutation was detected in a significantly lower

percentage of T-Ag positive cancers (32%) than in T-Ag negative cancers (57%). T-Ag can bind and inactivate *p53*<sup>[12]</sup>, resulting in disruption of chromosomal integrity and cell cycle checkpoints. Therefore, T-Ag expression may eliminate the selective pressure for *p53* mutations in a subset of gastric cancers.

A significant association was observed between T-Ag expression and allelic losses, being consistent with that in colorectal cancers<sup>[8]</sup>. It has been previously reported that the introduction of JCV into a diploid cell line leads to the rapid induction of CIN<sup>[33]</sup>. JCV can also induce CIN in a diploid colon cancer cell line RKO, which has wild-type *APC*, *p53*, and  $\beta$ -catenin genes<sup>[33]</sup>. These results suggest that T-Ag play a role in genomic damage during gastric carcinogenesis.

Significant association was also observed between T-Ag expression and aberrant methylation, being consistent with that in colorectal cancers<sup>[8]</sup>. A significant correlation has been found between EBV and methylation of multiple genes in gastric cancers<sup>[8,18,19]</sup>. These results suggest that JCV T-Ag and EBV play a similar role, at least in part, during gastric carcinogenesis.

Taken together, our results suggest that JCV T-Ag is involved in gastric cancers through multiple mechanisms of genetic and epigenetic alterations<sup>[34]</sup>. Further analysis is required to determine whether there is a plausible molecular mechanism by which JCV can induce genetic and epigenetic alterations in gastric carcinogenesis.

## COMMENTS

### Background

Gastric cancer is one of the most common cancer and leading cause of cancer-related death in the world. Understanding the molecular biological features of gastric cancer is necessary for early diagnosis and better prognosis. The potential role of viral infection in human cancer is receiving increasing attention.

### Research frontiers

A significant correlation has been found between Epstein-Barr virus (EBV) and methylation of multiple genes in gastric cancer. However, little is known about the significance of JC virus (JCV) T-antigen (T-Ag) expression in gastric cancers. In this study, the authors demonstrate that JCV T-Ag plays a key role in gastric carcinogenesis.

### Innovations and breakthroughs

This is the first study to report that JCV T-Ag plays a key role in gastric carcinogenesis. Furthermore, T-Ag expression was associated with nuclear/cytoplasmic localization of  $\beta$ -catenin, allelic losses and aberrant DNA methylation, suggesting that JCV T-Ag is involved in gastric carcinogenesis through multiple mechanisms of genetic and epigenetic alterations.

### Applications

JCV T-Ag expression could be future diagnostic and/or therapeutic targets in clinical settings. Understanding of a plausible molecular mechanism by which JCV can induce genetic and epigenetic alterations may represent a future strategy for therapeutic intervention in the treatment of patients with gastric cancer.

### Terminology

JCV: JCV is a polyomavirus that ubiquitously infects humans worldwide, and more than 80% of the adult population carries antibodies against the virus. EBV: EBV is a double-stranded DNA virus and takes a linear form in the viral particles. EBV was the first virus discovered from human neoplastic cells, a Burkitt's lymphoma cell lines. Microsatellite instability (MSI): MSI is a type of genetic instability characterized by length alterations within simple repeated microsatellite sequences.

### Peer review

This paper reports the characteristics of gastric cancer with JCV T-Ag expression. The authors showed that JCV T-Ag plays a key role in gastric carcinogenesis. The study sounds interesting and the information could be useful for other gastric cancer researchers.

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BRIEF ARTICLE

## Unsedated transnasal small-caliber esophagogastroduodenoscopy in elderly and bedridden patients

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### Abstract

**AIM:** To evaluate the safety of unsedated transnasal small-caliber esophagogastroduodenoscopy (EGD) for elderly and critically ill bedridden patients.

**METHODS:** One prospective randomized comparative study and one crossover comparative study between transnasal small-caliber EGD and transoral conventional EGD was done (Study 1). For the comparative study, we enrolled 240 elderly patients aged > 65 years old. For the crossover analysis, we enrolled 30 bedridden patients with percutaneous endoscopic gastrostomy (PEG) (Study 2). We evaluated cardiopulmonary effects by measuring arterial oxygen saturation (SpO<sub>2</sub>) and calculating the rate-pressure product (RPP) (pulse rate × systolic blood pressure/100) at baseline, 2 and 5 min after endoscopic intubation in Study 1. To assess the risk for endoscopy-related aspiration pneumonia

during EGD, we also measured blood leukocyte counts and serum C-reactive protein (CRP) levels before and 3 d after EGD in Study 2.

**RESULTS:** In Study 1, we observed significant decreases in SpO<sub>2</sub> during conventional transoral EGD, but not during transnasal small-caliber EGD (0.24% vs -0.24% after 2 min, and 0.18% vs -0.29% after 5 min,  $P = 0.034$ ,  $P = 0.044$ ). Significant differences of the RPP were not found between conventional transoral and transnasal small-caliber EGD. In Study 2, crossover analysis showed statistically significant increases of the RPP at 2 min after intubation and the end of endoscopy (26.8 and 34.6 vs 3.1 and 15.2,  $P = 0.044$ ,  $P = 0.046$ ), and decreases of SpO<sub>2</sub> (-0.8% vs -0.1%,  $P = 0.042$ ) during EGD with transoral conventional in comparison with transnasal small-caliber endoscopy. Thus, for bedridden patients with PEG feeding, who were examined in the supine position, transoral conventional EGD more severely suppressed cardiopulmonary function than transnasal small-caliber EGD. There were also significant increases in the markers of inflammation, blood leukocyte counts and serum CRP values, in bedridden patients after transoral conventional EGD, but not after transnasal small-caliber EGD performed with the patient in the supine position. Leukocyte count increased from  $6053 \pm 1975/L$  to  $6900 \pm 3392/L$  ( $P = 0.0008$ ) and CRP values increased from  $0.93 \pm 0.24$  to  $2.49 \pm 0.91$  mg/dL ( $P = 0.0005$ ) at 3 d after transoral conventional EGD. Aspiration pneumonia, possibly caused by the endoscopic examination, was found subsequently in two of 30 patients after transoral conventional EGD.

**CONCLUSION:** Transnasal small-caliber EGD is a safer method than transoral conventional EGD in critically ill, bedridden patients who are undergoing PEG feeding.

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**Key words:** Aged; Aspiration pneumonia; Gastrointestinal endoscopy; Critical illness

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## INTRODUCTION

Small-caliber endoscopy of the upper gastrointestinal tract was developed and for transnasal esophagogastroduodenoscopy (EGD) and has been used frequently in the past decade<sup>[1-4]</sup>. Transnasal small-caliber EGD has improved the safety of the endoscopic examination, and has fewer adverse effects on cardiopulmonary function<sup>[5-10]</sup> and autonomic nerve function<sup>[11]</sup>, compared with transoral conventional EGD. Transnasal small-caliber EGD provides good operability compared to that of transoral conventional EGD<sup>[12]</sup>, and requires no special training<sup>[13]</sup>. For these reasons, unsedated transnasal small-caliber EGD is used routinely in endoscopic examinations of the upper gastrointestinal tract, often in preference to transoral conventional EGD.

Although we perform endoscopy on elderly and critically ill patients with increasing frequency, we have little information concerning the relative safety of transnasal small-caliber and transoral conventional EGD in these patient groups. Elderly patients do not frequently gag or choke during transoral conventional EGD. If transoral conventional EGD is safe and comfortable for the elderly, the value of transnasal small-caliber EGD is limited, since the smaller charge-coupled device in the endoscope may potentially reduce diagnostic accuracy for minute gastric lesions. The comparative safety of the two techniques should therefore be considered carefully for the treatment of elderly patients with higher risks for gastric cancer and cardiopulmonary diseases.

We compared the safety and tolerability of transnasal and transoral EGD in patients aged > 65 years old and in chronically bedridden elderly patients who required percutaneous endoscopic gastrostomy (PEG) feeding. We used a prospective comparative study and a crossover analysis to evaluate changes in hemodynamic and pulmonary function, and the risk of endoscopy-related aspiration pneumonia during EGD by each method.

## MATERIALS AND METHODS

These following prospective and crossover studies were conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of Izumo City General Medical Center. In Study 1, we obtained written informed consent from all the participants. In Study 2, written informed consent was obtained from the key family members of the enrolled patients.

### Study 1

We compared changes in cardiopulmonary function

during unsedated transnasal small-caliber and transoral conventional EGD in patients aged > 65 years old.

**Patients:** Between July 2006 and April 2007, we enrolled 240 elderly patients (> 65 years old), who received EGD for their abdominal symptoms and for their annual medical check in the Izumo City General Medical Center, into the prospective comparison study. The first consecutive 120 patients were assigned to the transnasal small-caliber EGD (TN) group and the second consecutive 120 patients to the transoral conventional EGD (TO) group.

**Endoscopic procedure:** A single experienced endoscopist (M.Y.), certified by the Japan Gastroenterological Endoscopy Society, performed all of the examinations. Patients in the TN group were examined with a small-caliber videoendoscope (EG-530N; Fujinon Toshiba ES Systems Co. Ltd., Tokyo, Japan), 5.9 mm in diameter. Patients in the TO group were examined with a conventional endoscope (GIF-H260; Olympus Medical Systems, Tokyo, Japan), 9.8 mm in diameter. Patients who needed endoscopic biopsy and/or received a time-consuming chromoendoscopic examination were excluded from this study.

Patients in the TO group received throat anesthesia by application of 5 mL 2% lidocaine viscous (Xylocaine viscous; Astra Zeneca, Osaka, Japan) for 5 min. Nasal anesthesia was started in patients in the TN group by spraying a solution of 0.4% lidocaine and 0.5% naphazoline into the nostril. The patients were then instructed to inhale 2% lidocaine jelly (Xylocaine jelly; Astra Zeneca), and a 20 Fr catheter covered with lidocaine jelly was inserted into the deeper nasal cavity for 5 min. All the EGDs were performed with the patient in the left lateral recumbent position, and without administration of scopolamine butylbromide.

**Cardiopulmonary monitoring:** Systolic and diastolic blood pressure (BP), pulse rate (PR), and oxygen saturation by pulse oximetry (SpO<sub>2</sub>) were measured at the following four points during the endoscopic examination, to measure these parameters at the stabilized condition of patients: (1) baseline; (2) at 2 min and (3) at 5 min after endoscopic intubation; and (4) at the end of the examination. The values of BP, PR and SpO<sub>2</sub> were measured simultaneously with high sensitivity by the newly developed measuring system, Bedside Monitor PetiTelemo DS-7001 (Fukuda Denshi Co. Ltd., Tokyo, Japan). The rate-pressure product (PR × systolic BP/100) was also calculated as shown in previous reports<sup>[14,15]</sup>. Changes in each value were compared between TN and TO groups.

### Study 2

In the patients treated with PEG tube feeding, we performed periodic PEG tube exchanges during endoscopic observation every 6 mo. For these observations, an endoscopic method, transoral conventional or transnasal small-caliber was selected randomly for each patient by an envelop method, and switched at the next exchange (a

crossover design). Changes in cardiopulmonary parameters and markers of inflammation were measured during EGD, and blood leukocyte counts and serum C-reactive protein (CRP) concentrations were measured before and after EGD.

**Patients:** Between March 2007 and April 2008, 30 patients who were undergoing PEG feeding were enrolled. Reasons for PEG feeding included dysphagia caused by cerebral infarction, cerebral hemorrhage, subarachnoid hemorrhage, and other conditions. Their PEG tube exchanges were carried out routinely under endoscopic observation every 6 mo, using an instrument (transnasal small-caliber or transoral conventional endoscope) that was assigned randomly to each patient at enrollment. At the next tube exchange 6 mo later, the alternate method was used for each patient. The 30 patients were thereby divided into one initially transnasal, secondarily transoral (TN-TO,  $n = 15$ ) group, and one initially transoral, secondarily transnasal (TO-TN,  $n = 15$ ) group. In all of the enrolled patients, a bumper type kit (Ponsky Non-Balloon Replacement Gastrostomy Tube; Bard Limited, Salt Lake City, UT, USA) was used as a PEG tube. Each procedure for the PEG tube exchanges was not done when the patients had infectious diseases such as pneumonia, or urinary or hepato-biliary tract infection. Before PEG tube exchange, chest X-ray examination, urinalysis, and laboratory blood tests including peripheral blood count, inflammatory markers and hepato-biliary enzymes were done to confirm the absence of infectious diseases. In this study, PEG tube exchanges were not performed in patients who showed increases of leukocyte count or CRP and fever elevation before the procedure.

**Endoscopic procedure:** A single experienced endoscopist (M.Y., as in Study 1) performed all of the examinations, using the same endoscopes and pre-medication regimens as in Study 1. The procedure for PEG tube exchange by transnasal endoscope was similar to that by transoral endoscope. All the EGDs were performed with the patient in the supine position, and without administration of scopolamine butylbromide.

**Cardiopulmonary monitoring:** During each endoscopy procedure, systolic and diastolic BP, PR, SpO<sub>2</sub> and RPP were determined at the following three points: (1) baseline; (2) at 2 min after endoscopic intubation; and (3) at the end of the examination. Changes in each value were compared between TN and TO endoscopic studies.

**Evaluation of inflammatory response:** As markers of inflammation, body temperature, leukocyte counts and serum concentrations of CRP were measured. Body temperature was measured before and every 6 h after the endoscopy-guided exchange of PEG tube. Leukocyte counts and CRP were performed routinely before and 3 d after the procedure. If suspicious pneumonia was found, these parameters were examined more frequently. Further examination of pneumonia such as chest X-ray

**Table 1** Characteristics of patients enrolled in Study 1 (mean  $\pm$  SD)

	TN group	TO group	P value
Age (range, yr)	77.0 $\pm$ 7.7 (65-97)	76.7 $\pm$ 7.2 (65-95)	NS
Gender (M:F)	30:66	53:49	NS
Total procedure time (min)	8.46 $\pm$ 4.32	6.03 $\pm$ 2.50	0.001
Baseline cardiopulmonary parameters			
Systolic BP	131.4 $\pm$ 19.3	135.4 $\pm$ 21.8	NS
Diastolic BP	67.6 $\pm$ 12.3	70.5 $\pm$ 12.8	NS
RPP	100.2 $\pm$ 27.4	98.82 $\pm$ 25.7	NS
SpO <sub>2</sub>	98.1 $\pm$ 1.4	98.2 $\pm$ 1.4	NS

NS: Not significant; M: Male; F: Female.

examination and/or computed tomography (CT) was done when respiratory symptoms, fever elevation, and increased leukocyte count and/or CRP were found.

**Increased occurrence of aspiration pneumonia:** The incidence of aspiration pneumonia was also compared between the TN and TO groups. Pulmonary aspiration was defined by witnessed aspiration or tracheal suctioning of secretions including saliva as shown by previous studies on PEG procedures<sup>[16,17]</sup>. In patients with post-procedure elevation of inflammation markers and/or fever, aspiration pneumonia was evaluated by chest X-ray examination and/or CT. Pneumonia was diagnosed by the presence of new infiltrates of the lung associated with fever.

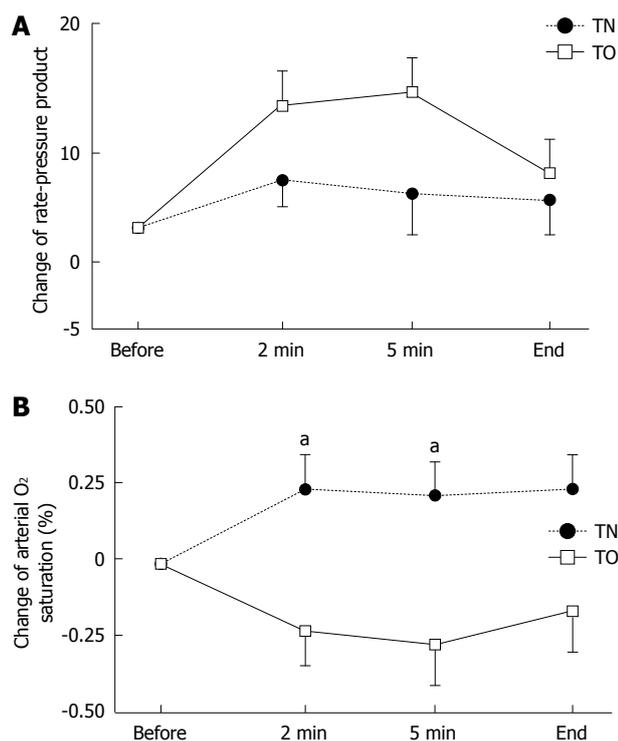
### Statistical analysis

Data for each comparison between groups were analyzed using the  $\chi^2$  and Wilcoxon signed rank tests. The latter test was done only when the Friedman test showed significant differences. Categorical data were compared using Student's *t* test or, where unequal variances occurred, Welch's test.  $P < 0.05$  was considered to be significant. All statistical analyses were performed using SPSS version 12.0 (SPSS Japan, Tokyo, Japan).

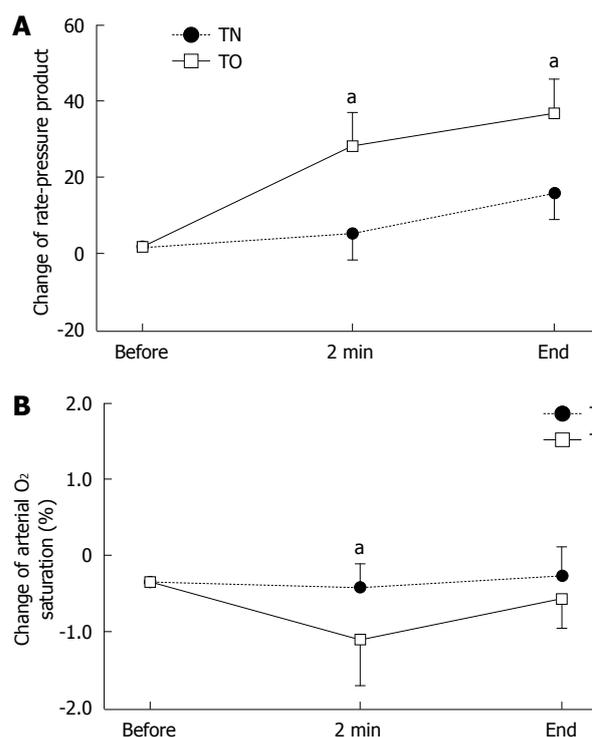
## RESULTS

### Study 1

Ninety-six and 102 elderly patients were analyzed finally as the TN and TO groups, respectively. Twenty-four and 18 patients were excluded since complete data could not be obtained, mainly because of the delay in measurement in the TN and TO groups, respectively. Characteristics of the enrolled patients in each group are shown in Table 1. Mean ages of these patients were 77.0 and 76.7 years, respectively. Total procedure time for EGD was 8.46  $\pm$  4.32 min in the TN group, and 6.03  $\pm$  2.50 min in the TO group. This difference in procedure time was statistically significant ( $P < 0.001$ ). No serious complications in cardiopulmonary function occurred in this study, although two patients experienced mild epistaxis during pre-medication for transnasal EGD. The baseline cardiopulmonary parameters, RPP and SpO<sub>2</sub>, did not show any significant differences (Table 1).



**Figure 1** Time course of changes in RPP and SpO<sub>2</sub> (Study 1). A: RPP tended to increase more in the TO group than in the TN group, although this difference did not reach statistical significance; B: SpO<sub>2</sub> fell significantly at 2 and 5 min after the start of transoral endoscopic examination. Bars show SE. <sup>a</sup>*P* < 0.05.



**Figure 2** Time course of changes in RPP and SpO<sub>2</sub> (Study 2). A: RPP increased significantly more in the TO group than in the TN group. B: SpO<sub>2</sub> fell significantly at 2 min after the start of examination in the TO group. Bars show SE. <sup>a</sup>*P* < 0.05.

	TN-TO group	TO-TN group	<i>P</i> value
Age (yr)	84.3 ± 7.9	80.7 ± 15.8	NS
Gender (M:F)	3:12	4:11	NS
Background pathological condition for PEG feeding			
Cerebral infarction	10	11	
Cerebral hemorrhage	2	3	
Subarachnoid hemorrhage	1	0	
Others	2	1	

	TN-EGD	TO-EGD	<i>P</i> value
Total procedure time (min)	4.36 ± 3.46	4.42 ± 3.18	NS
Baseline cardiopulmonary parameter			
Systolic BP	125.5 ± 25.3	122.6 ± 28.4	NS
Diastolic BP	70.1 ± 17.8	64.9 ± 20.1	NS
RPP	107.7 ± 44.6	101.3 ± 37.9	NS
SpO <sub>2</sub>	98.7 ± 1.7	95.4 ± 17.5	NS

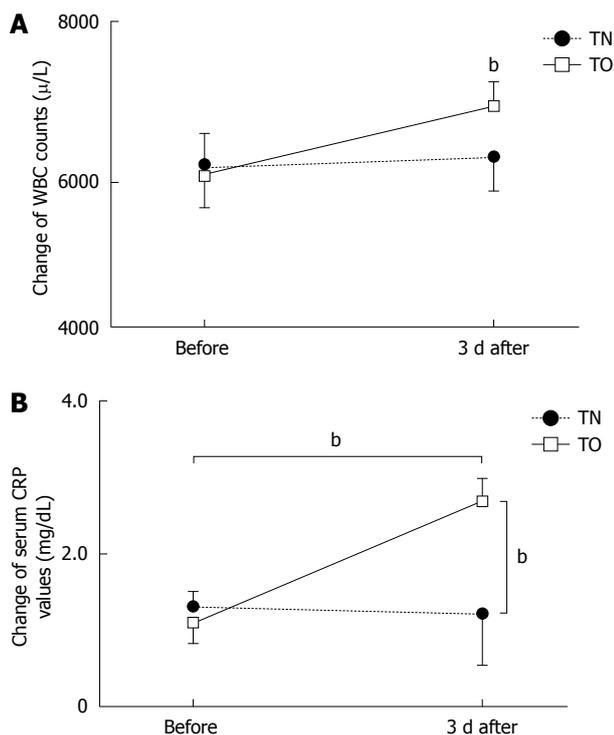
Changes in BP, PR and RPP during endoscopy did not differ significantly between the TN and TO groups, although these parameters tended to increase more in the TO group (Figure 1A). Values for SpO<sub>2</sub> in the TO group decreased significantly during EGD (-0.24% and -0.29% after 2 and 5 min) compared with those in the TN group (+0.24% and +0.18% after 2 and 5 min, *P* < 0.05). These findings suggested that transoral EGD affects pulmonary function more severely than transnasal EGD does in this group of patients (Figure 1B).

**Study 2**

We enrolled 30 patients who required PEG feeding: 15 in the TN-TO group and 15 in the TO-TN group. Characteristics of these patients are shown in Tables 2 and 3. Categorical data did not differ significantly between the TN-TO and TO-TN groups. Baseline cardiopulmonary parameters, RPP and SpO<sub>2</sub>, did not differ significantly.

Higher values of systolic and diastolic BP were found in the TO group than in the TN group after intubation for endoscopy, but these differences did not reach statistical significance. Values for RPP during and at the end of endoscopy were significantly higher in the TO group (26.8 and 34.6) than those in the TN group (3.1 and 15.2, *P* < 0.05), as shown in Figure 2A. The SpO<sub>2</sub> also significantly decreased at 2 min after the start of transoral EGD (-0.8%) in comparison with transnasal EGD (-0.1%, *P* < 0.05) (Figure 2B). Thus, in the bedridden patients with PEG feeding, examined in the supine position, transoral EGD disturbed cardiopulmonary function more strongly than did transnasal EGD.

Markers of inflammation also changed significantly in conjunction with endoscopy. Peripheral leukocyte count increased from 6053 ± 1975/L to 6900 ± 3392/L at 3 d after transoral EGD, as shown in Figure 3A (*P* < 0.001); and CRP values increased from 0.93 ± 0.24 mg/dL to 2.49 ± 0.91 mg/dL (*P* < 0.001) (Figure 3B). These data



**Figure 3** Changes in leukocyte counts (A) and CRP values (B) (Study 2). Counts and CRP increased significantly by the third day after transoral endoscopy (TO), but did not change after transnasal endoscopy (TN). Bars show SE. <sup>b</sup>*P* < 0.001.

also showed statistically significant differences at 3 d after PEG tube exchange between transnasal and transoral EGD (*P* < 0.001).

Aspiration pneumonia, possibly caused by the endoscopic examination, was subsequently found in two of 30 patients after transoral EGD. In these two cases, a significant drop in SpO<sub>2</sub> values was found during transoral EGD, and the elevation of inflammation makers (CRP values and leukocyte counts) was also found after the procedure with transoral EGD but not with transnasal EGD. Additionally, the witnessed aspiration or tracheal suctioning of secretions including saliva was observed in these two cases of transoral EGD. Both of these patients recovered after the administration of antibiotics.

## DISCUSSION

Transnasal small-caliber endoscopy, recently accepted for screening of the upper gastrointestinal tract, presents advantages apart from image quality, a noteworthy feature of transoral conventional endoscopy. Unsedated transoral EGD is well known to increase BP and PR, with an increased cardiopulmonary work load<sup>[2,4-6]</sup>. Recently, small-caliber endoscopes have been developed and used for EGD with the transnasal route. Several investigators have demonstrated that transnasal EGD with small-caliber endoscopes is feasible and tolerable<sup>[7-11]</sup>, since transnasal small-caliber EGD is less stimulative to the uvula, palatine arches and posterior part of the tongue, and does not induce gag reflex<sup>[6]</sup>. Therefore, the feasibility and tolerability of unsedated transnasal small-caliber

EGD strongly support its value as a standard screening procedure. However, the studies on transnasal small-caliber EGD were done mainly on relatively young patients aged < 60 years old. Elderly patients are believed to tolerate easily transoral EGD without excessive gagging or choking. Therefore, the clinical advantage of transnasal small-caliber EGD for this population may not outweigh the advantage of higher image quality obtained with conventional EGD. To determine the value of the transnasal small-caliber endoscope for screening older patients, we must first establish its tolerability and safety for this group. We have found no studies that specifically have addressed this issue.

In the first part of our study, we found no significant difference between transnasal and transoral endoscopy with respect to hemodynamic parameters. On the other hand, SpO<sub>2</sub>, a measure of pulmonary function, significantly declined during transoral endoscopy, although the decrease was small. The small decrease in SpO<sub>2</sub> is not clinically important for elderly patients in good physical condition. However, for elderly patients with cardiopulmonary diseases and resulting decreased basal SpO<sub>2</sub> value, further small decreases in SpO<sub>2</sub> are prone to have serious consequences. Patients long confined to bed (including most who require PEG feeding) may become susceptible to infectious pulmonary diseases as a result of swallowing disturbance or micro-aspiration that accompanies decreased SpO<sub>2</sub>. For safety in the periodic tube exchange, endoscopy-guided re-intubation of the PEG kit is recommended for these patients, because wrong replacement of the feeding tube can cause serious complications, such as peritonitis. This procedure requires that the patient remains supine, which increases the risk for aspiration of saliva and refluxed gastric contents. Transoral endoscopy may stimulate salivary secretion and thereby increase the risk of aspiration pneumonia. In addition, the supine position may influence specifically hemodynamic and/or pulmonary parameters during endoscopy. Previous studies have assessed only the hemodynamic changes that occur when PEG tube insertion is monitored with transnasal small-caliber endoscopy<sup>[18,19]</sup>. We have therefore measured pulmonary function and two markers of inflammation, as well as hemodynamic parameters, in this endoscopic examination (i.e. with the patient in the supine position). We consistently found advantages in the use of the transnasal small-caliber endoscope, with respect to both hemodynamic and pulmonary parameters. Significant increases in leukocyte counts and CRP values, which indicate systemic inflammatory disease, occurred only after transoral conventional endoscopy. Aspiration pneumonia was found in two cases after 30 transoral endoscopic procedures for the replacement of the PEG tube. PEG tube exchange is done in the supine position, therefore, the patients may have a higher risk of aspiration. However, no pneumonia was found after the procedure using the transnasal small-caliber endoscope. The transoral conventional endoscope may stimulate salivary secretion and further increase the risk of aspiration. Additionally, the oral cavity and saliva of bedridden patients with PEG feeding is prone to be infected by bacteria that may cause pneumonia. Use of the transnasal small-

caliber endoscope may therefore reduce the risk of this potentially serious complication of transoral conventional EGD in bedridden patients.

In conclusion, SpO<sub>2</sub> decreased significantly in elderly patients during transoral conventional EGD, but not during transnasal small-caliber EGD. Compared to transoral conventional EGD, transnasal small-caliber endoscopy may reduce the risk of aspiration pneumonia, when the patient must be examined in the supine position. Therefore, transnasal small-caliber EGD is a safer method than transoral conventional EGD in critically ill patients, such as those who are bedridden and undergoing PEG feeding.

## COMMENTS

### Background

Unsedated transnasal small-caliber esophagogastroduodenoscopy (EGD) is often used to examine the upper gastrointestinal tract. Its efficacy for elderly and critically ill patients, however, has not been fully evaluated. To evaluate the tolerability of transnasal EGD for elderly and critically ill bedridden patients, a prospective randomized comparative and a crossover study were undertaken.

### Research frontiers

Elderly patients, including those who are bedridden, do not frequently gag or choke during transoral conventional EGD. If transoral conventional EGD is safe and comfortable for them, the value of transnasal small-caliber EGD could be limited, because the smaller charge-coupled device in the endoscope may potentially reduce diagnostic accuracy for minute gastric lesions. This is believed to be the first published study concerned with the tolerability and safety of transnasal small-caliber endoscopy for critically ill patients.

### Innovations and breakthroughs

Other studies evaluating the tolerability and safety of transnasal endoscopy have focused mainly on younger patients, with a mean age < 50 years old. The data and viewpoints originated from elderly and critically ill patients and have not been published elsewhere.

### Applications

Significant decreases were observed in SpO<sub>2</sub> saturation during conventional transoral EGD, but not during transnasal small-caliber EGD. Significant increases were also found in the markers of inflammation in bedridden patients after transoral conventional EGD, but not after transnasal small-caliber EGD performed with the patient in the supine position.

### Peer review

This is a prospective study that compared the consequences of using two endoscopic devices (conventional and small caliber) in critically ill patients. The importance of this study is related to the fact that the use of small caliber endoscopes in diagnosis is becoming more common because of its tolerability and lack of sedation.

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BRIEF ARTICLE

## Association between MDM2-SNP309 and hepatocellular carcinoma in Taiwanese population

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of MDM2-SNP309 *vs* wild-type T/T genotype in patients with HCC was not significant (OR = 1.265, 95% CI = 0.074-21.77) after adjustment for sex, hepatitis B or C virus infection, age, and cardiovascular disease/diabetes. Nevertheless, there was a trend that GG genotype of MDM2-SNP309 might increase the risk in HCC patients infected with hepatitis virus (OR = 2.568, 95% CI = 0.054-121.69). Besides, the homozygous MDM2-SNP309 genotype did not exhibit a significantly earlier age of onset for HCC.

**CONCLUSION:** Current data suggest that the association between MDM2-SNP309 GG genotype and HCC is not significant, while the risk may be enhanced in patients infected by hepatitis virus in Taiwan.

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**Key words:** MDM2 protein; Hepatocellular carcinoma; Taiwan; Tumor suppressor protein p53

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### Abstract

**AIM:** To investigate the risk association and compare the onset age of hepatocellular carcinoma (HCC) patients in Taiwan with different genotypes of MDM2-SNP309.

**METHODS:** We analyzed MDM2-SNP309 genotypes from 58 patients with HCC and 138 cancer-free healthy controls consecutively. Genotyping of MDM2-SNP309 was conducted by restriction fragment length polymorphism assay.

**RESULTS:** The proportion of homozygous MDM2-SNP309 genotype (G/G) in cases and cancer-free healthy controls was similar (17.2% *vs* 16.7%). Multivariate analysis showed that the risk of G/G genotype

### INTRODUCTION

Hepatocellular carcinoma (HCC) is a prevalent type of cancer. It represents the fifth most prevalent cancer worldwide, and accounts for the top three causes of death in the Asia-Pacific region<sup>[1,2]</sup>. The risk factors associated with HCC include age, sex, alcohol, diet, and infection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV)<sup>[2]</sup>. Most newly diagnosed HCC is reported in Asia (> 70%), in which chronic HBV infection accounts for 75% of cases worldwide<sup>[3]</sup>. Significantly, 55% of HCC cases are reported from the Chinese population<sup>[4]</sup>. Although the incidence rate of HCC is plausibly linked to geographic area and geo-economic conditions, it is possible that gene polymorphism may be associated with the risk of HCC<sup>[5,6]</sup>.

Single nucleotide polymorphism (SNPs) occur frequently in human genomes. SNPs can be detected once per 1000 bp in DNA sequences, on average, and may affect gene transcription and amino acid composition if they are located in gene regions. Several lines of evidence have shown that SNPs in genes such as small inducible cytokine B14 precursor (*SCYB14*), glial cell-derived neurotrophic factor family receptor  $\alpha$  1 (*GFR $\alpha$ 1*), corticotropin-releasing hormone receptor 2 (*CRHR2*), glucose-regulated protein 78 (*GRP78*), heat shock protein A1B (*HSPA1B*), DNA-methyltransferase-3B (*DNMT3B*),  $\alpha$ -fetoprotein (*AFP*) and *p53* R72P are associated significantly with HCC<sup>[6-12]</sup>. These SNPs localize in promoter regions, coding sequences, or even introns of individual genes, which suggests that the expression level and functions of affected genes can influence the incidence rate of HCC.

MDM2 oncoprotein is a direct negative regulator for the p53 tumor suppressor protein, which accounts for 50% of human cancers if deleted or with loss-of-function<sup>[13,14]</sup>. Overexpression of MDM2 by up to fourfold in transgenic mice that harbor wild-type p53 leads to complete tumorigenesis<sup>[15]</sup>. MDM2 overexpression also is associated with poor survival and is a useful predictive factor for poor prognosis in humans with liver cancer<sup>[16,17]</sup>. A genetic polymorphism located in intron 1 of the MDM2 gene, so called MDM2-SNP309 (a change from T to G, rs2279744) can enhance the binding of Sp1 general transcription factor to this promoter region and increase MDM2 gene transcription<sup>[18]</sup>. It has been suggested that this SNP is associated with the risk and early onset age of various human cancers<sup>[19]</sup>. Some studies have shown that MDM2-SNP309 is associated with the risk of HCC in Japanese and Moroccan patients with chronic hepatitis C, and Korean patients with chronic hepatitis B<sup>[20-22]</sup>. Although > 50% of HCC cases are reported from Asia, it remains largely unknown whether MDM2-SNP309 influences the risk and onset age of HCC in other countries in this region, except for Japan and Korea.

In this study, we initiated a hospital-based case-control study to investigate the risk association between MDM2-SNP309 and HCC in Taiwanese patients. We also examined whether HCC onset was earlier in patients with homozygous MDM2-SNP309 (G/G) compared to wild-type MDM2-SNP309 (T/T).

## MATERIALS AND METHODS

### **Patients and cancer-free healthy controls**

We studied 58 patients with HCC diagnosed by cancer specialists, and 138 cancer-free healthy adults enrolled from Taipei City Hospital Ren Ai Branch, Taiwan during 2007. All volunteers signed the consent form and filled out the structured questionnaire before providing their blood samples. All patients and 50 cancer-free healthy controls were tested for hepatitis B and C by anti-HBsAg, HBsAg, Anti-HBc IgG, and anti-HCV. Several risk factors associated with HCC were included in the questionnaire, including age, sex, alcohol intake, frequency of exercise, and cardiovascular diseases/diabetes. There was no other cancer type diagnosed in each patient. The sub-

jects were born in the Taiwan Island except five patients and four cancer-free healthy controls who had emigrated from mainland China. This study was approved by institutional review committee board of National Yang-Ming University and Taipei City Hospital.

### **MDM2-SNP309 genotyping**

Analysis of MDM2-SNP309 genotyping was conducted by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), as described previously<sup>[23]</sup>. Genomic DNA was extracted from 200  $\mu$ L whole blood sample using the Qiagen Mini Blood DNA Extraction kit (Valencia, CA, USA). The DNA fragment that contained the MDM2 SNP309 was amplified by PCR using the forward (5'-CGGGAGTTCAGGGTAAAGGT-3') and reverse primer (5'-AGCAAGTCCGGTGCTTACCTG-3') (Protech Inc., Taipei, Taiwan). Each PCR reaction was conducted using 100 ng genomic DNA, 0.2  $\mu$ mol/L primer, 200  $\mu$ mol/L dNTP, 1.5 mmol/L MgCl<sub>2</sub>, 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl and 1 U Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA). The thermal cycler conditions were 94°C for 1-5 min; 40 cycles with denaturing at 94°C, annealing at 59°C, and elongation at 72°C for 30 s each; one cycle at 72°C for 10 min. Subsequently, 10  $\mu$ L of the PCR product was digested with 1 U *Msp*A1I restriction enzyme (New England Biolabs, Ipswich, MA, USA) at 37°C for 30-60 min. The T/T, T/G and G/G genotypes were identified as 233 bp/88 bp, 233 bp/187 bp/88 bp, and 187 bp/88 bp running on the 3% NuSieve agarose gel, respectively. The genotypes were confirmed by direct sequencing of the PCR products by the Sequencing Core Facility of Genomic Research Center in National Yang-Ming University.

### **Statistical analysis**

Whether the frequency of MDM2-SNP309 genotype obeyed the Hardy-Weinberg equilibrium was determined by an on-line public statistical tool (<http://www.genes.org.uk/software/hardy-weinberg.shtml>). Two-sample *t* test was used to evaluate the difference in age and body mass index (BMI) between cases and controls. Differences in sex, hepatitis frequency, HBV and HCV infection, alcohol intake, exercise, and incidence of cardiovascular diseases and diabetes between cases and controls were determined by Fisher's exact test. Multivariate logistic regression analysis was used to calculate the OR and 95% CI to determine the association between HCC risk and MDM2-SNP309 genotypes. The Kaplan-Meier survival analysis was used to describe the onset age of HCC, and the log-rank test was used to compare the median onset age between patients with GG and those with TT genotypes in MDM2 SNP309. *P* < 0.05 was considered statistically significant in all tests. All statistical analyses were performed with Statistical Analysis System ver. 9.1 (SAS Institute, Cary, NC, USA).

## RESULTS

We investigated 58 HCC patients and 138 cancer-free healthy controls to evaluate the risk association between

**Table 1** The demography of HCC patients and cancer-free healthy controls from Taiwan *n* (%)

Characteristics <sup>1</sup>	Cases (58)	Controls (138)	<i>P</i> value
Age (yr)			
mean	65.90	40.20	< 0.0001
SD	10.14	15.24	
Gender			
Male	45 (77.6)	42 (30.2)	< 0.0001
Female	13 (22.4)	96 (69.8)	
Hepatitis			
Yes	49 (84.5)	10 (7.2)	< 0.0001
No	9 (15.5)	79 (57.25)	
Unknown	0	49 (35.5)	
HBV <sup>2</sup>			
+	29	10	0.0002
-	28	40	
HCV <sup>3</sup>			
+	20	0	< 0.0001
-	37	50	
Alcohol intake			
Yes	26 (44.8)	81 (58.7)	0.0851
No	32 (55.2)	57 (41.3)	
Cardiovascular diseases and/or disorders			
Yes	23 (39.7)	26 (18.7)	0.0035
No	35 (59.6)	116 (81.3)	
BMI	22.7 ± 3.13	22.59 ± 3.44	0.8341

<sup>1</sup>Age and BMI (Body Mass Index) were determined by *t* test. Other parameters were analyzed by Fisher's exact test; <sup>2,3</sup>Two HCC cases were infected with both HBV and HCV. HCC: Hepatocellular carcinoma.

MDM2-SNP309 and HCC. The characteristics of these blood donors are summarized in Table 1. The mean ages were significantly different between cases and controls ( $65.9 \pm 10.14$  years *vs*  $40.2 \pm 15.24$  years) at the time that they joined this study. These participants were enrolled consecutively without pre-selection for age, therefore, this difference may confirm that HCC usually is found in elderly individuals. In HCC patients, > 80% had been infected with HBV or HCV, and two cases were infected with both HBV and HCV. In addition, the frequency of HCC patients infected with HBV was slightly higher than that of those infected with HCV. Other factors that may affect the incidence of HCC are also summarized in Table 1. Except for BMI and alcohol intake, other confounding factors exhibited a significant difference between HCC cases and cancer-free healthy controls. They were adjusted for the multivariate logistic regression model thereafter.

The frequency of MDM2-SNP309 distributed in wild-type (TT), heterozygous (TG) and homozygous (GG) genotypes are shown in Table 2. The genotype distributions in HCC cases and cancer-free healthy controls slightly departed from Hardy-Weinberg equilibrium (HCC,  $\chi^2 = 4.426$ ,  $P = 0.035$ ; controls,  $\chi^2 = 3.907$ ,  $P = 0.048$ ). It is impossible to predict the genotype of each blood donor, therefore, it is expected that this inconsistency may have been due to the small sample size. Although the frequency of wild-type MDM2-SNP309 genotype was lower in HCC cases than in cancer-free healthy controls, there was no significant difference found in the frequency of homozygosity between these two groups (Table 2). To determine the risk association between HCC and MDM2-SNP309

**Table 2** The risk evaluation of MDM2 SNP309 genotypic frequencies on the development of HCC *n* (%)

SNP309	Cases	Controls	OR (95% CI) <sup>1</sup>	<i>P</i> value
TT	11 (19)	35 (25.3)	1	
TG	37 (63.8)	80 (58)	1.016 (0.152-6.8)	0.99
GG	10 (17.2)	23 (16.7)	1.265 (0.074-21.767)	0.87
TG+GG	47 (81)	103 (57.5)	1.037 (0.757-6.862)	0.97
TT+TG	48 (82.8)	115 (83.3)	1	
GG	10 (17.2)	23 (16.7)	1.25 (0.123-12.66)	0.85

<sup>1</sup>The multivariate logistic regression model was used to calculate odds ratio adjusted for gender, infection of hepatitis virus (B or C type), age, cardiovascular disease and/or diabetes.

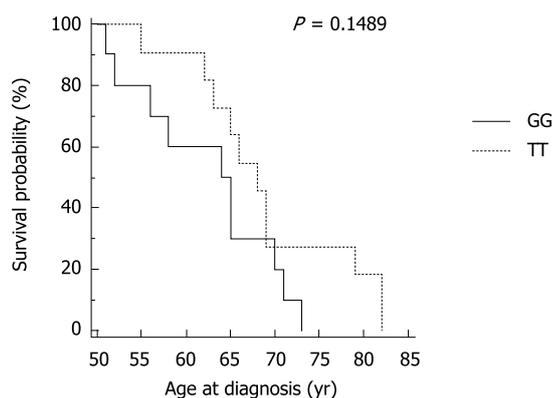
**Table 3** Estimation of odds ratio for different genotypes of MDM2-SNP309 in hepatitis viral infected patients with HCC *n* (%)

Hepatitis <sup>1</sup>	SNP309	Cases	Controls	OR <sup>2</sup>	95% CI	<i>P</i> value
Positive	TT	7 (14.3)	1 (10)	1.000		
	TG	32 (65.3)	7 (70)	2.340	0.11-49.655	0.59
	GG	10 (20.4)	2 (20)	2.568	0.054-121.687	0.63
	TG+GG	42 (85.7)	9 (90)	2.376	0.115-48.896	0.57
Negative	TT	4 (44.4)	19 (24)	1.000		
	TG	5 (55.6)	45 (57)	0.512	0.046-5.712	0.59
	GG <sup>3</sup>	0 (0)	15 (19)	-	-	-
	TG+GG	5 (55.6)	60 (76)	0.470	0.041-5.387	0.54

<sup>1</sup>Include HBV or HCV infected patients with HCC; <sup>2</sup>OR was adjusted for age, gender and cardiovascular diseases and/or diabetes; <sup>3</sup>OR was not estimated because the number in case was zero.

genotypes, multivariate logistic regression analysis showed that the OR of heterozygous (TG) and homozygous (GG) SNP309 genotypes was 1.016 (95% CI = 0.152-6.8,  $P = 0.987$ ) and 1.265 (95% CI = 0.074-21.767,  $P = 0.87$ ) compared to wild-type SNP309 genotype, respectively (Table 2). These OR values were adjusted for age, sex, infection with HBV or HCV, cardiovascular disease and/or diabetes. Comparison of homozygous MDM2-SNP309 (GG) genotype with common (TT) or heterozygous (TG) MDM2-SNP309 carriers showed that the adjusted OR was 1.247 (95% CI = 0.123-12.66,  $P = 0.852$ ). The TG or GG genotype of MDM2-SNP309 versus TT variant form exhibited an adjusted OR of 1.037 (95% CI = 0.757-6.682,  $P = 0.97$ ) (Table 2). These OR values are lower than those reported previously in Japanese, Korean and Moroccan populations<sup>[20-22]</sup>. Thus, current statistical results showed that risk association between MDM2-SNP309 genotype and HCC was not significant in the Taiwanese population.

We next investigated whether HBV or HCV infection influenced the effects of MDM2-SNP309 genotypes on patients with HCC. As shown in Table 1, 49 cases infected with HBV or HCV were analyzed by the logistic regression model. It revealed that the homozygous (GG) MDM2-SNP309 genotypes exhibited increased risk over the common (TT) genotype of MDM2-SNP309 (adjusted OR = 2.568, 95% CI = 0.054-121.687,  $P = 0.632$ ) (Table 3). The adjusted OR of TG or GG of MDM2-SNP309 genotype was 2.376 compared to TT genotype (95% CI =



**Figure 1** Comparison of age at diagnosis in HCC patients with wild-type (TT) and homozygous (GG) MDM2-SNP309 by the Kaplan-Meier method and the log-rank test. The survival fraction was the cumulative case-free survival rate against age at diagnosis of patients with HCC with different MDM2-SNP309 genotypes.

0.115–48.896,  $P = 0.575$ ). The broad range of 95% CI was due to the small sample size, while it seems plausible that the risk association between MDM2-SNP309 and patients with HCC may have been enhanced by viral hepatitis.

We compared the age at diagnosis for HCC patients who had different MDM2-SNP309 genotypes, and the median ages for TT, TG and GG genotypes were 68 (range 55–82), 66 (range 38–87) and 64.5 (range 51–73) years old, respectively. The arithmetic mean ages and SD for TT, TG and GG were  $69.1 \pm 8.23$ ,  $64.3 \pm 11.1$ , and  $62.5 \pm 7.88$  years old, respectively. Although the mean age of patients with homozygous MDM2-SNP309 genotype (GG) was 6.6 years lower than that of patients with wild-type MDM2-SNP309 genotype (TT), there was no significant difference between them by *t* test ( $P = 0.0844$ , 95% CI = -0.9835–14.1654). Besides, the Kaplan-Meier survival analysis showed that comparison of the age at diagnosis for HCC patients with GG and TT genotypes was not significantly different by log-rank test ( $P = 0.1489$ , Figure 1). Therefore, the current results suggest that MDM2-SNP309 is not associated with onset age of HCC in the Taiwanese population. However, a larger sample size may be necessary to confirm this observation.

## DISCUSSION

HCC commonly occurs in the Asia-Pacific region, and it also accounts for high morbidity and mortality in this area<sup>[2]</sup>. Although the incidence rate of male HCC patients is high in the Asia-Pacific region (14–36 per 100 000 men), it exhibits significant variance in different countries. The highest incidence rate of male HCC occurs in China and Taiwan, which is 58 and 53 per 100 000 men, respectively<sup>[2]</sup>. The main etiological agents that result in high incidence of HCC in the Asia-Pacific region include high infection rate with HBV and HCV, cirrhosis, family history, environmental contamination, diet, and  $\alpha$ -fetoprotein<sup>[4,24,26]</sup>. MDM2-SNP309 is a novel predictor for Japanese and Korean patients with HCC infected by HCV and HBV, respectively<sup>[20,21]</sup>. In this study, we examined the risk association between Taiwanese HCC

patients and MDM2-SNP309. Although the multivariate analysis showed that the association between MDM2-SNP309 and HCC in the Taiwanese population was not significant, there was a trend that homozygous (GG) or heterozygous (TG) MDM2-SNP309 genotype exhibited a higher risk in the subset of HCC patients infected with HBV or HCV. This is consistent with previous reports in Japanese and Korean patients with HCC, at least in part. Besides, our data failed to prove that MDM2-SNP309 could accelerate the development of HCC, although the median age at diagnosis of patients with homozygous MDM2-SNP309 genotype (GG) was 3.5 years lower than that of patients with the common genotype (TT). This result also agrees with the studies in the Japanese and Moroccan but not in the Korean population<sup>[20–22]</sup>. Therefore, the risk association and the effects of age onset between MDM2-SNP309 genotypes and HCC are likely to be dependent on the selected patient subgroups.

MDM2 negatively regulates p53 tumor suppressor protein. It is reasonable to expect that over-produced MDM2 will repress p53 function on cancer prevention. This hypothesis has been demonstrated in an MDM2 overexpression transgenic mouse model that develops systemic tumors. The putative effect of MDM2-SNP309 is to enhance the transcription of the MDM2 gene, and to affect the p53 regulatory pathway for tumor development. Several lines of evidence have demonstrated an association between MDM2-SNP309 and various sporadic or hereditary human cancers, including risk and earlier age onset. Nevertheless, non-supportive data are also reported to disagree that there is a risk association between MDM2-SNP309 and human cancers, even when the same cancer types are studied<sup>[27–36]</sup>. One of the reasons is likely to be the different subgroups of patients and races. For instance, a recent meta-analysis has demonstrated that the G allele of MDM2-SNP309 may affect breast cancer in the Chinese population rather than non-Chinese population<sup>[37]</sup>. Although HCC is a common cancer type in the Asia-Pacific region, its association with MDM2-SNP309 has only been reported in Japanese patients with chronic hepatitis C and in Korean patients chronically infected with hepatitis B virus<sup>[20,21]</sup>. HCC has the second highest cancer mortality in Taiwanese subjects who are vulnerable to be infection with HBV or HCV (<http://crs.cph.ntu.edu.tw/>). To the best of our knowledge, this is the first study to investigate the Taiwanese population to analyze the effects of MDM2-SNP309 genotypes on HCC development.

The sample size is the primary limitation of our study. The participants were from a single hospital, and most patients who suffered from HCC had a lack of enthusiasm to provide blood samples for analysis. Besides, the age distribution of cases and cancer-free healthy controls was significantly different. The patients were enrolled consecutively, therefore, this bias was not due to selection and was adjusted for in multivariate analysis. Another limitation is that sex distribution and incidence of hepatitis between cases and controls were not comparable. In HCC patients, the ratio of men to women was 3.46, and 84.5% of patients also had hepatitis. The mean ages at diagnosis for HBV- and HCV-infected HCC patients were  $59.53 \pm$

10.72 years and  $70.63 \pm 6.39$  years, respectively ( $P = 0.0002$ ). Male HCC patients were infected mainly by HBV (64.9%), while female HCC patients were infected by HCV (66.7%). Furthermore, the male/female ratio was 6.0 for HCC patients with HBV infection, while it was 1.375 for those with HCV infection. All of these results are similar to a previous study of 18 423 Taiwanese HCC patients enrolled from 1981 to 2001<sup>[2,38]</sup>. Therefore, the characteristics of HCC patients did not exhibit a significant discrepancy compared to a large cohort study in Taiwan, even though a small sample size was used in this study.

Several potential risk factors were also considered in this study. Frequent alcohol intake is considered to be an etiological agent for the Chinese population ( $OR = 1.88$ )<sup>[4]</sup>. In our study, there was no significant difference between cases and controls regarding alcohol intake. However, identification of alcohol intake is dependent on self-interpretation by participants, and it may lead to apparent bias. It has been reported that metabolic syndrome, such as obesity and diabetes may affect the incidence of HCC<sup>[39-41]</sup>. We adjusted for these factors in the multivariate logistic regression model to evaluate the risk association between MDM2-SNP309 and HCC in the Taiwanese population. Nevertheless, it remains important to collect more samples to evaluate the potential risk factors for HCC in the presence of various MDM2-SNP309 genotypes in the future.

In summary, this hospital-based case-control study showed that there was no significant association between MDM2-SNP309 and HCC in the Taiwanese population. In the subset with hepatitis B or C, the homozygous or heterozygous MDM2-SNP309 genotype tended to influence the incidence rate of HCC. Homozygous MDM2-SNP309 genotype did not significantly accelerate the development of HCC, even though the median age at diagnosis of patients with homozygous SNP309 was 3.5 years lower than that of patients with wild-type SNP309. In the future, we expect to use a larger sample size to further confirm the effect of MDM2 SNP309 on HCC in the Taiwanese population.

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## COMMENTS

### Background

The incidence and mortality rate of hepatocellular carcinoma (HCC) are among the highest in Taiwanese cancer patients according to the data from the Taiwan Cancer Registry (<http://crs.cph.ntu.edu.tw/main.php?Page=N1>). Recently, it has been reported that a single nucleotide polymorphism (SNP) in the promoter region of MDM2 oncogene, MDM2-SNP309, is associated with the risk of HCC in Japanese and Korean patients infected with hepatitis C and hepatitis B virus, respectively. However, it remains unclear whether this observation commonly occurs in other neighboring Asian populations.

### Research frontiers

To the best of our knowledge, this is the first study to investigate the associa-

tion between MDM2-SNP309 and HCC in Taiwanese, a population close to the Han Chinese, which also has a high incidence and mortality of HCC. The data showed that there was no significant association between HCC risk and MDM2-SNP309 in the Taiwanese population, while the association tended to increase in patients with hepatitis B or C virus infection.

### Innovations and breakthroughs

The incidence and mortality rate of HCC in Taiwan are comparable to those in Japan and Korea. Previous studies have shown that MDM2-SNP309 is associated with the risk of HCC in Japanese patients with chronic hepatitis C, and Korean patients with chronic hepatitis B. In this study, the authors demonstrated that MDM2-SNP309 genotype may not affect the risk of HCC in the Taiwanese population, except in those with a history of viral hepatitis, regardless of whether hepatitis B or C. This conclusion may be important for previous research groups to reevaluate whether hepatitis can increase the risk effect of MDM2-SNP309 on HCC. It is also an important parameter for other research groups that are dedicated to investigate the association between HCC and genetic polymorphism in the MDM2 gene and its related signaling pathways.

### Applications

The results are expected to provide information about MDM2-SNP309 genotyping for routine health and newborn screening for HCC in subjects with or without viral hepatitis.

### Terminology

MDM2-SNP309 is a genetic polymorphism that corresponds to nucleotide 309, starting from first nucleotide of intron 1 of the MDM2 gene.

### Peer review

This was a well-designed and well-conducted study on the association between polymorphisms in MDM2 gene promoter and HCC. The results are clearly presented and discussed. These results may help dissect the molecular alterations involved in HCC development in Taiwan.

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BRIEF ARTICLE

## A better parameter in predicting insulin resistance: Obesity plus elevated alanine aminotransferase

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with insulin resistance. The effects are synergistic. Coexistence of them is better than metabolic syndrome in predicting insulin resistance.

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Chen PH, Chen JD, Lin YC. A better parameter in predicting insulin resistance: Obesity plus elevated alanine aminotransferase. *World J Gastroenterol* 2009; 15(44): 5598-5603 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5598.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5598>

### Abstract

**AIM:** To investigate the association of obesity and elevated alanine aminotransferase with insulin resistance and compare these factors with metabolic syndrome.

**METHODS:** We enrolled a total of 1308 male workers aged from 22 to 63 years. Data was extracted from the workers' periodic health check-ups in hospitals. All cases were from the community of northern Taiwan. This was a cross-sectional observational study from July to September in 2004. We grouped all cases into four groups, based on the quartile of homeostasis model assessment. The top fourth quartile group was defined as the group with insulin resistance. We performed multivariate logistic regression analysis for the odds ratio of the risk factors for insulin resistance.

**RESULTS:** Compared with metabolic syndrome, the coexistence of both factors had a 4.3-fold (95% CI: 2.7-6.8) increased risk, which was more than metabolic syndrome with a 3.6-fold (95% CI: 2.6-5.0) increased risk. The two factors had a synergistic effect. The synergistic index of obesity and elevated alanine aminotransferase (ALT) was 2.1 (95% CI: 1.01-4.3).

**CONCLUSION:** Obesity and elevated ALT are associated

### INTRODUCTION

Insulin resistance is a key feature of type 2 diabetes mellitus and it plays an important role in cardiovascular diseases<sup>[1]</sup>. Predicting insulin resistance is useful in preventing diabetes or cardiovascular disease. Insulin resistance can be directly measured with a hyperinsulinemic euglycemic clamp<sup>[2]</sup>. In addition, several parameters from less invasive methods have been demonstrated to be related to, or to predict, insulin resistance, such as obesity<sup>[3-5]</sup>, alanine aminotransferase (ALT) level<sup>[6]</sup>, metabolic syndrome, chronic inflammation status<sup>[5]</sup>, nonalcoholic fatty liver disease (NAFLD)<sup>[7,8]</sup>, and adiponectin level<sup>[9]</sup>. Some of these factors are easily measured (e.g. body weight and ALT) and the others are more complex (e.g. metabolic syndrome), not available in most laboratories (e.g. adiponectin), or are technique-dependent (e.g. sonographic NAFLD).

In Taiwan, the national health program provides a periodic examination for every adult older than forty. The program includes measurements of body height/weight, and ALT level. Measurement of insulin resistance, however, is not included in the program because of its inconvenience, although it is important for preventing cardiovascular disease.

Obesity is an ongoing problem in Taiwan and world-

wide<sup>[10,11]</sup>. It is associated with several conditions, of which insulin resistance might be the most important<sup>[12]</sup>.

Alanine aminotransferase (ALT) is a widely used laboratory test and abnormal ALT levels are common<sup>[13,14]</sup>. Elevated ALT has also been noted to be related to insulin resistance.

Besides being non-invasive, both obesity and ALT measurements are easily obtained and are inexpensive. Our purpose was to confirm the belief that that obesity and elevated ALT are important parameters of insulin resistance. We reported that the coexistence of both factors has a synergistic effect and the coexistence might be better than metabolic syndrome for predicting insulin resistance. The possible mechanism will be explored in the article. This result is helpful for preventing cardiovascular disease in public health.

## MATERIALS AND METHODS

Data from a total of 1308 male workers' health check-up records were analyzed. Data were collected *via* a questionnaire, including age (years), smoking status (packs per day), and alcohol consumption status (times per week). Anthropometrical data collected included systolic and diastolic blood pressure (mmHg), body weight (kg), height (cm), calculated body mass index, BMI (kg/m<sup>2</sup>), and waist circumference (cm). The laboratory measurements were conducted by Beckman Coulter auto-analyzer (model DxC 800 Synchron), and included triglyceride (mg/dL), high-density lipoprotein (HDL, mg/dL), aspartate aminotransferase (AST, U/L), alanine aminotransferase (ALT, U/L), and fasting plasma glucose (mg/dL). The insulin level (U/mL) was measured by the Abbott autoanalyzer (model AxSym). Insulin resistance was defined as the top quartile of HOMA<sub>IR</sub> (homeostasis model assessment)<sup>[2]</sup>. Obesity was defined as BMI  $\geq 27$  according to the modified criteria in Taiwan<sup>[11]</sup>. Abdominal obesity (or central obesity) was defined as a waist circumference  $\geq 90$  cm in men, according to the modified criteria from ATPIII for Asia subjects<sup>[15]</sup>. Elevated blood pressure, elevated triglyceride, low high-density lipoprotein, and elevated fasting glucose were defined based on previous ATPIII criteria for metabolic syndrome as systolic blood pressure  $\geq 130$  mmHg or diastolic blood pressure  $\geq 85$  mmHg, triglyceride  $\geq 150$  mg/dL, high density lipoprotein  $< 40$  mg/dL, and fasting glucose  $\geq 100$  mg/dL. Elevated ALT was defined as ALT  $> 40$  U/L (according to our laboratory and any cause, such viral hepatitis or alcoholism, was not ruled out). Smoking was defined as smoking more than one pack per day. Alcohol consumption was defined as drinking more than once per week. The Student's *t* test and chi-square test were used for analyzing continuous variables and categorical variables, respectively. Multivariate logistic regression was used to evaluate the relationship between insulin resistance and the risk factors. We introduced three models for multivariate logistic regression analysis for the odds ratio of the risk factors for insulin resistance. Model 1 showed the odds ratio of the risk factors

among which metabolic syndrome is regarded as five separate components. Metabolic syndrome is regarded as a single factor in model 2. Elevated ALT and obesity are regarded as two separate risk factors in model 1 and model 2. The coexistence of the two factors is regarded as a single factor and is compared with metabolic syndrome in model 3. To understand the synergistic effect of the risk factors, we introduced the synergistic index to see if the synergistic effect of obesity and elevated ALT was statistically significant<sup>[16]</sup>. This analytical study, limited to health check-up data, followed all the ethical criteria for human research<sup>[17]</sup>. The study protocol (TYGH09702108) was reviewed and approved by the Ethics Committee of the Taoyuan General Hospital.

## RESULTS

A total of 1308 male examinees were enrolled in the present study. The baseline characteristics of the study population stratified by HOMA<sub>IR</sub> are presented in Table 1. The mean age of the total population was 38.7 years (ranging from 22-63 years); increasing age with the higher HOMA<sub>IR</sub> was noted. A similar increasing trend was also presented in the other parameters except that a decreasing trend was noted in the HDL parameter. We also examined the significance of the trend of the increasing ALT level with insulin resistance through ANOVA analysis.

The abnormality prevalence of the four stratified categories according to the HOMA quartile, and the whole samples, are shown in Table 2. Similarly, the abnormality prevalence rates of all parameters (except smoking and drinking) increase with the higher HOMA<sub>IR</sub>.

In Table 3, after controlling for age, metabolic syndrome, hypercholesterolemia, elevated low-density lipoprotein, drinking, and smoking status, obesity has a 2.5-fold (95% CI: 1.7-3.6) and elevated ALT has a 2.1-fold (1.4-3.0) increased risk of insulin resistance in model 1. Each of these is more than at least three components of metabolic syndrome (abdominal obesity, elevated blood pressure, and hypo-HDL cholesterolemia). In model 2, obesity has a 3.0-fold (2.1-4.2) and elevated ALT has a 2.1-fold (1.5-2.9) increased risk of insulin resistance. Metabolic syndrome has a 2.6-fold (1.8-3.7) increased risk. On their own, both risk factors are similar to, or even more significant than metabolic syndrome. In model 3, we compared the coexistence of obesity and elevated ALT with metabolic syndrome. The coexistence of the two factors has a 4.3-fold (2.7-6.8) increased risk, far more than metabolic syndrome that has a 3.6-fold (2.6-5.0) increased risk. To examine the relationship between obesity and elevated ALT, we presented the synergistic effect of both factors in Table 4. It showed the synergistic index is 2.1 and it is statistically significant (95% CI: 1.0-4.3). Different odds ratios of obesity and elevated ALT in Tables 3 and 4 were noted due to different controlling factors.

**Table 1 Basic characteristics of subjects, stratified by HOMA<sub>IR</sub> (mean ± SD)**

	The four sub-groups stratified by HOMA <sub>IR</sub> <sup>1</sup>				Total (1308, 100%)
	Q1 (327, 25%)	Q2 (326, 25%)	Q3 (328, 25%)	Q4 (327, 25%)	
Age (yr)	38.1 ± 10.0	38.5 ± 9.7	38.7 ± 10.0	39.7 ± 10.3	38.7 ± 10.0
Systolic blood pressure (mmHg)	124.4 ± 13.9	125.8 ± 14.7 <sup>a</sup>	128.0 ± 15.1 <sup>a</sup>	132.5 ± 16.0 <sup>a</sup>	127.7 ± 15.2
Diastolic blood pressure (mmHg)	77.3 ± 10.0	77.6 ± 10.4 <sup>a</sup>	79.6 ± 11.2 <sup>a</sup>	82.1 ± 11.7 <sup>a</sup>	79.1 ± 11.0
Body mass index (kg/m <sup>2</sup> )	22.8 ± 2.5	23.7 ± 2.5 <sup>a</sup>	25.1 ± 2.7 <sup>a</sup>	27.0 ± 3.4 <sup>a</sup>	24.7 ± 3.3
Waist circumference (cm)	83.6 ± 6.6	85.8 ± 6.1 <sup>a</sup>	88.9 ± 6.7 <sup>a</sup>	92.6 ± 7.5 <sup>a</sup>	87.7 ± 7.5
ALT (U/L)	22.4 ± 16.1	25.5 ± 18.1 <sup>a</sup>	36.1 ± 48.4 <sup>a</sup>	40.2 ± 26.4 <sup>a</sup>	31.0 ± 31.0
AST (U/L)	23.9 ± 11.1	24.2 ± 16.0 <sup>a</sup>	27.6 ± 20.2 <sup>a</sup>	28.0 ± 11.5 <sup>a</sup>	25.9 ± 15.3
Fasting glucose (mg/dL)	95.1 ± 7.6	97.9 ± 7.9 <sup>a</sup>	99.4 ± 7.9 <sup>a</sup>	102.5 ± 8.5 <sup>a</sup>	98.7 ± 8.4
Fasting insulin (U/mL)	3.6 ± 1.0	6.1 ± 0.9 <sup>a</sup>	8.9 ± 1.1 <sup>a</sup>	15.8 ± 6.5 <sup>a</sup>	8.6 ± 5.7
HOMA <sub>IR</sub>	0.8 ± 0.2	1.5 ± 0.2 <sup>a</sup>	2.1 ± 0.2 <sup>a</sup>	3.9 ± 1.7 <sup>a</sup>	2.1 ± 1.5
Triglyceride (mg/dL)	113.9 ± 86.8	127.6 ± 86.2 <sup>a</sup>	157.9 ± 105.7 <sup>a</sup>	202.8 ± 156.9 <sup>a</sup>	150.6 ± 117.6
HDL (mg/dL)	52.8 ± 12.5	50.2 ± 9.8 <sup>a</sup>	47.4 ± 10.7 <sup>a</sup>	44.4 ± 8.9 <sup>a</sup>	48.7 ± 11.0

<sup>1</sup>The subjects were stratified into four groups according to HOMA<sub>IR</sub>; Q1 means the 1st quartile, Q2 means the 2nd, etc. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HDL: High-density lipoprotein; HOMA<sub>IR</sub>: Insulin resistance assessed by homeostasis model assessment = fasting insulin (U/mL) × glucose (mmol/L)/22.5. <sup>a</sup>P < 0.05 vs Q1.

**Table 2 Abnormality prevalence distribution, stratified by HOMA<sub>IR</sub> n (%)**

Abnormality <sup>1</sup>	The four sub-groups stratified by HOMA <sub>IR</sub>				Total 1308 (100)
	Q1 327 (25)	Q2 326 (25)	Q3 328 (25)	Q4 327 (25)	
Obesity	20 (6.1)	29 (8.9)	81 (24.7) <sup>a</sup>	153 (46.8) <sup>a</sup>	283 (21.6)
Elevated ALT	25 (7.6)	36 (11.0)	74 (22.6) <sup>a</sup>	118 (36.1) <sup>a</sup>	253 (19.3)
Metabolic syndrome components					
Abdominal obesity	45 (13.8)	74 (22.7) <sup>a</sup>	126 (38.4) <sup>a</sup>	188 (57.5) <sup>a</sup>	433 (33.1)
Elevated blood pressure	110 (33.6)	118 (36.2)	136 (41.5) <sup>a</sup>	187 (57.2) <sup>a</sup>	551 (42.1)
Elevated fasting glucose	16 (4.9)	22 (6.7)	37 (11.3) <sup>a</sup>	62 (19.0) <sup>a</sup>	137 (10.5)
Elevated triglyceride	58 (17.7)	83 (25.5) <sup>a</sup>	132 (40.2) <sup>a</sup>	195 (59.6) <sup>a</sup>	468 (35.8)
Hypo-HDL cholesterolemia	39 (11.9)	47 (14.4)	71 (21.6) <sup>a</sup>	108 (33.0) <sup>a</sup>	265 (20.3)
Alcohol consumption	83 (25.4)	56 (17.2)	74 (22.6)	73 (22.3)	286 (21.9)
Cigarette smoking	122 (37.3)	118 (36.2) <sup>a</sup>	108 (32.9)	109 (33.3)	457 (34.9)

<sup>1</sup>Obesity: Body mass index ≥ 27 kg/m<sup>2</sup>; Elevated ALT: ALT > 40 U/L; Abdominal obesity: waist circumference ≥ 90 cm in male adults; Elevated blood pressure: blood pressure ≥ 130/85 mmHg; Elevated fasting glucose: fasting glucose ≥ 100 mg/dL; Elevated triglyceride: triglyceride ≥ 150 mg/dL; Hypo-HDL cholesterolemia: HDL cholesterol < 40 mg/dL; Hypercholesterolemia: total cholesterol ≥ 200 mg/dL; Elevated LDL: LDL cholesterol ≥ 130 mg/dL; Alcohol consumption: more than once per week; Cigarette smoking: more than one pack per day. <sup>a</sup>P < 0.05 vs Q1 (χ<sup>2</sup> test).

**Table 3 OR of risk factors, presented as OR (95% CI)**

Model <sup>1</sup>	Model 1	Model 2	Model 3
Both obesity and elevated ALT	-	-	4.3 (2.7-6.8)
Obesity	2.5 <sup>2</sup> (1.7-3.6)	3.0 <sup>2</sup> (2.1-4.2)	-
Elevated ALT	2.1 <sup>2</sup> (1.4-3.0)	2.1 <sup>2</sup> (1.5-2.9)	-
Metabolic syndrome	-	2.6 <sup>2</sup> (1.8-3.7)	3.6 (2.6-5.0)
Abdominal obesity	1.6 <sup>2</sup> (1.2-2.2)	-	-
Elevated blood pressure	1.7 <sup>2</sup> (1.2-2.4)	-	-
Elevated fasting glucose	2.8 <sup>2</sup> (1.8-4.4)	-	-
Elevated triglyceride	2.2 <sup>2</sup> (1.6-3.1)	-	-
Hypo-HDL cholesterolemia	1.6 <sup>2</sup> (1.1-2.2)	-	-
Alcohol consumption	1.2 (0.8-1.7)	1.2 (0.8-1.6)	1.2 (0.9-1.7)
Cigarette smoking	0.9 (0.7-1.2)	0.9 (0.6-1.2)	0.9 (0.6-1.2)

<sup>1</sup>Risk factors of metabolic syndrome were regarded as five individual factor in model 1 and as a single factor in model 2; both obesity and elevated ALT were regarded as a single factor in model 3; <sup>2</sup>Significant odds ratio (OR), adjusted for age.

## DISCUSSION

Obesity is a worsening problem in Taiwan and worldwide<sup>[10]</sup>. Due to the variety of definition criteria, the prevalence rate varies. In Taiwan, the prevalence rate of

**Table 4 Synergistic effect of obesity and elevated ALT on insulin resistance<sup>1</sup>**

	OR (95% CI)	SI	95% CI
Neither obesity nor elevated ALT	1.0 Reference	No risk	-
Obesity only	2.3 (1.5-3.5)	One risk only	-
Elevated ALT only	1.8 (1.1-2.9)	One risk only	-
Obesity plus elevated ALT	5.5 (3.2-9.3)	2.1	(1.01-4.3)

<sup>1</sup>Insulin resistance is defined as the 4th quartile of the HOMA<sub>IR</sub>. SI: Synergistic index.

obesity (defined as BMI ≥ 27 in this article), surveyed in 2000-2001 were 15.9% and 10.7% in men and women separately, and the rate has progressively increased<sup>[11]</sup>. The prevalence rate of obesity in our study, which was conducted after 2001, is higher and is compatible with the trend of increasing prevalence. Obesity is associated with several comorbidities<sup>[10]</sup>, including coronary heart disease, stroke, NAFLD, and type 2 diabetes mellitus. Insulin resistance is present and important in the above clinical problems.

In our study, obesity alone is an important risk factor of insulin resistance. Similar results have also been published in other studies<sup>[3-5]</sup>. In contrast, weight reduction improves insulin resistance<sup>[18,19]</sup>, even if it is achieved by surgery<sup>[20]</sup>. However, not all obese subjects have insulin resistance<sup>[12]</sup>. Although the exact mechanism is still not fully understood, many factors have been found to induce insulin resistance through different pathways, such as increased non-esterified fatty acids (NEFAs), adipocyte dysfunction, leptin, adiponectin, chronic inflammatory status, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and fat distribution<sup>[10,12,21]</sup>. Among these factors, NEFAs, released from dysfunctional adipocytes, might be the single most critical factor in modulating insulin sensitivity<sup>[10,12,21]</sup>.

ALT level is a widely used laboratory test in Taiwan. Elevated ALT is a common laboratory abnormality in a healthy population. The statistical database from the healthy population in the United States in 2003 showed the prevalence rate of abnormal transaminase was 20.8%, similar to previous findings (19.3%)<sup>[13]</sup>. Another study showed the prevalence rate in the male adolescent population was 12.4%, which was lower, but the population was younger<sup>[14]</sup>.

Our results show elevated ALT is a risk factor of insulin resistance, independent of metabolic syndrome. This conclusion has been also proposed in previous articles<sup>[6,22,23]</sup>. In Pima, in Indians with normal glucose tolerance, elevated ALT was associated with a decline in clamp-derived whole body insulin sensitivity, according to longitudinal analysis, and it predicted the development of type 2 diabetes in a prospective analysis<sup>[6]</sup>. This was extended to those with impaired glucose tolerance in another study<sup>[22]</sup>, in which a population of African-Americans and white subjects were analyzed. Burgert *et al.*<sup>[23]</sup> also noted a relationship between elevated ALT and reduced insulin sensitivity in obese children. Our study showed a similar result in Taiwanese adult males using community-based data. A semi-quantified result was also noted in a previous article<sup>[22]</sup>. Our study also showed the trend of a significantly increasing level of ALT in the more severe insulin-resistant group.

The causes of elevated ALT may be NAFLD, viral hepatitis, alcohol, or medications<sup>[24]</sup>. NAFLD is also a common clinical problem, and it has been proven to cause elevated ALT<sup>[25]</sup>. NAFLD was regarded as an explanation of the association between elevated ALT and insulin resistance in previous studies<sup>[6,26]</sup>. It has also been reported that hepatitis C induced insulin resistance<sup>[27]</sup>. This might be a partial explanation of the relationship between elevated ALT and insulin resistance. In contrast, alcohol intake in sufficient amounts can improve insulin resistance<sup>[8,28]</sup>. Many kinds of medication can induce elevated ALT, but there is a lack of evidence that medication leads to insulin resistance. One study in 1983 reported aspirin induced insulin insensitivity, but there were only eight subjects in the study<sup>[29]</sup>. The C-reactive protein (CRP) level might provide another explanation. A previous study indicated that the CRP level was higher

in subjects with elevated ALT than those with normal ALT<sup>[30]</sup>. Another study showed a high CRP level was related to insulin resistance<sup>[31]</sup>. From these studies, we might conclude that subjects with elevated ALT have a higher CRP level and further, are more insulin-resistant. The exact mechanism is, however, not well-understood. Further evidence is required.

Interestingly, there is a dramatically higher risk of insulin resistance in subjects with both elevated ALT and obesity. This result has seldom been discussed in the literature.

There are several studies concerning the association between obesity and elevated ALT, but the role of insulin resistance had not been identified in these studies<sup>[32,33]</sup>. In studies on the association between ALT level and insulin resistance, there was no analysis identifying the effect of obesity, but a significant relationship between body weight and ALT level were shown in the data<sup>[6,22]</sup>. Elevated ALT was associated with insulin resistance in obese subjects in previous studies, but it lacked comparative analysis between obese and non-obese subjects<sup>[23,30]</sup>.

What mechanism leads to the synergistic effect of obesity and elevated ALT? As discussed earlier, adipocyte dysfunction is regarded as the link between obesity and insulin resistance<sup>[21]</sup>, and its associated release of NEFAs (or free fatty acids) was regarded as the single most critical factor modulating insulin sensitivity<sup>[1,10,12,21]</sup>. In the model, adipocyte dysfunction results in increased circulating NEFAs, which further mediates insulin resistance. The excessive build-up of NEFAs in the liver leads to NAFLD, of which elevated ALT level is the best marker even within the reference interval<sup>[34]</sup>. Obesity coexisting with elevated ALT points to excessive NEFAs release and adipocyte dysfunction. This shows the existence of the link "from obesity to insulin resistance". Besides, in the cell module, TNF- $\alpha$ , and its effects on down-regulation of the nuclear hormone receptor peroxisome proliferator activated receptor- $\gamma$  (PPAR $\gamma$ ) strongly contributed to the effects of inflammatory cytokines on adipocytes<sup>[21]</sup>. Thus the inflammatory status results in adipocyte dysfunction. As elevated ALT is a reflection of systemic inflammation<sup>[6]</sup>, we concluded that coexistence of elevated ALT and obesity means the existence of inflammatory status, the excessive release of NEFAs, and their result: adipocyte dysfunction connecting obesity to insulin resistance.

Based on the models, most subjects with both obesity and elevated ALT should have insulin resistance. In our study, the actual prevalence rate of insulin resistance in the sub-group with both obesity and elevated ALT was almost 70% (not shown in table). This is compatible with our hypothesis.

The limits of this study deserve mention. First, insulin resistance was defined as the top quartile of HOMA<sub>IR</sub> (homeostasis model assessment), instead of a direct measurement. This might be less representative of insulin resistance, but more cases are available because of the more simple classification. Secondly, although our results showed a trend of increasing

ALT level with more severe insulin resistance, it still lacks an exact quantified relationship. More evidence is needed to confirm if there is a better cutoff value of ALT level or quantified relationship between ALT level and insulin resistance. Thirdly, cigarette smoking and alcohol consumption were recorded as more than one pack per day/once per week, instead of exact quantified data; therefore, it cannot be concluded that there was no relationship between smoking/drinking and insulin resistance based on our results.

In conclusion, obesity and elevated ALT have been shown to be associated with insulin resistance. The coexistence of both factors points to the existence of a linking mechanism between obesity and insulin resistance. The results suggest a synergistic effect on insulin resistance. The coexistence of these parameters might be better than metabolic syndrome for evaluating insulin resistance in clinical practice.

## COMMENTS

### Background

Insulin resistance is a key feature of type 2 diabetes mellitus and it plays an important role in cardiovascular diseases. Direct measurement of insulin resistance with hyperinsulinemic euglycemic clamp is invasive and inconvenient. For public health, less invasive methods are required. Many parameters have been proposed. Metabolic syndrome is one of the most popularly-used parameter.

### Research frontiers

This study proposed that obesity plus elevated alanine aminotransferase (ALT) might be better than metabolic syndrome for predicting insulin resistance due to its being non-invasive and convenience. This article also explored the mechanism linking obesity, elevated ALT, and insulin resistance.

### Applications

This study highlights the applications on public health since the measurements of obesity and ALT are easily obtained.

### Peer review

The content of the article will be interesting not only for the gastroenterologists, but also for other specialists. For understanding the quantified relationship between body weight or ALT level and insulin resistance, further investigations are required.

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BRIEF ARTICLE

## Does testosterone prevent early postoperative complications after gastrointestinal surgery?

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testosterone at later times after surgery was a better predictor of complications.

**CONCLUSION:** Patients with low testosterone level were prone to higher postoperative complications, which was evident in both sexes. However, further studies are necessary to support this result.

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**Key words:** Stomach neoplasms; Testosterone; Sex differences; Postoperative complications

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Sah BK, Chen MM, Peng YB, Feng XJ, Yan M, Liu BY, Fan QS, Zhu ZG. Does testosterone prevent early postoperative complications after gastrointestinal surgery? *World J Gastroenterol* 2009; 15(44): 5604-5609 Available from: URL: <http://www.wjg-net.com/1007-9327/15/5604.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5604>

### Abstract

**AIM:** To investigate the role of sex hormones in the early postoperative complications of gastrointestinal diseases.

**METHODS:** A total of 65 patients who underwent operations for gastric and colorectal diseases (mainly malignant diseases) were included in the study. Peripheral venous blood samples were collected at different times for analysis of estradiol, testosterone and progesterone. The only study endpoint was analysis of postoperative complications.

**RESULTS:** Patients of both sexes were uniform but postoperative complication rate was significantly higher in female patients ( $P = 0.027$ ). There was no significant association of estradiol and progesterone with postoperative complications. Testosterone levels in complicated patients were significantly lower than in uncomplicated patients ( $P < 0.05$ ). Area under the receiver operating characteristic curve showed that a lower value of testosterone was a predictor for higher complication rate ( $P < 0.05$ ), and a lower value of

### INTRODUCTION

There have been numerous reports of disparity in pathophysiology of different types of disease between male and female subjects, however, the reports have been quite diverse. Female sex is an independent predictor of mortality in patients with enterococcal bloodstream infections<sup>[1]</sup>. Women, in addition, might have a higher mortality among patients with necrotizing soft tissue infection<sup>[2]</sup>. Although most studies have suggested increased susceptibility to infectious complications among men, generally they have demonstrated a higher mortality rate for women from infections and sepsis<sup>[3-6]</sup>, but again, this is not universal<sup>[7-9]</sup>. On the other hand, male sex is an independent risk factor for the development of nosocomial bloodstream infection<sup>[10]</sup>, and is associated with in-hospital mortality in septic surgical patients<sup>[11]</sup>. Male sex is associated with anastomotic leakage in colorectal surgery<sup>[12]</sup>, as well as in rectal surgery<sup>[13-15]</sup>.

Female sex is an independent risk factor for early

postoperative complications of gastric cancer surgery<sup>[16,17]</sup>. Early postoperative complications of gastric cancer surgery are significantly more common in female patients, especially mortality and infection rate. Similarly, the duration of postoperative stay is significantly longer in female patients and they have more severe complications than male patients<sup>[17]</sup>.

We observed a significantly higher complication rate in female than male patients, therefore, we assumed that sex hormones may have influenced this disparity. Therefore, we conducted a prospective study to investigate the possible role of sex hormones in early postoperative complications.

## MATERIALS AND METHODS

### Patient characteristics

A total of 65 patients who underwent operations for gastric and colorectal diseases (mainly malignant diseases) from 2008 to 2009 were included in the study. We applied TNM classification and Duke's classification for gastric cancer and colorectal cancer, respectively. In gastric cancer patients, the majority was diagnosed with loco-regional advanced stage, and few patients were diagnosed as late stage (Table 1). There was one case of gastric polyp disease, one of malignant gastrointestinal stromal tumor, and one of mesenteric metastasis. The median age of the patients was 61 years (range: 22-82 years). All the operations were performed by senior consultants of a single department at Rui Jin Hospital, School of Medicine, Shanghai Jiao Tong University.

All the patients with early and resectable advanced gastric cancer (without significant distant metastases) underwent gastrectomy with D2 lymphadenectomy. Thirty partial and 17 total gastrectomies were performed for gastric cancer. One patient underwent total gastrectomy for gastric polyp disease and partial gastrectomy was performed for one malignant gastrointestinal stromal tumor. Anterior resection was performed on three patients, and one patient underwent abdominoperineal resection for rectal cancer. Three patients had partial colectomy and one underwent subtotal colectomy for colon cancer. One patient case of mesenteric metastasis was resected along with partial enterectomy.

The only study endpoint was analysis of postoperative complications. Complications were recorded in accordance with the definitions reported by Copeland *et al*<sup>[18]</sup>. Other complications were recorded separately.

### Blood sampling

This study was approved by the Hospital Institutional Review Boards for human subject research. All samples were obtained with informed consent. Patients on hormonal therapy or chemotherapy were excluded. Similarly, the patients with concomitant immunological disease were excluded. Peripheral venous blood samples were collected from 65 patients before operation (baseline measurements) and on postoperative day (POD)1, POD3, POD5, and POD7 for extensive analysis of their

Table 1 Demographic data of the patients

Details	Frequency
Age group (yr)	
≤ 60	32
61-70	19
≥ 71	14
Sex	
Male	42
Female	23
No. of procedures	
Gastrectomy	49
Colorectal resection <sup>1</sup>	9
Gastro-jejunal anastomosis	2
Exploratory laparotomy	5
TNM classification (Gastric cancer)	
I A	8
I B	6
II	7
III A	10
III B	4
IV	12
Dukes classification (Colorectal)	
A	7
B	0
C	1

<sup>1</sup>Including one case of small bowel resection combined with mesenteric tumor.

variation at different times. Samples were collected into Venoject tubes containing EDTA.

### Laboratory tests

Assays for all parameters were performed in the laboratory of the Rui Jin Hospital. Venous blood was centrifuged at a speed 2500 r/min for 10 min, and serum was then preserved at -80°C, until further investigation. Estradiol, total testosterone and progesterone were measured using a Dxl 800 analyzer (Beckman-Coulter, Fullerton, CA, USA).

### Statistical analysis

The statistical analysis was performed with SPSS for Windows version 13.0 (SPSS, Inc, Chicago, IL, USA). Test of normality for all related variables were checked by the Shapiro-Wilk method, and a nonparametric statistical method (Mann-Whitney *U* test) was applied to the variables without a normal distribution. The means were compared by Student's *t* test in normally distributed cases. Differences in proportion were compared by Pearson's  $\chi^2$  test.  $P < 0.05$  was considered statistically significant.

## RESULTS

Fifteen patients with gastric disease and with non-gastric disease were compromised by different complications. There were several patients who had pyrexia of unknown origin with a high white blood cell count, but who did not have any diagnosis, mainly because of a lack of further investigation. The definition of postoperative complication introduced by Copeland *et al*<sup>[18]</sup> does not cover many complications that are common after gastrointestinal surgery, therefore, complications which

Table 2 Overall complications in both sexes		
Complications	Frequency	
	Male	Female
Overall	9	11
Intra-abdominal hemorrhage	1	0
Anastomotic leak	1	1
Abdominal infection	2	1
Pyrexia of unknown origin	7	9
Renal failure	1	0
Respiratory failure	0	1
Cardiac failure	0	1
Hypotension	0	1
MODS	0	1
Gastroplegia or enteroplegia	0	2
Anastomosis site bleeding	1	1
Pleural effusion	0	2
Seroperitoneum	2	1
Death	0	1

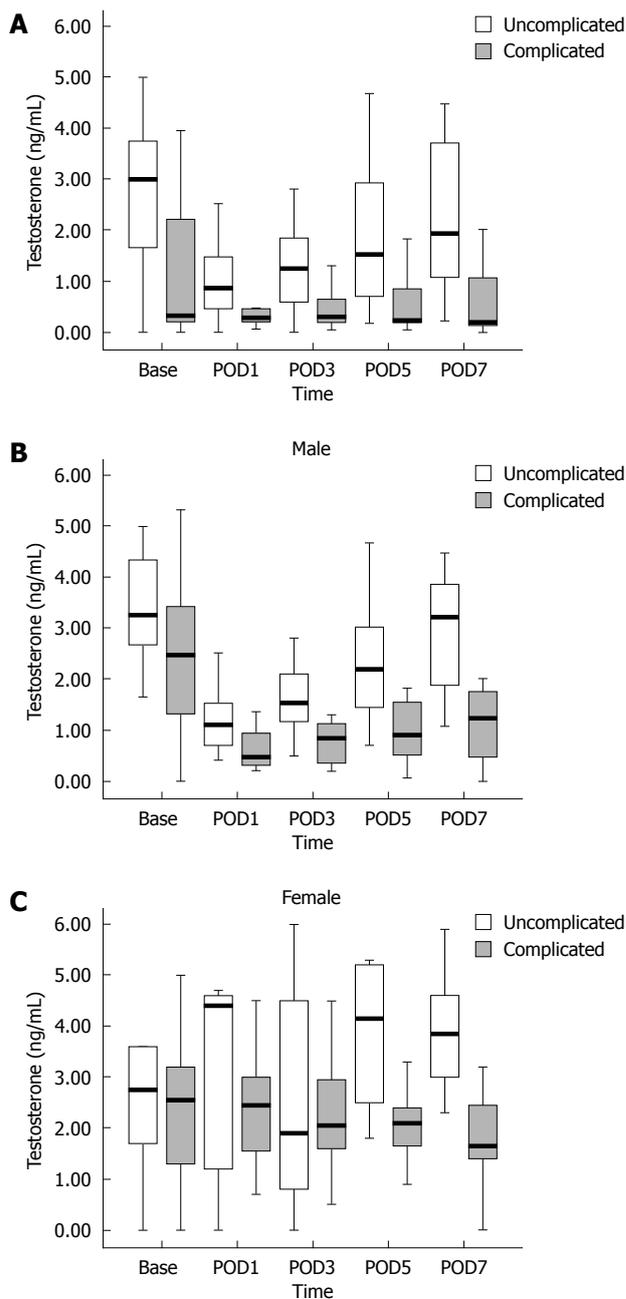
MODS: Multiple organ dysfunction syndrome.

Table 3 Normal range of different hormones		
	Male	Female (postmenopause)
Estradiol (pg/mL)	20-75	20-88
Progesterone (ng/mL)	0.1-0.84	0.08-0.78
Testosterone (ng/mL)	2.62-15.9	0.1-0.8

Table 4 Demographics and clinical characteristics according to sex			
	Male (n = 42)	Female (n = 23)	P value
Age (yr)	60.9	58.1	0.356
Physiological score	14	17	0.203
Operative severity score	16	18	0.365
Complications (%)	21.4	47.8	0.027
Baseline estradiol (pg/mL)	23	12	0.320
Baseline progesterone (ng/mL)	0.46	0.37	0.450
Baseline testosterone (ng/mL)	3.26	0.23	0.000

were confirmed clinically were also included. Details of all complications are summarized in Table 2. The sum of the individual complications was not equal to the total number of complications, and multiple complications were possible in a single patient. Patients of both sexes were uniform and there was no significant difference in age ( $P = 0.356$ ), physiological score ( $P = 0.616$ ) and operative severity score ( $P = 0.304$ ). However, there was a significant difference in complication rate between male and female patients ( $P = 0.027$ ). The complication rate for male and female patients was 21.4% and 47.8%, respectively. We analyzed the role of sex hormones in postoperative complications. Normal ranges of different sex hormones and demographics of patients according to sex are summarized in Tables 3 and 4, respectively.

There was no significant association of estradiol and progesterone with postoperative complications. Besides, the normal range of these hormones varies greatly at different times in a month in female patients, and there were not sufficient patients to stratify according to age



**Figure 1** Difference in testosterone level. A: Difference in mean testosterone level in complicated and uncomplicated patients; B: Male patients; C: Female patients.

or time period in women. Given the significant association between testosterone and postoperative complications, we focused additional analysis on this relationship. Testosterone decreased significantly after surgery in both sexes. Testosterone level in complicated patients was significantly lower ( $P < 0.05$ ) than in uncomplicated patients (Figure 1A).

Perioperative testosterone level was associated significantly with postoperative complication rate, and the result was similar for either overall complication rate or complications of gastric disease surgery alone (Table 5). Similarly, the difference was also significant after stratification of all complications according to sex. However, after stratification, we found there was no significant

**Table 5** Significance of difference in testosterone level at different times

Complication	Baseline	POD1	POD3	POD5	POD7
Overall	0.008	0.002	0.007	0.001	0.000
Gastric disease	0.025	0.008	0.026	0.005	0.005

**Table 6** Level of testosterone in different sexes according to outcome

Time	Complications (male)			Complications (female)		
	Absent	Present	P value	Absent	Present	P value
Base	3.66	2.74	0.104	0.23	0.29	0.689
POD1	0.91	0.87	0.141	0.40	0.23	0.268
POD3	1.54	0.85	0.046	0.19	0.20	0.948
POD5	2.20	0.91	0.040	0.41	0.21	0.038
POD7	3.22	1.24	0.030	0.38	0.16	0.007

**Table 7** Area under the ROC for testosterone

Time	Area	P value	95% CI	
			Lower bound	Upper bound
BASE	0.729	0.016	0.562	0.897
POD1	0.801	0.002	0.658	0.945
POD3	0.756	0.007	0.593	0.919
POD5	0.813	0.001	0.664	0.963
POD7	0.835	0.000	0.692	0.977

difference of testosterone level between complicated and uncomplicated patients during the first 3 d after surgery, in both sexes (Table 6). The median value of testosterone in complicated patients was approximately half of that in uncomplicated patients. The significance of the difference increased in later days after surgery, especially on POD5 and POD7. The result was similar in both sexes (Figure 1B and C).

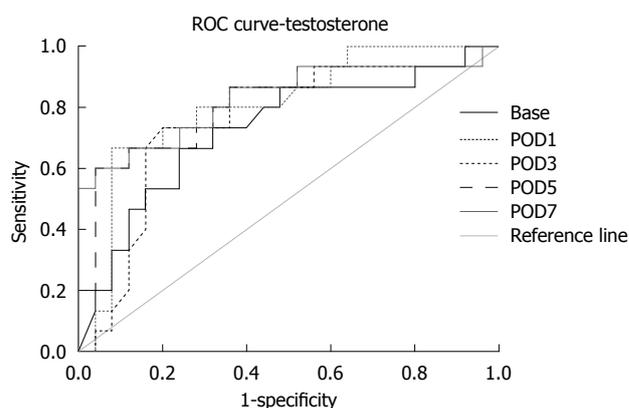
Area under the receiver operating characteristic curve showed that a lower value of testosterone was a predictor for higher complication rate (Table 7, Figure 2). A lower value of testosterone at a later time after surgery was a better predictor of complications (Table 8).

## DISCUSSION

Sex has been hypothesized to be a determinant of immunological variability after severe traumatic and surgical stress and, at least in animal models, accounts for differences in outcomes<sup>[3,7,19-22]</sup>. Some studies have demonstrated that altered levels of sex hormones rather than sex itself are related to postoperative complications. In particular, progesterone in male and testosterone in female subjects has an impact on survival. In both sexes, higher 17 $\beta$ -estradiol levels are associated clearly with shorter survival times<sup>[23]</sup>. However, we did not find a significant relationship between estradiol and postoperative complications. Unlikely, we observed a difference in progesterone level between complicated and uncomplicated patients. However, these observations were limited by the low number of patients, and variation of normal values

**Table 8** Sensitivity and specificity for different levels of testosterone

	Testosterone level	Sensitivity	Specificity
Base	$\leq 1.95$	70.0	68.9
POD1	$\leq 0.43$	60.0	84.4
POD3	$\leq 0.45$	73.3	80.0
POD5	$\leq 0.24$	60.0	90.0
POD7	$\leq 0.32$	66.7	92.0

**Figure 2** ROC curve for level of hormones at different times.

of this hormone at different times in female subjects made it difficult to obtain any conclusive result.

The uncertainty regarding the role of sex in influencing outcomes in humans may simply result from the clinical, phenotypic, and genetic diversity among populations, and the difficulty in detecting a difference due to these confounders. However, whether men and women respond differently to trauma or surgical insult, either in terms of the host response or the eventual outcome, is important for clinical care and the design of future research on anti-infective treatments or immunological regulators.

As mentioned above, our study was affected by the limitation of statistical calculation due to the low number of postoperative complications. Therefore, we could not analyze the differences in testosterone level in complicated and uncomplicated cases in different age groups, or its effect on different types of complications separately, because the number of patients and their relevant complications were low in those groups. It was still unknown whether the lower level of testosterone caused postoperative complications, or whether it was just a phenomenon observed in complicated patients. However, it was evident that even the preoperative level of testosterone was lower in patients who had complications after surgery. The testosterone level decreased significantly after surgery in all patients and it started to regain its preoperative level, or even higher, on POD3. However, the testosterone level in complicated patients did not regain its preoperative value, and moreover, it kept on decreasing. Even after controlling for sex by stratifying the group and looking at testosterone levels, the testosterone level decreased significantly in male and female patients with postoperative complications. Therefore, this observation

at least confirmed that the testosterone level had some kind of association with postoperative complications. However, without detailed investigation of the effects of other hormones, it was very hard to conclude that only testosterone was responsible for such disparity.

Although there were many limitations and unanswered questions in the present study, at least, our observations reflected that some kind of difference does exist between the sexes, which may be responsible for the disparity in postoperative complication rate. If a difference does indeed exist, it is possible that future studies will have to stratify for sex, infection source, and immunological phenotype. Similarly, future treatment strategy must be tailored for male and female patients separately, and different modes or treatment management will be necessary for the different sexes.

If there is an association between low testosterone level and postoperative complication rate, then serial tests of testosterone levels can easily identify the susceptible patients, and supplementary hormonal replacement therapy might be a novel treatment to prevent postoperative complications in such patients.

In conclusion, there was a significant association between testosterone and overall complications, and also with complications of gastric surgery. Association with testosterone was seen even after controlling for sex by stratifying the group and looking at testosterone levels. Postoperative complications were higher in patients who did not recover normal testosterone levels at POD5 or POD7. However, the results of this small-scale study need confirmation by a large cohort of patients, and prospective studies with hormone supplementation to reevaluate the role of testosterone in postoperative complications.

## COMMENTS

### Background

There have been numerous reports of disparity in pathophysiology of different types of disease between male and female patients, however, the reports have been quite diverse. Female sex is an independent risk factor for early postoperative complications of gastric cancer surgery. Early postoperative complications of gastric cancer surgery are significantly more common in female patients, especially mortality and infection rate. Similarly, the duration of postoperative stay is significantly longer in female patients, and they have more severe complications than male patients do.

### Innovations and breakthroughs

The authors found that there was a significant association between testosterone and overall complications, and also with complications of gastric surgery. Association with testosterone was seen even after controlling for sex by stratifying the group and looking at testosterone levels. Postoperative complications were higher in patients who did not recover normal testosterone levels at POD5 or POD7.

### Applications

By understanding the effect of sex hormones on postoperative complications, the present study might represent a future strategy for therapeutic intervention in the treatment of patients with gastrointestinal disease. If there is an association of low testosterone level with postoperative complication rate, then serial testing of testosterone level can easily identify the susceptible patients, and supplementary hormonal replacement therapy could be a novel treatment to prevent postoperative complications in these patients

### Terminology

Estradiol, testosterone and progesterone are sex hormones that are measured

to understand dysfunction in gonadal activity.

### Peer review

This study is unique and interesting. The authors studied the impact of sex hormones, especially testosterone, on the prevalence of complications after gastrointestinal surgery. This study gives us some valuable clues to explain why postoperative complications occur more frequently in female patients, as previously noted. It would also be useful in giving us a basis for clinical research to predict the occurrence of surgical complications.

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BRIEF ARTICLE

## Polymorphisms of some cytokines and chronic hepatitis B and C virus infection

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### Abstract

**AIM:** To study the relationship between the polymorphisms in some cytokines and the outcome of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection.

**METHODS:** Samples were obtained from 203 patients infected with HBV and/or HCV while donating plasma in 1987, and 74 controls were obtained from a rural area of North China. Antibodies to HBV or HCV antigens were detected by enzyme-linked immunoassay. The presence of viral particles in the serum was determined by nested reverse-transcriptase polymerase chain reaction (PCR). Hepatocellular injury, as revealed by alanine aminotransferase (ALT) and aspartate aminotransferase level, was detected by a Beckman LX-20 analyzer. DNA was extracted from blood cells. Then, the single nucleotide polymorphisms of IL-2-330, IFN- $\gamma$ +874, IL-10-1082/-592 and IL-4-589 were investigated by restriction fragment length polymorphism-PCR or sequence specific primer-PCR.

**RESULTS:** Persistent infection with HBV, HCV, and HBV/HCV coinfection was associated with IL-2-330 TT genotype and T allele, IFN- $\gamma$ +874 AA genotype, and IL-10-1082 AA genotype. The clinical outcome of HBV and/or HCV infection was associated with IL-2-330 TT genotype and T allele, IFN- $\gamma$ +874 AA genotype, and IL-10-1082 AA genotype. IL-2-330 GG genotype frequency showed a negative correlation with clinical progression, IL-10-1082 AA genotype frequency showed a positive correlation and IL-10-1082 AG genotype frequency showed a negative correlation with clinical progression. HCV RNA positive expression was associated with IL-10-1082 AA genotype and the A allele frequency. Abnormal serum ALT level was associated with IL-10-592 AC genotype frequency and IL-4-589 CC genotype, CT genotype, and the C allele.

**CONCLUSION:** These results suggest that polymorphisms in some cytokine genes influence persistent HBV and HCV infection, clinical outcome, HCV replication, and liver damage.

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**Key words:** Hepatitis B; Hepatitis C; Single nucleotide polymorphism; Disease susceptibility; Outcome studies; Cytokines

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### INTRODUCTION

The natural outcome in hepatitis B virus (HBV) and hepatitis C virus (HCV) infection varies dramatically among individuals. Infection with HCV is self-limited in a fortunate minority, while the majority of subjects develop persistent (chronic) infection<sup>[1,2]</sup>. Although infection with

HBV in adults progresses to the chronic phase in about 5%<sup>[3]</sup>, among those individuals with persistent HBV or HCV infection, the majority develop chronic hepatitis (CH), progressive fibrosis and even liver cancer<sup>[4,5]</sup>. However, there are some cases that never evolve into any significant liver disease within the patient's natural lifespan<sup>[6,7]</sup>. It remains unknown why patients infected with HBV and/or HCV frequently turn to be chronic and the outcome of HBV and/or HCV infection dramatically varies. Besides the pathogenesis of viral factors, the most important factor is the different immune response to HBV or HCV infection between individuals. For example, in patients infected with HBV/HCV, some show a strong reduction in CD4<sup>+</sup> T-helper (Th) and CD8<sup>+</sup> cytotoxic-T-lymphocyte (CTL) responses. This is likely to be crucial in clearance of acute viremia<sup>[8-10]</sup>. Another factor is thought to be immune tolerance to HBV/HCV infection. This contributes to explaining the different susceptibility to HBV/HCV infection. Many manifestations show that the immunity level of host correlates with relevant gene polymorphisms, especially with single nucleotide polymorphisms (SNPs) in the promoter region that regulates gene expression. Furthermore, the gene polymorphisms probably determine the outcome of the infection. This study investigated the screened Th1 cytokines, interleukin (IL)-2 (-330), interferon (IFN)- $\gamma$  (+874), and Th2 cytokines, IL-10 (-1082, -592) and IL-4 (-589) as candidate genes, to investigate the outcome of HBV/HCV infection.

## MATERIALS AND METHODS

### Subjects

We recruited 277 Han Chinese individuals from a rural area of Northern China (137 male and 140 female, aged 30-70 years, mean age  $50.20 \pm 10.43$  years), including 203 that were infected with HBV and/or HCV when they donated plasma in 1987, and 74 controls who had cleared HBV and HCV spontaneously. Plasma samples were evaluated by nested reverse-transcriptase polymerase chain reaction (nRT-PCR) and PCR. We diagnosed and excluded 28 patients with fatty liver. The infections had been diagnosed in 1993. Written informed consent for enrolling in the study was obtained from all the subjects. Patients were classified into the following groups. (1) Controls: 74 individuals (34 male and 40 female, mean age  $48.61 \pm 9.39$  years) who were negative for HBV and HCV antibodies. (2) Persistent HCV infection: 55 individuals (28 male and 27 female, mean age  $49.42 \pm 10.01$  years) who were positive for HCV antibodies but negative for HBV antibodies. (3) Persistent HBV infection: 69 individuals (39 male and 30 female, mean age  $52.74 \pm 13.17$  years) who had hepatitis B surface antigen and/or anti-hepatitis B core and/or anti-hepatitis B e antibodies, without HCV antibodies. (4) Persistent HBV and HCV coinfection: 79 individuals (36 male and 43 female, mean age  $50.01 \pm 8.56$  years) who had antibodies to HBV and HCV.

### Experimental and clinical diagnosis

Antibodies to HBV or HCV antigens were detected with an enzyme-linked immunoassay according to the manufacturer's instructions (Shanghai Kehua Bio-engineering Co. Ltd). The presence of hepatitis C viral particles in serum was determined by nRT-PCR. Hepatocellular injury, as revealed by alanine aminotransferase and aspartate aminotransferase level, was detected by a Beckman LX-20 analyzer. Clinical outcomes were diagnosed by evidence of viremia, liver transaminases and abnormal ultrasonography. Patients were diagnosed into four progressive hepatitis groups. (1) Healthy group: individuals negative for HBV and HCV antibodies, with normal liver transaminase levels and ultrasonography. (2) Mild CH group: patients with antibodies to HBV and/or HCV with mild abnormal liver transaminase and ultrasonography, but no evidence of viremia. (3) Moderate/severe CH: patients with antibodies to HBV and/or HCV with evidence of viremia, abnormal liver transaminase and/or abnormal ultrasonography. (4) Cirrhosis group: patients with antibodies to HBV and/or HCV and significantly abnormal ultrasonography. The diagnosis was performed using established methods for confirming cirrhosis<sup>[11]</sup>, with or without evidence of viremia, which had abnormal liver transaminase levels.

### DNA extraction

Blood specimens were collected in sodium citrate sterile tubes. Genomic DNA was extracted from 2-mL samples of whole blood and subsequently stored at  $-20^{\circ}\text{C}$  for genotype analysis. Genomic DNA extraction was performed using the guanidine-HCl method<sup>[12]</sup>.

### Gene polymorphism analyses of IL-2-330, IL-4-589 and IL-10-592

**Gene amplification:** IL-2-T330G, IL-4-C589T and IL-10-A592C gene polymorphisms were typed by restriction fragment length polymorphism-PCR. The primer sequences used are listed in Table 1. The 25- $\mu\text{L}$  final PCR volume was as follows: 20 ng DNA, 2.5  $\mu\text{L}$  10  $\times$  PCR Buffer (100 mmol/L Tris-HCl, pH 8.3, 500 mmol/L KCl), 200  $\mu\text{mol/L}$  dNTPs (400  $\mu\text{mol/L}$  dNTPs for IL-4-C589T), 1.5 mmol/L MgCl<sub>2</sub> (2.0 mmol/L MgCl<sub>2</sub> for IL-2-T330G), 10 pmol each primer, 1 U *Taq* polymerase (TaKaRa Biotechnology Co. Ltd., Dalian, China). PCR was carried out in an AmpGene DNA thermal cycler 4800 (America PE Corporation). After the initial denaturation step (95 $^{\circ}\text{C}$  for 2 min), the 35 cycles specified in Table 1 were employed. After the final cycle, the samples were kept at 72 $^{\circ}\text{C}$  for 7 min. Successful amplification was confirmed by using 2% agarose gel stained with ethidium bromide. The product size of each cytokine was described as in Table 2.

**Restriction enzyme digestion:** Restriction enzyme digestion was performed in 10- $\mu\text{L}$  volumes as follows: 2 U restriction enzyme, 1  $\mu\text{L}$  10  $\times$  Buffer, and the appropriate PCR product (4  $\mu\text{L}$  IL-2-T330G, 3  $\mu\text{L}$  IL-

Table 1 The position, polymorphism and primer sequence in IL-2, IL-4 and IL-10 genes

SNP position	Polymorphism	Primer sequence 5'-3'	PCR conditions
IL-2 -330	T→G	F: 5'-TATTCACATGTTTCAGTGTAGTTCT-3' R: 5'-ACATTAGCCCACACTTAGGT-3'	94°C, 48°C, 72°C for 1 min
IL-4 -589	C→T	F: 5'-ACTAGGCCTCACCTGATACG-3' R: 5'-GTTGTAATGCAGTCCTCCTG-3'	94°C, 57°C, 72°C for 30 s
IL-10 -592	A→C	F: 5'-CCTAGGTCACAGTGACGTGG-3' R: 5'-GGTGAGCACTACCTGACTAGC-3'	94°C/30 s, 60°C/45 s, 72°C/60 s

Table 2 Polymorphism position, product size, restriction enzyme, digestion fragments in IL-2-330, IL-4-589 and IL-10-592

SNP position	Product size (bp)	Restriction enzyme	Digestion fragments (bp)
IL-2-330 T/G	150	MaeI (Bio Basic Inc.)	150 26 + 124
IL-4-589 T/C	252	BsmFI (New England BioLabs)	252 60 + 192
IL-10-592 C/A	412	RsaI (Bio Basic Inc.)	412 236 + 176

4-C589T and 1  $\mu$ L 10  $\times$  BSA, 5  $\mu$ L IL-10-A592C). The digestion reaction of IL-2-T330G and IL-10-A592C was incubated at 37°C for 10 h, and the IL-4-C589T reaction at 65°C for 15 h. The digestive products were visualized on 3% agarose gel stained with ethidium bromide. The wild or mutant genotype was characterized by enzyme digestion fragments, as in Table 2.

#### Gene polymorphism analysis of IL-10-1082 and IFN- $\gamma$ +874

IL-10-G1082A and IFN- $\gamma$ +T874A gene polymorphisms were detected by sequence specific primer-PCR technique. For each sample, two parallel reactions were performed. For IL-10-1082, the forward allele-specific primers were either 5'-ACTACTAAGGCTTCTTTGGGAA-3' (for IL-10 allele A) or 5'-CTACTAAGGCTTCTTTGGGAG-3' (for IL-10 allele G), and the antisense primer was 5'-CAGTGC-CAACTGAGAATTTGG-3'. The product size was 258 bp. A human growth hormone sequence was used as an internal control. The control primers were 5'-GCCTTCCCAAC-CATTCCCTTA-3' and 5'-TCACGGATTCTGTGTGTGTTTC-3', and the product size was 429 bp. The 20- $\mu$ L final reaction volume contained: 2.0  $\mu$ L 10  $\times$  PCR Buffer (100 mmol/L Tris-HCl, pH 8.3, 500 mmol/L KCl), 200  $\mu$ mol/L dNTPs, 2.0 mmol/L MgCl<sub>2</sub>, 3.75  $\mu$ mol/L specific primer, 0.09  $\mu$ mol/L control primer, 20 ng DNA, and 0.5 U *Taq* polymerase. PCR was carried out in an AmpGene DNA thermal cycler 4800. The cycling conditions were 95°C for 2 min, and 10 cycles for 1 min at 95°C, 50 s at 65°C and 50 s at 72°C, followed by 20 cycles for 1 min at 95°C, 50 s at 59°C, and 50 s at 72°C, followed by a final extension for 7 min at 72°C. The PCR products were visualized on 1.5% agarose gel stained with ethidium bromide.

For IFN- $\gamma$ +874, the forward allele-specific primers were 5'-TTCTTACAACACAAAATCAAATCT-3' (for allele T) or 5'-TTCTTACAACACAAAATCAAATCA-3' (for allele A), and the anti-sense primer was 5'-TCAA-CAAAGCTGATACTCCA-3'. The product size was

262 bp. A human growth hormone sequence was used as an internal control. The control forward primer was 5'-TATGATCTCTGGCTAAGGAATG-3' and the anti-sense primer was 5'-TCAACAAAGCTGATACTCCA-3'. The product size was 440 bp. The 20- $\mu$ L final reaction volume contained 2.0  $\mu$ L 10  $\times$  PCR buffer (100 mmol/L Tris-HCl, pH 8.3, 500 mmol/L KCl), 200  $\mu$ mol/L dNTPs, 2.0 mmol/L MgCl<sub>2</sub>, 0.5  $\mu$ mol/L specific primer, 0.1  $\mu$ mol/L former control primer, 20 ng DNA, and 0.5 U *Taq* polymerase. PCR was carried out in an AmpGene DNA thermal cycler 4800. The cycling conditions were 94°C for 2 min, and 10 cycles for 30 s at 94°C, 40 s at 60°C and 40 s at 72°C, followed by 25 cycles for 30 s at 94°C, 40 s at 56°C, and 50 s at 72°C, followed by a final extension for 7 min at 72°C. The PCR products were visualized on 1.5% agarose gel stained with ethidium bromide.

#### Statistical analysis

The data were tested by the  $\chi^2$  test for the goodness of fit to the Hardy-Weinberg equilibrium (HWE) between the observed and expected genotype values. The frequencies of the alleles and genotypes among the groups were compared by the  $\chi^2$  test.  $P < 0.05$  was considered statistically significant. We calculated the OR and 95% CI.

## RESULTS

#### Hardy-Weinberg equilibrium (HWE) and characteristic distribution of age and sex

An HWE test was performed for all cytokine polymorphisms investigated in this study (Table 3). The distribution of these observed genotypes was not significantly different from the expected distribution according to HWE, except the IL-10-1082 polymorphisms, which revealed a deviation from HWE because of the lower frequency of wild-type homozygosity (the observed *vs* the expected frequency was 1.08% *vs* 10.91%). In IL-2-330, IL-4-589, IL-10-1082, IL-10-592 and IFN- $\gamma$ +874 gene polymorphisms, there were no significant differences in age and sex ( $P > 0.05$ ).

#### Genotype and allele distribution of IL-2-330, IFN- $\gamma$ +874, IL-4-589 and IL-10-1082 and -592 in persistent HBV and/or HCV infection

IL-2-330 TT, -330 GG and -330 TG are shown in Figure 1. The IL-2-330 polymorphisms showed obvious association with persistent HBV and/or HCV infection. IL-2-330 TT was associated with an increased risk, but IL-2-330 GG with a reduced risk of persistent HBV and/or HCV

Table 3 HWE test for cytokines

Locus	Observed homozygosity (%)		Expected homozygosity (%)		P value
	Wild-type genotype	Mutant genotype	Wild-type genotype	Mutant genotype	
IL-2-330	40.79	14.08	40.14	13.43	> 0.05
IFN- $\gamma$ +874	11.55	35.02	14.65	38.10	> 0.05
IL-4-589	3.25	50.54	6.94	54.24	> 0.05
IL-10-592	42.60	10.83	43.40	11.64	> 0.05
IL-10-1082	1.08	35.02	10.91	44.85	< 0.05

Table 4 Polymorphisms of IL-2-330, IFN- $\gamma$ +874, IL-10-1082 and -592 and IL-4-589 and HBV and HCV infection *n* (%)

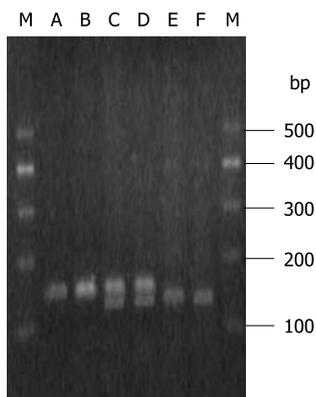
Genotype allele	Controls ( <i>n</i> = 74)	HCV infection ( <i>n</i> = 55)	HBV infection ( <i>n</i> = 69)	HBV/HCV coinfection ( <i>n</i> = 79)	$\chi^2$	P
IL-2-330						
TT	22 (29.7)	24 (43.6)	33 (47.8)	34 (43.0)		
GG	19 (25.7)	6 (10.9)	4 (5.8)	10 (12.7)	14.24	0.03
TG	33 (44.6)	25 (45.5)	32 (46.4)	35 (44.3)		
T	77 (52.0)	73 (66.4)	98 (71.0)	103 (65.2)	12.33	0.01
G	71 (48.0)	37 (33.6)	40 (29.0)	55 (34.8)		
IFN- $\gamma$ +874						
TT	7 (9.5)	5 (9.1)	9 (13.0)	11 (13.9)		
AA	14 (18.9)	23 (41.8)	25 (36.2)	35 (44.3)	16.15	0.01
TA	53 (71.6)	27 (49.1)	35 (50.7)	33 (41.8)		
T	67 (45.3)	37 (33.6)	53 (38.4)	55 (34.8)	4.87	0.18
A	81 (54.7)	73 (66.4)	85 (61.6)	103 (65.2)		
IL-10-1082						
GG	1 (1.4)	2 (3.6)	0 (0.0)	0 (0.0)		
AA	16 (21.6)	21 (38.2)	27 (39.1)	33 (41.8)	13.05	0.04
GA	57 (77.0)	32 (58.2)	42 (60.9)	46 (58.2)		
G	59 (39.9)	36 (32.7)	42 (30.4)	46 (29.1)	4.65	0.20
A	89 (60.1)	74 (67.3)	96 (69.6)	112 (70.9)		
IL-10-592						
AA	34 (45.9)	20 (36.4)	29 (42.0)	35 (44.3)		
CC	9 (12.2)	6 (10.9)	9 (13.0)	6 (7.6)	2.83	0.83
AC	31 (41.9)	29 (52.7)	31 (44.9)	38 (48.1)		
A	99 (66.9)	69 (62.7)	89 (64.5)	108 (68.4)	1.10	0.78
C	49 (33.1)	41 (37.3)	49 (35.5)	50 (31.6)		
IL-4-589						
CC	5 (6.8)	1 (1.8)	1 (1.4)	2 (2.5)		
TT	38 (51.4)	28 (50.9)	35 (50.7)	39 (49.4)	4.41	0.62
CT	31 (41.9)	26 (47.3)	33 (47.8)	38 (48.1)		
C	41 (27.7)	28 (25.5)	35 (25.4)	42 (26.6)	0.26	0.97
T	107 (72.3)	82 (74.5)	103 (74.6)	116 (73.4)		

infection. The OR and 95% CI for IL-2-330 TT compared with -330 GG in HCV infection, HBV infection and HBV/HCV coinfection were 3.46 (1.17-10.20), 7.14 (2.13-23.81) and 2.93 (1.15-7.46), respectively. However, IL-2-330 TT/GG did not differ significantly between patients with HBV and/or HCV infection. The IL-2-330 T allele was associated with an increased risk, but the IL-2-330 G allele was associated with a reduced risk of chronic HCV and/or HBV infection. The OR and 95% CI for the IL-2-330 T allele compared with -330 G allele in HCV infection, HBV infection, and HBV/HCV coinfection were 1.82 (1.09-3.03), 2.26 (1.39-3.69) and 1.73 (1.10-2.73), respectively. However, the IL-2-330 T/G allele did not differ significantly between HBV and/or HCV infection (Table 4).

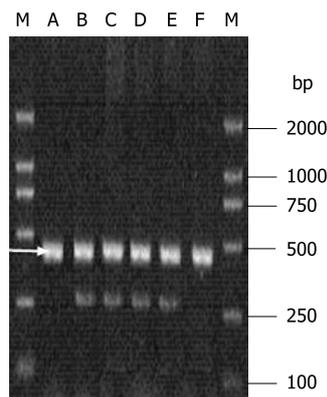
IFN- $\gamma$ +874 TT, +874 AA and +874 TA are shown in Figure 2. The IFN- $\gamma$ +874 polymorphisms showed

a significant association with persistent HBV and/or HCV infection. IFN- $\gamma$ +874 AA was associated with an increased risk, but IFN- $\gamma$ +874 TA with a reduced risk of persistent HBV and/or HCV infection. The OR and 95% CI for IFN- $\gamma$ +874 AA compared with +874 TA in HCV infection, HBV infection, and HBV/HCV coinfection were 3.23 (1.43-7.25), 2.70 (1.24-5.92) and 4.02 (1.88-8.55), respectively. However, IFN- $\gamma$ +874 AA/TA did not differ significantly between HBV and/or HCV infection. The IFN- $\gamma$ +874 T/A alleles did not differ significantly between all groups (Table 4).

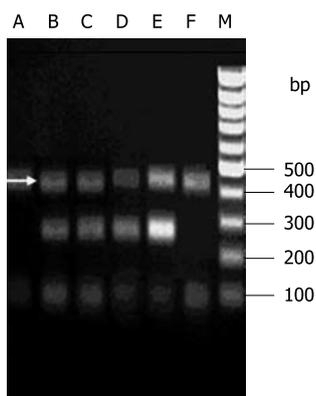
IL-10-1082 GG, -1082 AA and -1082 GA are shown in Figure 3. The IL-10-1082 polymorphisms showed a significant association with persistent HBV and/or HCV infection. IL-10-1082 AA was associated with an increased risk, but -1082 AG with a reduced risk of persistent HBV and/or HCV infection. The OR and 95%



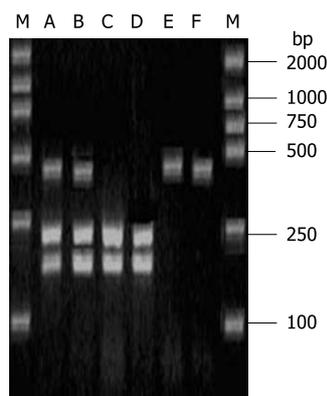
**Figure 1 IL-2-330 T/G SNP.** M: Marker; A and B: TT wild-type genotype; C and D: TG genotype; E and F: GG mutant genotype.



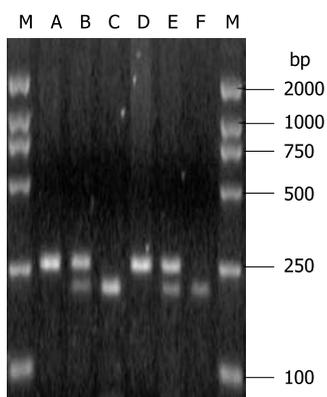
**Figure 2 IFN- $\gamma$ +874 T/A SNP.** M: Marker; A and B: AA mutant genotype; C and D: TA genotype; E and F: TT wild-type genotype;  $\rightarrow$ : Internal control gene.



**Figure 3 IL-10-1082 A/G SNP.** M: Marker; A and B: AA mutant genotype; C and D: AG genotype; E and F: GG wild-type genotype;  $\rightarrow$ : Internal control gene.



**Figure 4 IL-10-592 A/C SNP.** M: Marker; A and B: AC genotype; C and D: AA wild-type genotype; E and F: CC mutant genotype.



**Figure 5 IL-4-589 C/T SNP.** M: Marker; A and B: TT mutant genotype; C and D: CT genotype; E and F: CC wild-type genotype.

CI for IL-10-1082 AA compared with -1082 AG in HCV infection, HBV infection, and HBV/HCV coinfection were 2.34 (1.07-5.10), 2.29 (1.10-4.78) and 2.56 (1.25-5.21), respectively. However, IL-10-1082 AA/AG did not differ significantly between the subjects with HBV and/or HCV infection. The IL-10-1082 A/G alleles did not differ obviously between any of the subjects (Table 4).

The IL-10-592 polymorphisms are shown in Figure 4. The IL-10-592 A/C polymorphisms did not differ significantly between any of the groups (Table 4).

IL-10-589 TT, -589 CC and -589 TC are shown in Figure 5. IL-4-589 T/C polymorphisms did not differ significantly between any of the groups (Table 4).

#### **Association of IL-2-330, IFN- $\gamma$ +874, IL-4-589 and IL-10-1082 and -592 with clinical outcome of HBV and/or HCV infection**

The IL-2-330 polymorphisms showed a significant association with the outcome of persistent HBV and/or

HCV infection. IL-2-330 TT was associated with an increased risk, but -330 GG with a reduced risk of mild CH, moderate/severe CH, and cirrhosis. The OR and 95% CI for IL-2-330 TT compared with -330 GG genotype in patients with mild CH, moderate/severe CH, and cirrhosis were 3.42 (1.45-8.13), 3.29 (1.10-9.80), and 11.11 (1.32-90.91), respectively. However, IL-2-330 TT/GG did not differ significantly between patients with mild CH, moderate and severe CH, and cirrhosis. The IL-2-330 T allele was associated with an increased risk, but the -330 G allele was associated with a reduced risk of mild CH, moderate and severe CH, or cirrhosis. The OR and 95% CI for the -330 T allele compared with -330 G allele for patients with mild CH, moderate/severe CH and cirrhosis were 1.83 (1.20-2.80), 1.70 (1.02-2.82) and 2.75 (1.32-5.63), respectively. However, IL-2-330 polymorphisms did not differ significantly between patients with mild CH, moderate/severe CH, and cirrhosis (Table 5).

The IFN- $\gamma$ +874 polymorphisms showed a significant association with the clinical outcome of persistent HBV and/or HCV infection. IFN- $\gamma$ +874 AA was associated with an increased risk, but +874 TA with a reduced risk of mild CH, moderate/severe CH, and cirrhosis. There was significantly decreased IFN- $\gamma$ +874 AA genotype frequency in cirrhosis compared with moderate/severe CH patients. The OR and 95% CI for IFN- $\gamma$ +874 AA compared with +874 TA in patients with mild CH, moderate/severe CH, and cirrhosis were 3.09 (1.51-6.33), 3.85 (1.70-8.70) and 3.14 (1.08-9.17), respectively. The IFN- $\gamma$ +874 T/A alleles did not differ between any of the groups (Table 5).

The IL-10-1082 polymorphisms showed a significant association with the clinical outcome of persistent HBV

Table 5 Polymorphisms of IL-2-330, IFN- $\gamma$ +874, IL-4-589, IL-10-1082 and -592 and clinical outcome *n* (%)

Genotype	Control ( <i>n</i> = 74)	Mild CH ( <i>n</i> = 122)	Moderate/severe CH ( <i>n</i> = 57)	Cirrhosis ( <i>n</i> = 24)	$\chi^2$	<i>P</i>
IL-2-330						
TT	22 (29.7)	54 (44.3)	23 (40.4)	13 (54.2)	13.46	0.04
GG	19 (25.7)	14 (11.4)	6 (10.5)	1 (4.2)		
TG	33 (44.6)	54 (44.3)	28 (49.1)	10 (41.7)	11.69	0.01
T	77 (52.0)	163 (66.8)	74 (64.9)	36 (75.0)		
G	71 (48.0)	81 (33.2)	40 (35.0)	12 (25.0)		
IFN- $\gamma$ +874						
TT	8 (10.8)	14 (11.5)	5 (8.8)	4 (16.7)	14.78	0.02
AA	14 (18.9)	48 (39.3)	26 (45.6)	9 (37.5)		
TA	52 (70.3)	60 (49.2)	26 (45.6)	11 (45.8)	5.68	0.13
T	68 (45.9)	90 (36.9)	36 (31.6)	19 (39.6)		
A	80 (54.1)	154 (63.1)	78 (68.4)	29 (60.4)		
IL-10-1082						
GG	1 (1.4)	1 (0.8)	1 (1.8)	0 (0.0)	11.40	0.07
AA	16 (21.6)	44 (36.1)	24 (42.1)	13 (54.2)		
GA	57 (77.0)	77 (63.1)	32 (56.1)	11 (45.8)	5.85	0.12
G	59 (39.9)	79 (32.4)	34 (29.8)	11 (22.9)		
A	89 (60.1)	165 (67.6)	80 (70.2)	37 (77.1)		
IL-10-592						
AA	34 (45.9)	51 (41.8)	22 (38.6)	11 (45.8)	2.50	0.87
CC	9 (12.2)	11 (9.0)	8 (14.0)	2 (8.3)		
AC	31 (41.9)	60 (49.2)	27 (47.4)	11 (45.8)	0.92	0.82
A	98 (66.2)	163 (66.8)	71 (62.3)	33 (68.8)		
C	50 (33.8)	81 (33.2)	43 (37.7)	15 (31.3)		
IL-4-589						
CC	3 (4.0)	3 (2.4)	3 (5.3)	0 (0.0)	4.58	0.60
TT	38 (51.4)	69 (54.8)	24 (42.1)	11 (45.8)		
CT	33 (44.6)	54 (42.9)	30 (52.6)	13 (54.2)	2.46	0.48
C	39 (26.4)	58 (23.8)	36 (31.6)	13 (27.1)		
T	109 (73.6)	186 (76.2)	78 (68.4)	35 (72.9)		

and/or HCV infection. IL-10-1082 AA was associated with an increased risk, but -1082 AG with a reduced risk of mild CH, moderate/severe CH, and cirrhosis. There was a significantly increased IL-10-1082 AA genotype frequency in cirrhosis compared with mild CH. IL-10-1082 AA showed a positive correlation with clinical outcome ( $\gamma = 0.99$ ,  $P = 0.00$ ), but the IL-10-1082 AG showed a negative correlation with clinical outcome ( $\gamma = -0.99$ ,  $P = 0.00$ ). The OR and the 95% CI for IL-10-1082 AA compared with -1082 AG in patients with mild CH, moderate/severe CH, and cirrhosis were 2.03 (1.03-3.98), 2.70 (1.24-5.88) and 4.26 (1.59-11.36), respectively. The IL-10-1082 G/A allele did not differ obviously in every group (Table 5).

The polymorphisms of IL-10-592 A/C and IL-4-589 C/T did not differ significantly in every group (Table 5).

#### **Association of IL-2-330, IFN- $\gamma$ +874, IL-4-589 and IL-10-1082 and -592 polymorphisms with HCV replication and abnormal alanine aminotransferase (ALT) level**

IL-10-1082 AA was associated with an increased risk, but -1082 AG was associated with a reduced risk of HCV RNA replication. The OR and 95% CI for IL-10-1082 AA compared with -1082 AG was 3.36 (1.67-6.76). IL-10-1082 A was associated with an increased risk, but -1082 G with a reduced risk of HCV RNA replication. The OR and 95% CI for IL-10-1082 A compared with -1082 G was 1.67 (1.08-2.57). The polymorphisms of

IL-2-330, IFN- $\gamma$ +874, IL-10-592 and IL-4-589 showed no association with HCV RNA replication (Table 6).

IL-10-592 AC showed an increased risk, but -592 AA showed a reduced risk of abnormal ALT. The OR and 95% CI for IL-10-592 AC compared with -592 AA was 2.83 (1.21-6.63). The IL-10-592 A/C alleles were not associated with abnormal ALT (Table 6).

The IL-4-589 CT/CC showed an increased risk, but -589 TT showed a reduced risk of abnormal ALT level. The OR and 95% CI for IL-10-589 CT and -589 CC compared with -589 TT genotype were 3.43 (1.47-8.00) and 8.25 (1.74-39.22), respectively, in patients with abnormal ALT. IL-4-589 C showed an increased risk, but -589 T showed a reduced risk of abnormal ALT level (Table 6). The OR and 95% CI for IL-10-589 C compared with -589 T was 2.31 (1.36-3.93).

The polymorphisms of IL-2-330, IFN- $\gamma$ +874 and IL-10-1082 were not significantly associated with abnormal ALT (Table 6).

## **DISCUSSION**

The natural outcome of HBV and HCV infection varies dramatically among individuals. HCV infection is self-limited in a fortunate minority, while the majority of subjects develop persistent (chronic) infection<sup>[1,2]</sup>. Although infection with HBV in adults progresses to the chronic phase in about 5%<sup>[3]</sup>, among those individuals with persistent HBV or HCV infection, the majority develop

Table 6 Polymorphisms of IL-2-330, IFN- $\gamma$ +874, IL-4 -589 and IL-10-1082 and -592 and HCV RNA and ALT level *n* (%)

	HCV RNA -	HCV RNA +	$\chi^2$	<i>P</i>	ALT < 80 U/L	ALT $\geq$ 80 U/L	$\chi^2$	<i>P</i>
IL-2-330								
TT	29 (37.2)	39 (37.1)			102 (41.8)	11 (33.3)		
GG	14 (17.9)	14 (13.3)	0.83	0.66	35 (14.3)	4 (12.1)	1.35	0.51
TG	35 (44.9)	52 (49.5)			107 (43.9)	18 (54.5)		
T	93 (59.6)	130 (61.9)	0.20	0.66	311 (63.7)	40 (60.0)	0.24	0.62
G	63 (40.4)	80 (38.1)			177 (36.3)	26 (39.4)		
IFN- $\gamma$ +874								
TT	13 (16.7)	10 (9.5)			29 (11.9)	3 (9.1)		
AA	28 (35.9)	45 (42.9)	2.36	0.31	85 (34.8)	12 (36.4)	0.23	0.89
TA	37 (47.4)	50 (47.6)			130 (53.3)	18 (54.5)		
T	63 (40.4)	70 (33.3)	1.92	0.17	188 (38.5)	24 (36.4)	0.12	0.74
A	93 (59.6)	140 (66.7)			300 (61.5)	42 (63.6)		
IL-10-1082								
GG	1 (1.3)	2 (1.9)			3 (1.2)	0 (0.0)		
AA	14 (17.9)	44 (41.9)	12.27	0.00	81 (33.2)	16 (48.5)	3.25	0.20
GA	63 (80.8)	59 (56.2)			160 (65.6)	17 (51.5)		
G	91 (58.3)	147 (70.0)	5.36	0.02	166 (34.0)	17 (25.8)	1.79	0.18
A	65 (41.7)	63 (28.8)			322 (66.0)	49 (74.2)		
IL-10-592								
AA	41 (52.6)	44 (41.9)			110 (45.1)	8 (24.2)		
CC	6 (7.7)	11 (10.5)	2.10	0.35	27 (11.1)	3 (9.1)	6.32	0.04
AC	31 (39.7)	50 (47.6)			107 (43.9)	22 (66.7)		
A	113 (72.4)	138 (65.7)	1.88	0.17	327 (67.0)	38 (57.6)	2.30	0.09
C	43 (27.6)	72 (34.3)			161 (33.0)	28 (42.4)		
IL-4-589								
CC	3 (3.8)	5 (4.8)			6 (2.5)	3 (9.1)		
TT	48 (61.5)	55 (52.4)	1.53	0.47	132 (54.1)	8 (24.2)	12.46	0.02
CT	27 (34.6)	45 (42.9)			106 (43.4)	22 (66.7)		
C	33 (21.2)	55 (26.2)	1.24	0.27	118 (24.2)	28 (42.4)	9.97	0.00
T	123 (78.8)	155 (73.8)			370 (75.8)	38 (57.6)		

CH, progressive fibrosis and even liver cancer<sup>[4,5,13]</sup>. However, some cases will never progress to any significant liver disease<sup>[6,7]</sup>. It remains unknown why the infection frequently turns into CH, and why the outcome with HBV and/or HCV infection varies dramatically. Cytokines play a crucial role in regulating the immune and inflammatory responses. Polymorphisms in the regulatory regions of the cytokine genes may influence their expression. Therefore, as genetic predictors of the disease susceptibility or clinical outcome, the polymorphisms of cytokine genes are potentially important.

IL-2 is a Th1 cytokine and has a powerful immunoregulatory effect on the stimulation of proliferation and activation of most T lymphocytes, natural killer cells, and B lymphocytes<sup>[14]</sup>. Some evidence has shown that the IL-2 levels of patients with moderate/severe CH and cirrhosis are decreased significantly more than those of healthy controls<sup>[15-17]</sup>. Other authors have observed that increased IL-2 expression is correlated with the grading/staging of chronic hepatitis C<sup>[18]</sup>. In this study, we found that the IL-2-330 SNP was associated with persistent HBV and/or HCV infection. The subjects with persistent HBV and/or HCV infections had significantly lower IL-2-330 GG genotype frequency and higher IL-2-330 TT genotype frequency than the controls. The subjects with IL-2-330 TT had a 3.46, 7.14 and 2.93 times higher risk of infection with HCV, HBV, or HBV/HCV. The subjects with chronic HCV and/or HBV infections had higher IL-2-330 T allele and lower -330 G allele frequencies,

respectively. The patients with the -330 T allele had a 1.82, 2.26 and 1.73 times higher risk of infection with HCV, HBV and HBV/HCV, respectively. At the same time, we also found an association between IL-2-330 SNP and clinical outcome of mild CH, moderate/severe CH and cirrhosis. Compared with the controls, the patients with mild CH, moderate/severe CH and cirrhosis had significantly higher IL-2-330 TT genotype frequency and lower -330 GG genotype frequency. IL-2-330 GG showed a negative correlation with clinical outcome ( $\gamma = -0.92$ ,  $P = 0.01$ ). Individuals with -330 TT genotype had a 3.42, 3.29 and 11.11 times higher risk of developing into mild CH, moderate/severe CH, and cirrhosis, respectively. Subjects with IL-2-330 T had a 1.83, 1.70 and 2.75 times higher risk of developing into mild CH, moderate/severe CH, and cirrhosis, respectively. These results indicate that the IL-2-330 TT genotype and the -330 T allele are not only the potential susceptibility gene for HBV and/or HCV infections, but also the gene that potentially determines clinical outcome. Moreover, the clinical outcome of HBV and/or HCV infection is associated with IL-2 level, as determined by the IL-2-330 polymorphisms. A plausible explanation for the association of IL-2-330 TT genotype and T allele with persistent infection and clinical outcome is derived from experiments that have shown that patients with the IL-2-330 TT genotype and T allele, with lower IL-2 production<sup>[5]</sup>, unsuccessfully eradicate the virus and become persistently viremic, which further results in susceptibility to HBV and/or HCV infection.

IFN- $\gamma$  is a Th1 cytokine and plays an important role in modulating almost all the immune responses, such as T-cell differentiation, anti-proliferative, antitumor, and antiviral activities<sup>[19,20]</sup>. Pravica *et al.*<sup>[21]</sup> have found that IFN- $\gamma$ +874 T/T genotype is often regarded as indicating high IFN- $\gamma$  production, and +874 A/A genotype as indicating low production. Some authors have found that plasma IFN- $\gamma$  concentrations in chronic hepatitis C patients decrease significantly<sup>[22,23]</sup>. Other authors have documented a correlation between the increase of IFN- $\gamma$  expression and grading in chronic hepatitis C infection<sup>[18,24,25]</sup>. In this study, compared with the controls, the IFN- $\gamma$ +874 AA genotype was found more frequently and the +874 TA genotype was found less frequently in subjects with persistent HBV and/or HCV infection. Subjects with +874 AA genotype have a 3.23, 2.70 and 4.02 times higher risk of HCV infection, HBV infection, and HBV/HCV coinfection, respectively. No difference was found in the IFN- $\gamma$ +874 T/A alleles among the groups. However, Liu *et al.*<sup>[26]</sup> have found that the frequency of IFN- $\gamma$ +874 A allele was significantly higher in patients with chronic HBV infection than in the controls (OR = 2.25, 95% CI = 1.69-2.99,  $P < 0.0001$ ). Moreover, compared with the controls, the patients with mild CH, moderate/severe CH, and cirrhosis showed a higher IFN- $\gamma$ +874 AA frequency and a lower IFN- $\gamma$ +874 TA frequency. Individuals with IFN- $\gamma$ +874 AA had a 3.09, 3.85 and 3.14 times higher risk of developing mild CH, moderate/severe CH, and cirrhosis, respectively. Compared with moderate/severe CH patients, cirrhosis patients showed a significantly decreased +874 AA genotype frequency. This probably resulted from the lower number of samples of cirrhosis. These results indicate that IFN- $\gamma$ +874 AA genotype is not only the potential susceptibility gene for HBV and/or HCV infection, but also the gene that potentially determines clinical outcome. A plausible explanation is that IFN- $\gamma$ +874 AA genotype produces a low level of IFN- $\gamma$ , which results in the cytolytic defect that promotes viral persistence and hepatic persistent infection that determines the clinical outcome of HBV and/or HCV infection.

IL-10 is an important anti-inflammatory cytokine secreted from Th2 cells. The levels of IL-10 production determine immune regulation, and the balance between the inflammatory and humoral responses. There are three confirmed SNPs in the IL-10 gene promoter. The -1082 GG genotype is associated with high IL-10 production, the -1082 GA with intermediate production, and the -1082 AA with low production. Polymorphisms at position -819 and -592 have no independent influence on IL-10 production<sup>[27]</sup>. There are contradictory reports about the exact effect of IL-10 promoter polymorphisms on the natural outcome of HBV and HCV infection. Some authors have found that plasma IL-10 level is decreased significantly in chronic hepatitis C patients<sup>[22,28]</sup>. Others have reported that the level of IL-10 in patients with chronic hepatitis C is higher than that in healthy individuals ( $P < 0.05$ ). The value of IL-10 showed a significant positive correlation with ALT<sup>[29,30]</sup>. In the present study, IL-10-1082 AA was associated with an increased

risk, but -1082 AG was associated with a reduced risk of persistent HBV and/or HCV infection. Subjects with IL-10-1082 AA had a 2.34, 2.29 and 2.56 times higher risk of persistent HCV infection, HBV infection and HBV/HCV coinfection, respectively. At the same time, IL-10-1082 AA is associated with an increased risk, but -1082 AG is associated with a reduced risk of mild CH, moderate/severe CH, and cirrhosis. Also, IL-10-1082 AA showed a positive correlation with clinical outcome ( $\gamma = 0.99$ ,  $P < 0.01$ ), but -1082 AG showed a negative correlation with clinical outcome ( $\gamma = -0.99$ ,  $P < 0.01$ ). Individuals with the -1082 AA genotype had a 2.03, 2.70 and 4.26 times higher risk of developing mild CH, moderate/severe CH, and cirrhosis, respectively. These results indicate that the IL-10-1082 AA genotype is not only a potential susceptibility gene for HBV and/or HCV infection, but also potentially determines clinical outcome. IL-10-1082 AA and -1082 A were associated with an increased risk, but IL-10-1082 AG and -1082 G were associated with a reduced risk of HCV RNA replication (OR = 3.36, 1.67, respectively). This result indicates that IL-10-1082 AA and -1082 A are associated with HCV RNA replication. The evidence shows that during persistent HCV infection, IL-10 may compromise the cellular immune response to the virus<sup>[31-33]</sup>. A plausible explanation is that IL-10 is an important anti-inflammatory cytokine. IL-10-1082 AA and -1082 A lead to lower IL-10 production<sup>[27]</sup> and persistent infection, which determines the clinical outcome of chronic infection. The IL-10-1082 polymorphisms had no significant association with plasma ALT level, which agrees with the results of Abbas *et al.*<sup>[34]</sup>. The IL-10-592 AC genotype frequency was significantly higher in patients with abnormal ALT (OR = 2.83). This result indicates that the IL-10-592 AC genotype is associated with abnormal ALT level and hepatocellular injury. Hepatocellular injury is probably caused by the decreased production of the anti-inflammatory cytokine IL-10, which results from linkage of the IL-10-592 and IL-10-1082 genes.

IL-4 is a Th2 cytokine and plays an important role in humoral immunity. IL-4 has the function of inhibiting the production of IFN- $\gamma$  and downregulating the differentiation of Th1 cells<sup>[35]</sup>. IL-4 has the function of modulating inflammatory responses by downregulating production of pro-inflammatory mediators and preventing inflammatory injury of the liver<sup>[36,37]</sup>. It has been described that the IL-4-589 T compared with the -590 C allele increases the strength of the IL-4 promoter<sup>[38]</sup>, which leads to high production of IL-4. The evidence has shown that IL-4 plays a key role in HBV and HCV infection pathogenesis. Some have reported that IL-4 levels are lower in patients infected with HCV<sup>[39]</sup>. Others have reported that IL-4 is significantly higher in chronic HCV infection with abnormal ALT than with normal ALT level<sup>[38,39]</sup>. In the present study, IL-4-589 polymorphisms were not significantly associated with persistent HBV and/or HCV infection or clinical outcome. However, we found that the IL-4-589 CT genotype and -589 CC genotype frequency was significantly higher than that

of -589 TT genotype frequency in patients with abnormal ALT level. Individuals with IL-4-589 CT and -589 CC genotype had a 3.43 and 8.25 times higher risk of abnormal ALT. Higher -589 C allele and lower -589 T allele frequencies in patients with abnormal ALT were observed; individuals with the -589 C allele had a 2.31 times higher risk of abnormal ALT. This result indicates that the IL-4-589 CC and -589 CT genotype and the -589 C allele are associated with liver inflammatory injury. Such injury is probably caused by the decreased production of anti-inflammatory cytokines such as IL-4 and IL-10.

In summary, the polymorphisms of IL-2-330, IFN- $\gamma$ +874, IL-10-1082 and -592 and IL-4-589 showed a significant association with the outcome of chronic HBV and/or HCV infection. The IL-2-330 TT/T allele, IFN- $\gamma$ +874 AA and IL-10-1082 AA were associated with increased risk, but IL-2-330 GG/G allele, IFN- $\gamma$ +874 TA and IL-10-1082 AG were associated with reduced risk of persistent HBV and/or HCV infection, and of developing mild CH, moderate/severe CH, and cirrhosis. The IL-10-1082 AA/A allele was associated with an increased risk, but the IL-10-1082 AG/G allele was associated with a reduced risk of HCV RNA replication. IL-10-592 AC and IL-4-589 CT/CC showed increased risk, but IL-10-592 AA and IL-4-589 TT showed reduced risk of abnormal ALT.

## COMMENTS

### Background

The natural outcome in hepatitis B virus (HBV) and hepatitis C virus (HCV) infection varies dramatically among individuals. Some infections are self-limited; some individuals progress to persistent HBV or HCV infection; and some develop chronic hepatitis, fibrosis and even liver cancer. The mechanisms are unknown. Besides viral pathogenic factors, a more important factor is the different immune response to HBV or HCV infection between individuals. T helper (Th)1 and Th2 cytokines determine host immunity level, which is regulated by polymorphisms in the promoter region of the Th1 and Th2 cytokines. Interleukin (IL)-2 (-330), interferon (IFN)- $\gamma$  (+874), IL-10 (-1082, -592), IL-4 (-589) should have some association with the outcome of HBV and/or HCV infection.

### Research frontiers

It remains unknown why subjects infected with HBV and/or HCV frequently develop chronic infection, and why the outcome of infection varies dramatically. Among infections with HBV/HCV, some show a strong reduction in CD4<sup>+</sup> T-helper and CD8<sup>+</sup> cytotoxic-T-lymphocyte responses. This is likely to be crucial in clearance of acute viremia. It is thought that immune tolerance to HBV/HCV infection may explain the different susceptibility to HBV/HCV infection. Many manifestations show that the immunity level of the host correlates with relevant gene polymorphisms, especially with single nucleotide polymorphisms in the promoter region, which regulates gene expression. Furthermore, the gene polymorphisms probably determine the outcome of the infection.

### Innovations and breakthroughs

The polymorphisms of IL-2-330, IFN- $\gamma$ +874, IL-10-1082 and -592 and IL-4-589 showed a significant association with the outcome of chronic HBV and/or HCV infection. The IL-2-330 TT/T allele, IFN- $\gamma$ +874 AA and IL-10-1082 AA were associated with increased risk, but the IL-2-330 GG/G allele, IFN- $\gamma$ +874 TA and IL-10-1082 AG were associated with reduced risk of persistent HBV and/or HCV infection, and of developing mild chronic hepatitis (CH), moderate/severe CH, and cirrhosis. The IL-10-1082 AA/A allele was associated with increased risk, but the IL-10-1082 AG/G allele was associated with reduced risk of HCV RNA replication. IL-10-592 AC and IL-4-589 CT/CC showed increased risk, but IL-10-592 AA and IL-4-589 TT showed reduced risk of abnormal ALT.

### Applications

These results suggest that polymorphisms in some cytokine genes influence persistent HBV and/or HCV infection, clinical outcome, HCV replication and

liver damage. Some cytokines, including IL-2, IFN- $\gamma$  and IL-10 might be beneficial to the clinical outcome of HBV and/or HCV infection. Some cytokines, including IL-4, might result in liver damage. These results provide a scientific basis for the further treatment of patients with chronic HBV and HCV infection and viral hepatitis.

### Terminology

IL-2 is a Th1 cytokine and has a powerful immunoregulatory effect on the stimulation of proliferation and activation of most T lymphocytes, natural killer cells, and B lymphocytes. IFN- $\gamma$  is a Th1 cytokine and plays an important role in modulating almost all the immune responses, such as T-cell differentiation, and anti-proliferative, antitumor, and antiviral activities. IL-10 is an important anti-inflammatory cytokine secreted from Th2 cells. The levels of IL-10 production determine immunoregulation, and the balance between the inflammatory and humoral responses. IL-4 is a Th2 cytokine and plays an important role in humoral immunity. IL-4 has the function of inhibiting the production of IFN- $\gamma$  and downregulating differentiation of Th1 cells.

### Peer review

This is interesting paper. These results suggest that the IL-10-1082 polymorphisms are associated with Hepatitis C viral replication and the IL-10-592 and IL-4-589 polymorphisms influence liver damage.

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CASE REPORT

## Asymptomatic lymphangioma involving the spleen and retroperitoneum in adults

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### Abstract

Lymphangioma, a benign neoplasm of the lymphatic system, is common in children but rare in adults. Its clinical manifestations include abdominal pain, nausea, vomiting and a palpable mass. However, abdominal sonography or abdominal computed tomography (CT) scan can also incidentally reveal lymphangioma. A larger or symptomatic lymphangioma is treated with total resection to prevent recurrence, infection, torsion and enlargement. Although lymphangioma rarely becomes malignant, its prognosis is generally good. We report a cystic lymphangioma of the spleen and retroperitoneum, which was incidentally found in a 56-year-old man who was hospitalized due to a colon mass. Physical examination showed no specific findings. Abdominal CT revealed a 5.7 cm, non-enhanced multilobulated cystic mass with multiple septa in the spleen and a 10 cm lobulated cystic mass in the para-aortic area. Splenectomy and retroperitoneal resection of the cystic mass were conducted. The endothelium

of splenic and retroperitoneal cyst was immunohistochemically stained with D2-40 antibody. The patient was finally diagnosed with splenic cystic and retroperitoneal cavernous lymphangioma.

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**Key words:** Lymphangioma; Retroperitoneum; Spleen

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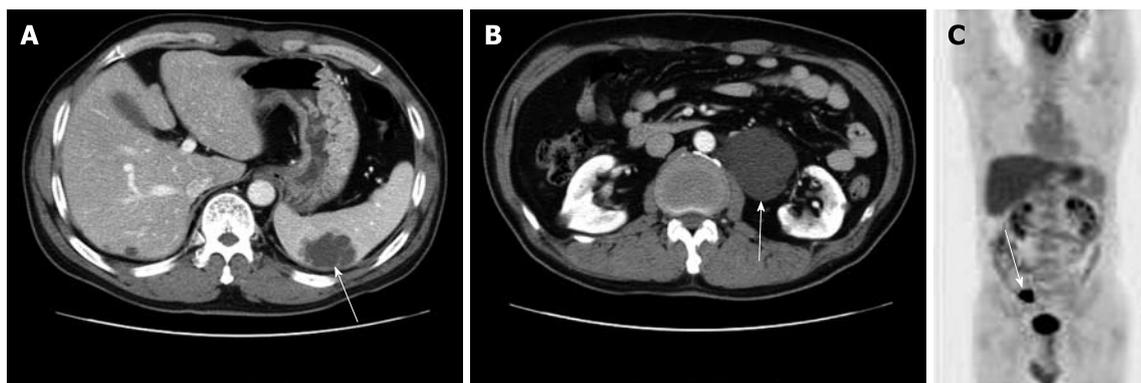
Chung SH, Park YS, Jo YJ, Kim SH, Jun DW, Son BK, Jung JY, Baek DH, Kim DH, Jung YY, Lee WM. Asymptomatic lymphangioma involving the spleen and retroperitoneum in adults. *World J Gastroenterol* 2009; 15(44): 5620-5623 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5620.asp>  
DOI: <http://dx.doi.org/10.3748/wjg.15.5620>

### INTRODUCTION

Lymphangioma, a very uncommon neoplasm, is mainly found in children but rarely in adults<sup>[1,2]</sup>. In 1885, Frink reported the first lymphangioma in the spleen. Cases of splenic and retroperitoneum lymphangioma are rarely reported in adults. In our case, lymphangioma involved both the spleen and the retroperitoneum. Cases like ours, involving two organs, are extremely rare<sup>[3-5]</sup>. The clinical manifestations of lymphangioma include abdominal pain, fever, fatigue, weight loss and hematuria. However, it is sometimes asymptomatic<sup>[6,7]</sup>. We report, here, a case of a 56-year-old man who was hospitalized due to a colon mass. Lymphangioma in his spleen and retroperitoneum was incidentally found in abdominal computed tomography (CT). This case is very rare because lymphangioma was found both in his spleen and in his retroperitoneum.

### CASE REPORT

A 56-year-old man was hospitalized at a private clinic due to a suspected colon cancer detected at colonoscopy. He



**Figure 1** Enhanced abdominal CT and F-18 FDG Torso PET CT images of lymphangioma. A: A 5.7 cm lobulated & septated mass without enhancement in the spleen (white arrow); B: A 10 cm lobulated cystic mass in the paraaortic area (white arrow); C: A hypermetabolic lesion in the sigmoid colon mass (white arrow) and maxSUV 23 but no hypermetabolic lesion in the spleen and retroperitoneum.

had no history of any medical disease, and no weight loss, abdominal pain and dyspepsia. Physical examination revealed no palpable mass, tenderness or rebound tenderness in his abdomen with normal bowel sound. Laboratory studies had no specific findings except for creatinine (1.4 mg/dL). Abdominal X-ray did not reveal calcification, but abdominal CT showed a 5.7 cm non-enhanced cystic mass with septum in the spleen and a 10 cm lobulated cystic mass in the left paraaortic area (Figure 1A and B). PET CT revealed a hypermetabolic lesion in the sigmoid colon where the maxSUV was 23. However, no hypermetabolic lesion was found in the spleen or in the left paraaortic area (Figure 1C).

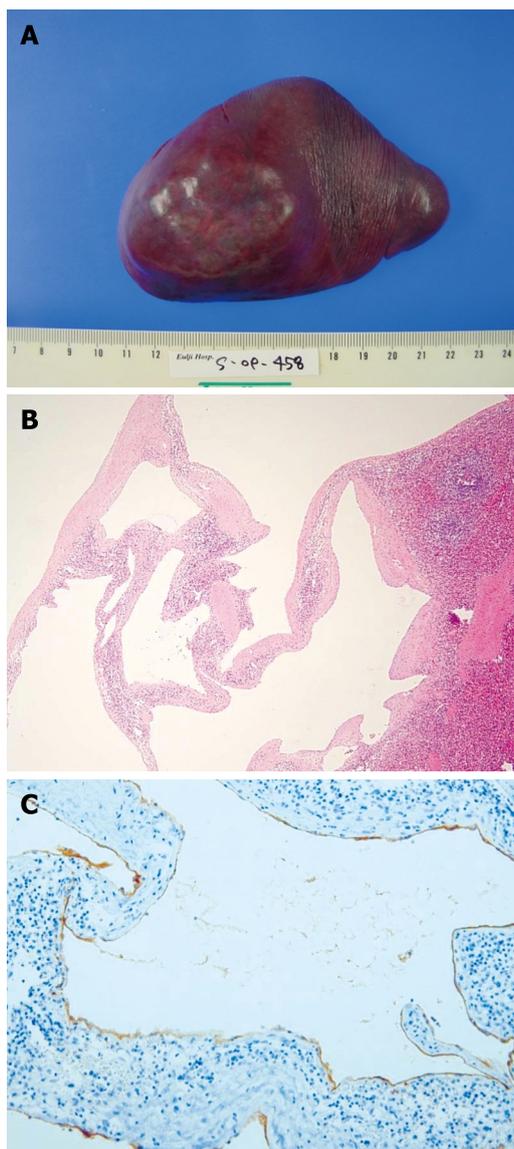
A 5 cm × 3 cm colon polyp with a neck and an intramural adenocarcinoma invading the lamina propria were found in the sigmoid colon. Splenectomy and resection of the retroperitoneal mass were conducted, during which a 4.5 cm × 3.5 cm bulging mass was exposed in the lower pole of the spleen (Figure 2A). The cut surface was composed of a multilobular cyst with a smooth inner surface. Histology showed thin fibrous walls of the cyst, which were different in size with no bleeding or necrosis (Figure 2B). In immunohistochemical study, the endothelium of splenic cystic mass was stained with D2-40 antibody (Figure 2C) and finally diagnosed as a cystic lymphangioma. The retroperitoneal mass was 4.5 cm × 4.5 cm × 2.5 cm in size, and its outer surface was lobular and yellow to brown in color (Figure 3A). The cut surface showed a sponge-like and ill-defined cyst.

Histology showed small cysts interspersed among fat, which were different in size, but had no calcification, bleeding, or necrosis (Figure 3B). In immunohistochemical study, the endothelium of retroperitoneal cystic mass was stained with D2-40 antibody (Figure 3C). The retroperitoneal mass was finally diagnosed as a cavernous lymphangioma. The patient was discharged 10 d after operation without complication.

## DISCUSSION

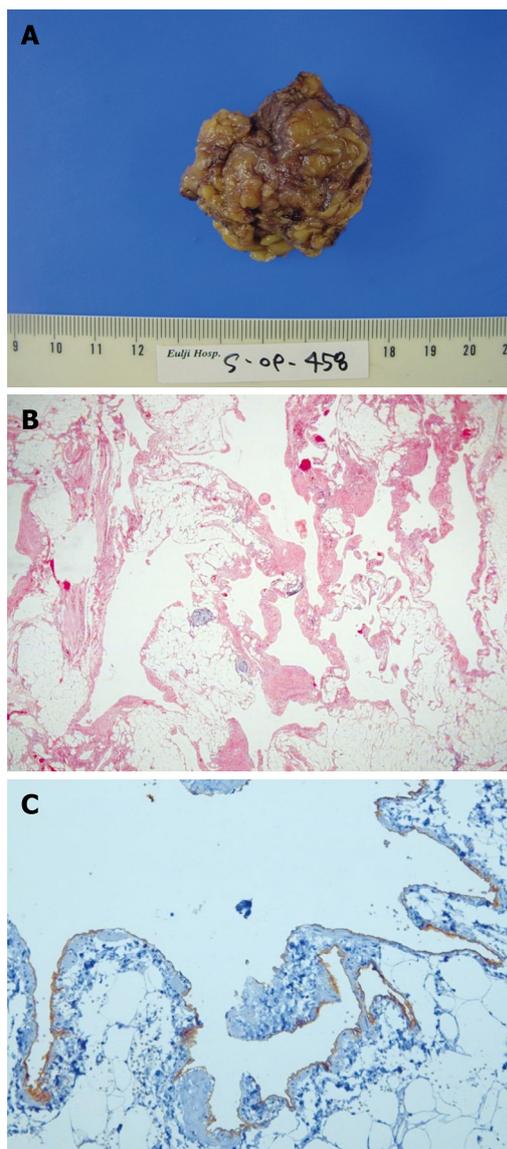
Cystic lymphangioma is a benign neoplasm composed of a malformation of the lymphatic system<sup>[1,2]</sup>. The incidence of lymphangioma occurring after 20 years of age

is very low. Lymphangioma generally occurs under the age of 2 years. There is no difference in the incidence between males and females<sup>[2]</sup>. The most commonly involved sites are the neck (75%) and axilla (20%). Lymphangioma is infrequently encountered in the mediastinum, adrenal gland, kidney, bone, omentum, gastrointestinal track, retroperitoneum, spleen, liver and pancreas<sup>[2,5]</sup>. There are two hypotheses for the causes and pathogenesis of lymphangioma, namely abnormal congenital development and bleeding or inflammation of the lymphatic system which causes an obstruction leading to additional lymphangiomas. The clinical manifestations of lymphangioma are left upper quadrant pain, abdominal distension, loss of appetite, nausea, vomiting and a palpable mass. However, lymphangioma can also occur without any symptoms<sup>[6,7]</sup>. Our case had no symptoms of lymphangioma and no palpable mass in the abdomen, and is unique because lymphangioma was found both in his spleen and in his retroperitoneum. Even if there are reports on cystic lymphangioma involving mesentery and retroperitoneum<sup>[2]</sup>, no report is available on lymphangioma involving both the spleen and the retroperitoneum in an asymptomatic patient. Lymphangioma involving more than two abdominal organs is rarely reported<sup>[2]</sup>. In our case, renal vein penetrated through the retroperitoneal lymphangioma, but retroperitoneal lymphangioma could be resected without impairment of the kidneys. Abdominal sonography can show a lowly attenuated mass and a highly attenuated inner part of lymphangioma, while abdominal CT may demonstrate multiple lowly attenuated, thin-walled, well-demarcated cysts and calcification in the cyst wall. The cystic surface can be divided by a septum and multiple lobules<sup>[7]</sup>. MRI may reveal a highly attenuated space in multiple lobules. Only a radiological image may have limitations in its diagnosis<sup>[6,7]</sup>. In this case, abdominal CT revealed a 5.7 cm cystic mass in the spleen and a 10 cm cystic mass composed of lobules in the paraaortic area. PET CT did not show highly metabolized lesions in the spleen or in the retroperitoneum of our case. Generally, lymphangioma is divided into capillary, cavernous and cystic types, with the cystic type being the most common<sup>[5]</sup>. Histology can show dilated lymphatic vessels surrounded by flat endothelial cells between fat, fibrotic



**Figure 2** Gross and microscopic images of splenic lymphangioma. A: A 4.5 cm × 3.5 cm bulging cystic mass in lower pole; B: HE staining of splenic lymphangioma, × 20; C: Immunostaining with D2-40 antibody (× 200) for cystic lining cells.

and lymphatic structures. Immunohistochemical staining can show a lymphangioma which is positive for keratin and HBME, and is negative for FV III-Rag, CD 31, CD 34 because it develops from the middle lobe<sup>[8]</sup>. Lymphatic vessel endothelial receptor-1, vascular endothelial growth factor-3, prox-1, and monoclonal antibody D2-40 are the markers for specific endothelial cells of lymphatic tissues, which are used in immunochemical studies of lymphangioma. In this case, the endothelium of splenic and retroperitoneal cystic mass was stained with D2-40 antibody and the splenic and retroperitoneal cystic mass was finally diagnosed as splenic cystic and retroperitoneal cavernous lymphangioma, based on the microscopic findings. Surgical treatment is needed to prevent recurrence, infection, rupture, and bleeding<sup>[5]</sup>. Conservative treatment modalities include aspiration, drainage and sclerosis, with a high risk of recurrence. However, an incidentally found small cyst needs not be resected, and the current treatment is



**Figure 3** Gross and microscopic images of retroperitoneal lymphangioma. A: A 4.5 cm × 4.5 cm × 2.5 cm retroperitoneal mass; B: HE staining of retroperitoneal lymphangioma, × 20; C: Immunostaining with D2-40 antibody (× 200) for cystic lining cells.

a partial splenectomy to remove it<sup>[5]</sup>. The post-operative recurrence rate is low and the rate of transformation into malignancy is very low as well. Complications are peritonitis, bleeding, abscess and torsion<sup>[9]</sup>. The natural regression rate is very low. If multiple lymphangiomas progress rapidly and are invasive, their prognosis could be poor<sup>[10]</sup>. In this case, a 5.7 cm splenic lymphangioma and a 10 cm lymphangioma were surgically removed and the patient was discharged 10 d later without any complication. Our case may be the first reported case of lymphangioma involving both the spleen and the retroperitoneum in an asymptomatic patient.

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CASE REPORT

## Acute myelogenous leukemia and acute leukemic appendicitis: A case report

Po-Jen Hsiao, Shih-Ming Kuo, Jia-Hong Chen, Hsuen-Fu Lin, Pau-Ling Chu, Shih-Hua Lin, Ching-Liang Ho

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### INTRODUCTION

Acute myeloid leukemia (AML) affects middle-aged adults. When the disease involves soft tissue, it is called granulocytic sarcoma (GS). GS can present in the gastrointestinal tract but involvement of the appendix is uncommon. Furthermore, infiltration of the appendix by leukemic cells is also a rare manifestation of leukemia relapse. Herein, we report a 75-year-old man with AML-M2 who had been in partial remission for 1 year, and who presented with symptoms mimicking acute appendicitis as the initial manifestation of leukemia relapse. Subsequent pathological examination confirmed the diagnosis.

### CASE REPORT

A 75-year-old man was admitted for evaluation of right lower quadrant abdominal pain and fever for 3 d. He had partial remission of AML-M2 for 1 year, after chemotherapy with low dose cytarabine. His past history included hypertensive cardiovascular disease with congestive heart failure, coronary artery disease, and chronic obstructive pulmonary disease. Physical examination showed rebound tenderness over the right lower quadrant. The leukocyte count was  $35 \times 10^3/L$ , with 15% neutrophils, 26% lymphocytes, 1% monocytes, 2% eosinophils, 0% basophils, and 56% immature cells. Hemoglobin and platelet counts were 9.9 g/dL and  $64 \times 10^3/\mu L$ , respectively. C-reactive protein was 7.98 mg/dL. Abdominal computed tomography showed thickening of the appendiceal wall and periappendicular fat stranding (Figure 1). The diagnosis of acute appendicitis was made, and appendectomy was performed immediately after admission. Grossly, the appendix was gray in color and soft in consistency. Microscopically, the sections showed transmural infiltrates of myeloblasts, which were positive for myeloperoxidase, CD43 and CD34 immunohistochemical stains (Figure 2). Hence, AML-M2 with involvement of the appendix was diagnosed. Thereafter the patient received chemotherapy with low-

### Abstract

Acute myelogenous leukemia (AML) can involve the gastrointestinal tract but rarely involves the appendix. We report a male patient who had 1 year partial remission from AML and who presented with apparent acute appendicitis as the initial manifestation of leukemia relapse. Pathological findings of the appendix revealed transmural infiltrates of myeloblasts, which indicated a diagnosis of leukemia. Unfortunately, the patient died from progression of the disease on the 19th d after admission. Although leukemic cell infiltration of the appendix is uncommon, patients with leukemia relapse can present with symptoms mimicking acute appendicitis.

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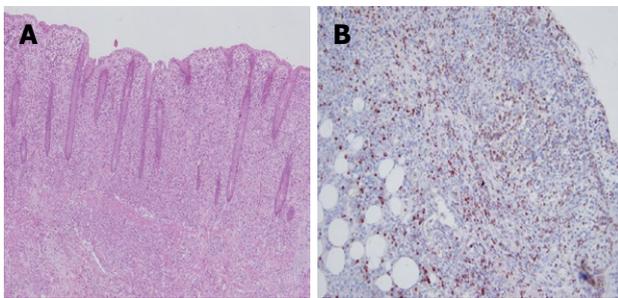
**Key words:** Acute myeloid leukemia; Appendicitis; Appendectomy; Granulocytic sarcoma

**Peer reviewer:** Mr. Morten Hylander Møller, Department of Anaesthesiology and Intensive Care Medicine, Copenhagen University Hospital Herlev, Skolevej 14B, Holte 2840, Denmark

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**Figure 1** Abdominal computed tomography reveals appendicular wall thickening and periappendicular fat stranding.



**Figure 2** Cross-section of the appendix specimen. A: Leukemia involving the appendix is characterized by transmural infiltrates of myeloblast cells (HE,  $\times 200$ ); B: These tumor cells are immunoreactive to myeloperoxidase protein (HE,  $\times 200$ ).

dose cytarabine (20 mg/kg for 12 d). However, he died from progressive disease complicated by septic shock and acute respiratory failure on the 19th d after admission.

## DISCUSSION

The incidence of GS is estimated to be 3% in living adult patients with AML and 4.7% in children<sup>[1,2]</sup>. Rappaport initially introduced the concept of acute lymphoblastic leukemia infiltrating the appendix<sup>[3]</sup>. In a review of the literature, AML involving the appendix has been described<sup>[1,3,4]</sup>. As a presentation of AML in adults, GS can involve various sites throughout the body but it is rare in the gallbladder and appendix. Bowel infiltration by leukemic cells, described initially in the 19th century, was thought to be a rare condition until autopsy studies in the 1960s and 1970s, which indicated a prevalence of this presentation in 10% to 53.3% of leukemia patients<sup>[5-8]</sup>. It has been reported that appendiceal involvement by leukemic cells occurs in approximately 3 of 36 patients (8.3%)<sup>[6]</sup>. Seven leukemia patients with involvement of the appendix, including our own, were identified in the literature. The survival time varied as shown in Table 1<sup>[1,4,6]</sup>.

Surgical management of patients with leukemia and

**Table 1** Seven cases of leukemia infiltration of the appendix described in the literature

No.	Sex/age	Type	Treatment	Survival time (d)
1	F/77	M3	Surgery	30
2	M/71	M2	Surgery	49
3-6	NA	NA	3 surgery	Hours to days
7 (Our case)	M/75	M2	Surgery	19

M: Male; F: Female; NA: Not available.

acute abdomen has not been advocated because of the high rate of operative mortality in the past<sup>[4,6]</sup>. However, there is some support for surgical management of appendicitis in acute leukemia as the most effective method of therapy<sup>[1,4,9]</sup>. Systemic chemotherapy is necessary in this setting for additional radiation or surgery in patients with GS<sup>[10]</sup>.

In conclusion, we report a rare case of AML who had been in partial remission for 1 year and presented with symptoms of acute appendicitis as the initial manifestation of leukemia relapse. Although leukemic cell infiltration into the appendix is uncommon, our case highlights the importance of differential diagnosis of acute appendicitis including recognition of possible leukemic involvement. The physicians should be aware of these conditions.

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SCIENTIST'S STORY

## So you aspire to be a Professor?

Nicholas J Talley

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“The truth is that life is delicious, horrible, charming, frightful, sweet, bitter, and that is everything.” Anatole France (François-Anatole Thibault, 1844-1924).

When I was in medical school and early on in residency, I dreamed of undertaking research and becoming an academic but I did not really know how to start; in the end, I settled for trying to master internal medicine as I sweated to pass the formidable barrier examination for admission to Fellowship of the Royal Australasian College of Physicians (comprising both a written test and an even more challenging clinical “viva”). My entry into research was entirely fortuitous; having completed Basic Training I wanted a change (I like change), and I was lucky to learn from my father (a prominent Sydney gastroenterologist in private practice) that one of Australia’s most famous academic gastroenterologists, Professor Douglas (Doug) Piper, was looking for someone to undertake a PhD with him. Remarkably few had expressed an interest in doing so; a PhD would require 3 years of one’s life living on a National Health and Medical Research Council (NH&MRC) scholarship that would hardly keep body and soul together.



Figure 1 Nicholas J Talley, MD, PhD, FACP, FRACP, FRCP Chair.

Actually I was not sure I wanted to become a gastroenterologist (in fact, I favored neurology because my Dad was a gastroenterologist, and two Nicholas Talley’s in the specialty seemed a formula for confusion). However, I saw working with Doug as a real opportunity, so I took the risk and said yes, one of the best decisions of my life (Figure 1).

Professor Doug Piper was particularly interested in peptic ulcer disease, and our initial NH&MRC grant application (developed before the observation of spiral bacteria in the stomach had been announced), aimed to determine the natural history of peptic ulcer disease. This grant failed to attract funding, which at the time seemed quite devastating. Remarkably, this apparent setback turned out to be very fortunate, because we then were forced to look at alternative projects. Almost by chance we fell upon a common clinical problem; it had been long observed that most patients with ulcer-like symptoms had no ulcer crater or other obvious explanation for their complaints. Little was known about this mysterious and vague entity, then called non-ulcer dyspepsia, and the topic seemed ripe for a comprehensive clinical research project that would fulfill the requirements for a higher degree in Australia. As a clinician I became quite fascinated by the problem (I still am). After reading the proceedings from a Scandinavian symposium pointing out many of the gaps in knowledge (published by the makers of cimetidine, Smith Kline), I decided to pursue the topic. I was blissfully unaware of how difficult such a task might be, but had the gift of youthful enthusiasm and energy.

And so my odyssey began; with the help of Doug’s team I designed several protocols and set out to learn about the epidemiology, etiopathogenesis and treatment of non-ulcer dyspepsia. It was very hard the first year; there were the inevitable setbacks and it took a while to become used to research rather than full time practice. I

thought about quitting. But I learned that guts and grit count more than any other characteristic in the research setting. I undertook classes in clinical epidemiology and biostatistics which I loved, and learnt how to apply rigorous clinical research methods and avoid some of the obvious pitfalls. Momentum helps too; my first paper on the topic was published in 1985<sup>[1]</sup> and I caught the publishing bug (once you know how, it is great fun to collect, analyze and share your data with the world). The research efforts eventually spilled over into a two volume thesis entitled "Non-ulcer dyspepsia: A study of the psychosocial, environmental, clinical, therapeutic and prognostic aspects - with particular reference to a subgroup with dyspepsia of unknown cause (essential dyspepsia)." Doug used to say a thesis should be judged first by its weight and then by its quality, which I took to heart. The publication of several papers led to the offer of a fully paid Research Fellowship at Mayo Clinic in Rochester, Minnesota in the famous Gastroenterology Unit [headed then by a very prominent and brilliant expatriate Australian, Professor Sidney (Sid) Phillips]. Sid was an inspiring mentor who trained many of the most brilliant minds in motility research; I learned how to do physiological research and joined the staff during one of the happiest times of my life.

As my career trajectory exploded upwards, a further boost came after presenting new work as a very raw Assistant Professor of Medicine at Digestive Diseases Week. After the oral symposium, Douglas Drossman and Grant Thompson strode up to me to introduce themselves. I was invited to join a new consensus group that would meet in Rome; the idea was to develop diagnostic criteria for the first time for all of the functional gastrointestinal disorders, including non-ulcer dyspepsia (the precursor of the famous Rome criteria!). I said yes, and have been involved ever since with what is now known as the Rome Foundation (and the Rome I, II and III criteria-Rome IV is currently being planned).

So if you want to eventually reach the rank of Professor and, more importantly, contribute to our field, the formula in my mind is uncomplicated. Try to choose a great mentor first and foremost; they are the ones eager

to impart their knowledge, leave a legacy and develop the science (you can find most of the talented names on PubMed today with ease - look at how much and where they have published as a guide). Search for someone who thinks outside the box and has a strong team working with them; teams tend to perform best in the research world. The project is important too, but without the right mentor your chance of major success greatly diminishes. Second, in my humble view brains matter much less than drive. Be prepared to work hard and realize you will have to overcome all sorts of roadblocks to succeed; if you are prepared to be persistent and flexible, you will make it. Do not neglect to read the literature widely and deeply in your chosen topic area; critically appraise the science, know intimately all of the relevant papers, and look for the gaps (there are always many!). I tell my Research Fellows I know they will become experts by the time they complete their projects. Third, continuously develop your skill set. Learn how to not only do the science but also how to analyze, present and write it up. Submit and personally present your work at major international meetings whenever you have the opportunity; this is very good exposure, allows you to learn the state of the art and will promote making professional contacts around the world who will likely prove invaluable. Scientific presentation is a skill that can be learned and improves with practice; seek out ways to become a better public speaker. Above all, dedicate yourself to publishing any and all important data you generate (if your work is not published in a peer review journal, it essentially does not exist!). Serendipity helps too, but chance usually only plays a role if you are well prepared. If you follow these simple strategies, academic opportunities and promotion are almost guaranteed; it is straightforward to become a Professor once you learn the trade, and most importantly the journey is great fun too.

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## Meetings

### Events Calendar 2009

January 12-15, 2009  
Hyatt Regency San Francisco, San Francisco, CA  
Mouse Models of Cancer

January 21-24, 2009  
Westin San Diego Hotel, San Diego, CA  
Advances in Prostate Cancer Research

February 3-6, 2009  
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)  
Second AACR Conference  
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009  
Hyatt Regency Boston, Boston, MA  
Translation of the Cancer Genome

February 8-11, 2009  
Westin New Orleans Canal Place, New Orleans, LA  
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009  
Hong Kong Convention and Exhibition Centre, Hong Kong, China  
19th Conference of the APASL  
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009  
Orlando, Florida  
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009  
Vienna, Austria  
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease  
[www.easl.ch/vienna2009](http://www.easl.ch/vienna2009)

March 13-14, 2009  
Phoenix, Arizona  
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009  
Marriott Wardman Park Hotel  
Washington, DC  
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009  
Glasgow, Scotland  
British Society of Gastroenterology (BSG) Annual Meeting  
Email: [bsg@mailbox.ulcc.ac.uk](mailto:bsg@mailbox.ulcc.ac.uk)

April 8-9, 2009  
Silver Spring, Maryland  
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009  
Colorado Convention Center, Denver, CO  
AACR 100th Annual Meeting 2009

April 22-26, 2009  
Copenhagen, Denmark  
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)  
<http://www.easl.ch/>

May 17-20, 2009  
Denver, Colorado, USA  
Digestive Disease Week 2009

May 29-June 2, 2009  
Orange County Convention Center  
Orlando, Florida  
45th ASCO Annual Meeting  
[www.asco.org/annualmeeting](http://www.asco.org/annualmeeting)

May 30, 2009  
Chicago, Illinois  
Endpoints Workshop: NASH

May 30-June 4, 2009  
McCormick Place, Chicago, IL  
DDW 2009  
<http://www.ddw.org>

June 17-19, 2009  
North Bethesda, MD  
Accelerating Anticancer Agent Development

June 20-26, 2009  
Flims, Switzerland  
Methods in Clinical Cancer Research (Europe)

June 24-27 2009  
Barcelona, Spain  
ESMO Conference: 11th World Congress on Gastrointestinal Cancer  
[www.worldgicancer.com](http://www.worldgicancer.com)

June 25-28, 2009  
Beijing International Convention Center (BICC), Beijing, China  
World Conference on Interventional Oncology  
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009  
Snowmass, CO, United States  
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009  
Aspen, CO, United States  
Molecular Biology in Clinical Oncology

August 1-7, 2009  
Vail Marriott Mountain Resort, Vail, CO, United States  
Methods in Clinical Cancer Research

August 14-16, 2009  
Bell Harbor Conference Center, Seattle, Washington, United States  
Practical Solutions for Successful Management  
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009  
Beijing International Convention Center (BICC), Beijing, China  
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists (IASGO)  
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009  
Taipei, China  
Asian Pacific Digestive Week  
<http://www.apdwcongress.org/2009/index.shtml>

October 7-11, 2009  
Boston Park Plaza Hotel and Towers, Boston, MA, United States  
Frontiers in Basic Cancer Research

October 13-16, 2009  
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States  
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

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Versailles, France  
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October 30-November 3, 2009  
Boston, MA, United States  
The Liver Meeting

November 15-19, 2009  
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States  
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009  
London, UK  
Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

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The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of

balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

#### Organization as author

- 4 **Diabetes Prevention Program Research Group.** Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

#### Both personal authors and an organization as author

- 5 **Vallancien G,** Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 7 **Geraud G,** Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

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- 10 **Sherlock S,** Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 12 **Breedlove GK,** Schorfheide AM. Adolescent pregnancy. 2nd ed. Wicczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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#### Electronic journal (list all authors)

- 15 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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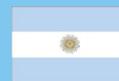
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## Emerging role of microRNAs in liver diseases

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### Abstract

MicroRNAs are a class of small non-coding RNAs that are found in plants, animals, and some viruses. They modulate the gene function at the post-transcriptional level and act as a fine tuner of various processes, such as development, proliferation, cell signaling, and apoptosis. They are associated with different types and stages of cancer. Recent studies have shown the involvement of microRNAs in liver diseases caused by various factors, such as Hepatitis C, Hepatitis B, metabolic disorders, and by drug abuse. This review highlights the role of microRNAs in liver diseases and their potential use as therapeutic molecules.

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**Key words:** MicroRNA; Hepatitis; Fatty liver; Fibrosis; Cirrhosis

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### INTRODUCTION

The recent discovery of several types of non-coding RNAs

revealed that the transcriptomes of higher eukaryotes are much more complex than originally anticipated. The non-coding RNAs, including microRNAs (miRNA), piwi interacting RNAs, small nucleolar RNAs, small interfering RNAs, long non-coding RNAs and antisense RNAs, have newly-discovered roles in the biology of health and diseases. These RNAs serve as modulators of genes involved in various biological pathways, such as development, cell differentiation, cell proliferation, cell death, chromosome modifications, virus pathogenesis, and oncogenesis<sup>[1-3]</sup>. Among these RNAs, miRNAs are small RNA molecules of approximate 21 nucleotides that are present in most eukaryotes<sup>[4]</sup>. Since the discovery of the first miRNA, lin-4, in 1993<sup>[5]</sup>, more than 500 miRNAs have been reported in the mammalian genome<sup>[6]</sup>.

It is postulated that about 1%-5% of genes in animals encode miRNAs, while 10%-30% of protein-coding genes are predicted miRNA targets<sup>[7,8]</sup>, and have a specific miRNA signature in normal or cancer cells<sup>[9,10]</sup>. Although our understanding of the specific roles of miRNAs in cellular functions is only beginning, several studies have shown that miRNAs play a pivotal role in the most critical biological events, including development, proliferation, differentiation, cell fate determination, apoptosis, signal transduction, organ development, hematopoietic lineage differentiation, host-viral interactions, and carcinogenesis<sup>[4,11-13]</sup>.

### Overview of miRNAs

Despite the emerging critical role of miRNAs, the mechanism of their action is yet to be fully understood. The widely-known mode of gene regulation by miRNAs occurs at the post-transcriptional level by either specific inhibition of translation or induction of mRNA cleavage<sup>[14]</sup>. The essential role of miRNAs in cellular physiology was elegantly shown in mice lacking the Dicer enzyme that is required for the processing of the precursor miRNAs into the mature form<sup>[15]</sup>.

Until now, the major focus of miRNA research was on their role in the cytoplasm, however recent reports indicate a diverse role of miRNAs, such as regulation of target genes by acting at 5' UTR<sup>[16]</sup>, regulation of DNA methylation<sup>[17,18]</sup> and import of the mature miRNA into the nucleus, suggesting other functional modes<sup>[19]</sup>. It is becoming clear that miRNAs not only regulate gene expression at the post-transcriptional level, but they are also capable of modifying chromatin (Figure 1).

In this review, we discuss the role of the newly emerging class of RNA, miRNAs, in different types of liver diseases.

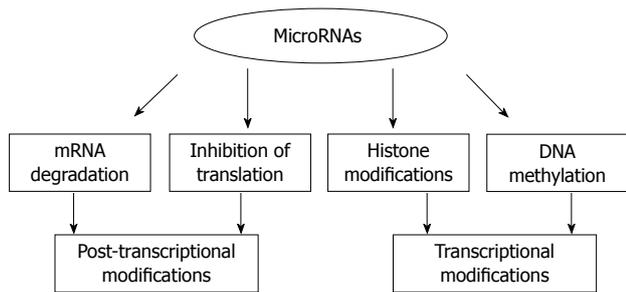


Figure 1 Various modes of gene regulation by miRNAs.

## MiRNAs in Liver Diseases

Improvements in the characterization and functional analysis techniques for miRNAs has not only uncovered their role in various cellular processes, but also revealed abnormal patterns of miRNA expression in various diseases, such as cancer<sup>[20]</sup>, viral infection<sup>[21]</sup>, inflammation<sup>[22]</sup>, diabetes<sup>[23]</sup>, cardiovascular<sup>[24]</sup>, and Alzheimer<sup>[25]</sup>. In this review, we focus on the involvement of miRNAs in liver diseases caused by various factors, such as viral hepatitis, fatty liver due to alcohol abuse or metabolic syndrome, drug-induced liver disease, and by auto-immune processes (summarized in Table 1).

### MiRNAs in host-viral response and viral hepatitis

Viruses are equipped with complex machinery to exploit the host biosynthetic pathways and to defend against host cellular responses<sup>[26]</sup>. Recent studies have revealed the involvement of miRNA-mediated RNA-silencing pathways during viral-host cell interactions<sup>[27]</sup>. However, little is known about the role of cellular miRNAs against viral infection in eukaryotic organisms. Interestingly, viruses not only exploit the cellular miRNAs, but also encode their own miRNAs, adding another layer of complexity<sup>[28,29]</sup>. Therefore, it is important to study the abundance and distribution of miRNAs within the host cell during viral replication and latency, which will help us to understand the molecular regulation by the host cellular system as well as the simultaneous attempts by viruses to overcome the host defense.

**Role of miRNAs in immune response:** The interaction of parenchymal and immune cells plays a unique role in the liver in response to liver insults by viruses, bacteria, toxins, or antigens. There is compelling evidence that characterizes miRNAs as key regulators of innate and adaptive immune responses<sup>[22,30]</sup> and, therefore, they might play a role in inflammatory, autoimmune, or viral diseases of the liver. The importance of miRNAs in the liver immune system is highlighted by the fact that mice lacking Dicer 1 function in the liver were unable to produce mature miRNA and showed progressive hepatocyte damage, apoptosis, and portal inflammation<sup>[31]</sup>.

The innate immune response is the first line of defense against noxious agents and is mediated by adaptors, such as the toll like receptors (TLRs), which recognize specific molecular patterns. Signals triggered by TLRs are involved in most liver diseases (from viral to inflam-

Table 1 Expression of miRNAs in liver diseases

Liver	Upregulated	Downregulated	Ref.
HCV	miR-215, miR-16, miR-199, miR-155, miR-146	miR-122, miR-320, miR-191	[53]
HBV	miR-181a, miR-200b and miR-146a	miR-15a	[56]
Drug overdose	miR-711 (liver), Plasma: miR-122 miR-15a, miR-21, miR-101b, miR-148a and miR-192	miR-29b(liver), miR-710 (plasma)	[62]
NAFLD	miR-122, miR-34a, miR-31, miR-103, miR-107, miR-194, miR-335-5p, miR-221 and miR-200a	miR-29c, miR-451, miR-21	[69,70]
ALD	miR-212, miR-320, miR-486, miR-705, and miR-1224	miR-27b, miR-214, miR-199a-3p, miR-182, miR-183, miR-200a, and miR-322	[71,72]
PBC	miR-328 and miR-299	miR-122a and miR-26a	[73]
Liver fibrosis	miR-27a, miR-27b, miR-30,	miR-9, miR-721 and miR-301	[80]
HCC	miR-18, miR-21, miR-221, miR-222, miR-224, miR-373 and miR-301	miR-122, miR-125, miR-130a, miR-150, miR-199, miR-200 and let-7 family members	[81,90-95]

HCV: Hepatitis C virus; HBV: Hepatitis B virus; NAFLD: Non-alcoholic fatty liver disease; ALD: Alcoholic liver disease; PBC: Primary biliary cirrhosis; HCC: Hepatocellular carcinoma.

matory conditions) as well as in liver regeneration<sup>[32]</sup>. Several miRNAs (such as miR-155 or miR-146a/b) have been implicated in the regulation of TLR-induced signaling and might associated with liver pathophysiology. In particular, miR-155 was upregulated in monocytes and macrophages both upon TLR2, TLR3, TLR4, and TLR9 stimulation and after exposure to cytokines such as tumor necrosis factor (TNF)- $\alpha$  and interferons (IFN)<sup>[33-35]</sup>. The exact function of this miRNA in macrophages is not completely clear, but it might exert a positive regulation on TNF- $\alpha$  release, through targeting genes involved in nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling or enhancing TNF- $\alpha$  translation<sup>[34]</sup>. miR-146 is also upregulated in response to TLR2, TLR4, and TLR5 stimulation in monocytes<sup>[33]</sup>. This miRNA seems to decrease the release of inflammatory mediators, such as interleukin (IL)-1 $\beta$  or IL-8<sup>[36]</sup>, possibly by downregulating IL-1 receptor-associated kinase 1 and TNF receptor-associated factor 6, and act as a negative regulator of TLR signaling<sup>[33]</sup>. Most of the studies describing the importance of these miRNAs in the innate immune response have been performed in macrophages or monocytes *in vitro*, and therefore the elucidation of specific miRNA functions *in vivo* in Kupffer cells, the resident liver macrophage, still awaits confirmation.

Neutrophil leukocyte infiltration in the liver is also a part of the innate immune response, which is common to liver injury, hepatic stress, or systemic inflammation signals<sup>[37]</sup>. Regulators of granulocyte function and activation, such as miR-223, might also be able to play a role in liver

disease. Several reports have shown that the transcription factor C/EBP $\alpha$  binds to the miR-223 promoter and enhances its expression<sup>[38-40]</sup>. Given the importance of antigen presentation in the liver, the involvement of miRNAs in this function could be also of interest. In line with this, Hashimi *et al.*<sup>[41]</sup> have recently shown that inhibition of both miR-21 and miR-34a in monocyte-derived dendritic cells led to a decrease in endocytic capacity and altered differentiation.

Apart from their role in the innate immune responses, miRNAs are associated with many aspects of both normal and abnormal immune adaptive responses, making them likely actors in autoimmune liver diseases or, particularly, viral hepatitis<sup>[22,30]</sup>. Virtually every T and B cell type seems to be associated with a different miRNA expression profile, and therefore an interesting avenue of research is to identify the miRNAs signature associated with specific liver diseases. Regarding specific miRNA functions, miR-181 has been described as a key quantitative regulator of T cell response to antigens<sup>[42]</sup>. MiR-150 plays a crucial role in B cell differentiation because its over-expression results in a selective defect in B cell development that blocks the transition from pro-B cell to pre-B cell<sup>[43]</sup>. Of note, miR-155 is implicated in both T and B cell function and differentiation promoting T helper 1 vs type 2 differentiation<sup>[44,45]</sup> and is essential in the production of antigen-specific antibodies<sup>[46]</sup>. These data highlight the importance of this miRNA as a central actor in immune regulation.

**Hepatitis C virus (HCV) infection:** HCV is an enveloped RNA virus of the Flavivirus family, and is capable of causing both acute and chronic hepatitis in humans by infecting liver cells. It is a major cause of chronic liver disease, with about 170 million people infected worldwide<sup>[47]</sup>. Up to 70% of patients will have persistent infection after inoculation, making it a fatal disease. The disease varies widely, from asymptomatic chronic infection to cirrhosis and hepatocellular carcinoma (HCC)<sup>[47]</sup>. Since the discovery of HCV, the treatment of hepatitis C has considerably improved by the use of pegylated interferons with ribavirin; however, more than 50% of people still do not respond to current medication and thus there is need for better drug therapy.

Recent studies have shown the involvement of miRNAs in the regulation of HCV infection. MiR-122 is first identified liver-specific cellular miRNA, which has been shown to enhance the replication of HCV by targeting the viral 5' non-coding region<sup>[48]</sup>. It appears that HCV replication is associated with an increase in expression of cholesterol biosynthesis genes that are regulated by miR-122 and hence is considered as a potential target for antiviral intervention<sup>[49]</sup>.

To date, there are some controversies around the role of miR-122 in HCV infection. Recently, Henke *et al.*<sup>[50]</sup> showed that miR-122 stimulates HCV translation by enhancing the association of ribosomes with the viral RNA. The findings describing the role of miR-122 in HCV replication are of special interest, not only because of its novel mode of action, but because they also provide the first evidence of miRNAs linked to

infectious disease. The development of therapeutic products to inhibit the miR-122 is believed to be an attractive approach to treat HCV patients.

By contrast, Sarasin-Filipowicz *et al.*<sup>[51]</sup> found that liver biopsies from chronic hepatitis C patients undergoing IFN therapy revealed no correlation between miR-122 expression and viral load. Moreover, they found markedly decreased miR-122 levels in people who did not have any virological response during later IFN therapy. These reports indicate that the role of miR-122 in HCV viral replication is still controversial and awaits future research.

While some host miRNAs are beneficial for the virus, others inhibit viral replication. Studies by Pedersen *et al.*<sup>[52]</sup> demonstrated that IFN- $\beta$  stimulation, an antiviral cytokine, induces numerous cellular miRNAs, specifically eight miRNAs (miR-1, miR-30, miR-128, miR-196, miR-296, miR-351, miR-431 and miR-448) that have sequence-predicted targets within the HCV genomic RNA. Overexpression of these miRNAs in infected liver cells considerably attenuated viral replication. From this report it seems that host miRNAs have evolved to target viral genes and inhibit their replication, and thus might represent part of the host antiviral immune response. Very recently, Peng *et al.*<sup>[53]</sup> carried out a computational study of hepatitis C virus associated miRNAs-mRNA regulatory modules in human livers. They found differential profiles of cellular miRNAs that target the genes involved in chemokine (16 genes such as *CXCL12 etc.*), B cell receptor, PTEN (13 genes), IL-6 ERK/ MAPK (18 genes; Ras, Erk3 and STAT3 *etc.*) and JAK/STAT signaling pathways, suggesting a critical role of miRNAs in the replication, propagation, and latency of virus in the host cell. Specifically, they found that miR-122, miR-320, and miR-191 were downregulated, whereas miR-215, miR-16, miR-26, miR-130, miR-199 and miR-155 were upregulated. These findings suggest that miRNAs have the potential to become novel drug targets in virally induced infectious or malignant diseases.

**Hepatitis B virus (HBV) infection:** HBV infection is a global problem and it can cause acute or chronic hepatitis B, liver cirrhosis, and, in some instances HCC<sup>[54]</sup>. The available therapy is only partially effective against the virus and development of better therapy remains an important issue. Little is known about the involvement of miRs during HBV infection. Considering the fact that cellular miRNAs play an important role in viral pathogenesis, it is likely that they have a role in HBV infection.

A differential miRNAs expression pattern was found in the livers of HBV and HCV infected individuals with hepatocellular cancer<sup>[55]</sup>. A total of nineteen miRNAs were clearly differentiated between HBV and HCV groups, out of which thirteen miRNAs were downregulated in the HCV group, whereas six showed a decreased expression in the HBV group. Some of the differentially regulated miRNAs between the HCV and HBV groups were miR-190, miR-134, miR-151, miR-193, miR-211, and miR-20. Interestingly, in the same study, it was shown that pathway analysis of targeted genes using infection-associated miRNAs could differentiate the genes into two groups. For instance,

in HBV-infected livers, pathways related to cell death, DNA damage, recombination, and signal transduction were activated, and those related to immune response, antigen presentation, cell cycle, proteasome, and lipid metabolism were activated in HCV-infected livers<sup>[55]</sup>.

In a second study, the profiling of cellular miRNAs of a stable HBV expressing cell line HepG2.2.15 and its parent cell line HepG2, showed that eighteen miRNAs were differentially expressed between the two cell lines<sup>[56]</sup>. Of them, eleven were upregulated and seven were downregulated. For instance, miR-181a, miR-200b, and miR-146a were found to be upregulated and miR-15a was downregulated. It remains to be evaluated if differentially regulated miRNAs could be exploited as potential biomarkers to differentiate between HCV and HBV in the initial stages of pathogenesis.

### **MiRNAs in drug-induced liver injury**

Drug-induced liver injury is a serious clinical health problem and is the leading cause of drugs being removed from the market<sup>[57]</sup>. Acetaminophen overdose is the most common cause of fulminant liver failure. Many studies have been carried out to identify reliable and sensitive early blood markers for liver injury using high throughput technologies<sup>[58]</sup>. However, these efforts have so far failed to yield markers that are superior compared to the existing aminotransferase-based markers, and the need for better and stable biomarkers persists. Increasing attention is being paid to circulating miRNAs as potential biomarkers because of their stability over enzymes or proteins<sup>[59-61]</sup>. However, not much is known about the roles of miRNAs in drug-induced liver injury. A study by Wang *et al*<sup>[62]</sup> showed differential miRNAs profiling in a mouse model of acetaminophen overdose. Significant differences were found in the levels of miRNAs in both liver tissues and plasma between control and acetaminophen overdosed animals. Using miRPortal ([www.miRportal.net](http://www.miRportal.net)), pathways involving antigen processing and presentation, apoptosis, B cell receptor signaling pathways, cytokine-cytokine receptor interaction, and cell cycles were found to be potential targets of miRNAs that showed decreased levels in the liver after acetaminophen exposure. In contrast, pathways involved in various signaling transduction and cell-cell interactions, such as gap-junctions, focal adhesion, and MAPK signaling pathways were potentially affected by miRNAs with increased levels in the liver<sup>[62]</sup>.

Interestingly, it was observed that most miRNAs that were found to decrease in the liver exhibited increased levels in the plasma in overdosed mice. For instance, miR-122 and miR-192 showed higher levels in plasma, but they were decreased in tissue, whereas miR-710 and miR-711 were elevated in liver tissues; however, they displayed lower levels in plasma after acetaminophen overdose. Moreover, liver specific miRNAs, such as miR-122 and miR-192, showed dose and time-dependent changes in the plasma that paralleled serum aminotransferase levels and liver injury on histopathology. These findings suggest the potential of using specific circulating miRNAs as sensitive and informative biomarkers for drug-induced liver injury.

### **MiRNAs in non-alcoholic fatty liver disease (NAFLD)**

NAFLD is the most common form of chronic liver disease worldwide and a major public health concern in the modern society<sup>[63]</sup>. It is characterized by excess fat accumulation in liver, which ranges from simple steatosis to steatohepatitis, and cirrhosis in the absence of heavy alcohol consumption<sup>[64]</sup>. The major risk factors for the development of NAFLD include metabolic syndrome, such as obesity, type 2 diabetes mellitus, and dyslipidemia<sup>[65]</sup>. Although progress has been made in understanding the factors causing NAFLD, more needs to be done to dissect the underlying regulatory networks. Compelling evidences indicate the role of miRNAs in energy metabolism and liver functions<sup>[66,67]</sup>. For instance, miR-143 has been shown to play a role in adipose differentiation<sup>[68]</sup>.

MiR-122, which was found to be upregulated in NAFLD, is known to regulate genes involved in fatty acid biosynthesis<sup>[69]</sup>, and administration of this miRNA antagonist into mice resulted in reduced levels of plasma cholesterol, increased hepatic fatty acid oxidation, and decreased synthesis of hepatic fatty acid and cholesterol.

Li *et al*<sup>[70]</sup> reported that eight miRNAs (miR-34a, miR-31, miR-103, miR-107, miR-194, miR-335-5p, miR-221 and miR-200a) were upregulated and three miRNAs (miR-29c, miR-451, miR-21) were downregulated in the livers of ob/ob mice. These findings suggested a connection between miRNAs and metabolic disorders, and the role of miRNA in NAFLD deserves further investigation.

### **Role of miRNAs in alcoholic liver disease (ALD)**

ALD, alcoholic hepatitis, and cirrhosis represent a major segment of liver diseases worldwide. A recent report by Dolganiuc *et al*<sup>[71]</sup> analyzed the miRNA expression profile in a murine model of ALD. Liver samples from mice fed an ethanol-containing diet (Lieber-DeCarli) showed features of alcoholic steatohepatitis and had an increased expression of miR-320, miR-486, miR-705, and miR-1224. On the other hand, decreased expression was found for several miRNAs, including miR-27b, miR-214, miR-199a-3p, miR-182, miR-183, miR-200a, and miR-322. Furthermore, livers from mice with alcoholic and with non-alcoholic fatty liver shared common alterations in their miRNA profiles (such as upregulation of miR-705 and miR-1224). However, the functions and physiological roles of these miRNAs in ALD are yet to be determined. Tang *et al*<sup>[72]</sup> have suggested a novel mechanism for ethanol-induced intestinal permeability. They found that ethanol intake resulted in the induction of miR-212, which in turn downregulated the Zonula occludens (ZOP) protein. The decrease in ZOP protein levels led to intestinal barrier disruption and thus increased intestinal permeability. The interest of this finding lies in the association of intestinal permeability with the development of ALD, therefore linking miR-212 with the pathogenesis of this disease.

### **Primary biliary cirrhosis (PBC)**

Recent studies indicate that the role of miRNAs is yet to be explored in liver diseases associated with autoimmune

aggression. In PBC, altered hepatic miRNA expression was found<sup>[73]</sup>. miRNA profiling of explanted livers from subjects with PBC revealed differential expression of thirty-five independent miRNAs compared to controls. Downregulation of miR-122a and miR-26a, and increased expression of miR-328 and miR-299, were confirmed by PCR in the PBC livers. The role of miRNAs in autoimmune hepatitis is yet to be studied.

### Liver fibrosis

Chronic hepatitis contributes to liver fibrosis, which has been linked to fibrosis due to fibrin deposition by hepatic stellate cells (HSC)<sup>[74]</sup>. The relationship between miRNAs and fibrosis has been shown in the context of specific diseases, such as diabetic kidney sclerosis<sup>[75]</sup> or myocardial fibrosis<sup>[76]</sup>, and some of the mechanisms described could be involved in common pathways of fibrosis in other diseases. A recent report by Kato *et al*<sup>[77]</sup> has described that transforming growth factor (TGF)- $\beta$  activates Akt kinase by enhancing the expression of both miR-216a and miR-217. These miRNAs, in turn, target PTEN, an inhibitor of Akt activation that has also been described to play a role in hepatic stellate cell activation<sup>[78]</sup>. Hepatic stellate cell activation has been identified as the major driver of liver fibrosis. Activation of HSC is associated with upregulation of various signaling pathways. In cirrhotic rat livers, HSC activation was associated with mitochondrial damage indicated by increased Bcl-2 and downregulated caspase-9 levels. The miRNA profile of the same rat HSC indicated upregulation of thirteen and downregulation of twenty-two, signaling pathways by miRNAs<sup>[79]</sup>. In another in vitro study, overexpression of miR-27a and miR-27b resulted in reversal of the activated phenotype of stellate cells to a more quiescent phenotype with increased fat accumulation and decreased proliferation<sup>[80]</sup>. The effects of miR-27a and miR-27b were mediated by regulation of the retinoid X receptor in the hepatic stellate cells.

Although there are only a few studies in the literature on miRNA genes and HCV-induced liver fibrosis, given the critical role of miRNA, its possible that they play a critical role in HCV infection, in the progression of fibrosis, and in the prediction of treatment response. Jiang *et al*<sup>[81]</sup> showed increased miRNA expression in cirrhotic and hepatitis-positive liver samples and suggested that important changes in miRNA expression occur during the development of chronic viral hepatitis and cirrhosis.

### HCC

HCC is the third-leading cause of death from cancer and the fifth most common malignancy worldwide<sup>[82]</sup>. Liver cancer is a complex disease with exceptional heterogeneity in cause and outcome, involving epigenetic and chromosomal instability<sup>[83]</sup> and abnormalities in the expression of both coding and non-coding genes, including miRNAs<sup>[84,85]</sup>. The major etiologies of HCC include chronic liver disease due to chronic hepatitis B or hepatitis C virus infection, alcoholic steatohepatitis, metabolic disorders such as nonalcoholic steatohepatitis or insulin resistance, hereditary hemochromatosis, and

immune-related diseases such as PBC and autoimmune hepatitis<sup>[86]</sup>.

There are several studies in the literature showing specific miRNA signatures in HCC formation<sup>[87-89]</sup> and progression that could be exploited as potential cancer biomarkers. Briefly, miR-18, miR-21, miR-221, miR-222, miR-224, miR-373, and miR-301 were reported to be upregulated<sup>[81,90,91]</sup> whereas miR-122, miR-125, miR-130a, miR-150, miR-199, miR-200, and let-7 family members<sup>[92-95]</sup> were found to be downregulated in HCC by most studies. These miRNAs target the genes involved in cell cycle and cell death regulation, including cyclin-dependent inhibitors *p27/CDKN1B* and *p57/CDKN1C*, or the PI3K antagonist phosphatase and tensin homolog (PTEN). Budhu *et al*<sup>[96]</sup> defined twenty miRNAs, as a HCC metastasis signature, and showed that the twenty-miRNA-based signature was capable of predicting survival and recurrence of HCC in patients with multinodular or solitary tumors, including those with early-stage disease. miR-219-1, miR-207, and miR-338 were the most highly upregulated, whereas miR-34a, miR-30c-1, and miR-148a were highly downregulated in metastasis cases. Target Scan analysis revealed that *SPTBN2*, *GTF2H1*, *PSCD3*, *VAMP3*, and *SLC20A2* are the target genes which were affected by multiple miRNAs related to metastasis and might be the part of signaling pathways that significantly contribute to this phenotype. Moreover, this miRNA signature was an independent and significant predictor of patient prognosis when compared to other available clinical parameters<sup>[96]</sup>. This study suggested that these twenty miRNAs can assist in HCC prognosis and might have clinical relevance. Further functional studies of these miRNAs could help to elucidate the mechanism leading to HCC metastasis. Deregulation of these twenty miRNAs in metastatic HCC implies that altered expression of their target genes might contribute to the development or recurrence of metastasis, and miR-122 is one example of this metastasis signature<sup>[97]</sup>.

## THE POTENTIAL OF MiRNAs IN DIAGNOSTICS, DISEASE PROGNOSIS, AND THERAPY

Increasing evidence indicates that miRNAs play an important role in a wide range of liver diseases, ranging from cancers and viral hepatitis, to metabolic diseases. The unique expression profile of miRNAs in different types and at different stages of cancer, and in other diseases, suggest that these small molecules can be exploited as novel biomarkers for disease diagnostics and might present a new strategy for miRNA gene therapy.

Recently, Kota *et al*<sup>[98]</sup> showed that administration of miR-26a, (which is under-expressed in HCC cells and downregulates cyclins D2 and E2) using adeno-associated virus (AAV) resulted in the inhibition of cancer cell proliferation, induction of tumor-specific apoptosis, and dramatic protection from disease progression without toxicity in a mouse model of HCC, suggesting the potential therapeutic use of miRNAs. This study

showed that anti-miRNA compounds could be delivered *in vivo* safely and with efficacy; thus opening the way for translation of these basic research findings to clinical applications. It is also important to consider other factors, because some miRNA genes such as miR-1 undergo methylation-mediated regulation in HCC cell lines<sup>[99]</sup>, suggesting a strong link between the DNA methylome and the miRNAome. In particular, there are some reports showing that the expression profiles of miRNA differ between malignant hepatocytes, malignant cholangiocytes, and benign liver cancer<sup>[90]</sup>, suggesting that miRNA profiling could be used as molecular diagnostic markers in liver disease.

## CONCLUSION

It is increasingly evident that miRNAs play a novel and important role in regulation of gene expression. In recent years, research focusing on small molecules such as miRNAs has intensified to understand their role in health and disease. The excitement of exploring their regulatory potential will remain for years to come, given the observation that miRNAs could be ideal therapeutic targets for many diseases. We are at the beginning of understanding of the diverse roles of miRNAs in fine-tuning biological pathways, and in future years we will have a better picture of this complex regulatory mechanism. Presently, there is need for miRNAs-based diagnostics and gene therapy; however, these techniques are in their infancy.

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## Acute pancreatitis in pregnancy

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### Abstract

Acute pancreatitis (AP) is a rare event in pregnancy, occurring in approximately 3 in 10000 pregnancies. The spectrum of AP in pregnancy ranges from mild pancreatitis to serious pancreatitis associated with necrosis, abscesses, pseudocysts and multiple organ dysfunction syndromes. Pregnancy related hematological and biochemical alterations influence the interpretation of diagnostic tests and assessment of severity of AP. As in any other disease associated with pregnancy, AP is associated with greater concerns as it deals with two lives rather than just one as in the non-pregnant population. The recent advances in clinical gastroenterology have improved the early diagnosis and effective management of biliary pancreatitis. Diagnostic studies such as endoscopic ultrasound, magnetic resonance cholangiopancreatography and endoscopic retrograde cholangiopancreatography and therapeutic modalities that include endoscopic sphincterotomy, biliary stenting, common bile duct stone extraction and laparoscopic cholecystectomy are major milestones in gastroenterology. When properly managed AP in pregnancy does not carry a dismal prognosis as in the past.

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**Key words:** Acute pancreatitis; Pregnancy; Pancreatitis in pregnancy

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### INTRODUCTION

Acute pancreatitis (AP) is a common problem with an annual incidence of 5 to 80 per 100000 of the general population. The incidence of AP in pregnancy varies and is approximately 1 in 1000 to 1 in 10000 births<sup>[1]</sup>. The wide variation in the incidence is influenced by the prevalence of its most important etiological factor i.e. gallstone disease. While biliary pancreatitis complicated 1 in 3300 pregnancies at a large public hospital in Dallas, Texas<sup>[2]</sup>, in southern California 1 in 1500 women were affected<sup>[3]</sup>. In a retrospective study from USA spanning 10 years Legro and Laifer<sup>[4]</sup> identified 25 cases of AP in pregnancy. Eleven of these 25 patients were diagnosed in the first trimester of pregnancy when physicians had to clearly distinguish between hyperemesis gravidarum and AP. In another study 43 pregnant women out of 147197 were diagnosed with AP<sup>[2]</sup>; 19% of these were diagnosed in the first trimester, 26% in the second, and 53% in the third trimester (one was postpartum), demonstrating that AP was more common with advancing gestational age, paralleling the frequency of gallstone diseases in pregnancy.

Older reviews of AP in pregnancy reported maternal and fetal mortality rates as high as 20% and 50% respectively<sup>[2,5-9]</sup>. The above data from the pre-endoscopic retrograde cholangiopancreatography (ERCP), pre-laparoscopic cholecystectomy era are not valid anymore. Contemporary reports document a much improved outcome of AP in pregnancy, when the management of AP secondary to gallstones has undergone substantial changes<sup>[10,11]</sup>. Hernandez *et al*<sup>[11]</sup> in 2007, based on a single center experience spanning 10 years, reported 34 episodes of

AP with no maternal deaths and a fetal loss of only 4.7%. Date *et al*<sup>[12]</sup> in 2008 compared conservative and surgical management of cholecystitis in pregnancy and noted no difference in fetal mortality (2.2% *vs* 1.2%,  $P = 0.57$ ), and there was no maternal mortality. The major changes are the availability of many options in abdominal imaging and less invasive therapeutic options. In addition to abdominal ultrasound (US) we have endoscopic ultrasound (EUS), magnetic resonance cholangiopancreatography (MRCP) and ERCP. The introduction of laparoscopic cholecystectomy in 1986 is a milestone that has reduced the morbidity of surgical intervention by open abdominal surgery even in high-risk pregnant patients. Above all the safe applications of therapeutic ERCP, endoscopic sphincterotomy (ES) have permitted delaying cholecystectomy to safer periods in pregnancy or postpartum.

The etiological associations of AP during pregnancy are similar to those in the general population. AP in pregnancy is most often associated with gallstone disease or hypertriglyceridemia. Gallstones are the most common cause of AP during pregnancy responsible for more than 70% of cases<sup>[2]</sup>. The incidence of gallstone related diseases including acute cholecystitis and biliary pancreatitis complicating pregnancy is 0.05%-0.8%<sup>[13]</sup>. Even in patients who had prior cholecystectomy, a biliary etiology may exist. The prevalence of microlithiasis after cholecystectomy is 5%-10%<sup>[14,15]</sup>. The pathogenesis of AP in gallstone disease is attributed to lodging or impaction of a stone or microlithiasis in the ampulla of Vater initiating premature activation of intracinar trypsinogen to trypsin.

## AP OF BILIARY ETIOLOGY IN PREGNANCY

The prevalence rate of gallstones varies with ethnicity. Native American Indians, Mexicans, Latin Americans and Pima Indians all have a high incidence while the incidence is lower in Asia and Africa. Many studies have reported a high incidence of gallstones in the population from northern states of India<sup>[16,17]</sup>. Gallbladder disease is strongly related to the metabolic syndrome, a problem that is growing in incidence all over the world<sup>[18-21]</sup>. Rapid weight loss is a recently recognized factor for microlithiasis and gallstones<sup>[22]</sup>. Although pregnancy itself is a risk factor, the risk increases with parity<sup>[23]</sup>. Weight gain and hormonal changes predispose pregnant women to biliary sludge and gallstone formation<sup>[24]</sup>. Identification of a biliary etiology for AP is important because as in the non-pregnant patient recurrence of AP episodes will occur in one-third to two-thirds of patients unless gallstones are removed<sup>[10,11,25-27]</sup>.

### **Pathogenesis of increased prevalence of gallstone in pregnancy**

Cholesterol secretion in the hepatic bile increases in the second and third trimester compared to bile acids and phospholipids, leading to supersaturated bile; in addition, fasting and postprandial gallbladder volumes are greater, with reduced rate and volume of emptying.

This large residual volume of supersaturated bile in the gallbladder of the pregnant patient leads to the retention of cholesterol crystals and eventual gallstones. The formation of biliary sludge and stones is strongly associated with frequency and number of pregnancies<sup>[25]</sup>.

Up to 10% of patients develop stones or sludge over the course of each pregnancy, with obesity and increased serum leptin being risk factors<sup>[28]</sup>. After delivery gallbladder motility becomes normal when sludge as well as stones may disappear<sup>[26,27]</sup>.

In evaluating pregnant patients with AP the four important questions to be answered are (1) does the patient have AP (establishing the diagnosis and ruling out other causes)? (2) if it is AP, what is the predicted severity? (3) is there a biliary etiology? and (4) what is the trimester of pregnancy?<sup>[29]</sup> The answer to the last question determines the choice of imaging studies and mode of therapy.

In the management initial blood tests are done to establish the diagnosis of AP and to assess the severity. Serum amylase and lipase levels are reliable markers of AP during pregnancy. The serum lipase level is unchanged during pregnancy, and the amylase level is either normal or only mildly elevated<sup>[30]</sup>. The alterations in blood chemistry in normal pregnancy do not hinder the assessment of severity. Elevation of serum alanine aminotransferase levels to > 3 times the upper limit of normal is a very sensitive biochemical marker of biliary pancreatitis<sup>[31,32]</sup>. Any abnormality of liver enzymes and bilirubin as well as rapid change in the levels should suggest a biliary etiology.

## IMAGING STUDIES

Abdominal ultrasound (AUS) with no radiation to the fetus is the initial imaging technique of choice to identify a biliary etiology. Gallstones as a potential cause of AP are identified by AUS in most cases<sup>[33]</sup>. However, it is insensitive for the detection of common bile duct stones or sludge. When a common bile duct (CBD) stone is suspected based on AUS or biochemical abnormalities EUS, a semi-invasive procedure of the biliary tree is an accurate modality for detecting common bile duct stones<sup>[34]</sup>. However EUS requires expensive equipment, intravenous sedation and technical expertise. EUS can be considered the best imaging study to evaluate CBD, although not for gallbladder stones. In expert hands small gallstones as well as sludge can be picked up by EUS, however it is operator dependent. EUS is appropriate prior to the consideration of therapeutic ERCP in patients where non-invasive imaging such as MRCP is not available, contraindicated or inconclusive. EUS has a high positive predictive value nearing 100% in detecting CBD stones and in many instances EUS is superior to MRCP<sup>[35]</sup>. EUS entails no radiation exposure and is extremely safe apart from a minimal sedation related risk. If a common bile duct stone is detected, an ERCP with sphincterotomy can be performed following the EUS study during the same sedation.

Magnetic resonance imaging (MRI) and MRCP provide multi-planar large field of view images of the

body with excellent soft-tissue contrast and images of bilio-pancreatic duct systems. MRCP does not require any contrast injections and has no risk of renal injury. MRCP is a preferred method of evaluating CBD in many clinical situations. There is paucity of data on the safety of MRI in the first trimester of pregnancy<sup>[36-38]</sup>. Some authors have raised concerns of thermal injury to the fetus in first trimester<sup>[39,40]</sup>.

According to the Safety Committee of the Society for Magnetic Resonance Imaging<sup>[41]</sup>, MR procedures are indicated in pregnant women if other non-ionizing forms of diagnostic imaging studies are inadequate, or if the examination provides important information that would otherwise require exposure to ionizing radiation [i.e. X-Ray computerized tomography (CT), *etc.*]. Gallstone pancreatitis is generally associated with small gallbladder stones and sludge<sup>[42]</sup>. Small ductal stones in particular, located in the distal CBD could be missed by MRCP<sup>[43]</sup>. Claustrophobia remains the major barrier in the use of MRCP and MRI.

CT scan of the abdomen is the most commonly used imaging modality in diagnosing and later on in assessing severity of AP among adults. It is not recommended in pregnant patients because of the fear of radiation exposure to the fetus<sup>[44]</sup>. In general CT is not the preferred modality of imaging in all trimesters of pregnancy in view of a small radiation risk to the fetus.

ERCP solely as a diagnostic study has lost its value because of the risks of radiation, incidence of AP post procedure, and the availability of safer procedures such as EUS or MRCP. ERCP increases the risk of complications and death from 5% to 10% and 0.1% to 0.2% respectively<sup>[45]</sup>. However, the clinical usefulness of therapeutic ERCP when indicated is unchallenged. Persistent biliary obstruction worsens the outcome, increases the severity of AP, and predisposes to bacterial cholangitis. ERCP along with ES helps to extract impacted gallstones and drain infected bile in severe AP<sup>[46]</sup>. Several reports have shown that ERCP can be carried out successfully in the management of symptomatic choledocholithiasis in pregnancy<sup>[47,48]</sup>. A major concern of this procedure is harmful ionizing radiation to the fetus. Tham *et al*<sup>[49]</sup> reported their experience with ERCP in pregnancy (15 patients over 5 years) with fetal dose radiation measurement. The fetal radiation dose could be reduced to a level less than that considered teratogenic. Kahaleh *et al*<sup>[50]</sup> looked at 17 ERCPs performed in pregnant women between January 1995 and August 2003. They reported a mean gestational age of 18.6 wk, mean fluoroscopy time of 14 s and an estimated fetal radiation exposure of 40 mrad. By limiting fluoroscopy time, shielding the pelvis and fetus with lead and avoiding direct X-ray films, the fetal radiation dose can be reduced to far below the maximum permissible doses. Performing MRCP or EUS before ERCP helps to identify patients who require therapeutic ERCP thus reducing the number of ERCP<sup>[51]</sup>.

## MANAGEMENT

Several recommendations below are mostly based on

expert opinion only and not confirmed by double blind/randomized controlled trials. The difficulties in performing such studies in critically ill pregnant patients are obvious.

### Nutrition

Although successful outcomes can be achieved in obstetric patients requiring parenteral nutrition, the frequency of maternal complications secondary to centrally inserted central venous catheters (TPN) is greater than that reported in non-pregnant patients<sup>[52]</sup>. Peripherally inserted central catheters may be preferable when parenteral nutrition is required during pregnancy. Enteral nutrition by naso-jejunal feeding is preferable to TPN<sup>[11]</sup> in patients with severe AP. Enteral nutrition is physiological, helps the gut flora maintain the gut mucosal immunity, reduced translocation of bacteria, while simultaneously avoiding all the risks of TPN.

### Antibiotics

The topic of prophylactic use of antibiotics is very controversial and the choice of antibiotic in pregnancy is difficult. However, in suspected cholangitis there is no controversy with regards to the need for appropriate antibiotic therapy. Patients with mild AP, normal CBD size with no evidence for cholangitis do not need antibiotics. In a pregnant patient there are concerns with regard to the antibiotic being transplacentally transferred to the fetus with a risk of teratogenicity. Metronidazole passes freely across the placenta. However, recent studies do not show any association with an increased risk of teratogenic effects with metronidazole<sup>[53,54]</sup>. Imipenem (N-formimidoyl thienamycin), belonging to the carbapenem class of antibiotics, has a broad spectrum of activity. It is currently classified as a category C in terms of its risk to the fetus. Although limited animal studies have shown no teratogenic risk or adverse fetal effects, data in humans are not available<sup>[55]</sup>. Quinolones have been classified as category C because adverse effects have been noted in some animal studies. However, there are no adequate studies in humans; the benefits may outweigh the risks. Ampicillin-sulbactam and piperacillin/tazobactam are classified as category B with no evidence of risk in humans. Regardless of initial drug regimen, therapy should be modified to reflect the organisms recovered in blood cultures and the clinical status of the patient.

### Management of underlying cause

**Management of gallstones:** In a pregnant woman with gallstones and CBD stones a major decision is on choice of procedure to clear the CBD of stones. The second decision is on timing and approach to cholecystectomy<sup>[56]</sup>. Factors which influence the decision include the trimester of pregnancy, presence or absence of CBD dilatation, cholangitis, and the severity of AP. AP patients with gallstones need to be evaluated for early cholecystectomy to prevent recurrence of AP later on in the pregnancy when it could be more serious and dangerous<sup>[25-27]</sup>. It is a well respected surgical concept that the second trimester is the best period for surgery since during this period organogenesis is complete and the uterus

is not big enough to obliterate the surgical view for laparoscopic approach. It has also been recognized that cholecystectomy during the second trimester is safe for both the mother and the fetus<sup>[10,12,57]</sup>.

Laparoscopic cholecystectomy in pregnant women offers all of the advantages of laparoscopic surgery in non-pregnant patients - reduced hospital stay, decreased narcotic use and a quick return to a regular diet compared to open surgery in pregnant women<sup>[58]</sup>. In the second trimester the gravid uterus does not interfere with visualization of the operative field. The indications for surgery in pregnancy are severity of symptoms, obstructive jaundice, acute cholecystitis intractable to medical treatment and peritonitis.

Four retrospective studies comparing open cholecystectomy *vs* laparoscopic cholecystectomy did not find any significant difference in maternal or fetal outcomes<sup>[12]</sup>. Gouldman *et al*<sup>[59]</sup> reviewed the available world literature on laparoscopic cholecystectomy in pregnancy and found 107 patients who had cholecystectomy during pregnancy. Most had been performed in the second trimester, with 10 and 16 patients in the first and third trimesters, respectively. Premature labor was rare, with only 2 of the 16 reported patients (12.5%) in the third trimester developing preterm labor, and these were successfully treated with tocolytics. Overall results were good with excellent maternal (100%) and fetal (96%) survival. There is a recent view that states when surgical intervention is warranted, laparoscopic cholecystectomy can be safely performed in any trimester<sup>[60]</sup> but it is a minority view. Performance of cholecystectomy is desirable in the second trimester as organogenesis is complete, and spontaneous abortions are less frequent than in the first trimester<sup>[61]</sup>.

ERCP with sphincterotomy and clearance of bile duct stones is indicated in patients with severe AP, with cholangitis, with strong evidence of persistent biliary obstruction, and in those who are post cholecystectomy as well as patients who are poor candidates for surgical therapy<sup>[25]</sup>. Pregnant women in the first and third trimester who are not ideal candidates for cholecystectomy fall in the last category. Biliary sphincterotomy rather than cholecystectomy may be appropriate when CBD stones are detected and cholecystectomy has to be delayed because of pregnancy. The effectiveness of ES in preventing further episodes of biliary pancreatitis, as an alternative to cholecystectomy in high risk patients has been demonstrated<sup>[48,62-69]</sup>. The indication for ERCP in patients with severe pancreatitis without significant cholestasis is controversial. At this time there is no evidence that therapeutic ERCP is required in all patients with biliary sludge during pregnancy.

The role of therapeutic ES in the management of pregnant patients with AP without CBD stones continues to be controversial<sup>[70]</sup>. Some advocate biliary stent placement rather than performing sphincterotomy and stone extraction and therefore, eliminating complications that accompany sphincterotomy. Farca *et al*<sup>[71]</sup> placed 10-French biliary stents without sphincterotomy in 10 patients, all of whom had uncomplicated pregnancies with normal deliveries. All underwent repeat ERCP with stent extraction and sphincterotomy post-partum and 8 had stones

extracted. In 2 patients, the stent remained in place for 7 and 8 mo, respectively, without the development of occlusion and or cholangitis. However, stenting carries risks of stent occlusion and cholangitis and the need for a second procedure.

**Hyperlipidemic pancreatitis:** Hypertriglyceridemia is the second most common cause of AP, when the serum triglyceride is > 1000 mg/dL. In the third trimester of pregnancy, there is a three-fold rise in serum triglyceride levels<sup>[72]</sup>. This is thought to be due to estrogen-induced increases in triglyceride synthesis and very low-density lipoprotein secretion<sup>[72]</sup>. Hypertriglyceridemia may be more severe in persons with familial hyperlipidemia, predisposing them to develop pancreatitis<sup>[73]</sup>. Rarer causes of AP that need to be considered in the differential diagnosis are hyperemesis during the first trimester; hyperparathyroidism; preeclampsia; and genetic mutations<sup>[74-76]</sup> and acute fatty liver of pregnancy. AP can also complicate the course of thrombotic thrombocytopenic purpura during pregnancy<sup>[77]</sup> and pregnancy induced hypertension<sup>[78]</sup>. Medication and alcoholism are extremely rare causes of AP in pregnancy.

No formal recommendations exist for gestational hypertriglyceridemia treatment in pregnancy at present. Treatment of hyperlipidemic AP is mostly supportive. These treatments include low fat diet<sup>[79,80]</sup>, antihyperlipidemic therapy<sup>[79,80]</sup>, insulin<sup>[79-81]</sup> (to increase lipoprotein lipase activity), heparin<sup>[79-81]</sup> (to increase lipoprotein lipase activity), and even plasmapheresis<sup>[79,82]</sup>.

## CONCLUSION

AP in pregnancy remains a challenging clinical problem to manage, with a relatively limited but expanding evidence base. Among the various etiological factors for AP in pregnancy, gallstone disease is the most common one. Abdominal ultrasound, CT scan, EUS and MRCP are the available imaging studies in diagnosing a biliary etiology for AP. Potential radiation to the fetus is a major disadvantage with CT scan, restricting their use substantially. Diagnostic ERCP is to be avoided whenever possible owing to the associated risks including bleeding, perforation, pancreatitis, fetal radiation, while abdominal ultrasound, MRCP and EUS do not carry these risks. The general management of AP in pregnancy is supportive and includes hospitalization, intravenous fluids, analgesia, and bowel rest. Laparoscopic cholecystectomy is ideally performed in the second trimester when the risk to fetus is the least and only limited technical problems exist as a result of an enlarging uterus. Whenever laparoscopic cholecystectomy is not feasible and the index of suspicion for a stone in the CBD is high based on AUS, MRCP or by EUS, ES or stenting serves to prevent recurrence of AP and allows postponement of laparoscopic cholecystectomy to a more suitable period. Hyperlipidemic pancreatitis and AP due to other etiologies are rare. The outcome of pregnant patients with AP has substantially improved with technical advances in imaging and therapeutic endoscopy.

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## Hepatitis C virus in Pakistan: A systematic review of prevalence, genotypes and risk factors

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### Abstract

In Pakistan more than 10 million people are living with Hepatitis C virus (HCV), with high morbidity and mortality. This article reviews the prevalence, genotypes and factors associated with HCV infection in the Pakistani population. A literature search was performed by using the keywords; HCV prevalence, genotypes and risk factors in a Pakistani population, in Pubmed, PakMediNet and Google scholar. Ninety-one different studies dating from 1994 to May 2009 were included in this study, and weighted mean and standard error of each population group was calculated. Percentage prevalence of HCV was  $4.95\% \pm 0.53\%$  in the general adult population,  $1.72\% \pm 0.24\%$  in the pediatric population and  $3.64\% \pm 0.31\%$  in a young population applying for recruitment, whereas a very high  $57\% \pm 17.7\%$  prevalence was observed in injecting drug users and  $48.67\% \pm 1.75\%$  in a multi-transfused population. Most prevalent genotype of HCV was 3a. HCV prevalence was moderate in the general population but very high in injecting drug users and multi-transfused populations. This data suggests that the major contributing factors towards increased HCV prevalence include unchecked blood transfusions and reuse of injection syringes. Awareness programs are required to decrease the future burden of HCV in the Pakistani population.

**Key words:** Hepatitis C virus; Prevalence; Genotypes; Blood transfusions; Injections

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### INTRODUCTION

Hepatitis C virus (HCV) was discovered in 1989 as the major causative agent of non-A, non-B hepatitis<sup>[1]</sup>. It belongs to the *Flaviviridae* family and is a plus-stranded RNA virus<sup>[2]</sup>. About 200 million people are infected with HCV worldwide, which covers about 3.3% of the world's population<sup>[3,4]</sup>. HCV infection leads to chronic hepatitis in 50% to 80% of individuals<sup>[5]</sup>. It was estimated by the WHO in 2004 that the annual deaths due to liver cancer caused by HCV and cirrhosis were 308 000 and 785 000 respectively<sup>[6]</sup>.

Pakistan is a developing country of 170 million people with low health and educational standards. According to the human development index of the United Nations, it was ranked 134th out of 174 countries<sup>[7]</sup>. In Pakistan 10 million people are presumed to be infected with HCV<sup>[8]</sup>. Public health authorities are creating awareness about hepatitis through print and electronic media<sup>[9]</sup>, but still tremendous efforts are required to increase the awareness regarding various risk factors involved in HCV transmission. In developing countries, due to non-implementation of international standards regarding blood transfusion, reuse of needles for ear and nose piercing, reuse of syringes, injecting drug users, tattooing, shaving from barbers, unsterilized dental and surgical instruments are the main source of transmission of HCV. This article briefly presents the prevalence, genotypes and risk factors associated with HCV transmission in the Pakistani population.

### LITERATURE SEARCH

Articles were searched for in Pubmed, Google scholar and PakMediNet (for non-indexed Pakistani journals),

by using the key words; HCV in Pakistan, prevalence of HCV in Pakistan, epidemiological patterns of HCV in Pakistan, HCV in multi-transfused Pakistani population, HCV in general Pakistani population, HCV in Pakistani injecting drug users (IDUs) population, HCV in Pakistani health care workers, sexual transmission of HCV, injection use in Pakistan, blood banks/transfusion in Pakistan, awareness about HCV in Pakistani population and HCV genotypes in Pakistan. Inclusion criteria entailed the studies demonstrating the prevalence, genotypes and risk factors of HCV in the Pakistani population while studies with incomplete references were excluded. Two hundred and eighty-one different articles/abstracts/reports were obtained from the literature search, out of which 91, published from 1994 to May 2009, were included in this study.

## ANALYSIS

Table 1 includes various reports showing the percentage prevalence of HCV in different groups. Weighted mean of each population was calculated by using the formula:

$$\bar{x} = \frac{\sum_{i=1}^n \omega_i x_i}{\sum_{i=1}^n \omega_i}$$

Standard error of mean was calculated by using the formula:

$$SE_{\bar{x}} = \frac{s}{\sqrt{n}}$$

Results of each population group are presented in the form of mean  $\pm$  SE with 95% confidence interval.

## HCV PREVALENCE IN VARIOUS GROUPS

### General population

Ten different studies showed that the percent prevalence of HCV in the general adult population was  $4.95\% \pm 0.53\%$ <sup>[10-19]</sup>, while six studies showed a percent prevalence of  $1.72\% \pm 0.24\%$  in the pediatric population<sup>[20-25]</sup>. In Pakistan, military recruits are screened for HCV before induction; six different studies showed a percent prevalence of  $3.64\% \pm 0.31\%$  in candidates for military recruitment<sup>[26-31]</sup>. About 5% of infants obtained HCV infection transmitted from a mother carrying both HCV antibody and HCV RNA<sup>[32]</sup>; four studies in pregnant women showed the percent prevalence to be  $4.54\% \pm 3.5\%$ <sup>[33-36]</sup>. Volunteer blood donors are the healthiest population in a community and HCV prevalence in these individuals is a true reflector of a general population's health<sup>[9]</sup>; twelve different studies showed the percent prevalence to be  $3.78\% \pm 0.41\%$  in blood donors<sup>[17,37-47]</sup>.

### IDUs

It was estimated that there were about 5 million drug users in Pakistan, out of which 15% were regular IDUs<sup>[48]</sup>. There has been an increased shift among addicts from inhalatory to injectable drugs due to decrease in quality and availability of heroin (common inhalatory drug used in Afghanistan and Pakistan)<sup>[49]</sup>. The effect of injecting drugs is more intense and satisfying, and young drug users who switch over to injectables usually adopt it as the

main route of their drug administration<sup>[50]</sup>. Approximately 50% of IDUs reported by Altaf *et al*<sup>[51]</sup> in 2007 were in a treatment program; the majority of them wanted to get rid of their addiction but could not do so due to non availability or high charges by rehabilitation centers. The main reason for relapse was the economic crisis which most addicts suffer because the rehabilitation centers are not involved in the development of vocational skills among addicts. Four different reports showed a high  $57\% \pm 17.7\%$  prevalence of HCV among the IDUs<sup>[52-55]</sup>.

### Multi-transfused population

Thalassemic and hemophilic patients require life-long blood transfusions, so it is necessary to obtain screened blood from a reputable source, because the multi-transfused population is more prone to blood-borne pathogens. Arif *et al*<sup>[56]</sup> reported that only 15.8% of parents of thalassemic children knew the importance of blood screening. Six different reports showed an HCV percent prevalence of  $48.67\% \pm 1.75\%$  among the thalassemic and hemophilic population<sup>[56-62]</sup>.

### Health care workers

Health care workers are at high risk of HCV infection because they are dealing with blood, blood-related products and instruments which may carry transmissible pathogens. Hamid *et al*<sup>[63]</sup> reported that recapping of syringes is the key factor for receiving needle stick injuries in health care workers and that transmission of HCV by needle stick injury ranges from 2% to 10%. Two different reports showed an HCV percent prevalence of  $5.2\% \pm 0.63\%$  in health care workers<sup>[64,65]</sup>.

### Sexual transmission

It has been reported from the US that up to 20% of new HCV infections are due to sexual activity<sup>[66]</sup>. Two different reports from the USA and Congo indicate low HCV prevalence among commercial sex workers<sup>[67,68]</sup>. The main problems in Pakistan are illiteracy, lack of awareness about sexually transmitted diseases and low use of condoms among the sex workers. Saleem *et al*<sup>[69]</sup> reported in 2005 that 17% of female sex workers, 3% of male sex workers and 4% of hijras (transgender men) consistently used condoms during the previous month; 67% of female sex workers were illiterate, 34% of female sex workers were suffering from sexually transmitted infections. Homosexual activities were very high among street children who are sexually victimized or indulge in such activities; later on they adopt commercial sex in order to raise their income. Condom usage among the male homosexual population was very low. Four different studies showed an interspousal percent prevalence of  $17.24\% \pm 7.98\%$ <sup>[70-73]</sup>.

## RISK FACTORS

### Unsafe and unnecessary needles

The reuse of syringes and needles was a major factor contributing towards increased HCV prevalence<sup>[74,75]</sup>. It was reported that there are several small groups involved in recycling and repacking of used unsterilized

**Table 1 Percentage prevalence of HCV among different communities in Pakistan**

Population type	Author	Region	Methods	Population size	HCV (%)	
General population	Luby <i>et al</i> <sup>[10]</sup> , 1997	Hafizabad	RIBA	309	6.50	
	Parker <i>et al</i> <sup>[11]</sup> , 2001	Lahore	EIA	417	6.70	
	Khokhar <i>et al</i> <sup>[12]</sup> , 2004	Islamabad	ELISA	47538	5.31	
	Muhammad <i>et al</i> <sup>[13]</sup> , 2005	Buner	ELISA	16400	4.57	
	Hashim <i>et al</i> <sup>[14]</sup> , 2005	Attock	ELISA	4552	4.00	
	Zaman <i>et al</i> <sup>[15]</sup> , 2006	Bahawalpur	ICT	6815	4.41	
	Alam <i>et al</i> <sup>[16]</sup> , 2006	Central Punjab	ELISA, ICT	2038	4.41	
	Chaudhary <i>et al</i> <sup>[17]</sup> , 2007	Rawalpindi	MEIA	1428	2.52	
	Hakim <i>et al</i> <sup>[18]</sup> , 2008	Karachi	ELISA,ICT,PCR	3820	5.20	
	Tunveer <i>et al</i> <sup>[19]</sup> , 2008	Lahore	ICT	203	1.48	
	Pediatric population	Agboatwalla <i>et al</i> <sup>[20]</sup> , 1994	Karachi	ELISA	236	0.44
		Khan <i>et al</i> <sup>[21]</sup> , 1996	Lahore	EIA,RIBA	538	4.09
		Parker <i>et al</i> <sup>[22]</sup> , 1999	Lahore	ELISA	538	1.30
Hyder <i>et al</i> <sup>[23]</sup> , 2001		Lahore	ELISA	171	0.58	
Jafri <i>et al</i> <sup>[24]</sup> , 2006		Karachi	ELISA	3533	1.60	
Aziz <i>et al</i> <sup>[25]</sup> , 2007		Karachi	EIA	380	1.40	
Recruitment		Ali <i>et al</i> <sup>[26]</sup> , 2002	Rawalpindi	ICT	5371	3.29
	Zakaria <i>et al</i> <sup>[27]</sup> , 2003	Karachi	ICT-ELISA	966	2.20	
	Masood <i>et al</i> <sup>[28]</sup> , 2005	Lahore	ELISA	4552	4	
	Mirza <i>et al</i> <sup>[29]</sup> , 2006	Mardan	ELISA	15550	3.69	
	Sharif <i>et al</i> <sup>[30]</sup> , 2006	Risalpur	ELISA	2558	3.40	
	Alam <i>et al</i> <sup>[31]</sup> , 2006	Sargodha	unknown	2038	4.41	
	Pregnant women	Zafar <i>et al</i> <sup>[33]</sup> , 2001	Lahore	PCR	300	4
Khokhar <i>et al</i> <sup>[34]</sup> , 2004		Islamabad	ELISA	503	4.80	
Jaffery <i>et al</i> <sup>[35]</sup> , 2005		Islamabad	ELISA,PCR	947	3.27	
Yousfani <i>et al</i> <sup>[36]</sup> , 2006		Hyderabad	ICT, ELISA	103	16.50	
Blood donors		Mujeeb <i>et al</i> <sup>[37]</sup> , 1996	Karachi	EIA	839	2.40
	Bhatti <i>et al</i> <sup>[38]</sup> , 1996	Rawalpindi	EIA	760	4.80	
	Mujeeb <i>et al</i> <sup>[39]</sup> , 2000	Karachi	ELISA	7047	2.40	
	Ali <i>et al</i> <sup>[40]</sup> , 2003	Quetta	ELISA	1500	1.87	
	Asif <i>et al</i> <sup>[41]</sup> , 2004	Islamabad	MEIA	3430	5.14	
	Ahmad <i>et al</i> <sup>[42]</sup> , 2004	Peshawar	MEIA	4000	2.20	
	Zaidi <i>et al</i> <sup>[43]</sup> , 2004	Peshawar	ELISA	49037	2.60	
	Chaudry <i>et al</i> <sup>[44]</sup> , 2005	Lahore	ELISA	890	6.06	
	Abdul Mujeeb <i>et al</i> <sup>[45]</sup> , 2006	Karachi	ELISA	7325	3.60	
	Chaudhary <i>et al</i> <sup>[17]</sup> , 2007	Rawalpindi	MEIA	1428	2.52	
	Sultan <i>et al</i> <sup>[46]</sup> , 2007	Different Areas	EIA	41498	4.99	
	Khattak <i>et al</i> <sup>[47]</sup> , 2008	Different Areas	ELISA	103858	4	
	IDU	Kuo <i>et al</i> <sup>[52]</sup> , 2006	Lahore	ELISA	351	88
		Achakzai <i>et al</i> <sup>[53]</sup> , 2007	Quetta	ELISA	50	60
		Altaf <i>et al</i> <sup>[54]</sup> , 2009	Karachi	ELISA	161	94
Platt <i>et al</i> <sup>[55]</sup> , 2009		Abbottabad	ELISA	102	8	
Platt <i>et al</i> <sup>[55]</sup> , 2009		Rawalpindi	ELISA	302	17.30	
Thalassemic		Bhatti <i>et al</i> <sup>[58]</sup> , 1995	Rawalpindi	ELISA	35	60
	Muhammad <i>et al</i> <sup>[59]</sup> , 2003	Peshawar	ELISA	80	36	
	Shah <i>et al</i> <sup>[60]</sup> , 2005	NWFP	ELISA	250	57	
	Hussain <i>et al</i> <sup>[61]</sup> , 2008	Islamabad/Peshawar	ELISA	180	41.70	
Hemophilia	Hussain <i>et al</i> <sup>[62]</sup> , 2003	Peshawar	ELISA	40	25	
	Malik <i>et al</i> <sup>[57]</sup> , 2006	Lahore	ELISA	100	56	
Health care workers	Mujeeb <i>et al</i> <sup>[64]</sup> , 1998	Karachi	EIA	114	4.40	
	Aziz <i>et al</i> <sup>[65]</sup> , 2002	Karachi	ELISA	250	5.60	
Sexual/spousal	Irfan <i>et al</i> <sup>[70]</sup> , 2004	Islamabad	PCR	23	4.30	
	Kumar <i>et al</i> <sup>[71]</sup> , 2004	Karachi	MEIA	50	18	
	Khokher <i>et al</i> <sup>[72]</sup> , 2005	Islamabad	ELISA	227	4.40	
	Qureshi <i>et al</i> <sup>[73]</sup> , 2007	Karachi	EIA/PCR	153	38	

HCV: Hepatitis C virus; RIBA: Recombinant strip immunosorbent assay; ICT: Immunochromatographic test; ELISA: Enzyme linked immunosorbent assay; MEIA: Micro-enzyme immunoassay; EIA: Enzyme immunoassay; PCR: Polymerase chain reaction.

syringes, which were available in various drug stores. It was difficult for the public to differentiate between new sterilized syringes and recycled unsterilized syringes<sup>[76]</sup>. Janjua *et al*<sup>[77]</sup> reported that 68% of individuals received injections during the previous three months in Digri and Mirpur khas, two districts of Pakistan, out of which only 54% were from freshly opened syringes. The incidence

of sharing of injection equipment for the last injection was 8.5% in Hyderabad and 33.6% in Sukkur<sup>[54]</sup>.

In Pakistan, the number of estimated injections per person per year ranged from 8.2 to 13.6, which was the highest among developing countries, out of which 94.2% were unnecessary<sup>[51]</sup>. Household members who received four injections per year were 11.4% more prone

to HCV infection than who did not receive injection<sup>[78]</sup>. Khan *et al*<sup>[74]</sup> reported that if both oral and injectable medicine were equally effective, 44% of the Pakistani population preferred injectable medicine.

In 2000, the WHO recommended that countries should implement strategies to change the behavior of health care workers and patients in order to decrease the over-use of injections, to ensure the practice of sterile syringes and needles, and to properly destroy sharp waste after use<sup>[79]</sup>. It was reported that 59% of syringes were dumped into the general waste and not properly disposed of in the healthcare waste. Scavengers seeking valuable things from the waste are at high risk of receiving needle stick injuries from contaminated needles<sup>[76]</sup>.

### Blood transfusion

People in developing countries are mostly anemic, and are more prone to traumatic injuries and obstetric complications. Blood transfusion in these situations is life-saving. If blood is not properly stored or is carrying blood-borne pathogens, then the situation becomes more complicated. According to the WHO office in Pakistan about 1.2 to 1.5 million transfusions are carried out annually in Pakistan<sup>[80]</sup>. In 2000, Luby *et al*<sup>[81]</sup> reported that 50% of blood banks in Karachi recruited paid donors, 25% of donations were from volunteer donors and only 23% of the blood banks screened for HCV while 29% of them were storing blood outside the WHO recommended temperature.

In developing countries, blood transfusion is still a problem due to lack of organized infrastructure, continuous supply of electricity, and properly trained and educated staff. In Pakistan the main source of blood donations are replacement donors and the majority of these are friends and relatives of the patient. These donors donate blood due to fear of death of a relative, chance of further complication of disease or under family pressure. These donors mostly hide their health conditions from their relatives. Selection of donor and their proper screening are key factors to ensure safe transfusion. Safe blood donors are those whose donations are repeatedly negative on screening<sup>[17]</sup>. Sixty-six percent of Pakistanis are residents of rural areas where there is less access to blood transfusion services. To provide proper transfusion facilities to underdeveloped areas requires economic growth. Efforts are required to eliminate the transfusions from paid donors, to improve the safety of the blood supply<sup>[81]</sup>. These conditions can be overcome by development of a fair and organized system of blood screening and transfusion.

### Barbers

In third world countries like Pakistan, most of the barbers are illiterate and unaware of transmission of infectious agents through the repeated use of razors and scissors for different customers without sterilizing them first<sup>[82]</sup>. Janjua and Nizamy reported that only 13% of the barber community knew that hepatitis is a liver disease and that it could be transmitted by contaminated razors; 11.4% of them were cleaning razors with antiseptic solution while 46% of them were re-using razors<sup>[83]</sup>. Recent reports suggested that only 42% knew about hepatitis, 90% did

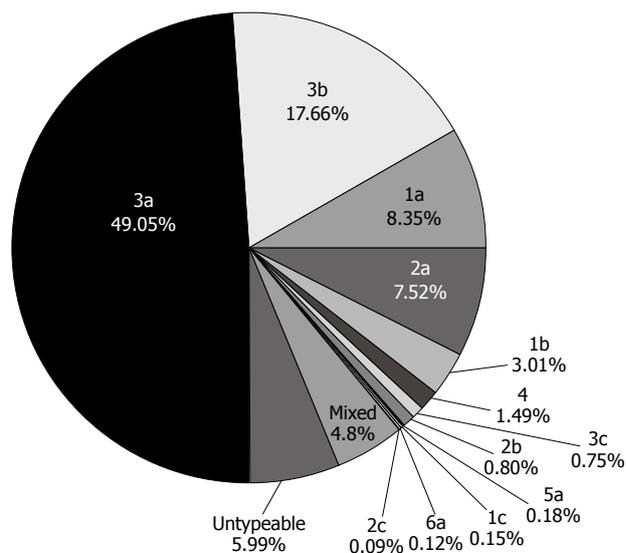


Figure 1 Hepatitis C virus (HCV) genotypes in Pakistan (2008)<sup>[95]</sup>.

not wash hands, 80% were not changing aprons and 66% were not changing towels after each customer. Circumcision is a very important religious procedure performed during early infancy both in rural and urban areas, and the barber community performing this procedure are mostly unaware of transmission of hepatitis by contaminated instruments<sup>[84]</sup>. Bari *et al*<sup>[85]</sup> identified the risk factors involved in transmission of HCV and reported that 70% and 48% of HCV patients had histories of facial and armpit shaving from barbers respectively.

### Awareness

In the Pakistani population there was moderate knowledge about HCV infection whereas awareness about various HCV risk factors was very low<sup>[31,86,87]</sup>. In a survey conducted at a family medicine clinic in Karachi, most of the participants had some educational background and were living in Karachi city. It was reported that 61% of participants believed that HCV was a viral disease, 49% believed that it could be transferred by needles and injections, 5.3% believed that it could be transmitted by ear and nose piercing, and 20.6% knew that it can cause cancer<sup>[86]</sup>. Kuo *et al*<sup>[82]</sup> reported in 2006 that HCV awareness was only 19% in the IDU population of Lahore and Quetta. Zuberi *et al*<sup>[88]</sup> reported that knowledge about HCV infection was related to the educational background of the participants. Public awareness programs are required to decrease the future burden of HCV infection in the Pakistani population.

## GENOTYPES

HCV is classified into eleven different genotypes, of which six are the major genotypes and these genotypes are further classified into many subtypes<sup>[89,90]</sup>. In 1997 it was reported in a small study that 87% of the individuals in Pakistan had genotype 3<sup>[91]</sup>. In 2004, a panel of 30 top gastroenterologists of the country met at a conference and reported that 75%-90% of HCV patients in Pakistan had genotype 3a<sup>[8]</sup>. Qazi *et al*<sup>[92]</sup> reported in 2006 that 71%

of patients had genotype 3 while only 10% had genotype 1. In 2007 it was reported that 81% of individuals had genotype 3 while only 9.5% had genotype 1<sup>[93]</sup>. Hakim *et al*<sup>[18]</sup> reported in 2008 that 51% of HCV patients had genotype 3a; 24% had 3a/3b co-infection and 16% had genotype 3b, while similar results were also reported by Afridi *et al*<sup>[94]</sup> who stated that 50% of HCV patients had genotype 3a followed by 3b and 1a. The most detailed study was conducted by Idrees and Riazuddin in 2008, who performed genotyping of 3351 patients and reported that genotype 3a was the most prevalent genotype in Pakistan; their results are summarized in Figure 1<sup>[95]</sup>.

## CONCLUSION

This study reviewed the seroprevalence of HCV among various population groups, along with risk factors and genotypes in Pakistan. HCV prevalence was observed in nearly 5% of the general population which is in parallel with the WHO estimates of HCV in Pakistan. High prevalence was observed in IDUs and the multi-transfused population, suggesting that the reuse of syringes was common among the injecting drug users, and that blood transfusions were not properly screened. Most prevalent genotype of HCV was 3a. The majority of HCV-positive patients had a history of facial and armpit shaving by barbers suggesting that the barbers shop was the key place for viral transmission. Condom usage was very low among the commercial sex workers and there was low awareness about sexually transmitted diseases amongst this group. In Pakistan, the number of estimated injections per person per year was very high because most Pakistanis think that injectable drugs are more efficacious than oral drugs. There was low awareness in people about the various risk factors associated with HCV transmission. Treatment of hepatitis is very expensive and is creating a huge burden on the country's economy. More emphasis should be given to the preventive measures of the disease in order to decrease the future health and economic burden; these include screened blood transfusions, proper sterilization techniques in clinics and hospitals, use of disposable syringes and razor blades. The government should take aggressive steps to create awareness among the general public by the use of media or by modifying the school syllabus.

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ORIGINAL ARTICLE

## Shift work aggravates metabolic syndrome development among early-middle-aged males with elevated ALT

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### Abstract

**AIM:** To examine whether shift work accelerates metabolic syndrome (MetS) development among early middle-aged males with elevated alanine aminotransferase (e-ALT).

**METHODS:** A retrospective, observational follow-up study on MetS development at a 5-year interval was conducted using health examination data. Nine hundred and ninety six male employees not fulfilling MetS criteria at screening were enrolled. Age, MetS-components, liver enzymes, serological markers for viral hepatitis, abdominal ultrasound, insulin resistance status, lifestyles, and workplace factors were analyzed.

**RESULTS:** The prevalence of elevated serum ALT (> 40 U/L, e-ALT) at baseline was 19.1%. There were 381 (38.3%) workers with long-term exposures to day-night rotating shift work (RSW). 14.2% of subjects developed MetS during follow-up. After 5 years, the workers with e-ALT had significantly unfavorable changes in MetS-components, and higher rates of MetS development, *vs* subjects with normal baseline ALT levels. Workers with both baseline e-ALT and 5-year persistent RSW (pRSW) exposure had the highest rate of MetS development. Also, e-ALT-plus-pRSW workers had a significant increase in MetS-components at

follow-up, compared with the other subgroups. After controlling for potential confounders, e-ALT-plus-pRSW workers posed a significant risk for MetS development (odds ratio, 2.7; 95% confidence interval, 1.4-5.3, *vs* workers without baseline e-ALT nor pRSW).

**CONCLUSION:** We suggest that all early middle-aged male employees with e-ALT should be evaluated and managed for MetS. Particularly in terms of job arrangements, impacts of long-term RSW on MetS development should be assessed for all male employees having baseline e-ALT.

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**Key words:** Alanine aminotransferase; Early middle aged; Male; Metabolic syndrome; Shift work

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### INTRODUCTION

Elevated serum alanine aminotransferase (ALT) is a common abnormality of health examinations among the middle-aged working population<sup>[1,2]</sup>. Nowadays, it is unavoidable that a large number of asymptomatic workers with elevated ALT (e-ALT), regardless of the underlying cause, are asked to do rotating shift work (RSW) on 24-h production lines<sup>[3]</sup>. In previous studies, e-ALT<sup>[4,5]</sup> and shift work<sup>[6,7]</sup> had been independently assessed for their associations with metabolic syndrome (MetS), which has been linked with cardiovascular disease (CVD)<sup>[8,9]</sup>, one of the leading death causes among working populations<sup>[10]</sup>. However, for early middle-aged workers with e-ALT in baseline conditions, few long-term follow-up studies assessed the association between RSW and MetS development. In terms of workplace health management and job arrangement, surveys for

the impact of RSW on MetS development among the subjects having e-ALT urgently required. In Taiwan, periodic health checkups are compulsorily for employees in many workplaces; thus we had the opportunity to utilize a retrospective follow-up study to assess the impact of RSW on MetS development among early-middle-aged workers having baseline e-ALT.

## MATERIALS AND METHODS

### Participants

In accordance with the Labor Health Protection Regulation of the Labor Safety and Health Act, 1203 male workers of an electronic manufacturing company underwent compulsory health checkups in 2002 and 2007. Final analysis of this follow-up study only included the subjects not fulfilling MetS criteria in 2002. Among the 1203 workers undergoing health examinations in both 2002 and 2007, 207 employees were excluded from study because they were screened previously with MetS. Final records of a total of 996 male workers constituted the cohort for the study and endpoint analysis. Hepatic virus infection is highly prevalent among Taiwanese adults<sup>[2]</sup>, our analysis included the hepatic virus carriers without MetS, and we controlled these factors in our final multivariate analysis. Most of the male employees of this electronics manufacturing company were residents of northern Taiwan.

The procedures and measurements of the health examinations took place in the morning, following an overnight fast, at a health-care unit of the factory. This health examination was open to all registered employees during every working day, 07:30 am to 09:30 am, for a 1-mo span. We suggested that the health checkups for the rotating shift workers be performed on the 3rd to 6th day of their day-duty. All the employees were requested to avoid drastic physical exercises (i.e. long-distance running, heavy weight lifting training etc) during the 3 d before undergoing the health examination.

Subjects' identities were anonymous and unlinked to the data. This analytical study, limited within health checkups records, followed the ethical criteria for human research, and the study protocol (TYGH09702108) was reviewed and approved by the Ethics Committee of the Tao-Yuan General Hospital, Taiwan.

### Demographics, job types, lifestyle data, and biological measurements

In 2002, a questionnaire about baseline personal history, including smoking habits, physical exercise, drinking, and dietary habits, was completed by the examinees. The day-night RSW was determined from the self-reported questionnaires in 2002 and 2007. The shifts on the 24 h production line were scheduled on a three-team/two-shift plan (6 d-shifts-three rest days-six night-shifts-three rest days, *etc.*). The day and night shifts started at 07:30 am and 19:30 pm, respectively.

Physical examination and blood tests were performed on all participants in both 2002 and 2007. The participants arrived at the health care unit in the morning, between

07:30 am and 09:30 am, after an overnight 8 h fast. The physical examination records included measurements of waist circumference, weight, height, and blood pressure. Waist circumferences were measured midway between the lowest rib and the superior border of the iliac crest. After being seated for 5 min, sitting blood pressure was measured with the dominant arm using digital automatic sphygmomanometers (model HEM 907, Omron, Japan), twice at a 5-min intervals; the mean of the reading was used for data analysis. After the physical examination, participants were placed in a reclined position, and venous blood (20 mL) from an antecubital vein of the arm was taken for subsequent tests. Blood specimens were centrifuged immediately thereafter, and shipped in the frozen state, using dry ice, to a central clinical laboratory in the Tao-Yuan General Hospital (certified by ISO 15189 and ISO 17025). Glucose [glucose oxidase method, intra- and inter-assay coefficient of variation (CV) < 5%], triglyceride (enzymatic method, intra- and inter-assay CV < 5%), high-density lipoprotein (HDL) (enzymatic method, intra- and inter-assay CV < 5%), ALT, aspartate aminotransferase (Japan Society of Clinical Chemistry method, intra- and inter-assay CV < 5%) assays were conducted by a Hitachi autoanalyzer model 7150 (Hitachi, Tokyo, Japan). hepatitis B virus (HBV) surface antigen, anti-HBV surface antibody and anti-HCV antibody were measured by the microparticle enzyme immunoassay using the AxSym System instrument (Abbott, Illinois, USA), with the intra-assay and inter-assay CV < 10%. Insulin was determined with an AxSym autoanalyzer (Abbott, Illinois, USA) radioimmunoassay, with the intra-assay and inter-assay CV < 10%.

### Abdominal sonographic examinations

Institutional Review Board of Tao-Yuan General Hospital did not agree to perform liver biopsies on apparently healthy subjects. Sonographic diagnosis for fatty liver is widely accepted in many epidemiological surveys<sup>[11]</sup>; therefore, we used this noninvasive method to diagnose fatty liver. In 2002, abdominal ultrasound examinations were performed using convex-type real-time electronic scanners (Toshiba SSA-340 with 3.75 MHz convex-type transducer) by three gastrointestinal specialists. These gastrointestinal specialists had 10-15 years of experience in abdominal ultrasound examinations, and were blind to the medical history as well as to the blood test results of the examinees.

### Definitions

**MetS:** The pre-MetS status was defined as one or two<sup>[12]</sup>, and the MetS designation was made if three or more of the following five criteria were fulfilled: Central obesity (waist circumference > 90 cm based on Taiwanese criteria<sup>[13]</sup>); Elevated blood pressure (defined as systolic blood pressure  $\geq$  130 mmHg or diastolic blood pressure  $\geq$  85 mmHg; Hyperglycemia, hypo-HDL cholesterolemia and hypertriglyceridemia (defined based on modified National Cholesterol Education Program Adult Treatment Panel III criteria<sup>[14]</sup> as: fasting sugar  $\geq$  100 mg/dL, HDL < 40 mg/dL, and triglycerides  $\geq$  150 mg/dL). We specified

the subjects with MetS outcome as those who were without MetS in 2002 but with MetS in 2007.

**e-ALT:** e-ALT was defined as ALT > 40 U/mL, according to the standard reference limits used at Tao-Yuan General Hospital and other by studies<sup>[15]</sup>.

**Insulin resistance:** Insulin resistance was calculated by the equation of homeostasis model assessment,  $HOMA_{IR} = \text{fasting insulin (U/mL)} \times \text{glucose (mmol/L)} / 22.5$ , and defined as the top quartile of  $HOMA_{IR}$ <sup>[16]</sup>.

**Persistent RSW (pRSW):** According to the Labor Health Protection Regulation of the Labor Safety and Health Act, periodical health examination ought to be performed on all registered employees working more than 1 year. These examinations took place annually for workers in this company. In the questionnaire, workers answered “yes” to “my job is a rotating shifting work”, indicating that the workers had done a rotating job for at least 1 year. Generally, this working pattern continues except for reasons of family events or promotions in this kind of factory. Those who did RSW for at least 1 year in the beginning and at the end of our follow-up were likely to have continued doing the same types of jobs. For the reasons mentioned above, we arbitrarily defined in our analysis of job type as: “pRSW” exposure was defined when the answers to the question in 2002 and 2007 were both day-night rotating shift work. Work information obtained through a self-administered questionnaire has been generally accepted in previous investigations<sup>[6,17]</sup>.

**Lifestyle factors:** For defining “ever been a smoker”, the question started with “Do you smoke? (1 Never, 2 Currently smoke, 3 Did but quit now)” and was followed by questions about quantity. We defined “ever been a smoker” as saying “yes” to question 2 or 3 and consuming at least 10 cigarettes daily for over 1 year. For defining “used to having snacks”, the question started with “Are you used to having snacks before sleeping?” and “Are you used to having snacks between meals?” and followed with questions about quantity. We defined the habits of having snacks in the present study as more than three times per week. The standard portions of examples for snacks (fruit products, milk products, fried food, nuts, beans products, meat, and alternatives) were explained to examinees in questionnaires. We defined “having routine physical exercise” as answering “doing exercises more than three times every week”. For defining “habitual drinker”, the question started with “Do you consume alcohol as usual? (1 No, 2 Yes)” and was followed by questions about frequency and quantity. We defined “habitual drinker” in this study as saying “yes” to question 2 and consuming it at least once per week for over 1 year.

**Sonographic fatty liver:** The definition of ultrasonic fatty liver was based on a comparative assessment of image brightness relative to the kidneys, under identical criteria<sup>[18]</sup>.

### Statistical analysis

Baseline characteristics and abnormal rates were compared between four subgroups divided by e-ALT and pRSW using ANOVA and Tukey’s test for continual variables and  $\chi^2$  test for categorical variables. Modes and ranges represented the initial, the 5th year and the changes of MetS-component numbers, were also tested by ANOVA and Tukey’s test. Baseline and the 5th year abnormality rates were compared using the  $\chi^2$  test. Multivariate logistic regression, adjusted for age, baseline insulin resistance status, MetS-components, pRSW exposure, exercise, smoking, drinking and diet behaviors, viral hepatitis infection, and fatty liver was used to estimate the adjusted odds ratios (OR) and 95% confidence intervals (CI) of risk factors for predicting MetS development. A two-sided *P*-value < 0.05 was considered statistically significant. SAS version 8.0 (SAS Institute, Cary, NC, USA) was used for all statistical analyses. Excel 2000 (Microsoft®, USA) was used for the statistical figure.

## RESULTS

### General and follow-up characteristics of the study population

Job descriptions are shown in Table 1. According to the characteristics of automatic production lines in such factories, most employees do light manual labor jobs or sedentary work. Tables 2 and 3 present baseline data of the overall and the four subgroups divided by initial e-ALT and pRSW exposures. Our sample population had an initial mean age of 32.1 years (SD 5.9 years); with total prevalence rates of e-ALT and pRSW of 19.1% and 38.3%, respectively. There were 14.2% (141/996) subjects who developed MetS within 5 years. At baseline, there were significant differences in age among the four subgroups; the subgroups with pRSW exposures were younger than the other two subgroups without pRSW exposures (30.9 and 31.4 years *vs* 32.8 and 32.6 years, Table 2). Baseline measurements of MetS-components, liver enzymes and insulin were significantly unfavorable in the two subgroups with e-ALT (Table 2). As shown in Table 3, baseline abnormality rates of MetS-components, hepatic virus infections, insulin resistance, and fatty liver were higher in the two groups with baseline e-ALT than in the subgroups with normal baseline ALT, though the baseline prevalence rates of central obesity and hyperglycemia were not significantly different among the four groups. Having snacks before bedtime and smoking habits prevailed in the group of pRSW with normal ALT, whereas other lifestyle parameters were not significantly different among the four groups. The overwhelming majority of pRSW workers (92.4%) were on-site workers (Tables 1 and 3).

### Changes of MetS-components and its numbers within follow up

Most of the 5th year and 5-year changes of abnormality rates for MetS-component were unfavorable in the two subgroups with baseline e-ALT, and particularly in the subgroup with e-ALT-plus-pRSW (Table 4). The

**Table 1 Job contents for different job types/e-ALT of the workers in this investigation *n* (%)**

Job title	Routine work activities	<i>n</i> = 996	e-ALT: no; pRSW: no ( <i>n</i> = 496)	e-ALT: no; pRSW: yes ( <i>n</i> = 310)	e-ALT: yes; pRSW: no ( <i>n</i> = 119)	e-ALT: yes; pRSW: yes ( <i>n</i> = 71)
Operator	On-site work	232 (23.3)	101	97	17	17
Technician	On-site work	422 (42.4)	158	178	40	46
Engineer	On-site work	159 (16.0)	131	2	24	2
Team leader	On-site work	85 (8.5)	33	29	18	5
Specialist	On-site work	22 (2.2)	16	1	5	0
Deputy manager	Management	42 (4.2)	31	1	10	0
General manager	Management	19 (1.9)	15	0	4	0
Director	Management	3 (0.3)	3	0	0	0
Guard	Security	8 (0.8)	6	1	1	0
Clerk	Office work	4 (0.4)	2	1	0	1

e-ALT: Elevated alanine aminotransferase; pRSW: Persistent rotating shift work.

**Table 2 Baseline characteristics of subjects stratified by e-ALT<sup>1</sup> and pRSW status *n* (%)**

Measurement (SD)	Total population ( <i>n</i> = 996)	e-ALT: no; pRSW: no ( <i>n</i> = 496)	e-ALT: no; pRSW: yes ( <i>n</i> = 310)	e-ALT: yes; pRSW: no ( <i>n</i> = 119)	e-ALT: yes; pRSW: yes ( <i>n</i> = 71)	<i>P</i> value <sup>2</sup>
Age (yr)	32.1 (5.9)	32.8 (6.0)	30.9 (5.8)	32.6 (5.3)	31.4 (5.7)	0.0001
Body mass index (kg/m <sup>2</sup> )	23.4 (2.9)	23.2 (2.8)	22.7 (2.8)	25.0 (2.5)	25.0 (3.0)	< 0.0001
Waist (cm)	78.3 (7.6)	78.1 (7.4)	76.4 (7.4)	82.5 (6.3)	81.3 (8.1)	< 0.0001
Systolic blood pressure (mmHg)	119.2 (14.1)	118.2 (13.8)	118.2 (14.3)	123.0 (14.0)	123.5 (14.1)	0.0002
Diastolic blood pressure (mmHg)	72.9 (9.0)	72.3 (8.6)	72.3 (8.9)	75.4 (10.0)	75.7 (9.3)	0.0002
Fasting blood glucose (mg/dL)	94.5 (14.8)	95.2 (18.6)	92.9 (8.1)	95.1 (8.1)	94.5 (15.2)	0.1797
Triglycerides (mg/dL)	113.1 (86.3)	109.3 (97.8)	101.0 (55.6)	149.3 (96.0)	132.2 (74.7)	< 0.0001
HDL cholesterol (mg/dL)	48.6 (11.0)	49.3 (10.9)	49.6 (11.4)	45.2 (10.8)	44.9 (7.9)	< 0.0001
Alanine aminotransferase (U/L)	31.0 (29.4)	21.7 (7.7)	21.1 (8.4)	68.8 (42.6)	75.8 (54.3)	< 0.0001
Aspartate aminotransferase (U/L)	25.8 (17.9)	21.9 (5.1)	21.8 (5.5)	39.3 (23.3)	48.5 (47.8)	< 0.0001
Insulin (mg/dL)	8.5 (6.2)	8.0 (4.9)	7.4 (5.2)	11.4 (7.8)	11.6 (11.4)	< 0.0001

<sup>1</sup>ALT > 40 mg/dL; <sup>2</sup>ANOVA was conducted, using Tukey's test.

**Table 3 Baseline abnormality rates of subjects stratified by e-ALT<sup>1</sup> and pRSW status**

Abnormalities	Total population ( <i>n</i> = 996)	e-ALT: no; pRSW: no ( <i>n</i> = 496)	e-ALT: no; pRSW: yes ( <i>n</i> = 310)	e-ALT: yes; pRSW: no ( <i>n</i> = 119)	e-ALT: yes; pRSW: yes ( <i>n</i> = 71)	<i>P</i> value <sup>2</sup>
MetS-component abnormality (%)						
Central obesity	3.6 (36/996)	2.8	3.2	5.9	7.0	0.1595
Hyperglycemia	20.7 (206/996)	22.0	18.4	22.7	18.3	0.5658
Hypertriglyceridemia	17.5 (174/996)	15.1	13.2	34.5	23.9	< 0.0001
Elevated blood pressure	21.3 (212/996)	18.3	20.6	29.4	31.0	0.01
Hypo-HDL cholesterol	20.2 (201/996)	18.5	17.1	32.8	23.9	0.0018
Elevated alanine aminotransferase	19.1 (190/996)					
Hepatovirus B carrier	19.3 (192/996)	18.1	14.8	27.7	32.4	0.0005
Hepatovirus C carrier	1.2 (12/996)	0.4	0.3	4.2	5.6	< 0.0001
Fatty Liver	30.8 (307/996)	24.0	21.0	66.4	62.0	< 0.0001
Lifestyle factor (%)						
Ever been a smoker (≥ 1 yr)	42.7 (425/996)	36.9	51.6	39.5	49.3	0.0003
Having snacks before sleeping (≥ 1 d/wk)	42.5 (423/996)	37.7	51.9	39.5	39.4	0.0008
Having snacks between meals (≥ 1 d/wk)	40.9 (407/996)	40.5	39.4	47.1	39.4	0.5192
Physical exercise (≥ 3 times/wk)	34.6 (345/996)	38.5	31.6	31.9	25.4	0.0545
Habitual drinker (≥ 1 d/wk)	10 (99/996)	8.7	10.6	10.1	15.5	0.3196
Workplace factor (%)						
Persistent day-night rotating shift work	38.3 (381/996)					
On-site worker	92.4 (920/996)	88.5	99.1	87.4	98.6	< 0.0001
Development of MetS within 5 yr	14.2 (141/996)	11.1	8.7	27.7	36.6	< 0.0001

<sup>1</sup>ALT > 40 mg/dL; <sup>2</sup>χ<sup>2</sup> test was conducted for abnormalities and four liver function/shift work subgroups. MetS: Metabolic syndrome.

male workers having both baseline e-ALT and 5-year exposures to pRSW had the highest increasing rates of hyperglycemia (15.5% increased), hypertriglyceridemia

(21.1% increased) and elevated blood pressure (31.0% increased). They also displayed the highest rate of MetS development (36.6%) among four subgroups. As

**Table 4** Change of abnormalities based on a 5-year follow-up among the subgroups stratified by ALT and shift work (%)

MetS and its components	e-ALT initially	pRSW exposure	Baseline	P value <sup>1</sup>	5th yr	P value <sup>1</sup>	Δ 5th yr vs baseline
Central obesity	No	No	2.8	0.1595	22.2	0.0037	19.4
		Yes	3.2		20.0		16.8
	Yes	No	5.9	0.5658	33.6	< 0.0001	27.7
		Yes	7.0		33.8		26.8
Hyperglycemia	No	No	22.0	0.5658	16.7	< 0.0001	-5.2
		Yes	18.4		13.9		-4.5
	Yes	No	22.7	< 0.0001	26.9	< 0.0001	4.2
		Yes	18.3		33.8		15.5
Hypertriglyceridemia	No	No	15.1	< 0.0001	26.8	< 0.0001	11.7
		Yes	13.2		25.5		12.3
	Yes	No	34.5	0.01	42.0	0.1025	7.6
		Yes	23.9		45.1		21.1
Elevated blood pressure	No	No	18.3	0.01	48.0	0.1025	29.6
		Yes	20.6		50.0		29.4
	Yes	No	29.4	0.0018	55.5	< 0.0001	26.1
		Yes	31.0		62.0		31.0
Hypo-HDL cholesterol	No	No	18.5	0.0018	6.3	< 0.0001	-12.3
		Yes	17.1		8.1		-9.0
	Yes	No	32.8	0.0001	17.6	< 0.0001	-15.1
		Yes	23.9		23.9		0.0
Development of MetS within 5 yr	No	No		0.0001	11.1	< 0.0001	
		Yes			8.7		
	Yes	No		0.0001	27.7	< 0.0001	
		Yes			36.6		
MetS-component numbers (mode)	No	No	0	0.0001	1	< 0.0001	0
		Yes	0		1		0
	Yes	No	2	0.0001	1	< 0.0001	0
		Yes	1		2		1

<sup>1</sup>χ<sup>2</sup> test was conducted, four abnormalities of subgroups were constructed. Δ: Differences between 2007 and 2002; plus indicates increasing and minus indicates decreasing trend within follow-up.

**Table 5** Multivariate analysis for the risk of development of metabolic syndrome among male workers

Adjustment for initial characteristics		OR <sup>1</sup> (e-ALT: no vs pRSW: no)	95% CI	
Model 1	Age (yr)	e-ALT: no; pRSW: yes	0.8	0.5-1.3
		e-ALT: yes; pRSW: no	3.1	1.9-5.0
		e-ALT: yes; pRSW: yes	4.7	2.7-8.2
Model 2	Age, MetS-components, insulin resistance	e-ALT: no; pRSW: yes	0.8	0.5-1.3
		e-ALT: yes; pRSW: no	1.8	1.03-3.0
		e-ALT: yes; pRSW: yes	3.8	2.0-6.9
Model 3	Age, MetS-components, insulin resistance, hepatovirus infections	e-ALT: no; pRSW: yes	0.8	0.5-1.3
		e-ALT: yes; pRSW: no	1.9	1.1-3.2
		e-ALT: yes; pRSW: yes	4.0	2.1-7.4
Model 4	Age, MetS-components, insulin resistance, hepatovirus infections, fatty liver	e-ALT: no; pRSW: yes	0.8	0.5-1.3
		e-ALT: yes; pRSW: no	1.4	0.8-2.4
		e-ALT: yes; pRSW: yes	2.9	1.5-5.5
Model 5	Age, MetS-components, insulin resistance, hepatovirus infections, fatty liver, lifestyle and workplace factors	e-ALT: no; pRSW: yes	0.7	0.4-1.2
		e-ALT: yes; pRSW: no	1.3	0.7-2.3
		e-ALT: yes; pRSW: yes	2.7	1.4-5.3

<sup>1</sup>Each variant put into the model as categorical data. OR: Odds ratio; CI: Confidence interval.

shown on figure, the subgroup of e-ALT-plus-pRSW had a significantly increasing shift in numbers of MetS-component.

**Risk assessment for MetS development**

Table 5 shows the age-adjusted OR for MetS obtained by the step-by-step examinations. At the beginning, the age-adjusted OR of MetS development for the e-ALT workers without or with pRSW exposure

were 3.1 (95% CI: 1.9-5.0) and 4.7 (95% CI: 2.7-8.2) respectively. Combined with step by step examinations, after controlling for confounders of initial age, MetS-components, insulin resistance, liver status, lifestyle and workplace factors, the multivariate analysis indicated that the male workers with baseline e-ALT plus pRSW remained at a 2.7-fold (95% CI: 1.4-5.3) greater risk of developing MetS compared with those having neither initial e-ALT nor pRSW exposure. Male employees who

had initial e-ALT but without pRSW had an increased risk for progression to MetS when compared with those who initially had normal ALT. This result was significant but became insignificant when fatty liver was introduced as one of the controlling factors: adjusted OR of MetS development became to 1.3 (95% CI: 0.7-2.3) for the workers having initial e-ALT but without pRSW (Table 5).

## DISCUSSION

All the subjects in our sample population continued working in the company at least for 5 years, which reveals that our workers were in relatively stable occupational situations. Our conclusions from observations of a large and stable middle-aged working population will contribute to the surveys on MetS in such working groups.

### **e-ALT and MetS development**

The baseline e-ALT prevalence rate (19.1%) in our sample population was as high as that in other working populations<sup>[1]</sup>. Although not fulfilling MetS criteria at screening, the workers with e-ALT had significantly more unfavorable baseline measurements, as well as higher abnormality rates for most of the MetS-components (Tables 2 and 3), as compared with workers having normal baseline ALT. Similar findings were revealed in a previous cross-sectional study for young healthy men<sup>[19]</sup>. Our follow-up results (Table 4) demonstrated that the subjects initially having e-ALT tended display considerably raised abnormality rates of MetS-components within 5 years. Moreover, at the end of follow-up, the two subgroups with baseline e-ALT had significantly higher rates of MetS development than the other two subgroups with normal baseline ALT levels.

As a mortality predictor<sup>[20]</sup>, e-ALT represents many detrimental health conditions, including systemic inflammation<sup>[19,20]</sup>, which was reported to contribute to MetS development. In our step-by-step multivariate analysis (Table 5), the significant impacts of baseline e-ALT on MetS development among the workers without pRSW remained until we introduced an operator-dependent survey, fatty liver<sup>[21]</sup>, which has an extremely close relationship with e-ALT<sup>[15]</sup>, as a controlling factor. According to our statistical analysis, baseline e-ALT is associated with long-term development of manifold metabolic abnormalities and MetS among early middle-aged male workers. We suggest workplace MetS managements for all workers having e-ALT.

### **Changes of MetS-components among our male workers within 5 years**

Taking each MetS-component into consideration, most of the MetS-components abnormality rates increased as our population got older (Table 4); though some exceptions for hyperglycemia and hypo-HDL cholesterolemia were found in the subgroups with normal baseline ALT or without pRSW exposure.

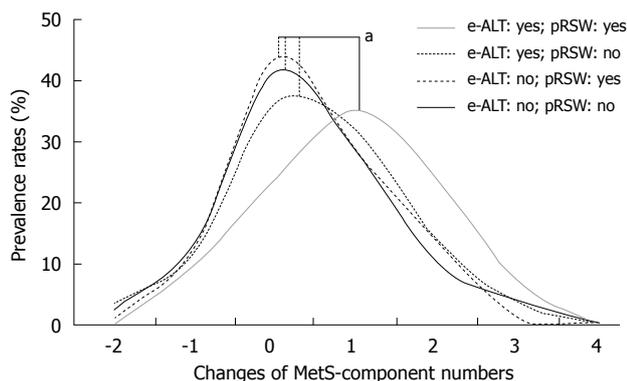
We demonstrated that the early middle-aged male workers with baseline e-ALT had increased abnormality rates of hyperglycemia, same as previous findings<sup>[22]</sup>.

Similarly, a significantly increased prevalence of hyperglycemia was found for workers with e-ALT-plus-pRSW, which was compatible with the findings that shift work can aggravate the insulin resistance<sup>[23]</sup>. In contrast, for the workers with normal baseline ALT levels, hyperglycemia showed an improving trend. These phenomena of improved blood sugar were dissimilar to the elderly population<sup>[24]</sup>, but have been shown in many follow-up observations for healthy adults<sup>[22,25]</sup>. Our early middle-age subjects with normal baseline ALT seemed to maintain the ability to stabilize blood sugar. In contrast, the subjects with baseline e-ALT displayed impaired abilities to improve their blood sugar levels, which might be related to pRSW exposure. Also, hypo-HDL was associated with an improving trend among our early-middle-aged males during follow-up, except for the e-ALT-plus-pRSW workers. This increasing trend of HDL has been mentioned among healthy adults<sup>[26]</sup>, but not found in the elderly<sup>[24]</sup>. As apparently healthy young adults get older, HDL might quantitatively increase in response to external stimuli<sup>[24,27]</sup> or internal challenges<sup>[28]</sup>, and this increase is likely to reach a plateau<sup>[29]</sup>. Our comparatively young healthy workers might not yet have reached their plateau, so they might have increasing HDL concentrations within our follow-up. However, when under chronic oxidative stress caused by simultaneous e-ALT<sup>[30]</sup> and shift work exposure<sup>[31]</sup>, our early middle-aged subjects might have significantly weaker capabilities for quantitatively increasing HDL.

### **Increasing MetS-component numbers and development of MetS among the workers with both baseline e-ALT and pRSW exposures**

Analyzing the number of MetS-components is informative in determining the CVD risk of the general population<sup>[9]</sup>. In addition, MetS-component numbers were reported in many convincing studies that closely linked them with damaged conditions of the whole human body: impaired cardiovascular conditions<sup>[32,33]</sup>, cognitive impairment<sup>[34]</sup>, and colon polyps<sup>[35]</sup>. Shift work is associated with the premature aging process<sup>[36]</sup>, and as demonstrated in our analyses (Table 4 and Figure 1), the e-ALT-plus-pRSW workers had significantly increased numbers of MetS-components in the end of follow-up. Thus, the impacts of shift work exposure on general health conditions of the e-ALT workers should receive close attention.

Some factors might confound our findings, such as: high prevalence of HBV and HCV among those with e-ALT at baseline, fatty liver might be linked with HCV carrier status, and the reduction in the amount of exercise in individuals who developed MetS. However, in the analysis carried out in consecutive steps for the male workers with e-ALT, all the other confounders did not remove the significance of pRSW on MetS development (Table 5, Model 3, 4 and 5). Our follow-up observations confirmed that persistent day-night RSW accelerates the progression toward MetS among the e-ALT workers. e-ALT can work with other risk factors to worsen the inflammatory state<sup>[30]</sup>, and shift work brings about long-term oxidative stress<sup>[31]</sup>, therefore a possible explanation for our male shift workers with e-ALT tending to develop



**Figure 1** Distributions of MetS-components number changes at 5-year follow-up, sub-grouped by elevated alanine aminotransferase and persistent rotating shift work. <sup>a</sup>ANOVA was conducted, using Tukey's test;  $P = 0.0015$ . e-ALT: Elevated alanine aminotransferase; pRSW: Persistent day-night rotating shift work; MetS: Metabolic syndrome.

MetS might be aggravated systemic chronic inflammatory reactions<sup>[37,38]</sup>.

Due to the dramatically rising occurrence rate of MetS, we strongly suggest screening, follow-up, and treatment of MetS for workers with both e-ALT and unavoidable long-term rotational shift work exposures.

#### Potential limitations

Some potential limitations of our analysis need to be considered.

Firstly, Taiwan National Health Insurance has provided comprehensive medical care, and for the pre-MetS subjects, it was possible that they could have had corrective management during our follow-up, and those actions might have led to protective effects<sup>[39]</sup>. In such a case, the main limitations of this observational investigation would be that we did not take into consideration the potential treatment effects for the at risk workers. Thus, our conclusion might be affected by the results of therapeutic management programs, and we might be underestimating risk factors for MetS development. Secondly, our shift workers with normal ALT were relatively young in our sample population (Table 2), as compared with workplace populations of other studies<sup>[6]</sup>. Physically, the advantage of youthfulness<sup>[6]</sup> might lead us to a statistically insignificant conclusion for the shift work exposures on MetS development among subjects with normal ALT (Table 5); the aging effect on MetS development is an important issue for such a young cohort, and requires further surveys. Lastly, we proposed that MetS development among our e-ALT shift workers might be closely linked to chronic inflammation; therefore direct inflammatory evidence<sup>[19]</sup> should be carefully surveyed in future investigations.

As for the increasing body of professional women occupying workplaces, it is important to compare the differences between genders in risk assessments for MetS development; thus all the present findings are worthy of being tested in future studies of female workers. In terms of health and safety, for the individuals who initially had MetS and were excluded of this study, it is necessary to assess their cardiovascular outcomes in future investigations.

In conclusion, for early middle-aged male workers, baseline and changes of MetS component abnormalities, as well as the development of MetS, are all associated with baseline e-ALT. In addition, long-term RSW exposures significantly aggravate MetS development among the workers having baseline e-ALT. We suggest all male workers having e-ALT should be carefully evaluated and managed for MetS. Particularly in terms of job arrangements, impacts of long-term RSW on MetS development should be assessed for all male employees having baseline e-ALT.

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## COMMENTS

### Background

Elevated serum alanine aminotransferase (ALT) is a common abnormality of health examinations among middle-aged working populations. It is unavoidable nowadays that a large number of asymptomatic workers with elevated serum ALT levels might be asked to do rotating shift work (RSW) on 24-h production lines. In terms of workplace health management and job assignment, a 5-year follow-up study assessing the association between RSW and metabolic syndrome (MetS) development was conducted in Taiwan for male workers.

### Research frontiers

In some studies, elevated ALT (e-ALT) and shift work had been independently assessed for their associations with MetS, which is associated with cardiovascular disease, one of the leading causes of death among working populations. This survey takes these two risk factors together into consideration and obtained significant findings.

### Innovations and breakthroughs

Workers who had both baseline e-ALT and persistent RSW (pRSW) exposures (e-ALT-plus-pRSW workers) had a significantly high risk for MetS development among middle-aged male workers. In addition, e-ALT-plus-pRSW workers had a significant increase in MetS-component numbers at the end of follow-up, compared with the other workers. Finally, e-ALT-plus-pRSW workers had a significant risk for MetS development.

### Applications

All the workers with e-ALT should be carefully evaluated and managed for MetS. Particularly, MetS risk assessment must be emphasized for male employees having e-ALT and facing long-term RSW exposures.

### Peer review

The public health expert reviewers agreed the important area of research given the amount of shift work performed around the globe; they also pointed out that, given the large number of individual records and individuals examined, this is an important 5 years retrospective analysis of associated factors.

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ORIGINAL ARTICLE

## Intraluminal *versus* infiltrating gallbladder carcinoma: Clinical presentation, ultrasound and computed tomography

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### Abstract

**AIM:** To compare clinical presentation and ultrasound (US) and computed tomography (CT) sensitivity between intraluminal and infiltrating gallbladder carcinoma (GBCA).

**METHODS:** This retrospective study evaluated 65 cases of GBCA that were categorized morphologically into the intraluminal-GBCA ( $n = 37$ ) and infiltrating-GBCA

( $n = 28$ ) groups. The clinical and laboratory findings, presence of gallstones, gallbladder size, T-staging, nodal status, sensitivity of preoperative US and CT studies, and outcome were compared between the two groups.

**RESULTS:** There were no significant differences between the two groups with respect to female predominance, presence of abdominal pain, serum aminotransferases level, T2-T4 staging, and regional metastatic nodes. Compared with the patients with intraluminal-GBCA, those with infiltrating-GBCA were significantly older ( $65.49 \pm 1.51$  years *vs*  $73.07 \pm 1.90$  years), had a higher frequency of jaundice (3/37 patients *vs* 13/28 patients) and fever (3/37 patients *vs* 10/28 patients), higher alkaline phosphatase ( $119.36 \pm 87.80$  IU/L *vs*  $220.68 \pm 164.84$  IU/L) and total bilirubin ( $1.74 \pm 2.87$  mg/L *vs*  $3.50 \pm 3.51$  mg/L) levels, higher frequency of gallstones (12/37 patients *vs* 22/28 patients), smaller gallbladder size (length,  $7.47 \pm 1.70$  cm *vs*  $6.47 \pm 1.83$  cm; width,  $4.21 \pm 1.43$  cm *vs*  $2.67 \pm 0.93$  cm), and greater proportion of patients with  $< 12$  mo survival (16/37 patients *vs* 18/28 patients). The sensitivity for diagnosing intraluminal-GBCA with and without gallstones was 63.6% and 91.3% by US, and 80% and 100% by CT, respectively. The sensitivity for diagnosing infiltrating-GBCA with and without gallstones was 12.5% and 25% by US, and 71.4% and 75% by CT, respectively.

**CONCLUSION:** In elderly women exhibiting small gallbladder and gallstones on US, especially those with jaundice, fever, high alkaline phosphatase and bilirubin levels, CT may reveal concurrent infiltrating-GBCA.

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**Key words:** Gallbladder neoplasms; Carcinoma; Ultrasound; Computed tomography

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## INTRODUCTION

Gallbladder carcinoma (GBCA) is the sixth most common gastrointestinal malignancy in the United States, following cancer of the colon, pancreas, stomach, liver, and esophagus<sup>[1,2]</sup>. Nevertheless, GBCA is relatively uncommon with an estimated annual incidence of 1 or 2 cases per 100,000 people<sup>[2]</sup>. The nonspecific nebulous symptoms associated with GBCA make the early diagnosis of this uncommon entity a challenge. Approximately 50% of cases of GBCA are found incidentally during cholecystectomy or surgery for other reasons<sup>[3,4]</sup>. GBCA is a highly aggressive neoplasm with a 5-year survival rate of only 5%<sup>[1-3]</sup>. Although most patients present with advanced disease, aggressive radical surgical resection of GBCA has been reported with encouraging results<sup>[3-5]</sup>. Furthermore, survival of GBCA can be improved if early detection of the tumor can be achieved so that curative resection can be performed. Ultrasound (US) is the main initial diagnostic tool for suspected biliary lesions. It may be helpful for detecting GBCA although the infiltrative morphology of some tumors and the presence of gallstones, inflammation and debris may preclude tumor detection<sup>[6-9]</sup>. Computed tomography (CT) has been reported as a comprehensive tool for imaging and staging of GBCA<sup>[7-11]</sup>.

To the best of our knowledge, the influence of different tumor morphology on clinical presentation, as well as on US and CT sensitivity of GBCA detection, have not been well studied. The purpose of this study was to analyze the differences in clinical presentation and sensitivity of preoperative US and CT assessment between GBCA presenting as an intraluminal mass and an infiltrating tumor.

## MATERIALS AND METHODS

### Patients

The computer data bank of our hospital was searched for cases from 1995 to 2008 with the index terms "malignant neoplasm of gallbladder and extrahepatic bile ducts" and "malignant neoplasm of gallbladder" (International Classification of Diseases, 9th Revision, clinical modification code = 156, 156.0). The medical records of all patients with these discharge diagnoses were reviewed. Patients who had surgery and pathologically proven GBCA, as well as preoperative US and/or CT were included in this case series. The patients were categorized into two groups according to the gross tumor morphology and histopathological features of the GBCA. The intraluminal-GBCA group comprised patients with intraluminal tumor growths that protruded into the gallbladder lumen, which grossly appeared as polypoid, fungating or large intraluminal masses. The infiltrating-GBCA group consisted of patients with infiltrating carcinomas that appeared grossly as focal or diffuse areas of wall thickening, or induration in the gallbladder wall. This retrospective study was approved by the institutional review board of our hospital and informed consent was waived due to the retrospective and anonymous nature of the analysis.

### Imaging evaluation

Transabdominal US was performed with various scan-

ners including the Aloka SSD-650 (Aloka Co. Ltd., Tokyo, Japan), and the Acuson 128 XP/10 and Sequoia 512 scanners (Acuson, Mountain View, CA, USA) using a 3.5-MHz sector transducer. Abdominal CT was performed using a ProSpeed scanner (GE Healthcare, Milwaukee, WI, USA) or a Somatom Plus 4 scanner (Volume Zoom; Siemens, Forchheim, Germany) from the liver dome to the pelvic floor. An intravenous bolus of 85-100 mL iodinated contrast material (60%-76% diatrizoate meglumine) was injected at a rate of 2-3 mL/s *via* the antecubital vein before scanning. All images were reconstructed at intervals of 5-10 mm. Starting in 2003, all CT images were interpreted on a high resolution monitor (MGD 521MK II; BarcoView, Kortrijk, Belgium) *via* the picture archiving and communication systems (PACS) system (Centricity Workstation, version 2.0; GE Healthcare). All CT images recorded in hard copy before 2003 were scanned into the PACS system for review, and all measurements were done by applying the tools provided by the Centricity Workstation. A detailed retrospective review of the medical records, US and CT images and pathological findings of each patient was conducted jointly by two radiologists, a surgeon and a pathologist, and any discordance was resolved by consensus.

### Measurements

The clinical features (age, sex and presenting symptoms and signs), laboratory data (serum aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and total bilirubin levels), and pathological findings (gallstones, tumor morphology, gallbladder size, histopathological evaluation of T-staging and nodal metastasis) were recorded. With pathology as the gold standard, the true-positive and false-negative results of preoperative US and CT were obtained, and the sensitivity of the imaging modalities was determined. The outcomes of our patients were also documented.

### Statistical analysis

The data are presented as mean  $\pm$  SD or number (%). The clinical, laboratory and pathological findings were analyzed and compared between the intraluminal-GBCA and infiltrating-GBCA group using the Wilcoxon rank sum test for continuous variables and Fisher's exact test or the  $\chi^2$  test for categorical variables. The sensitivity of preoperative US and CT for the diagnosis of GBCA between the two groups with and without gallstones also was analyzed. Statistical analysis was performed with SYSTAT software (SPSS, version 11.0, Chicago, IL, USA) and  $P < 0.05$  was considered statistically significant.

## RESULTS

### Clinical and laboratory information

Eight of 73 patients with a discharge diagnosis of GBCA were excluded from further study. Three of these patients refused surgery; three were considered poor candidates for surgery because of concurrent cardiac or pulmonary disease or old age; and the other two lacked preoperative US or CT images for review. A total of 65 patients who fulfilled the inclusion criteria were included. According to

the pathological findings, 37 patients (female:male = 23:14; mean age, 65.5 years) formed the intraluminal-GBCA group and the other 28 patients (female:male = 18:10; mean age, 73.1 years) formed the infiltrating-GBCA group.

The clinical and pathological data of both groups are compared in Table 1. Female preponderance was noted in both groups but the patients in the infiltrating-GBCA group were significantly older than those in the intraluminal-GBCA group ( $P = 0.02$ ). The presenting symptoms were, in combination or alone, right upper abdominal or epigastric pain or discomfort in 51 patients, jaundice in 23 and fever in 13. Ten patients were asymptomatic and were diagnosed incidentally. The infiltrating-GBCA group had a higher frequency of jaundice (46.4% *vs* 8.1%,  $P < 0.001$ ) and fever (35.7% *vs* 8.1%,  $P = 0.006$ ) than the intraluminal-GBCA group. There were no significant differences in the serum aspartate and alanine aminotransferase levels between the groups. However, the infiltrating-GBCA group had significantly higher levels of serum alkaline phosphatase (220.68 IU/L *vs* 119.36 IU/L,  $P = 0.03$ ) and total bilirubin (3.50 mg/dL *vs* 1.74 mg/dL,  $P = 0.011$ ) than the intraluminal-GBCA group.

### Pathological findings

The histological types in the intraluminal-GBCA group included 34 adenocarcinomas, one papillary adenocarcinoma, one sarcomatoid adenocarcinoma and one adenosquamous carcinoma. The maximal tumor length ranged from 1.1 to 3.8 cm (mean 2.3 cm). Among 34 intraluminal adenocarcinomas, five patients had no associated gallstones, while histopathological examinations revealed tumor development from the tubular or villotubular polypoid adenomas. The histological types in the infiltrating-GBCA group included 24 adenocarcinomas, two adenosquamous carcinomas, one clear cell carcinoma and one squamous cell carcinoma. The maximal thickness of the tumor ranged from 0.7 to 1.2 cm (mean 0.9 cm). Thirty-four of these 65 patients had gallstones and the number of stones ranged from one to 35. Twenty-six of these patients had > 3 stones with sizes ranging from 0.5 to 2.9 cm, and the other eight patients had 1-3 stones, with sizes varying from 0.3 to 2.7 cm. The infiltrating-GBCA group exhibited a significantly higher frequency of gallstones than the intraluminal group (78.6% *vs* 32.4%,  $P < 0.001$ ). Compared with the intraluminal-GBCA group, the infiltrating-GBCA group exhibited a significantly shorter gallbladder length (7.47 cm *vs* 6.47 cm,  $P = 0.032$ ) and width (4.21 cm *vs* 2.67 cm,  $P = 0.026$ ), and had a significantly lower frequency of T1 tumors (21.7% *vs* 3.6%,  $P = 0.038$ ). On the other hand, there were no significant differences between our two groups with respect to the frequencies of T2, T3, or T4 tumors, and the presence of regional metastatic lymph nodes.

### Preoperative US and CT

Comparison of preoperative US and CT diagnosis is summarized in Table 2. Twelve out of 37 (32.4%) patients in the intraluminal mass group had gallstones and 11 of them underwent US, with a sensitivity of 63.6% for preoperative diagnosis of GBCA, while five underwent CT, with a sensitivity of 80%. Twenty-two out of 28

**Table 1** Clinical and pathological data from 37 patients with GBCA presented as intraluminal mass, and 28 patients with GBCA presented as infiltrating tumor (mean  $\pm$  SD) *n* (%)

	Intraluminal mass	Infiltrating tumor	<i>P</i> value
Age (yr) <sup>2</sup>	65.49 $\pm$ 1.51	73.07 $\pm$ 1.90	0.020 <sup>3</sup>
Sex ratio (female) <sup>1</sup>	23 (62.1)	18 (64.3)	0.861
RUQ or epigastric pain or discomfort <sup>1</sup>	28 (75.7)	23 (82.1)	0.530
Jaundice <sup>1</sup>	3 (8.1)	13 (46.4)	< 0.001 <sup>a</sup>
Fever <sup>1</sup>	3 (8.1)	10 (35.7)	0.006 <sup>a</sup>
Incidental finding	7 (18.9)	3 (8.1)	0.364
Aspartate aminotransferase (IU/L) <sup>2</sup>	89.36 $\pm$ 101.14	74.37 $\pm$ 88.62	0.914
Alanine aminotransferase (IU/L) <sup>2</sup>	113.21 $\pm$ 103.97	78.87 $\pm$ 82.04	0.252
Alkaline phosphatase (IU/L) <sup>2</sup>	119.36 $\pm$ 87.80	220.68 $\pm$ 164.84	0.030 <sup>a</sup>
Total bilirubin (mg/dL) <sup>2</sup>	1.74 $\pm$ 2.87	3.50 $\pm$ 3.51	0.011 <sup>a</sup>
Presence of gallstones <sup>1</sup>	12 (32.4)	22 (78.6)	< 0.001 <sup>a</sup>
Gallbladder size			
Length (cm) <sup>2</sup>	7.47 $\pm$ 1.70	6.47 $\pm$ 1.83	0.032 <sup>a</sup>
Width (cm) <sup>2</sup>	4.21 $\pm$ 1.43	2.67 $\pm$ 0.93	0.026 <sup>a</sup>
T1 tumor <sup>1</sup>	8 (21.7)	1 (3.6)	0.038 <sup>a</sup>
T2 tumor <sup>1</sup>	11 (29.7)	9 (32.1)	0.835
T3 tumor <sup>1</sup>	17 (45.9)	16 (57.1)	0.267
T4 tumor <sup>1</sup>	1 (2.7)	2 (7.2)	0.581
Metastatic lymph nodes <sup>1</sup>	13 (35.1)	15 (53.6)	0.712
Survival < 12 mo <sup>1</sup>	16 (43)	18 (68)	0.017 <sup>a</sup>

<sup>1</sup>Fisher's exact test or  $\chi^2$  test; <sup>2</sup>Wilcoxon rank sum test; <sup>3</sup>Significant at 0.05; RUQ: Right upper quadrant; GBCA: Gallbladder carcinoma.

(78.6%) patients in the infiltrating-GBCA group had gallstones and 16 of them underwent US, with a sensitivity of only 12.5% for preoperative diagnosis of GBCA, while 14 underwent CT, with a sensitivity of 71.4%.

Twenty-five out of 37 (67.64%) patients in the intraluminal mass group had no gallstones and 23 of them underwent US, with a sensitivity of 91.5% for preoperative diagnosis of GBCA, while 20 underwent CT, with a sensitivity of 100%. Only six out of 28 (21.4%) patients in the infiltrating-GBCA group had no gallstones and four of them underwent US, with a sensitivity of only 25% for preoperative diagnosis of GBCA, while four underwent CT, with a sensitivity of 75%.

None of the 22 patients (15 without and seven with gallstones) in the intraluminal-GBCA group with both preoperative US and CT (Figures 1 and 2), had a false-negative result for GBCA. However, among 10 patients (two without and eight with gallstones) in the infiltrating-GBCA group, US and CT results were true-positive for GBCA in only four patients (Figure 3). The US results were false-negative for GBCA in the remaining six patients (five with gallstones), and CT was true-positive in four (Figures 4 and 5).

### Outcomes

In the intraluminal-GBCA group, T1 tumors were found in eight patients, T2 in 11, T3 in 17, and T4 in one. Follow-up ranged from 3 to 115 mo (average 28.8 mo) and 16 patients (43%) survived < 12 mo (average survival, 9 mo). In the infiltrating-GBCA group, a T1 tumor was found in only one patient, T2 in nine, T3 in 16, and T4

**Table 2** Comparison of preoperative US and CT diagnosis of 37 patients with GBCA presented as intraluminal mass, and 28 patients with GBCA presented as infiltrating tumor

	Intraluminal mass	Infiltrating tumor
With gallstones	12	22
Preoperative US	11	16
True positive	7	2
False negative	4	14
Sensitivity (%)	63.6	12.5
Preoperative CT	5	14
True positive	4	10
False negative	1	4
Sensitivity (%)	80.0	71.4
Without gallstones	25	6
Preoperative US	23	4
True positive	21	1
False negative	2	3
Sensitivity (%)	91.3	25.0
Preoperative CT	20	4
True positive	20	3
False negative	0	1
Sensitivity (%)	100	75

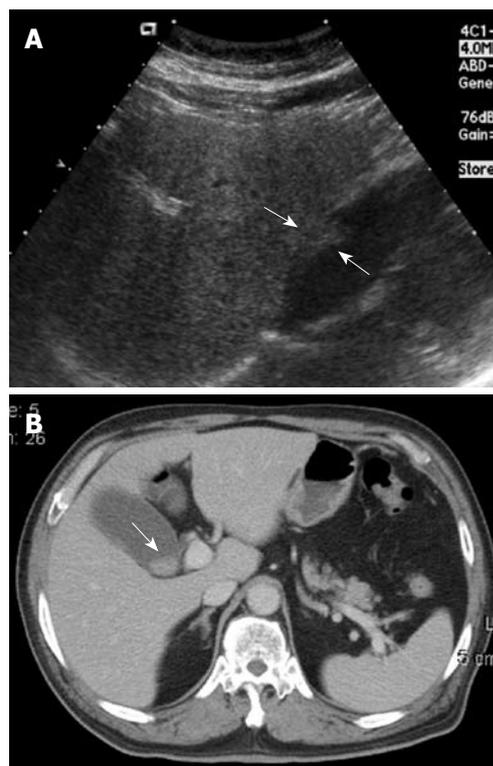
US: Ultrasound; CT: Computed tomography.

in two. Follow-up ranged from 1 to 160 mo (average 15.5 mo) and 19 patients (68%) survived < 12 mo (average survival, 4 mo). The percentage of patients with < 1-year survival in the infiltrating group was significantly higher than in the intraluminal-GBCA group ( $P = 0.017$ ).

## DISCUSSION

GBCA is a relatively uncommon cancer and is found incidentally in < 1% of 750 000 open cholecystectomies performed annually for gallstones in the United States<sup>[12]</sup>. Although GBCA is characterized by early local invasion of the liver and biliary tree, early diagnosis remains difficult because most patients present with nonspecific abdominal symptoms<sup>[2-5]</sup>. In the present study, most patients in both groups harbored T2 and T3 tumors and complained of chronic right upper quadrant or epigastric discomfort at presentation, but our results showed that intraluminal and infiltrating-GBCA might demonstrate different clinical manifestations. In patients with intraluminal-GBCA, T1 tumors were detected more commonly by US or CT incidentally as an asymptomatic gallbladder nodule, while patients with infiltrating GBCA exhibited a higher frequency of jaundice and fever, which mimicked acute cholecystitis. Although both groups showed similar abnormal mean levels of serum aminotransferases, patients with infiltrating-GBCA had significantly higher levels of serum alkaline phosphatase (220.7 IU/L *vs* 119.4 IU/L) and total bilirubin (3.5 mg/dL *vs* 1.7 mg/dL) than patients with intraluminal-GBCA.

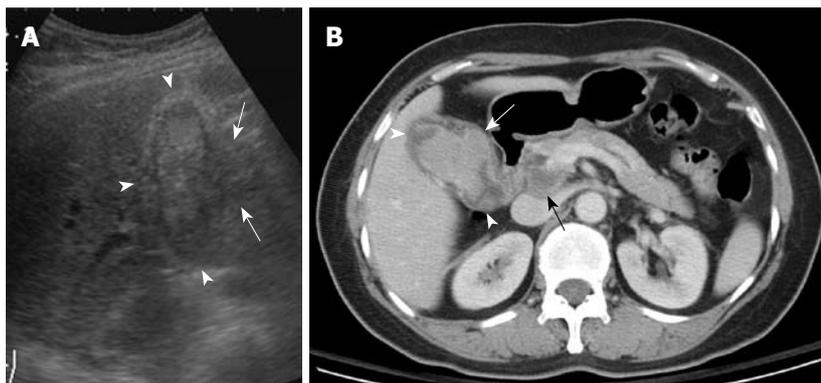
Epidemiological studies have shown that female sex, age, postmenopausal status, ethnic differences and gallstones are the most well-known factors for GBCA development<sup>[9,13-16]</sup>. A high prevalence of GBCA has been reported in New Mexico, Bolivia, Chile, Israel and Northern Japan, and a high prevalence of gallstones in these ethnic groups has also been found<sup>[14,15]</sup>. Chronic



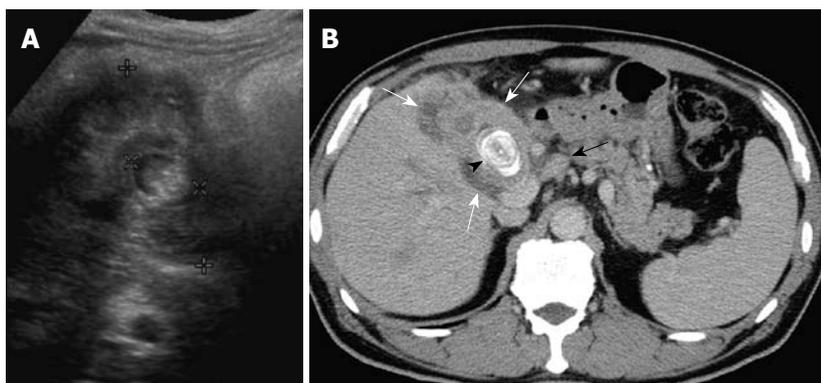
**Figure 1** A 68-year-old woman with intermittent dull right upper quadrant pain for 6 mo. A: Ultrasound (US) showed a sessile polypoid lesion (arrows) with a mildly uneven surface; B: Contrast-enhanced computed tomography (CT) showed an intraluminal polypoid lesion (arrow) with no apparent enlarged regional lymph node.

irritation of the gallbladder caused by gallstones and tumor-promoter activity in the bile in cholelithiasis patients may account for the development of GBCA<sup>[16,17]</sup>. In the present study, gallstones were identified in 34/67 patients (54%), with a lower incidence than in previous reports (64%-98%)<sup>[18-20]</sup>. Of note, our results showed that significantly more patients with infiltrating-GBCA (22/28 patients, 78.6%) harbored coexisting gallstones than patients with intraluminal-GBCA (12/37 patients, 32.6%). Coupled with the fact that patients with infiltrating-GBCA were significantly older (mean age, 73 years) and had significantly smaller gallbladders than those with intraluminal-GBCA, we concur with the postulation that gallstones with a long duration of gallbladder wall irritation may lead to mucosal dysplasia and subsequent neoplasia<sup>[19]</sup>.

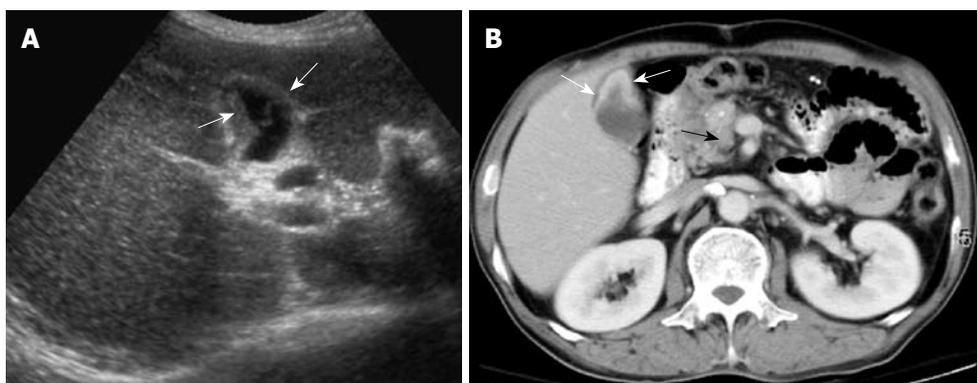
In contrast, more than two-thirds of patients with intraluminal-GBCA had no gallstones, and other predisposing factors of GBCA development including polypoid gallbladder lesions, choledochal cyst, pancreatobiliary duct anomalies, sclerosing cholangitis, porcelain gallbladder, cigarette smoking, typhoid carrier state, certain occupational and environmental carcinogens, and hormonal changes in women have also been described<sup>[9,13-15]</sup>. None of our patients had any history of congenital bile duct or pancreatobiliary lesions, porcelain gallbladder or sclerosing cholangitis, but they did have other less common factors such as chemical or carcinogen exposure. Gallbladder polyps that are > 1 cm, single,



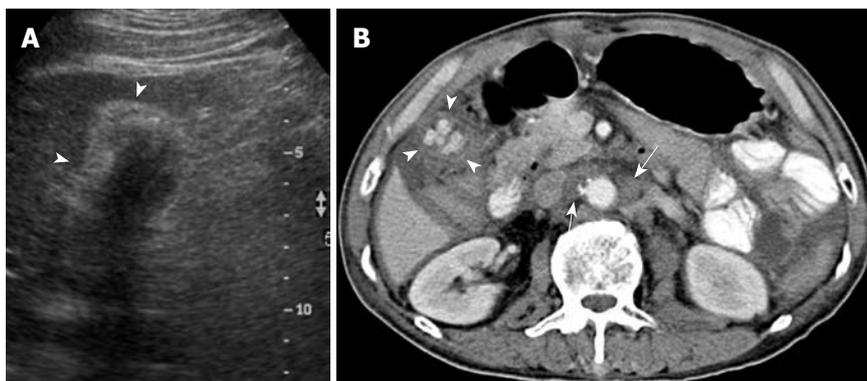
**Figure 2** A 78-year-old woman with right upper abdominal pain and progressive jaundice for 2 mo. A: US showed an intraluminal heteroechoic mass that occupied nearly the whole gallbladder (arrowheads), with focal extraluminal invasion (arrows); B: Contrast-enhanced CT showed a large lobulated mass within the gallbladder (arrowheads), with extracholecystic invasion (white arrow) and hepatoduodenal ligament lymph node metastasis (black arrow).



**Figure 3** A 77-year-old woman with right upper abdominal pain and jaundice for 1 mo. A: US showed a large gallstone (x cursors) encased by the diffusely thickened gallbladder wall, with a heteroechoic appearance (+ cursors); B: Contrast-enhanced CT showed diffuse, uneven wall thickening of the gallbladder (white arrows), with a large laminated gallstone (arrowhead) and hepatoduodenal ligament lymph node metastasis (black arrow).



**Figure 4** A 61-year-old man with dull right upper abdominal pain for 3 wk. A: US showed a relatively small gallbladder with focal wall thickening (arrows), which was misinterpreted initially as inadequate gallbladder distension; B: Contrast-enhanced CT was done because of persistent abdominal discomfort. It revealed relatively poor distension of the gallbladder with prominent enhancement of the focally thickened gallbladder wall (white arrows) and celiac lymph node metastasis (black arrow).



**Figure 5** A 78-year-old man admitted to the emergency room with right upper abdominal pain, fever and jaundice for 1 d. A: US showed a contracted gallbladder with a thickened wall (arrowheads) and multiple gallstones that obliterated underlying details, which mimicked chronic cholecystitis; B: Contrast-enhanced CT showed a relatively small gallbladder with uneven wall thickening (arrowheads), multiple gallstones and pericholecystic inflammatory stranding and fluid suggestive of gallbladder carcinoma (GBCA), with coexistent acute cholecystitis. Note the intercavaoortic and left para-aortic lymph node metastasis (white arrows).

sessile, and echogenic have been associated with a higher risk of malignancy, especially in patients > 60 years

old<sup>[15,21-23]</sup>. The present study showed that the maximal diameter of intraluminal-GBCA varied from 1.1 to 3.8

cm (mean 2.3 cm), while the mean age of the patients was 65.5 years. Of note, five of our patients with intraluminal adenocarcinoma had no gallstones, while histopathological examination revealed tumor development from tubular or villotubular polypoid adenomas. Furthermore, all eight T1 tumors in the intraluminal-GBCA group had diameters between 1.1 and 1.4 cm. Therefore, our results supported prophylactic cholecystectomy of a single intraluminal polyp or mass > 1 cm in diameter<sup>[15]</sup>.

Although many physicians express a relatively nihilistic approach to the treatment of GBCA, several studies have encouraged an aggressive surgical approach that might lead to improved survival<sup>[3-5]</sup>. Early detection and accurate preoperative diagnosis of GBCA is highly beneficial for surgical planning, especially in avoidance of laparoscopic surgery, which may induce recurrent tumors in the abdominal wall along the port track<sup>[24,25]</sup>. Abdominal US and CT are the most common modalities for assessing suspected hepatobiliary malignancies<sup>[6-11]</sup>. US allows the correct diagnosis in 70%-80% of advanced and 20%-30% of early gallbladder carcinoma cases<sup>[6-9]</sup>. The present study disclosed that US was fairly sensitive for detecting intraluminal-GBCA with gallstones (sensitivity 63.6%) and highly sensitive for intraluminal-GBCA without gallstones (sensitivity 91.3%). Intraluminal-GBCA may appear as a hypoechoic polypoid lesion (> 1 cm) that projects into the lumen, a fungating mass, or a large mass that replaces the gallbladder, on US<sup>[6-9]</sup>. In contrast, benign cholesterol or inflammatory polyps are usually multiple, hyperechoic and < 1 cm in diameter<sup>[6,21-23]</sup>. Intraluminal-GBCA with a fungate appearance or large mass can be distinguished by a heterogeneous echotexture and irregular border of the lesions, as well as intralesional or perilesional echogenic foci and acoustic shadowing caused by coexisting gallstones and tumoral calcification<sup>[7-9]</sup>. On the contrary, US has poor sensitivity for detecting infiltrating-GBCA, with and without gallstones (sensitivity of 12.5% and 25.0%, respectively). As demonstrated in the present study, possibly as a result of chronic gallbladder wall irritation by gallstones, and the infiltrating nature of the tumor, which might limit gallbladder expansion, the mean gallbladder size was significantly smaller in patients with infiltrating-GBCA than in those with intraluminal-GBCA. Subtle gallbladder wall thickening that leads to misinterpretation as chronic inflammatory changes, coexistence of acute cholecystitis and obliteration by concurrent gallstones or bile sludge are also factors that might hamper the US detection of infiltrating-GBCA<sup>[6]</sup>.

Similar to US, GBCA may appear as an intraluminal or infiltrating tumor upon CT<sup>[7-9]</sup>. Our study showed that CT was 80% and 100% sensitive, respectively, for detecting intraluminal-GBCA with and without gallstones. In contrast to US, CT offered 71.4% and 75% sensitivity, respectively, for detecting infiltrating-GBCA with and without gallstones. Focal or diffuse gallbladder wall thickening (maximal thickness from 0.7 to 1.2 cm, mean 0.9 cm) is the most common CT finding. This finding is nonspecific and may occur in a variety of conditions, including extracholecystic inflammatory processes such as hepatitis, pancreatitis, pyelonephritis, adenomyomatosis, and chronic

or xanthogranulomatous cholecystitis<sup>[24-27]</sup>. However, CT allows assessment of the condition of the liver, pancreas and kidney, and thus exclusion of secondary involvement of the gallbladder in extracholecystic inflammation is not difficult<sup>[24,25]</sup>. CT also is helpful for revealing adenomyomatosis with proliferation of the subserosal fat and intramural diverticula with small calculi, chronic cholecystitis with a double-layered small gallbladder and a weakly enhancing thin inner layer, and xanthogranulomatous cholecystitis with intramural hypoattenuated nodules in a thickened gallbladder wall<sup>[24-27]</sup>.

Our study was limited by its retrospective nature. First, all patients had surgically proven GBCA, and thus, it was not possible to establish a false-positive rate for US and CT. A prospective study is needed to validate the accuracy, specificity, and true- and false-predictive values of US and CT for intraluminal and infiltrating-GBCA, but due to the infrequency of GBCA, such a large-scale prospective study may be difficult. Second, only a small proportion of our patients underwent percutaneous cholangiography, radionuclide and magnetic resonance imaging studies and hence, the role of these studies in these two morphologically different types of GBCA could not be determined.

In summary, in contrast to intraluminal-GBCA, infiltrating-GBCA is overlooked easily on US. In elderly women with suspected small gallbladder and gallstones upon US, especially those with jaundice, fever, and high serum alkaline phosphatase and total bilirubin levels, CT is helpful in surveying underlying infiltrating-GBCA.

## COMMENTS

### Background

Gallbladder carcinoma (GBCA) is a relatively uncommon cancer with early local invasion of the liver and biliary tree, but early diagnosis remains difficult because most patients present with nonspecific abdominal symptoms. Ultrasound (US) and computed tomography (CT) have been reported as helpful tools for imaging and staging of GBCA. To the best of our knowledge, the influences of different tumor morphology on clinical presentation, as well as on US and CT sensitivity of GBCA detection have not been well studied.

### Research frontiers

US is the main initial diagnostic tool for suspected biliary lesions and may be helpful for detecting GBCA. GBCA may present as an intraluminal mass and an infiltrating tumor, and the infiltrative morphology of some tumors may render US assessment difficult. In addition, the presence of gallstones, inflammation and debris may preclude tumor detection by US. CT has been reported as a comprehensive tool for imaging and staging of GBCA, but its usefulness in patients with different tumor morphology has not been explored.

### Innovations and breakthroughs

The results demonstrated that, compared with patients with intraluminal-GBCA, patients with infiltrating-GBCA were significantly older, and had a significantly higher frequency of jaundice and fever, higher alkaline phosphatase and total bilirubin levels, higher frequency of gallstones, smaller gallbladder size and shorter survival. The sensitivity for diagnosing intraluminal-GBCA with and without gallstones was 63.6% and 91.3%, respectively, by US, and 80% and 100%, respectively, by CT. The sensitivity for diagnosing infiltrating-GBCA with and without gallstones was 12.5% and 25%, respectively, by US, and 71.4% and 75%, respectively, by CT.

### Applications

Intraluminal and infiltrating-GBCA exhibit different clinical presentations and features by US and CT. US and CT are fairly to highly sensitive for detecting intraluminal-GBCA with and without gallstones. Conversely, infiltrating-GBCA, especially when associated with gallstones, is overlooked easily by US. Further CT examination can

be useful in revealing underlying infiltrating-GBCA in elderly women with relatively small gallbladders and gallstones noted by US, particularly those with jaundice, fever, and high serum alkaline phosphatase and total bilirubin levels.

### Terminology

Intraluminal-GBCA denotes tumor growths that protrude into the gallbladder lumen, which appear grossly as polypoid, fungating or large intraluminal masses. Infiltrating-GBCA denotes infiltrating carcinomas that appear grossly as focal or diffuse areas of wall thickening, or induration of the gallbladder wall.

### Peer review

This is an interesting report of gallbladder cancer with differences in clinical presentation. Although it is of clinical significance, the presentation of the paper needs some modification.

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## Inhibitory effect of emodin and Astragalus polysaccharide on the replication of HBV

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**CONCLUSION:** Emodin and APS have a weak but persistent inhibitory effect on HBV replication *in vivo*, which may function as a supplementary modality in the treatment of hepatitis B infection.

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**Key words:** Astragalus polysaccharides; Emodin; Hepatitis; Hepatitis B virus; Lamivudine

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### Abstract

**AIM:** To evaluate the anti-viral effect of emodin plus Astragalus polysaccharide (APS) in hepatitis B virus (HBV) transgenic mice.

**METHODS:** Sixty HBV transgenic mice (HBV TGM) whose weight varied between 18 and 24 g were randomly divided into 3 groups, with 20 mice in each group. Group A was the normal control, where the mice were treated with physiological saline; group B was the positive control where the mice were treated with lamivudine solution (100 mL/kg per day). Group C was the experimental group where the mice were treated with physiological saline containing emodin and APS (57.59 mg/kg per day and 287.95 mg/kg per day, respectively). The mice were treated daily for 3 wk. After 1 wk recovery time, the mice were sacrificed and serum as well as liver tissues were collected for ELISA and histological examination.

**RESULTS:** After 21 d treatment, HBV DNA levels in group B and group C significantly declined when compared with group A ( $P < 0.05$ ). However, a significant increase in HBV DNA content was observed in group B, whereas this phenomenon was not observed in group C. A reduction in the contents of HBsAg, HBeAg and HBcAg in the mice from group B and C was observed when compared with group A.

### INTRODUCTION

Hepatitis B is a global health problem, and affects about 400 million people worldwide, particularly in Asian and Western Pacific countries<sup>[1]</sup>. It is the leading cause of cirrhosis and liver cancer in China<sup>[2]</sup>. The current treatment strategy for hepatitis B is to eradicate replication and infection of hepatitis B virus (HBV) *in vivo* using anti-virus drugs such as interferon and nucleoside analogs such as lamivudine<sup>[3]</sup>. However, because of their side effects, low antiviral potency, and long treatment period, the actual effects of these treatments are neither ideal nor adequate<sup>[4]</sup>.

Chinese herbal medicine has been used for chronic liver diseases for thousands of years in China, and their efficacy has been confirmed by modern biological technology in recent years<sup>[5,6]</sup>. Emodin (1,3,8-tri-hydroxy-6-methylanthraquinone) is an active component of herbal medicine derived from the genera Rheum, Polygonum, Rhamnus and Senna<sup>[7]</sup>. Our previous study showed that emodin could inhibit the replication of HBV in human hepatoma cells *in vitro*<sup>[8]</sup>, suggesting its potential antiviral effect in hepatitis B treatment. Astragalus polysaccharide (APS) is a bioactive chemical in Astragalus membranaceus (AM) which has been used in medicine for centuries in China and is believed to exhibit immune-stimulatory, anti-viral, anti-oxidation and anti-tumor

effects<sup>[9-12]</sup>. A previous study performed *in vivo* has shown that Astragalus-Polygonum Anti-Fibrosis Decoction, in which APS is one of the main components, significantly inhibited liver fibrosis induced by hepatitis B and suppressed hepatic inflammation<sup>[13]</sup>, illustrating that APS may be helpful in eradicating HBV replication *in vivo*.

In this study, we investigated the antiviral effects of emodin and APS in HBV transgenic mice and found that the combination of emodin and APS suppressed HBV replication in HBV transgenic mice. Although lamivudine had a stronger direct inhibitory effect on HBV replication, emodin and APS showed no HBV recurrence 7 d after the last treatment, suggesting a long-lasting effect and may prove to be a potential therapeutic modality for hepatitis B infections.

## MATERIALS AND METHODS

### Reagents

Emodin was purchased from Tianxingjian Bio. Co. (Xi'an, China), and was diluted to 5.80 mg/mL using dH<sub>2</sub>O just before administration. APS was purchased from Hongsheng Biotech. Co. (Xi'an, China) and was diluted to 28.80 mg/mL in H<sub>2</sub>O just before administration. Lamivudine was kindly provided by GlaxoSmithKline (GSK) China Co, and was diluted to 5 mg/mL in dH<sub>2</sub>O.

### Animals and drug administration

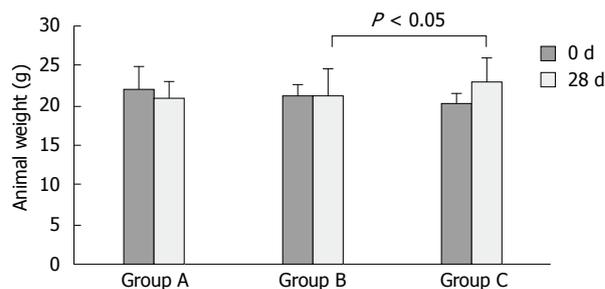
Sixty adult C57\_TgN (HBVadr2.0) SMMU mice weighing between 18-24 g with an equal number of males and females, were provided by the Laboratory Animal Center and Department of Cell Biology of the Second Military Medical University. The mice were randomly divided into three groups with 20 mice in each group. Group A was the normal control, where the mice were administered physiological saline; group B was the positive control where the mice were administered lamivudine solution (100 mL/kg per day). Group C was the experimental group where the mice were administered physiological saline containing emodin and APS (57.59 and 287.95 mg/kg per day, respectively). The mice were treated daily for 3 wk followed by one week of recovery time without any treatment. The mice were then sacrificed. Blood was sampled from the abdominal aorta, centrifuged at 4°C, and plasma was stored at -20°C for assays; liver tissues were collected for histological examination.

### Alteration in animal weight

The weight of each animal was measured just before the first drug administration and after sacrifice. The alteration in animal weight was calculated from these two values.

### Histological examination

Hepatic tissues were fixed using 4% paraformaldehyde phosphate-buffered saline (PBS, pH 7.4) and embedded in paraffin wax. After deparaffinization, 5 μm sections were stained with hematoxylin and eosin (HE), and liver condition was classified according to the standard formulae published by the China Medical Association in 1995.



**Figure 1** Animal weight alteration before and after the experiment. The weight of each mouse was determined before drug administration and before sacrifice after 28 d of experimentation, respectively. One-way ANOVA analysis indicated that there was no significant weight change between day 0 and day 28 in all three groups. However, increased weight was observed in group C compared with group B after 28 d of experimentation. Group A: Normal control; Group B: Lamivudine; Group C: Emodin and Astragalus polysaccharides.

### HBsAg & HBcAg expression in tissue

The surface antigen of the hepatitis-B-virus (HBsAg) and hepatitis B core antigen (HBcAg) were detected using immunohistochemical staining as previously described<sup>[13]</sup>. The stained slides were examined microscopically. Quantification of HBsAg and HBcAg positive cells was performed over several different areas of each section. The percentage of positive cells was evaluated by counting 100 cells (40 ×) in three consecutive tissue sections, and square scores were marked according to the following semi-quantitative criteria: 1: < 25% positive cells; 2: 26%-50% positive cells; 3: 51%-75% positive cells; 4: >75% positive cells. The intensity of the HBsAg staining within positive cells was evaluated, and the intensity scores were marked according to the following semi-quantitative criteria: 1: light yellow; 2: light brown; 3: chocolate brown. The final immunohistochemical reaction score (IRS) was calculated according to the formula IRS = positive staining scores × intensity scores.

### HBsAg and HBeAg quantification

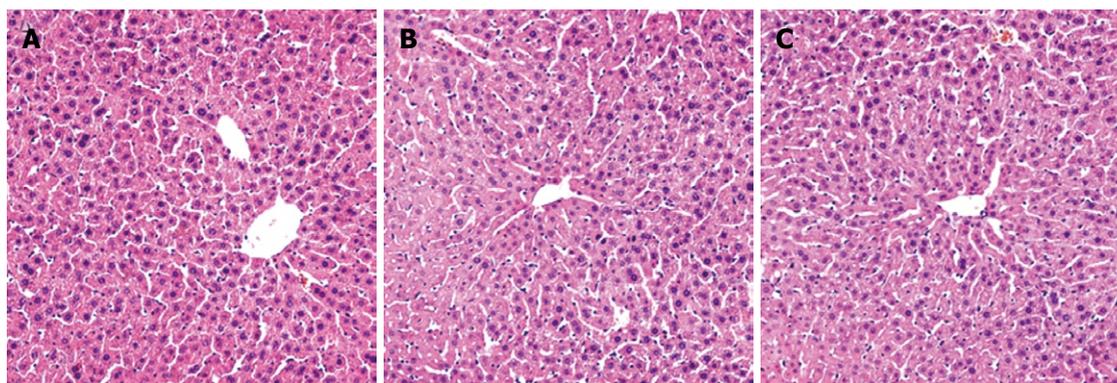
HBsAg and HBeAg (hepatitis B e antigen) levels in serum were determined using Enzyme-Linked Immunosorbent Assay (ELISA) (Huatai Biotechnologies Co, Shanghai, China) according to the manufacturer's instructions as previously described<sup>[14]</sup>.

### HBV DNA concentration determination

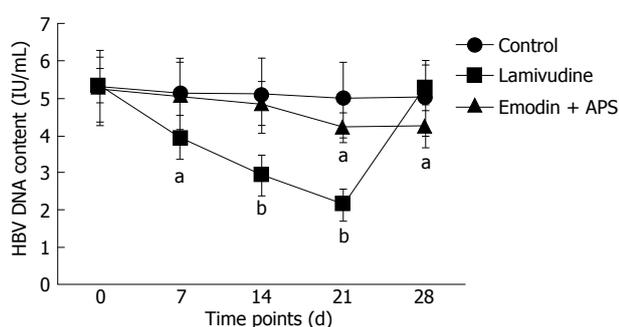
Blood was collected *via* the abdominal aorta after the mice were sacrificed. After centrifugation at 8000 r/min for 5 min, the serum was separated and stored at -20°C. HBV DNA content in serum was determined using real-time PCR (PG Biotech Co, Shenzhen, China) according to the manufacturer's instructions.

### Statistical analysis

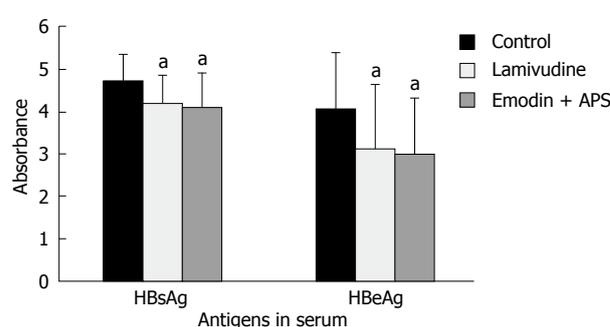
Data are presented as mean ± SE. Data was checked for normal distribution and equal variance. One-way ANOVA test analysis was carried out by SPSS 11.0 software. Differences were considered significant at *P* values of less than 0.05.



**Figure 2** HE staining of liver tissue from different groups (Original magnification,  $\times 200$ ). A: Normal control; B: Lamivudine; C: Emodin and Astragalus polysaccharides.



**Figure 3** Hepatitis B virus (HBV) DNA levels in serum. Mice were sacrificed at day 28 of the experiment. Blood was collected, serum was separated, and HBV DNA content was investigated using real-time PCR. Lower HBV DNA contents were observed in the lamivudine group except on day 28 of the experiment. Emodin + APS decreased serum HBV DNA content at day 21 and 28. Data was presented as mean  $\pm$  SE ( $n = 19$ ). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs the control group at the same time point.



**Figure 4** HBsAg and HBeAg levels in serum. Mice were sacrificed at day 28 of the experiment. Blood was collected and serum was separated. HBsAg and HBeAg levels in serum were determined using ELISA. Lower HBsAg and HBeAg levels were observed in the lamivudine and emodin + APS groups. Data was presented as mean  $\pm$  SE ( $n = 19$ ). <sup>a</sup> $P < 0.05$  vs the control group.

## RESULTS

### Mice weight alteration

One mouse in group A died due to improper blood collection during the experiment at the third week. The other mice in all three groups were healthy during the experiments and their behaviour was normal. There was no significant weight alteration in the mice before or after the experiments. However, an increase in weight was observed in group C when compared with group B after 28 d of experimentation (Figure 1).

### Histological examination

At the end of the experiment, there was no significant difference in the macro-appearance of the livers from mice in the three groups. The livers were pink, soft and their borders were even. Morphologically, the liver structure was intact, with few necrotic hepatocytes, limited inflammatory cell infiltration and fibrous tissue formation (Figure 2).

### HBV DNA content in serum was reduced by lamivudine, emodin and APS

The use of real-time PCR showed that lamivudine significantly decreased serum HBV DNA content after one week of administration, and this inhibitory effect lasted up to 21 d. HBV DNA content increased to

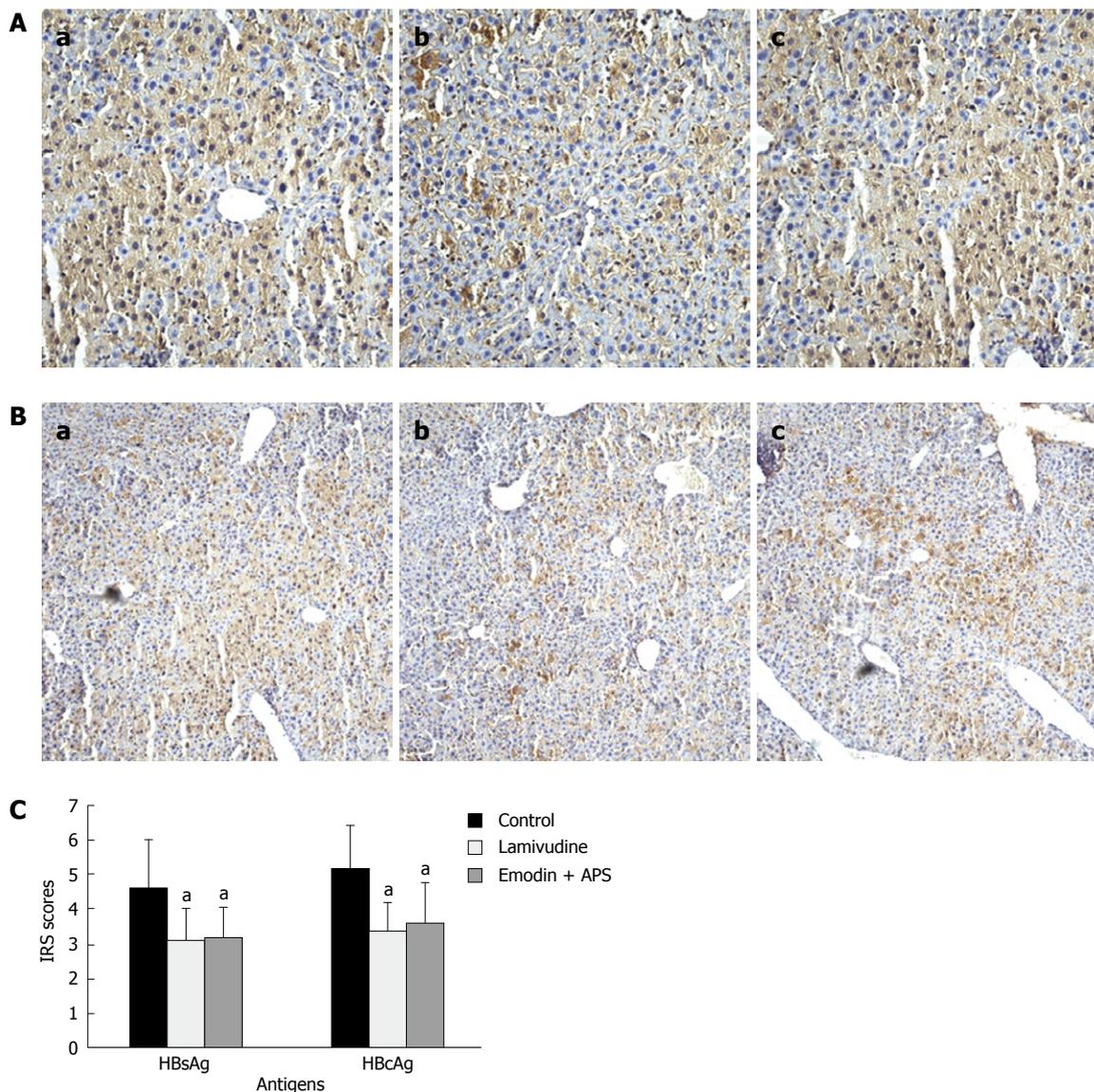
the original level when lamivudine administration was stopped for one week. However, emodin and APS did not decrease serum HBV DNA content after 7 or 14 d of administration (Figure 3), but reduced HBV DNA content after 21 d of administration, and this inhibitory effect lasted up to day 28, one week after administration ceased (Figure 3). There was no alteration in serum HBV DNA content in mice from the control group.

### Lamivudine and emodin + APS reduced HBsAg and HBeAg levels in serum

HBsAg and HBeAg levels in serum were determined using ELISA at day 28 of the experiment and showed that lamivudine and emodin + APS significantly decreased HBsAg and HBeAg levels in serum in the treated groups, compared with the control group ( $P < 0.05$ ). However, there was no significant difference in HBsAg and HBeAg levels between the lamivudine group and the emodin + APS group ( $P > 0.05$ ) (Figure 4).

### Lamivudine and emodin + APS inhibited HBsAg and HBcAg expression in hepatocytes

HBsAg and HBcAg expression in mouse liver tissue was also investigated using immunohistochemistry. HBsAg and HBcAg positive staining was brown or dark brown, and mainly localised within the cytoplasm. Both HBsAg and HBcAg were distributed throughout the liver tissue,



**Figure 5** HBsAg and HBcAg expression in hepatocytes (original magnification,  $\times 200$ ). Positive staining of HBsAg and HBcAg was brown or dark brown, and mainly localised in the cytoplasm. A: HBsAg; B: HBcAg; a: Control group; b: Lamivudine group; c: Emodin and Astragalus polysaccharides group; C: The immunohistochemical reaction score (IRS) was calculated according to the formula IRS = positive staining scores  $\times$  intensity scores. Lower HBsAg and HBcAg expression were observed in the lamivudine and emodin + APS groups. Data was presented as mean  $\pm$  SE. <sup>a</sup> $P < 0.05$  vs the control group.

especially in the portal area and around the central vein area (Figure 5A and B). The positive ratios of HBsAg staining in hepatocytes were 80% (control group), 75% (lamivudine group) and 80% (emodin + APS group), respectively, and the positive ratios of HBcAg staining were 55% (control group), 45% (lamivudine group) and 50% (emodin + APS group), respectively. There was no significant difference in the positive ratios of HBsAg and HBcAg staining in hepatocytes between these three groups ( $P > 0.05$ ). However, lamivudine and emodin + APS decreased the positive staining of HBsAg and HBcAg in hepatocytes after analysis according to the IRS ( $P < 0.05$ ) (Figure 5C).

## DISCUSSION

The transgenic mouse model used in this study was established by integration of HBV genome into mouse genome using a microinjection method, and it has been

confirmed that HBV genes can be stably expressed, replicated and packaged in the mouse<sup>[15]</sup>. The present study showed that administration of lamivudine for 3 wk significantly reduced serum HBV DNA content. However, after ceasing administration for 1 wk, HBV DNA returned to the original level. Similar results were obtained following administration of lamivudine to hepatitis B patients, suggesting that this transgenic mouse model can mimic HBV infection in man.

Using real-time PCR, we found that emodin + APS significantly reduced serum HBV DNA content, although this effect was weaker than that observed with lamivudine. Interestingly, the reduction in serum HBV DNA content in the emodin + APS group lasted longer, compared with the lamivudine group. This suggests that emodin + APS had a weaker but long-lasting antiviral effect on HBV.

HBV antigens including HBsAg, HBcAg and HBeAg are important markers for hepatitis B development in

patients<sup>[16]</sup>. Our results indicate that emodin + APS significantly decreased the expression of these antigens in both serum and liver tissue and lamivudine had a stronger inhibitory effect. The mechanism of this inhibition is still largely unknown. Previous studies have shown that emodin can inhibit several different viruses such as herpes simplex virus (type I and II), parainfluenza virus and Coxsackie virus<sup>[17-20]</sup>. More importantly, our previous study demonstrated that emodin can inhibit HBV replication *in vitro*<sup>[8]</sup>. Therefore it is not surprising that emodin can inhibit HBV *in vivo*. APS has been used for chronic liver disease in China for thousands of years, and is known to increase CD3<sup>+</sup> and CD4<sup>+</sup> T-cells and the ratio of CD4<sup>+</sup>/CD8<sup>+</sup> T-cells in mice, suggesting an immunoregulative effect<sup>[10]</sup>. Therefore, the combination of emodin and APS not only resulted in inhibition of HBV replication, but also regulation of the immunologic system to eradicate HBV *in vivo*. This may explain the weaker and long-lasting effects of emodin + APS.

In conclusion, for the first time, we demonstrated that emodin and APS had a weak but long-lasting inhibitory effect on HBV replication *in vivo*, which may provide a new therapeutic option for hepatitis B infection.

## COMMENTS

### Background

The current treatment strategy for hepatitis B is to eradicate the replication and infection of hepatitis B virus (HBV) using anti-virus drugs such as interferon and nucleoside analogs such as lamivudine. However, the actual effects of these treatments are neither ideal nor adequate. The aim of this study was to evaluate the anti-viral effect of emodin plus Astragalus polysaccharide (APS) in HBV transgenic mice.

### Research frontiers

In this study, the authors found that the combination of emodin and ASP suppressed HBV replication in HBV transgenic mice, which might be a potential alternative treatment method for HBV patients.

### Innovations and breakthroughs

This is the first time that the combination of emodin and ASP was found to suppress HBV replication *in vivo*, which is valuable for anti-HBV drug screening in the future.

### Applications

This study provides valuable experimental evidence for future anti-HBV drug studies. It may provide an alternative strategy for therapeutic intervention in the treatment of patients with HBV.

### Terminology

Emodin (1,3,8-tri-hydroxy-6-methylantraquinone) is an active component of herbal medicine derived from the genera *Rheum*, *Polygonum*, *Rhamnus* and *Senna*. APS is a bioactive chemical in *Astragalus membranaceus* (AM) which has been used in medicine for centuries in China.

### Peer review

This is a well performed experimental study showing for the first time a moderate effect on *in-vivo* HBV replication of two molecules derived from Chinese Traditional Medicine drugs.

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ORIGINAL ARTICLE

## Nicotinamide overload may play a role in the development of type 2 diabetes

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### Abstract

**AIM:** To investigate whether nicotinamide overload plays a role in type 2 diabetes.

**METHODS:** Nicotinamide metabolic patterns of 14

diabetic and 14 non-diabetic subjects were compared using HPLC. Cumulative effects of nicotinamide and *N*<sup>1</sup>-methylnicotinamide on glucose metabolism, plasma H<sub>2</sub>O<sub>2</sub> levels and tissue nicotinamide adenine dinucleotide (NAD) contents of adult Sprague-Dawley rats were observed. The role of human sweat glands and rat skin in nicotinamide metabolism was investigated using sauna and burn injury, respectively.

**RESULTS:** Diabetic subjects had significantly higher plasma *N*<sup>1</sup>-methylnicotinamide levels 5 h after a 100-mg nicotinamide load than the non-diabetic subjects ( $0.89 \pm 0.13 \mu\text{mol/L}$  vs  $0.6 \pm 0.13 \mu\text{mol/L}$ ,  $P < 0.001$ ). Cumulative doses of nicotinamide (2 g/kg) significantly increased rat plasma *N*<sup>1</sup>-methylnicotinamide concentrations associated with severe insulin resistance, which was mimicked by *N*<sup>1</sup>-methylnicotinamide. Moreover, cumulative exposure to *N*<sup>1</sup>-methylnicotinamide (2 g/kg) markedly reduced rat muscle and liver NAD contents and erythrocyte NAD/NADH ratio, and increased plasma H<sub>2</sub>O<sub>2</sub> levels. Decrease in NAD/NADH ratio and increase in H<sub>2</sub>O<sub>2</sub> generation were also observed in human erythrocytes after exposure to *N*<sup>1</sup>-methylnicotinamide *in vitro*. Sweating eliminated excessive nicotinamide (5.3-fold increase in sweat nicotinamide concentration 1 h after a 100-mg nicotinamide load). Skin damage or aldehyde oxidase inhibition with tamoxifen or olanzapine, both being notorious for impairing glucose tolerance, delayed *N*<sup>1</sup>-methylnicotinamide clearance.

**CONCLUSION:** These findings suggest that nicotinamide overload, which induced an increase in plasma *N*<sup>1</sup>-methylnicotinamide, associated with oxidative stress and insulin resistance, plays a role in type 2 diabetes.

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**Key words:** Type 2 diabetes; Nicotinamide; *N*<sup>1</sup>-methylnicotinamide; Insulin resistance; Oxidative stress; Liver; Sweat glands

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## INTRODUCTION

Type 2 diabetes, characterized by insulin resistance and oxidative stress<sup>[1,2]</sup>, has reached epidemic proportions not only among adults, but also among adolescents in the past few decades, which has led to the hypothesis that type 2 diabetes is a result of gene-environment (diet) interactions, because the human genome has not changed markedly in such a short time<sup>[3-5]</sup>. However, what the environmental/dietary risk factors are and how they function remain unclear.

Nicotinamide, the amide of nicotinic acid, is the precursor for the coenzymes  $\beta$ -nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), which function in many enzyme-catalyzed oxidation and reduction reactions. Therefore, nicotinamide homeostasis is vitally important for the body<sup>[6]</sup>. In humans, excess nicotinamide is methylated, oxidized or hydroxylated to  $N^1$ -methylnicotinamide, nicotinamide- $N$ -oxide or 6-hydroxynicotinamide, respectively, and then  $N^1$ -methylnicotinamide is further oxidized to the pyridones  $N^1$ -methyl-2-pyridone-5-carboxamide (2Py) and  $N^1$ -methyl-4-pyridone-3-carboxamide by aldehyde oxidase (AOX; EC 1.2.3.1). The major nicotinamide metabolites in human urine are 2Py and  $N^1$ -methylnicotinamide<sup>[7]</sup>. Although the nicotinamide metabolism pathway is well understood, however, the influence of the catabolic efficiency of excess nicotinamide on the body is not fully understood.

Since pellagra was found to be related to vitamin B<sub>3</sub> (niacin) deficiency in 1930s, more attention has been paid to the prevention of niacin deficiency. As a result, nicotinamide is used extensively as a food additive without having undergone full formal safety evaluation<sup>[8]</sup>, and the chronic effects of nicotinamide overuse are far from understood<sup>[9]</sup>. Nicotinic acid and nicotinamide frequently are reported to impair glucose tolerance and induce insulin resistance<sup>[10-12]</sup>. Recent evidence suggests that  $N^1$ -methylnicotinamide is involved in Parkinson's disease<sup>[9]</sup>. More importantly, the abrupt increase in prevalence of type 2 diabetes in the United States in the latter half of the 20th century<sup>[13]</sup> occurred roughly in parallel with the increase in per capita niacin content<sup>[14]</sup>. A similar situation occurred in China, in which food enrichment with niacin began in 1980s, followed by a sharp increase in the incidence of diabetes from 1% in 1980 to 5.5% in 2001<sup>[15]</sup>. However, whether type 2 diabetes pathogenesis involves nicotinamide overload is undetermined.

The present study aimed to compare nicotinamide metabolic patterns between diabetic and non-diabetic

subjects, and to investigate the relationship between nicotinamide overload and insulin resistance. We found that diabetic subjects exhibited slow  $N^1$ -methylnicotinamide clearance, and that  $N^1$ -methylnicotinamide could trigger oxidative stress and insulin resistance, which suggested that the association of nicotinamide overload with relatively slow  $N^1$ -methylnicotinamide detoxification and excretion might be at least partially responsible for the development of type 2 diabetes.

## MATERIALS AND METHODS

### Chemicals

Nicotinamide was purchased from Sigma (St. Louis, MO, USA). Nicotinamide tablets (50 mg/tablet) were purchased from Lisheng Pharma (Tianjin, China).  $N^1$ -methylnicotinamide was purchased from Takeda Chemical Industries (Osaka, Japan). Tamoxifen was from Kunshan Sanyou Pharmaceutical Adjuvant Factory (Kunshan, China). Olanzapine was obtained from Beijing Nordhuns Chemical Technology (Beijing, China).  $N^1$ -ethylnicotinamide and 2-Py were synthesized according to the methods described respectively by Hirayama *et al.*<sup>[16]</sup> and Holman *et al.*<sup>[17]</sup>.

### Nicotinamide load test in humans

This study was approved by the relevant ethics committee, and all the Chinese participants gave informed consent. Fourteen type 2 diabetic patients from eight families with a positive history (eight men and six women, mean age,  $55.3 \pm 10.2$ ; range, 36-75 years) and 14 age- and sex-matched healthy volunteers without a family history (mean age,  $53.8 \pm 10.8$ ; range, 39-75 years) participated in this study. Type 2 diabetes was defined as a fasting glucose level  $\geq 7.0$  mmol/L or current receipt of hypoglycemic medication. All subjects refrained from drugs, alcohol and caffeinated products for at least 12 h before the study. After an overnight fast, urine was collected and quantitated 1 h before, and 2, 4 and 5 h after loading with 100 mg nicotinamide. Venous blood was collected into sodium citrate tubes before and 5 h after nicotinamide loading, and separated by centrifugation (1500 g, 10 min). Aliquots of each plasma and urine sample were placed directly in liquid nitrogen and then transferred to  $-80^\circ\text{C}$  and  $-20^\circ\text{C}$ , respectively.

### Cumulative nicotinamide and $N^1$ -methylnicotinamide experiments

All animal experiments were conducted in accordance with institutional guidelines. Male Sprague-Dawley rats (180-220 g) were fed a standard rat chow. In nicotinamide experiment, rats were divided randomly into three groups ( $n = 6$  each) and fasted for 14 h before the experiment. In the two nicotinamide-treated groups, nicotinamide (100 or 400 mg/kg) was administered (intraperitoneally, ip) and repeated every 2 h for five doses. Glucose tolerance test was performed by injection of glucose (2 g/kg, ip) 2 h after the final nicotinamide injection. Blood glucose was measured 1 h after glucose injection. Blood was collected

by eye bleed into EDTA tubes under urethane anesthesia (1.5 g/kg, ip) 2 h after glucose administration. Plasma was separated by centrifugation (1500 g, 10 min). After plasma collection, the buffy coat and top fifth reticulocyte-rich layer of erythrocytes were discarded. Aliquots of plasma and erythrocytes, and harvested samples of liver and gastrocnemius muscle were plunged directly into liquid nitrogen and subsequently stored at -80°C until assay. The same protocol was used in the  $N^1$ -methylnicotinamide experiment, except that the two treated groups repeatedly received 100 or 400 mg/kg  $N^1$ -methylnicotinamide per injection and a total dosage of 0.5 or 2 g, respectively (each group,  $n = 6$ ).

### **AOX inhibition**

Rats in inhibitor-treated groups received subcutaneous tamoxifen (50 mg/kg) or olanzapine (20 mg/kg) twice daily for 4 d, and rats in each control group received vehicle only (each group,  $n = 6$ ). After an overnight fast, all the rats received nicotinamide (100 mg/kg, ip). Plasma samples were collected 5 h after nicotinamide administration as described above.

### **Chronic AOX inhibition**

Rats were divided initially into two groups: tamoxifen-treated (20 mg/kg per day, subcutaneously,  $n = 24$ ) and vehicle-treated (control,  $n = 8$ ). Eight control and eight tamoxifen-treated rats were sacrificed at the end of 7 wk. Liver samples were harvested and stored at -80°C for western blotting. The remainder of tamoxifen-treated rats was then divided into two groups treated with tamoxifen (20 mg/kg per day) with or without  $N^1$ -methylnicotinamide (100 mg/kg per day, subcutaneously), respectively. Two weeks after the treatment, glucose tolerance test was performed by injection of glucose (2 g/kg, ip) after an overnight fast. Blood glucose was measured before (fasting) and 1 h after glucose injection. Samples of plasma, liver and gastrocnemius muscle were harvested under urethane anesthesia (1.5 g/kg, ip) 2 h after glucose injection.

### **Thermal injury**

Rats in the burn group ( $n = 11$ ) were given a 40% total body surface area, full-thickness scald burn under ether anesthesia by immersion of the back in 95°C water for 15 s, as previously described<sup>[18]</sup>. Sham rats ( $n = 7$ ) were subjected to an identical procedure, except that they were immersed in 25°C water. All rats received glucose (2 g/kg, ip) 24 h after burning, with an overnight fast. Blood glucose was measured before (fasting) and 1 h after glucose dosing. Samples of plasma, liver and gastrocnemius muscle were harvested 2 h after glucose injection.

### **Sweat collection**

Five healthy young male volunteers aged 20-24 years participated in this study. After an overnight fast, whole body sweat was collected before and 1, 2 and 3 h after a single oral dose of 100 mg nicotinamide, by having the volunteers stay in a plastic bag during sauna treatment (80°C, 15 min), with stringent precautions to minimize

evaporative loss. Aliquots of sweat were placed in liquid nitrogen, and transferred to -80°C until analysis.

### **Assays of glucose, insulin and glycogen**

Blood glucose was measured using a glucometer (OneTouch Ultra; LifeScan Inc.). Plasma insulin was measured by radioimmunoassay using commercial kits (Beijing North Institute of Biological Technology, China). Muscle and liver glycogen contents were determined with Glycogen Assay Kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

### **H<sub>2</sub>O<sub>2</sub> assay**

Blood from healthy adult male volunteers was collected in EDTA-containing tubes and centrifuged (1500 g, 10 min). The plasma, buffy coat, and top fifth reticulocyte-rich layer of erythrocytes were discarded, and the remaining cells were washed three times in isotonic saline, and centrifuged (1500 g, 10 min). After the supernatant was discarded, the packed erythrocytes were transferred to Earle's Balanced Salt Solution presaturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> to make an erythrocyte suspension (20 μL packed erythrocytes/mL). Two hundred microliters of erythrocyte suspension was added to each well of a 96-well plate in the absence or presence of  $N^1$ -methylnicotinamide or 2Py at concentrations of 10 nmol/L to 100 μmol/L, and incubated for 3 h at 37°C. H<sub>2</sub>O<sub>2</sub> concentrations in the supernatant of cell cultures and in rat plasma were measured using an H<sub>2</sub>O<sub>2</sub> Assay Kit (Beyotime Biotechnology, Jiangsu, China).

### **NAD/NADH assay**

Human erythrocyte suspension (20 μL packed erythrocytes/mL Earle's Balanced Salt Solution) was incubated in 1.5-mL Eppendorf tubes with a 1.5-mm hole in the cover, for 4 h at 37°C in the presence or absence of  $N^1$ -methylnicotinamide (10 μmol/L). The tubes were centrifuged (1500 g, 15 min, 4°C) and the supernatant was discarded. Four hundred microliters of BioVision NAD/NADH Extraction Buffer (Mountain View, CA, USA) was added to each tube for 5 min to lyse the cells. The lysates were ultrafiltered using BioVision 10-kD cut-off filters (14000 g, 30 min, 4°C). Assays were performed using the NAD<sup>+</sup>/NADH Quantification Kits according to the manufacturer's instructions (BioVision). For rat tissue NAD/NADH assay, 20 mg frozen liver, 20 mg frozen muscle or 20 μL packed erythrocytes were homogenized in 400 μL BioVision NAD/NADH Extraction Buffer. The homogenate was ultrafiltered using BioVision 10-kD cut-off filters (14000 g, 30 min, 4°C). The assay was conducted following the manufacturer's instructions.

### **Western blotting**

Western blotting analysis of AOX was performed according to standard protocols. Briefly, 100 μg rat liver proteins were separated on 5%-8% SDS polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked in PBS that contained 0.1% Tween-20 and 5% non-fat dry milk for

30 min at room temperature, and incubated with antibody to AOX (1:250; BD Transduction Laboratories, Lexington, KY, USA) overnight at 4°C. Then, the membranes were washed by PBS-Tween followed by 1 h incubation at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and detected using the enhanced chemiluminescence (ECL) (Amersham Life Science).

#### Determination of *N*<sup>1</sup>-methylnicotinamide, nicotinamide and 2Py

*N*<sup>1</sup>-methylnicotinamide, nicotinamide and 2Py were analyzed using an HPLC system that consisted of an LC-9A pump (Shimadzu, Kyoto, Japan), a Rheodyne 7725i sample injector with a 20- $\mu$ L sample loop (Rheodyne LLC, Rohnert Park, CA, USA), a Hypersil ODS C18 column (Thermo, Bellefonte, PA, USA), a Waters 470 fluorescence detector (Milford, MA, USA) for *N*<sup>1</sup>-methylnicotinamide measurements, and a UV3000 detector (Thermo Separations Products, Fremont, CA, USA) for 2Py detection. *N*<sup>1</sup>-methylnicotinamide concentration was analyzed by detecting its fluorescent 1,6-naphthyrindine derivatives according to the method of Musfeld *et al.*<sup>[19]</sup>, using 366 nm excitation and 418 nm emission wavelengths, with the mobile phase (10 mmol/L sodium heptanesulfonate, 50 mmol/L triethylamine and 22% acetonitrile in water, pH 3.2 with 85% H<sub>3</sub>PO<sub>4</sub>) at a flow rate of 1 mL/min. Nicotinamide was quantitated by the same procedure after quantitative conversion of nicotinamide to *N*<sup>1</sup>-methylnicotinamide using iodomethane, according to the method of Clark<sup>[20]</sup>. For analyzing urinary 2Py, 50  $\mu$ L 85% H<sub>3</sub>PO<sub>4</sub> and 0.45 mL water were added to each urine sample (0.5 mL). After brief vortex mixing, the samples were centrifuged (12000 *g*, 10 min). The supernatant was filtered through a 0.45- $\mu$ m filter for HPLC analysis. Standard solutions were prepared for calibration, which contained 0, 0.1, 1, 10 and 100  $\mu$ g/mL of pure 2Py in normal urine. The mobile phase (5% methanol, 10 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 10 mmol/L triethylamine, pH 3.0 adjusted with 85% H<sub>3</sub>PO<sub>4</sub>) was delivered at 1 mL/min. UV detection was performed at 260 nm.

#### Statistical analysis

The data are presented as means  $\pm$  SD. Statistical differences in the data were evaluated by paired or unpaired Student's *t* test, or ANOVA as appropriate, and were considered significant at *P* < 0.05.

## RESULTS

#### Slow *N*<sup>1</sup>-methylnicotinamide clearance is a prominent feature of type 2 diabetes

The 5-h total urinary 2Py excretion after 100 mg nicotinamide load in the diabetic group was significantly less than that in the non-diabetic group (20.9  $\pm$  4.5 mg *vs* 24.3  $\pm$  4.1 mg, *P* < 0.05, Figure 1A and C), but urinary *N*<sup>1</sup>-methylnicotinamide excretion increased in the diabetic group (Figure 1B and D). The plasma *N*<sup>1</sup>-methylnicotinamide level 5 h after nicotinamide load was significantly higher in the diabetic than non-diabetic

group (Figure 2). It should be noted that there were no statistical differences in the basal urinary excretions of 2Py and *N*<sup>1</sup>-methylnicotinamide and the basal levels of plasma *N*<sup>1</sup>-methylnicotinamide between the two groups (Figures 1 and 2). These results suggest that slow plasma *N*<sup>1</sup>-methylnicotinamide clearance, which can be revealed by nicotinamide load test, may be a potential biomarker of type 2 diabetes.

#### High plasma *N*<sup>1</sup>-methylnicotinamide induces insulin resistance

We examined the effect of nicotinamide overload on rat glucose metabolism. Rats treated with cumulative doses of nicotinamide (2 g/kg) exhibited significantly higher levels of blood glucose and plasma insulin, but significantly lower muscle glycogen content than control rats after glucose load (Figure 3A). Another notable change after nicotinamide administration was that there was a marked increase in plasma *N*<sup>1</sup>-methylnicotinamide (Figure 3A), so we examined the effects of *N*<sup>1</sup>-methylnicotinamide. Cumulative doses of *N*<sup>1</sup>-methylnicotinamide (2 g/kg) had comparable effects to nicotinamide (Figure 3B), which suggested that the effects of nicotinamide overload might have been mediated by *N*<sup>1</sup>-methylnicotinamide.

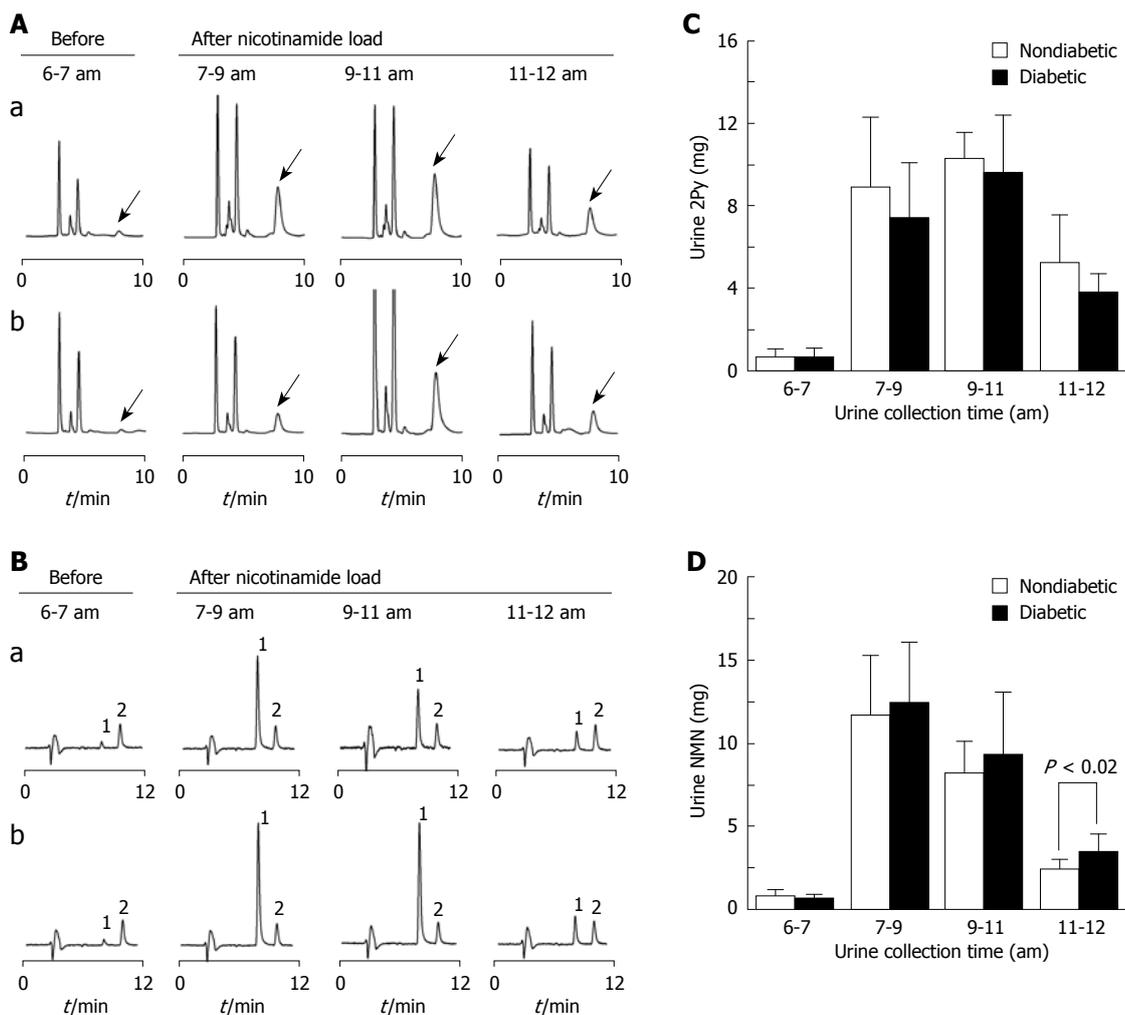
#### *N*<sup>1</sup>-methylnicotinamide triggers oxidative stress

Type 2 diabetes is associated with oxidative stress<sup>[1,2]</sup>. We therefore examined whether nicotinamide overload and high *N*<sup>1</sup>-methylnicotinamide levels were implicated in oxidative stress. The results showed that cumulative effects of nicotinamide (2 g/kg) or *N*<sup>1</sup>-methylnicotinamide (2 g/kg) led to a significant increase in rat plasma levels of H<sub>2</sub>O<sub>2</sub> (Figure 4A and B), a major reactive oxygen species (ROS) and common indicator of oxidative stress<sup>[2]</sup>. Such an enhancing effect was also observed in human erythrocytes *in vitro* at physiological concentrations of *N*<sup>1</sup>-methylnicotinamide (Figure 4C), whereas 2Py, the end product of nicotinamide, did not have an enhancing effect at the observed concentrations (10 nmol/L to 100  $\mu$ mol/L) (data not shown). These results indicate that high plasma *N*<sup>1</sup>-methylnicotinamide may induce systemic oxidative stress.

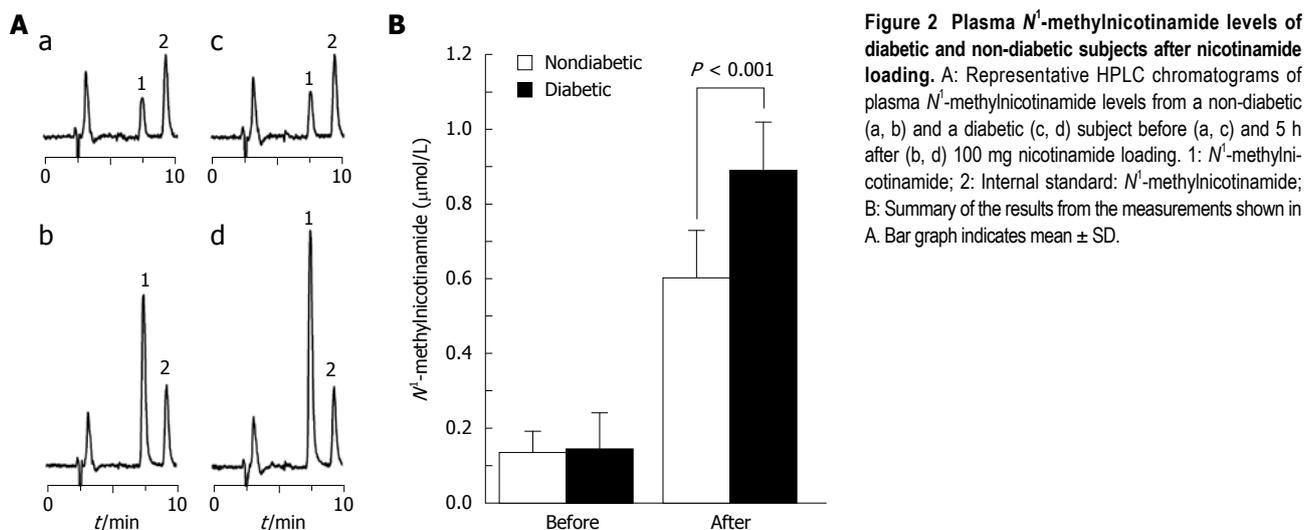
Increasing evidence has indicated that type 2 diabetes has abnormalities in the NAD<sup>+</sup>/NADH redox couple<sup>[21]</sup>. We therefore investigated whether *N*<sup>1</sup>-methylnicotinamide affected tissue NAD levels. Cumulative exposure to *N*<sup>1</sup>-methylnicotinamide significantly reduced rat muscle and liver NAD (NAD<sup>+</sup> + NADH) contents (Figure 4D and E). Notably, the erythrocytes of rats treated with cumulative doses of *N*<sup>1</sup>-methylnicotinamide (2 g/kg) exhibited a significant increase in NADH and decrease in NAD/NADH ratio (Figure 4F). A similar effect was observed in human erythrocytes *in vitro* (Figure 4G). These results suggest that *N*<sup>1</sup>-methylnicotinamide-induced oxidative stress may originate from imbalance in the NAD<sup>+</sup>/NADH redox couple.

#### AOX inhibition reduces *N*<sup>1</sup>-methylnicotinamide clearance

AOX is responsible for conversion of *N*<sup>1</sup>-methylni-



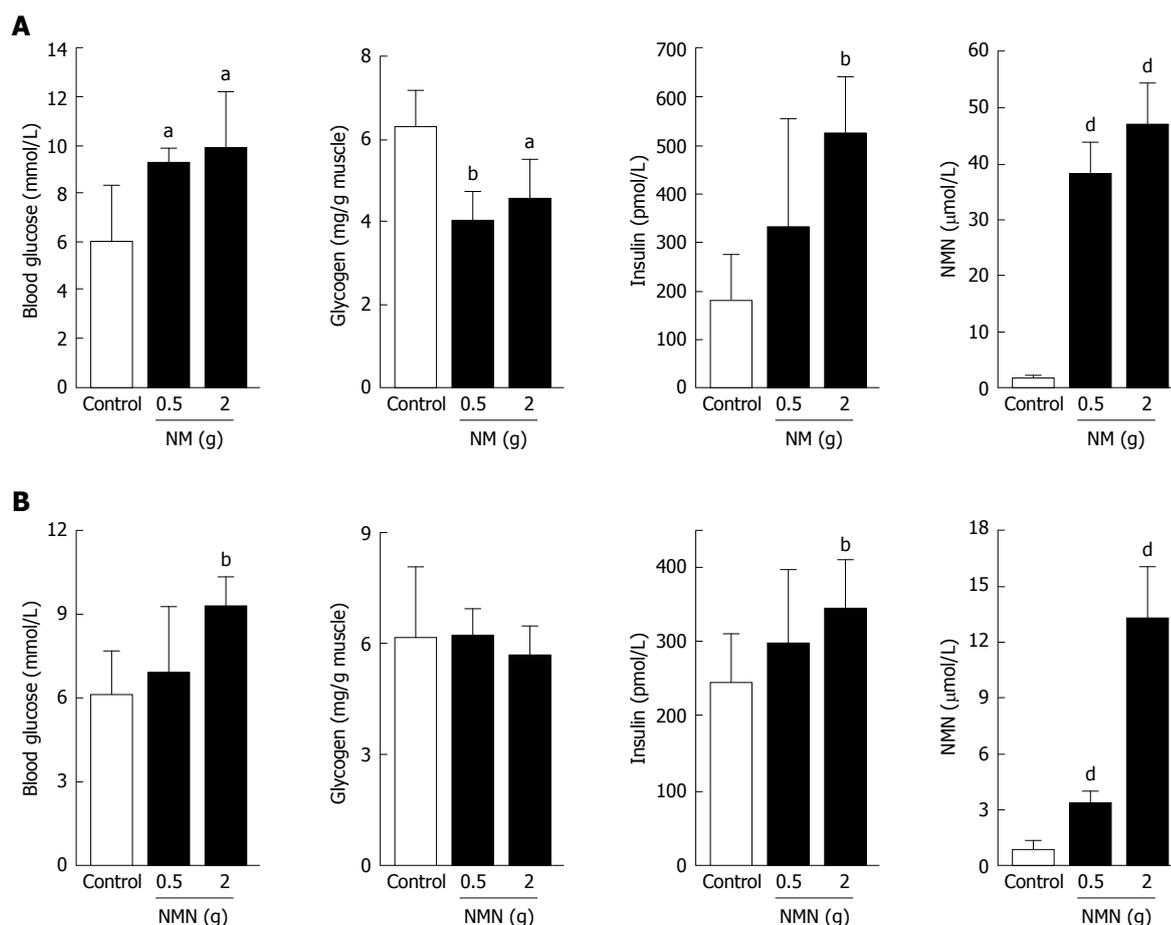
**Figure 1** Urinary excretion patterns of 2Py and *N*<sup>1</sup>-methylnicotinamide in diabetic and non-diabetic subjects. A and B: Representative HPLC chromatograms of urinary excretions of 2Py (indicated by arrow) and *N*<sup>1</sup>-methylnicotinamide (NMN) of a non-diabetic (Aa and Ba) and a diabetic (Ab and Bb) subject, before and after 100 mg nicotinamide loading at 7:00 am. Urine samples taken at given time were normalized to equal volumes before HPLC analysis; C and D: Summaries of the results from the measurements shown in A and B, respectively. Bar graphs indicate mean ± SD.



**Figure 2** Plasma *N*<sup>1</sup>-methylnicotinamide levels of diabetic and non-diabetic subjects after nicotinamide loading. A: Representative HPLC chromatograms of plasma *N*<sup>1</sup>-methylnicotinamide levels from a non-diabetic (a, b) and a diabetic (c, d) subject before (a, c) and 5 h after (b, d) 100 mg nicotinamide loading. 1: *N*<sup>1</sup>-methylnicotinamide; 2: Internal standard: *N*<sup>1</sup>-methylnicotinamide; B: Summary of the results from the measurements shown in A. Bar graph indicates mean ± SD.

cotinamide-to-2Py<sup>[22]</sup>. We examined changes in *N*<sup>1</sup>-methylnicotinamide clearance after rat AOX inhibition by the putative inhibitors tamoxifen and olanzapine<sup>[23]</sup>, both of which are known to impair glucose tolerance<sup>[24,25]</sup>.

The rats treated with tamoxifen (100 mg/kg per day) or olanzapine (40 mg/kg per day) for 4 d exhibited significantly higher plasma *N*<sup>1</sup>-methylnicotinamide levels than control rats 5 h after 100 mg/kg nicotinamide



**Figure 3** Effects of nicotinamide and *N*<sup>1</sup>-methylnicotinamide on glucose metabolism of rats. A: Changes in blood glucose, muscle glycogen, plasma insulin and plasma *N*<sup>1</sup>-methylnicotinamide in rats treated with or without cumulative nicotinamide (0.5 or 2 g/kg) after glucose loading; B: Comparable effects of cumulative *N*<sup>1</sup>-methylnicotinamide (0.5 or 2 g/kg). NM: Nicotinamide; NMN: *N*<sup>1</sup>-methylnicotinamide. <sup>a</sup>*P* < 0.05 vs control, <sup>b</sup>*P* < 0.01 vs control, <sup>d</sup>*P* < 0.001 vs control. Bar graphs show mean  $\pm$  SD.

loading (Figure 5A and B). Moreover, chronic tamoxifen treatment for 7 wk significantly reduced rat liver AOX protein expression (*P* < 0.05, Figure 5C). To further explore the role of nicotinamide overload in insulin resistance, we used tamoxifen plus *N*<sup>1</sup>-methylnicotinamide to mimic the conditions of relatively low AOX activity and excess nicotinamide intake. Rats treated with tamoxifen plus *N*<sup>1</sup>-methylnicotinamide exhibited significantly higher blood glucose and much lower liver glycogen content than those treated with tamoxifen alone after glucose loading (Figure 5D).

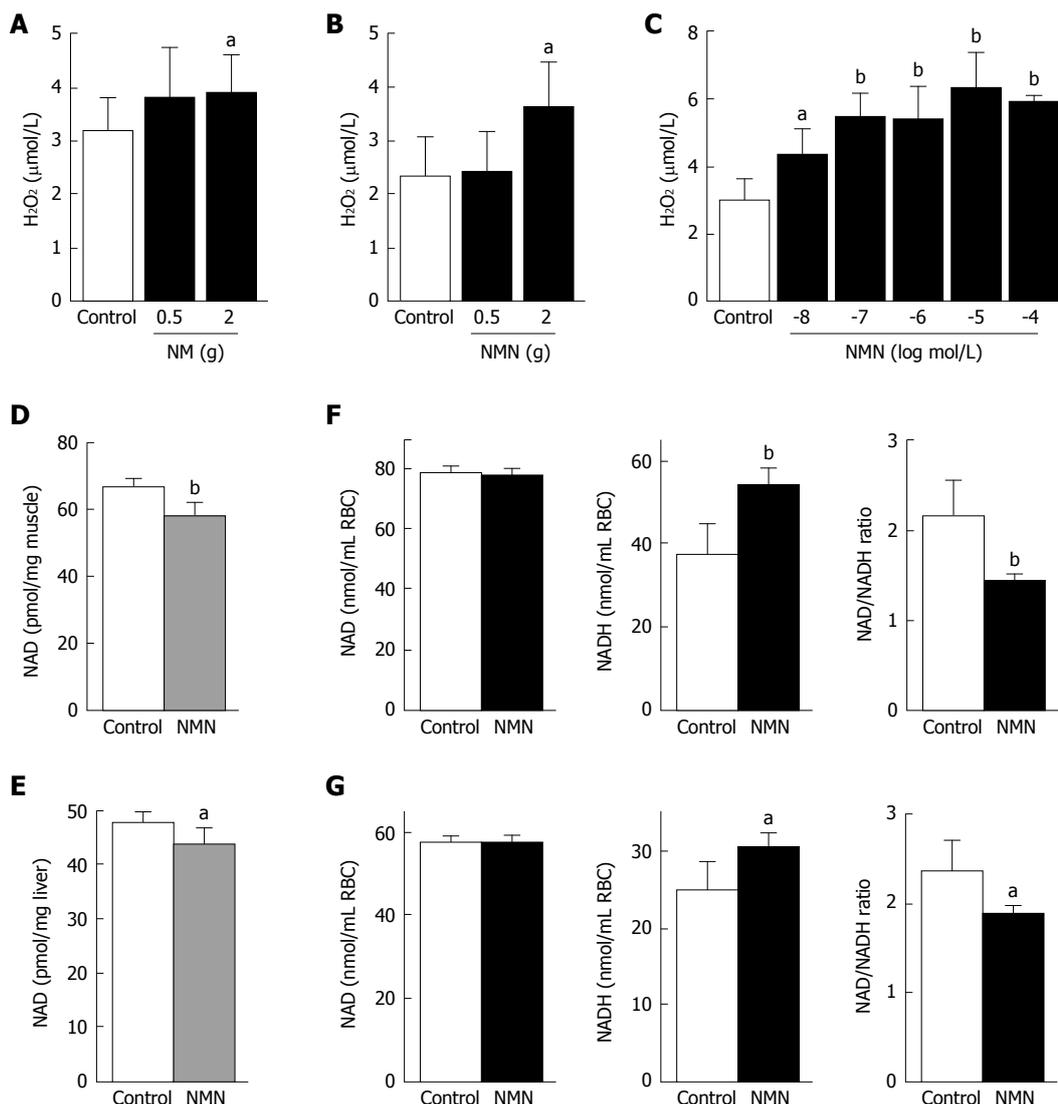
#### Skin involvement in *N*<sup>1</sup>-methylnicotinamide-mediated insulin resistance

If *N*<sup>1</sup>-methylnicotinamide is indeed involved in insulin resistance, then any factors related to *N*<sup>1</sup>-methylnicotinamide detoxification and excretion may affect insulin sensitivity. Of the factors, skin may be of particular significance because it takes part in *N*<sup>1</sup>-methylnicotinamide detoxification and excretion, respectively, through skin AOX<sup>[26,27]</sup> and sweat glands<sup>[28]</sup>. We thus explored the role of skin by analyzing human sweat excretion of nicotinamide and *N*<sup>1</sup>-methylnicotinamide after nicotinamide loading, or by assessing changes in plasma *N*<sup>1</sup>-methylnicotinamide

clearance of rats with severe skin damage. Importantly, we found that nicotinamide concentrations in the sweat 1 h after 100 mg nicotinamide loading was 5.3-fold higher than that in fasting sweat, whereas *N*<sup>1</sup>-methylnicotinamide concentrations were not significantly altered under such conditions (Figure 6A and B). This indicates that excess nicotinamide, without needing any conversion, is expelled through sweat. Moreover, this study found that rats with severe skin damage had a significant elevation in plasma *N*<sup>1</sup>-methylnicotinamide associated with high blood glucose and plasma insulin levels, but lower muscle glycogen content after glucose loading (Figure 6C).

## DISCUSSION

The main findings of this study were that: (1) nicotinamide overload elevates plasma levels of *N*<sup>1</sup>-methylnicotinamide associated with oxidative stress and insulin resistance; (2) the skin plays an important role in expelling excess nicotinamide and detoxifying *N*<sup>1</sup>-methylnicotinamide; and (3) diabetic subjects have slow plasma *N*<sup>1</sup>-methylnicotinamide clearance. These findings may contribute to explain the mechanism of oxidative stress and insulin resistance in a variety of clinical conditions.



**Figure 4** Effects of *N*<sup>1</sup>-methylnicotinamide on H<sub>2</sub>O<sub>2</sub> generation and NAD levels. A, B: Cumulative effects of nicotinamide (NM, 0.5 or 2 g/kg) and *N*<sup>1</sup>-methylnicotinamide (NMN, 0.5 or 2 g/kg) on rat plasma H<sub>2</sub>O<sub>2</sub> levels; C: H<sub>2</sub>O<sub>2</sub> concentrations in the supernatant of cultured human erythrocytes with or without 3 h exposure to different concentrations of NMN. For each concentration, *n* = 4; D, E: NAD (NAD<sup>+</sup> and NADH) contents in muscle (D) and liver (E) of rats treated with saline (control) or a cumulative dose of 2 g/kg NMN; F: NAD and NADH contents and NAD/NADH ratio in the erythrocytes (RBCs) of rats with or without cumulative exposure to NMN; G: NAD and NADH contents, and NAD/NADH ratio in human RBCs with (*n* = 4) or without (control, *n* = 4) 4 h exposure to 10 μmol/L NMN *in vitro*. <sup>a</sup>*P* < 0.05 vs control, <sup>b</sup>*P* < 0.01 vs control. Bar graphs indicate mean ± SD.

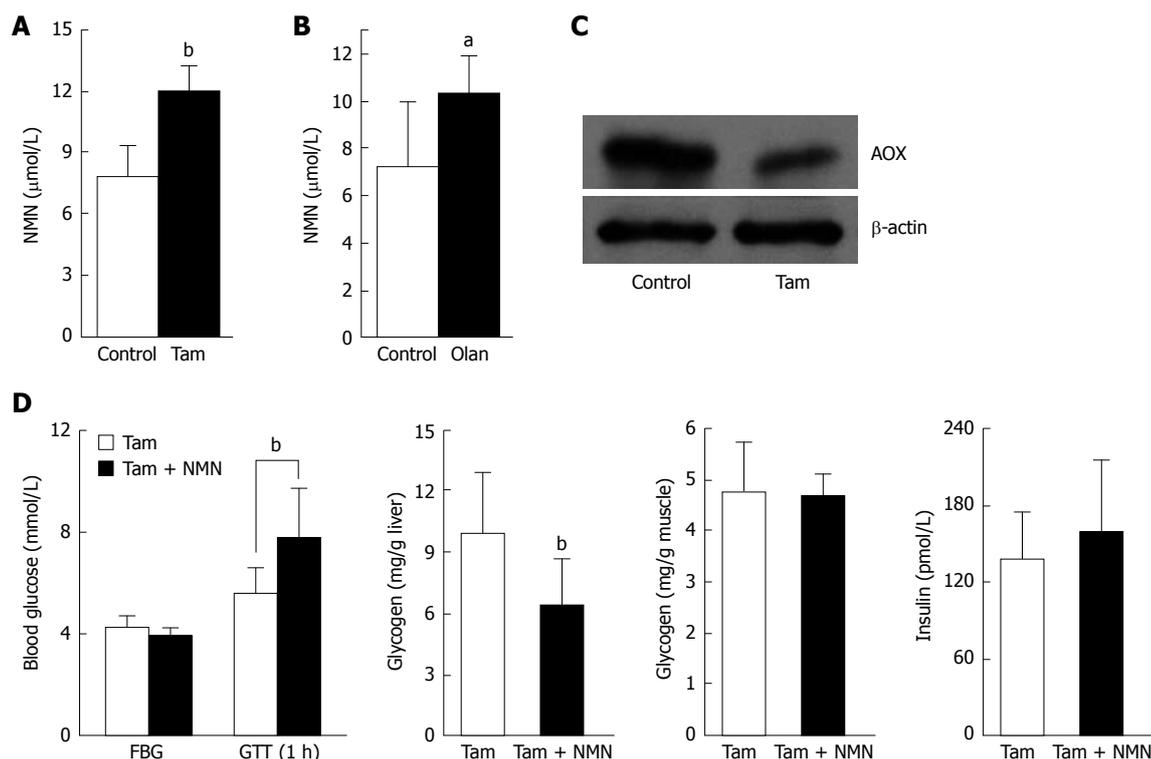
### *N*<sup>1</sup>-methylnicotinamide is a potent trigger of oxidative stress and insulin resistance

Insulin resistance and oxidative stress are the major hallmarks of type 2 diabetes<sup>[1,2]</sup>, but the mechanisms underlying the development of systemic oxidative stress and insulin resistance are unclear. Both nicotinic acid and nicotinamide have been reported to induce insulin resistance, which may lead to elevation of plasma insulin due to β-cell compensation<sup>[10-12]</sup>. Coincidentally, our data showed that nicotinamide overload induced acute insulin resistance in rats associated with high plasma levels of *N*<sup>1</sup>-methylnicotinamide; the methylation product of nicotinamide being more toxic than nicotinamide (> 6-fold)<sup>[29]</sup>. Importantly, diabetic subjects exhibited significantly higher plasma *N*<sup>1</sup>-methylnicotinamide levels than non-diabetic subjects after nicotinamide loading, which suggests its involvement in the toxic effects of nicotinamide overload. Indeed, this study

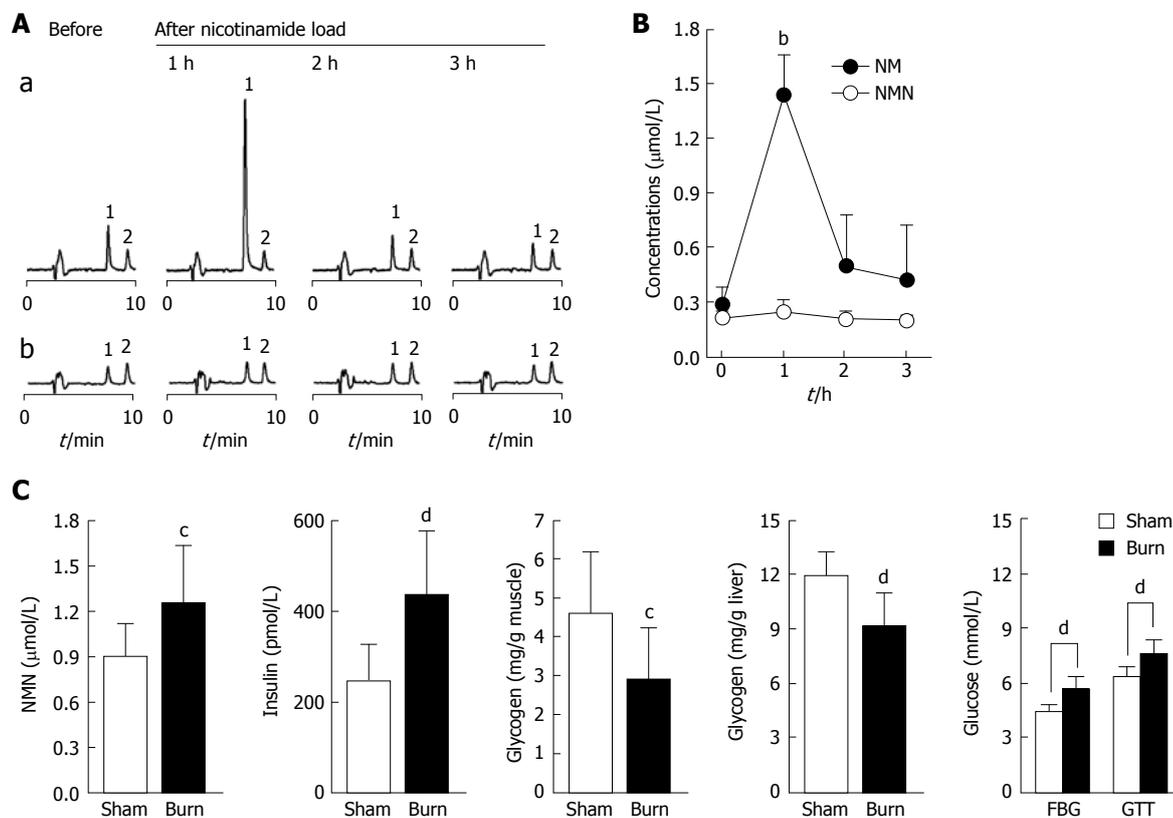
demonstrated that *N*<sup>1</sup>-methylnicotinamide mimicked the effect of nicotinamide overload, which suggested *N*<sup>1</sup>-methylnicotinamide mediation of the toxic effect.

Increasing evidence suggests that insulin resistance is due to an unfavorable internal environment because muscles resistant to insulin, when cultured *in vitro*, regain sensitivity to insulin<sup>[30-32]</sup>. Further evidence reveals that systemic oxidative stress may be responsible for triggering insulin resistance<sup>[1,33]</sup>. Consistent with previous research, this study found that *N*<sup>1</sup>-methylnicotinamide not only elevated plasma H<sub>2</sub>O<sub>2</sub> levels *in vivo*, but also directly stimulated H<sub>2</sub>O<sub>2</sub> generation of human erythrocytes *in vitro* at physiological concentrations, which indicates that *N*<sup>1</sup>-methylnicotinamide is a potent trigger of diabetic oxidative stress.

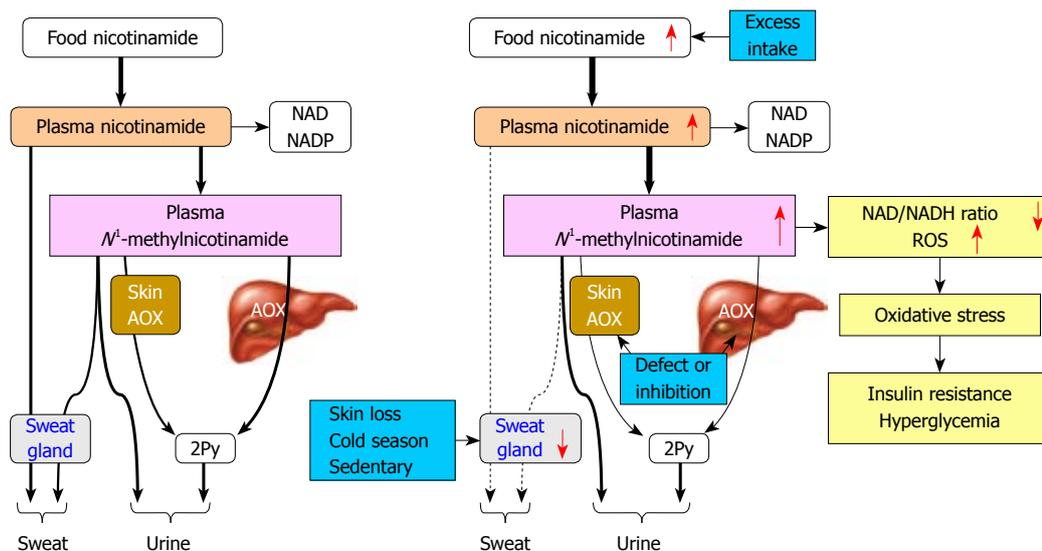
Oxidative stress may induce NAD depletion, a marker of cell injury<sup>[34,35]</sup>. Indeed, this study found that *N*<sup>1</sup>-methylnicotinamide-induced high plasma H<sub>2</sub>O<sub>2</sub> level was associated with a significant reduction in NAD content in



**Figure 5** Effects of aldehyde oxidase (AOX) inhibitors on plasma *N*<sup>1</sup>-methylnicotinamide levels and glucose metabolism in rats. A and B: Plasma *N*<sup>1</sup>-methylnicotinamide (NMN) levels 5 h after nicotinamide load (100 mg/kg, ip) in rats treated with or without AOX inhibitors tamoxifen (Tam, A) or olanzapine (Olan, B) (each group, *n* = 6). <sup>a</sup>*P* < 0.05 vs control, <sup>b</sup>*P* < 0.01 vs control; C: Liver AOX expression in rats with or without 7 wk tamoxifen treatment. The blot is representative of four independent experiments; D: Responses to a glucose tolerance test in rats after 9 wk treatment with tamoxifen with or without NMN (100 mg/kg per day) treatment in the last 2 wk. FBG: Fasting blood glucose; GTT (1 h): Blood glucose measured 1 h after glucose tolerance test. <sup>b</sup>*P* < 0.01 vs control. Bar graph indicates mean ± SD.



**Figure 6** Role of skin in nicotinamide metabolism and insulin resistance. A: Representative HPLC chromatograms showing changes of sweat nicotinamide (NM) and *N*<sup>1</sup>-methylnicotinamide (NMN) concentrations in a subject before and 1, 2 and 3 h after 100 mg nicotinamide loading. 1 and 2 in Aa are NM and internal standard *N*<sup>1</sup>-ethylnicotinamide, respectively; 1 and 2 in Ab are NMN and internal standard *N*-ethylnicotinamide, respectively; B: Summary of the measurements shown in A. <sup>b</sup>*P* < 0.0001 vs control; C: Comparison of plasma NMN and insulin levels, muscle and liver glycogen contents, and blood glucose between sham-burn (*n* = 7) and burn (*n* = 11) rats after glucose load. FBG: Fasting blood glucose; GTT: Blood glucose 1 h after glucose injection. Bar graphs show mean ± SD. <sup>c</sup>*P* < 0.05, <sup>d</sup>*P* < 0.01 vs control.



**Figure 7** Proposed model of the role of nicotinamide overload in the development of type 2 diabetes. Normally, if nicotinamide intake is slightly more than the body needs, excess nicotinamide will be detoxified rapidly and eliminated mainly via the  $N^1$ -methylnicotinamide to 2Py pathway, which involves liver and skin functions (left). Frequent excess nicotinamide intake, low  $N^1$ -methylnicotinamide detoxification, or sweat gland inactivity induces a substantial rise in plasma  $N^1$ -methylnicotinamide concentrations and residence time after each meal, and consequently induces oxidative stress and insulin resistance (right). The change trends are indicated by red arrows or line thickness.

the muscle and liver of rats. Then came the vital question: where did the excess systemic ROS originate? NADH-dependent ROS generation is an important source of intracellular ROS<sup>[34]</sup>. Accumulating evidence has indicated that diabetes shows a decreased cytosolic  $NAD^+$ / $NADH$  ratio in a variety of tissues<sup>[21]</sup>. Importantly, this study demonstrates that  $N^1$ -methylnicotinamide decreases  $NAD^+$ / $NADH$  ratio in rat erythrocytes *in vivo* and human erythrocytes *in vitro*. Thus, it is likely that diabetic oxidative stress is initiated by high plasma  $N^1$ -methylnicotinamide-induced imbalance in the  $NAD^+$ / $NADH$  redox couple. Many cellular processes are governed by the enzymes using  $NAD^+$ / $NADH$  as a cofactor<sup>[6,21,34]</sup>, therefore, it is not difficult to understand why a set of metabolic abnormalities happen in type 2 diabetes.

### Role of AOX in insulin resistance

Mammalian AOX is a molybdo-flavo enzyme involved in the detoxification of various endogenous and exogenous  $N$ -heterocyclic compounds<sup>[22,23]</sup>.  $N^1$ -methylnicotinamide is one of the substrates of AOX by which  $N^1$ -methylnicotinamide is oxidized to pyridones<sup>[7]</sup>, and thus, detoxified. AOX is expressed predominantly in the liver. Therefore, severe liver disease might be expected to reduce plasma  $N^1$ -methylnicotinamide clearance and subsequent insulin resistance. In fact, it is well known that liver cirrhosis is associated with high incidence of diabetes<sup>[36,37]</sup>. Pumpo *et al.*<sup>[38]</sup> have found that cirrhotic patients have high serum  $N^1$ -methylnicotinamide levels, both in basal values and after nicotinamide loading. Moreover, non-alcoholic steatohepatitis, the most prevalent liver disease in the western world, typically is associated with the metabolic syndrome that is characterized by insulin resistance, diabetes and hypertension<sup>[24]</sup>. Tamoxifen, a well-known inducer of non-alcoholic steatohepatitis<sup>[24]</sup>, is found to inhibit strongly AOX activity<sup>[23]</sup> and its expression (Figure 5C). Collectively,

it seems that the decrease in  $N^1$ -methylnicotinamide detoxification may be involved in hepatogenic insulin resistance.

AOX is also expressed in the skin<sup>[26]</sup>, which suggests skin involvement in  $N^1$ -methylnicotinamide detoxification. Moreover, as found in this study, human sweat glands can excrete excess nicotinamide. Therefore, decreased skin function may be implicated in  $N^1$ -methylnicotinamide-induced insulin resistance. In fact, severe burns may induce long-lasting insulin resistance, a well-documented but poorly understood phenomenon<sup>[31,39]</sup>. The present study demonstrated that severe burns significantly delayed  $N^1$ -methylnicotinamide clearance, which suggests that long-lasting post-burn insulin resistance may involve a decrease in nicotinamide detoxification and excretion.

### Relationship between lifestyle risk factors and nicotinamide overload

Environmental and lifestyle risk factors may trigger type 2 diabetes<sup>[3,4]</sup>. From our study, it emerges that the risk factors may involve nicotinamide overload. Firstly, excess nicotinamide may lead to high plasma  $N^1$ -methylnicotinamide, with subsequent oxidative stress and insulin resistance. In response to insulin resistance, the pancreatic  $\beta$  cells have to increase insulin secretion (hyperinsulinemia) compensatorily, which may eventually lead to  $\beta$ -cell failure and blood sugar levels being out of control. The result that tamoxifen-induced AOX inhibition plus  $N^1$ -methylnicotinamide impaired rat glucose tolerance (Figure 5D) implies that excess nicotinamide intake may be more harmful to those with a low  $N^1$ -methylnicotinamide clearance.

Secondly, dietary risk factors may be related to nicotinamide contents in foods. For example, meat, which is rich in nicotinamide, increases the risk of type 2 diabetes<sup>[40,41]</sup>. Moreover, food fortification with niacin may play a role in nicotinamide overload. If comparing the epidemic of type

2 diabetes in the United States<sup>[13]</sup> with the history of food fortification<sup>[14]</sup>, one can clearly see that the trend for rapid increase in the incidence of diabetes has occurred in parallel with the trend in mandatory niacin-fortification-induced increase in the per capita niacin consumption in the latter half of the 20th century. These facts may explain why the western dietary pattern, characterized by a high intake of meat and niacin-fortified foods, confers such a high risk of type 2 diabetes.

Thirdly, sedentary lifestyle risk factor may involve sweat gland inactivity. Sweat gland activity fluctuates according to ambient temperature; the most significant feature of the gland. Therefore, sweat gland inactivity is expected to slow nicotinamide catabolism and thereby increase the danger of developing insulin resistance. In fact, the cold season is known to worsen glucose metabolism<sup>[42,43]</sup>, whereas exercising sufficiently to sweat may reduce diabetic incidence<sup>[44]</sup>. Therefore, it is likely that sedentary lifestyle risk factors may be at least partially due to sweat gland inactivity by air-conditioned working/living environments.

It should be noted that large doses of nicotinic acid and nicotinamide may induce liver damage<sup>[45,46]</sup>, therefore, long-term investigation may be necessary to determine the relationship between chronic nicotinamide overload and non-alcoholic steatohepatitis. Historically, the epidemic of pellagra has been restricted mainly to those who have performed heavy industrial labor with poor nutrient supply<sup>[9]</sup>. Hence, the present study gives rise to an important social and public health issue as to whether foods need to be fortified with niacin (nicotinamide or nicotinic acid), when the people in developed countries have already been living in an age of over-nutrition and sweat gland inactivity.

In summary, it appears that gene-environment (diet) interactions may be a reflection, to some extent, of the outcome of combination of nicotinamide overload and relatively low  $N^1$ -methylnicotinamide detoxification and excretion. As summarized in Figure 7, the pathogenesis of type 2 diabetes may be at least partially due to long-term excess nicotinamide intake, and/or slowness in  $N^1$ -methylnicotinamide detoxification, and/or decrease in excess nicotinamide and  $N^1$ -methylnicotinamide excretion. This may lead to high plasma  $N^1$ -methylnicotinamide levels, and subsequently oxidative stress and insulin resistance. Therefore, reducing nicotinamide intake and facilitating excretion of nicotinamide metabolites may be a useful preventive and therapeutic intervention in type 2 diabetes.

## COMMENTS

### Background

Type 2 diabetes generally is accepted to be a result of gene-environment interaction, although the underlying mechanism is not clear. Of the environmental factors, diet appears to play a major role. In fact, the sharp increases in the incidence of diabetes in the United States in the latter half of the 20th century and in China in the past two decades of the 20th century followed food fortification with niacin (i.e. nicotinamide and nicotinic acid) beginning in the early 1940s in the United States and in the early 1980s in China. Moreover, niacin is reported frequently to impair glucose metabolism and cause liver injury. Thus, there is the possibility that the high prevalence of type 2 diabetes in these countries in the past few decades may involve niacin toxicity.

### Research frontiers

Type 2 diabetes is associated with increased systemic oxidative stress, a

factor responsible for the development of insulin resistance. How the systemic oxidative stress occurs is a major issue in type 2 diabetes.

### Innovations and breakthroughs

The present study demonstrated that the pathogenesis of type 2 diabetes may involve abnormal nicotinamide metabolism. Factors that induce nicotinamide overload and/or decrease in the detoxification and excretion of nicotinamide metabolites may lead to systemic oxidative stress and insulin resistance. The factors may include the frequent use of foods rich in nicotinamide and/or fortified with niacin, congenital enzymatic defects, liver diseases, and sweat gland loss or inactivity. The present study suggests that gene-environment interactions may reflect the outcome of increased nicotinamide intake and a decrease in its detoxification and excretion.

### Applications

Reducing nicotinamide intake and facilitating the excretion of excess nicotinamide may be a useful preventive and therapeutic intervention in type 2 diabetes.

### Peer review

This is an interesting study with human and experimental data, which investigated a clinically relevant issue, and gave an insight into the pathogenic mechanisms involved. The experiments were performed well and are presented well in the paper.

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## Capsule endoscopy in suspected small bowel Crohn's disease: Economic impact of disease diagnosis and treatment

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### Abstract

**AIM:** To model clinical and economic benefits of capsule endoscopy (CE) compared to ileo-colonoscopy and small bowel follow-through (SBFT) for evaluation of suspected Crohn's disease (CD).

**METHODS:** Using decision analytic modeling, total and yearly costs of diagnostic work-up for suspected CD were calculated, including procedure-related adverse events, hospitalizations, office visits, and medications. The model compared CE to SBFT following ileo-colonoscopy and secondarily compared CE to SBFT for initial evaluation.

**RESULTS:** Aggregate charges for newly diagnosed, medically managed patients are approximately \$8295. Patients requiring aggressive medical management costs are \$29508; requiring hospitalization, \$49074. At sensitivity > 98.7% and specificity of > 86.4%, CE is less costly than SBFT.

**CONCLUSION:** Costs of CE for diagnostic evaluation

of suspected CD is comparable to SBFT and may be used immediately following ileo-colonoscopy.

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**Key words:** Capsule endoscopy; Costs; Crohn's disease; Disease management

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### INTRODUCTION

Crohn's disease (CD) is a chronic, transmural inflammatory bowel disease that primarily involves the small bowel. Symptoms of CD are often non-specific and vary among afflicted individuals. Symptoms may include abdominal pain, diarrhea, vomiting, gastrointestinal bleeding, iron deficiency anemia, weight loss, and fevers<sup>[1]</sup>. Crohn's disease can also be associated with extra-intestinal manifestations such as skin rashes, arthritis, and uveitis<sup>[2]</sup>. While any segment of the gastrointestinal tract can be affected, the small intestine is involved in approximately 70% of patients and up to 30% of patients will have their disease limited solely to the small bowel, particularly the ileum<sup>[2]</sup>.

There is no single test that establishes the diagnosis of CD. Diagnosis is based on a combination of clinical, endoscopic, radiographic, and laboratory findings. The sequence of diagnostic tests attempting to establish the diagnosis of CD is often based upon the patient's presenting symptoms. For example, when small bowel CD is suspected clinically, ileo-colonoscopy along with small bowel barium radiography [e.g. small bowel follow-through (SBFT)] has traditionally been performed<sup>[3]</sup>. However, barium radiographic studies are limited by poor sensitivity for detecting early lesions of CD (e.g. aphthous erosions/

ulcerations) and ileo-colonoscopy evaluation is confined to only the most distal 5-15 cm of the ileum (terminal ileum). In addition, the non-specific symptoms of CD often lead to delays in obtaining a definitive diagnosis and therefore the institution of appropriate and targeted disease therapy<sup>[4-6]</sup>. Rath *et al*<sup>[7]</sup> found that 38% of CD patients had an interval of more than a year between onset of symptoms and definitive diagnosis.

Because of delays in diagnosing small bowel CD, the costs to payers that are associated with indeterminate tests, repeat endoscopic and radiographic procedures, frequent physician visits, and hospitalizations are likely to be substantial<sup>[8,9]</sup>. Thus, there is a need for an efficient and effective algorithm of diagnostic tests leading to a definitive diagnosis of CD. Optimizing such a diagnostic pathway should have substantial benefits for both patients and payers.

Until recently, the small bowel has been the most difficult part of the gastrointestinal tract to evaluate endoscopically. Since being introduced in 2001, small bowel capsule endoscopy (CE) has facilitated easy access for direct investigation of the entire small bowel mucosa and as a result has revolutionized the diagnosis and management of small bowel diseases including obscure gastrointestinal (GI) bleeding, iron deficiency anemia, CD, polyposis syndromes/tumors, and celiac disease. Because of its ability to examine the entire mucosa of the small intestine, capsule endoscopy has the potential for diagnosing suspected CD patients earlier and as a result, direct costs of care may be reduced.

The aim of this study was to derive and evaluate a decision analytic model to determine the economic benefit of CE compared to ileo-colonoscopy and SBFT for the evaluation of suspected CD of the small bowel.

## MATERIALS AND METHODS

### Model design

We developed a comprehensive model comparing the direct costs of performing CE for the diagnosis and management of suspected CD in a hypothetical cohort of 10 000 patients. Each arm of the model includes the yield of an initial ileo-colonoscopy immediately followed by SBFT or CE based on current clinical practice and guidelines (Figure 1)<sup>[10-16]</sup>. If no diagnosis was made after initial ileo-colonoscopy, we assumed that patients would then receive either a SBFT or CE. In a secondary analysis, we eliminated the initial ileo-colonoscopy to determine the relative global costs using SBFT or CE as an initial diagnostic strategy (Figure 2).

In constructing the model to reflect accepted standards of care for suspected CD we used guidance from clinical experts in CD (JAL, IMG) and the 2005 International Conference on Capsule Endoscopy (ICCE) consensus statement<sup>[10-17]</sup>. For economic comparators, we utilized the economic analysis of Goldfarb *et al*<sup>[18]</sup> concerning evaluation of suspected CD using CE and publications identified by a systematic literature review for guidance in building and populating the model.

The Goldfarb economic analysis compared CE to traditional modalities for the diagnosis of suspected small bowel CD<sup>[18]</sup>. Based on published diagnostic yields of 69.6% for CE *vs* 53.9% for a combined approach with ileo-colonoscopy and SBFT, their analysis determined CE to be a less costly strategy as long as its diagnostic yield was 64.1% or greater. The authors also reported CE to be less costly as a primary diagnostic tool in this clinical setting<sup>[18]</sup>. These data are provocative, but CE and ileo-colonoscopy/SBFT as mutually exclusive diagnostic strategies may not translate into every day clinical practice. In addition, the included trials were not subjected to a systematic review analysis to evaluate for study heterogeneity.

### Model assumptions and definitions

**Literature review:** To estimate key model variables we performed a systematic literature review to investigate the evidence concerning the use of CE for evaluating suspected CD. The MeSH search terms and strategies employed by third party payers were used for identifying relevant publications. Multiple MEDLINE, EMBASE, COCHRANE TRIALS, and BIOSIS searches were completed using MeSH search terms including “capsule endoscopy”, “Pillcam”, “wireless endoscopy”, “wireless capsule endoscopy”, “video endoscopy”, “video capsule endoscopy”, “M2A capsule endoscopy”, “endoscopy”, “ileum endoscopy”, “ileal endoscopy”, “small bowel imaging”, “small intestine imaging”, “Crohn’s disease”, “Crohn’s disease diagnosis”, “Crohn’s disease radiology”, “Crohn’s disease imaging”, “Crohn’s disease costs”, “Crohn’s disease payments”, “Crohn’s disease reimbursement”.

A total of 891 citations were derived from the published literature searches (January 1970 - January 2008). 792 (89%) were published in English and 141 (16%) were review articles; 29 were published in German, 28 in Spanish, 7 each in Japanese and French, and 5 in Italian. In all cases, we reviewed full manuscripts/reports and case reports with sufficient evaluable information but did not include published abstracts. The literature reporting on ultrasound, magnetic resonance (MR), and computed tomography (CT) enterography in CD continues to grow but remains inconsistent in estimates of diagnostic yield and cost; consequently, we chose not to compare these techniques.

### Patients

Using a decision analysis software (TreeAge Pro Suite 2006 Release 0.3, Williamstown, MA), we evaluated a hypothetical cohort of 10 000 patients who had suspected CD as defined by the 2005 ICCE Consensus conference<sup>[17]</sup>. Ileo-colonoscopy was negative and was assumed to effectively exclude lesions within the reach of a colonoscope. Therefore, CD lesions modeled were defined to be located proximal to the distal terminal ileum.

This model estimates the total expected global costs to a third party payer of the initial diagnostic work-up as well as expected follow-up costs of managing these patients for one year after diagnosis of CD, including procedure-related adverse events, subsequent hospitalizations, office

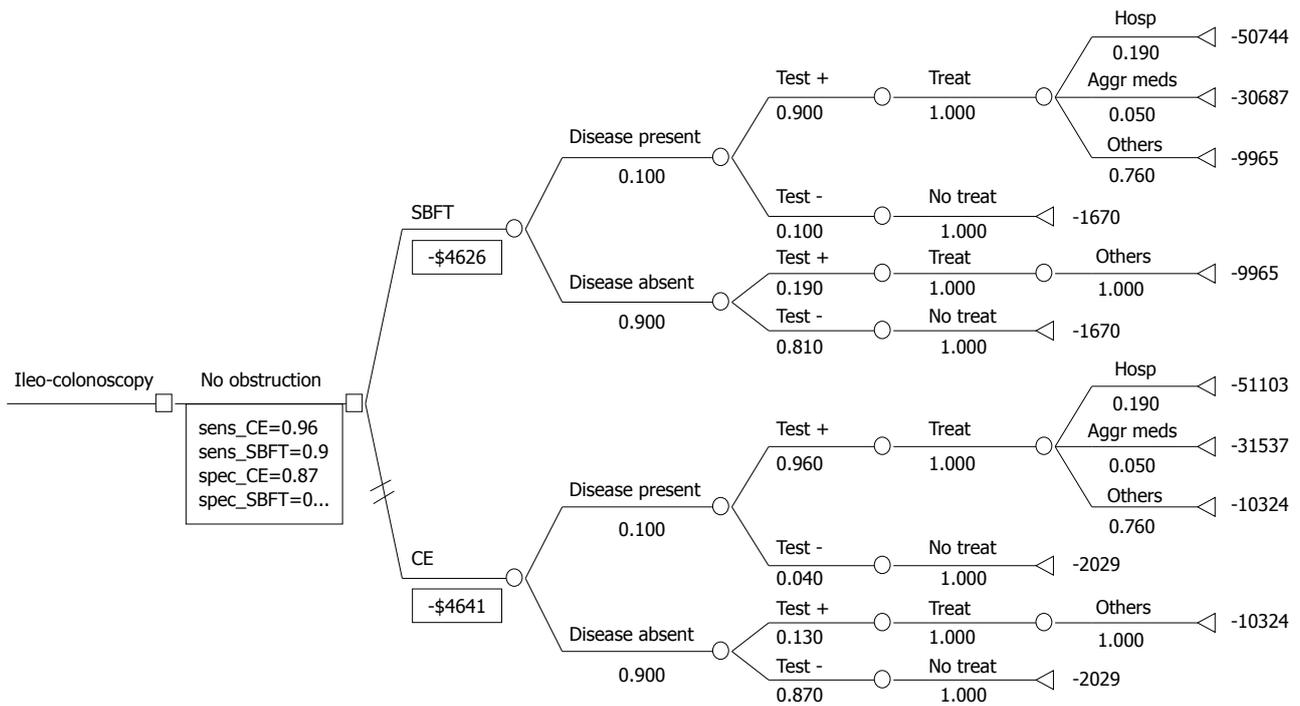


Figure 1 One-year cost model for diagnosis and management of suspected Crohn's disease (CD) of the small bowel with ileo-colonoscopy as first-line diagnostic technique. SBFT: Small bowel follow-through; CE: Capsule endoscopy.

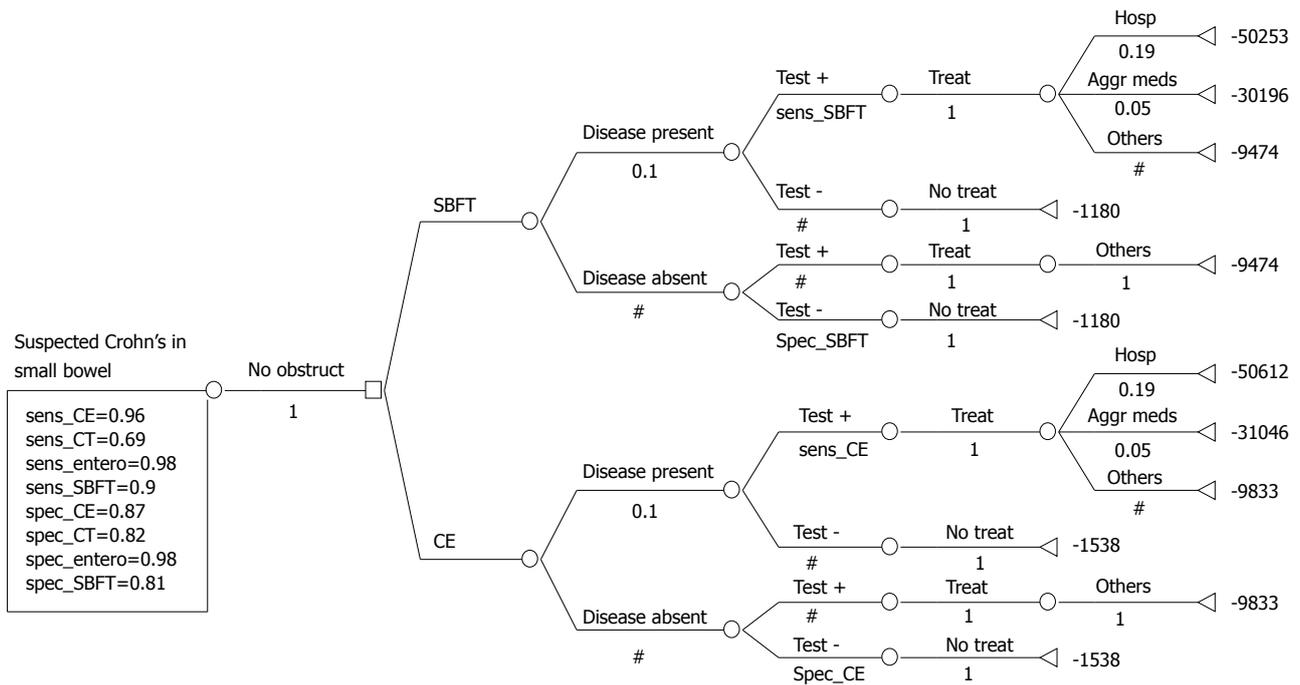


Figure 2 One-year cost model for diagnosis and management of suspected CD of the small bowel with ileo-colonoscopy/SBFT or capsule endoscopy as first-line diagnostic technique. CT: Computed tomography.

visits, laboratory tests, and medications. We chose this design because this economic model allows predictive modeling of the annual economic burden for a chronic disease like CD. Payers are concerned about the rate of medical resource utilization of both inpatient and outpatient services and are not generally interested in other burdens to the patient such as sick time off or other opportunity loss. In addition, because of enrollee turnover

rates in health plans, payers are most interested in models that study short-term (1-2 years) rather than long-term (> 2 years) economic burdens. Finally, payers are interested in imaging and diagnostic tests that are not additive (i.e. leads to treatment change without requiring further testing).

We developed a cost model based on the annual costs of care for diagnosis and management of a patient with suspected CD<sup>[19]</sup>.

Previously, Feagan *et al*<sup>[19]</sup> used medical claims data to identify and stratify patients with CD into three mutually exclusive disease severity groups: Group 1, required an inpatient hospitalization associated with a primary/secondary diagnosis of CD; Group 2, required aggressive pharmacotherapy, defined as chronic glucocorticoid (> 10 mg/d) or immunosuppressive drug (purine antimetabolites/methotrexate) therapy, for > 6 mo and/or anti-TNF monoclonal antibody therapy; or Group 3, all remaining patients (Table 1). As with prior economic evaluations and models reported in the biomedical literature, we used Medicare reimbursement data as surrogates of cost<sup>[18,19]</sup>. For tests and procedures, the model was populated with national average allowable 2007 Medicare reimbursement data (Table 2).

We were interested in determining total payer costs associated with the diagnostic evaluation and management of suspected CD. Therefore we evaluated typical hospitalization, outpatient, and medication costs associated with the care of a patient being evaluated for suspected CD of the small bowel. To estimate the added cost of anti-TNF monoclonal antibody therapy (e.g. Remicade<sup>®</sup>), we identified cost-effectiveness analyses on the use of these agents for treatment of aggressive CD. Recent literature has estimated that 25% of patients in this group would receive anti-TNF monoclonal antibody therapy<sup>[20,21]</sup>. Based on the reported shifts in utilization, we estimated an annual cost of \$65 000 for this class of drugs and calculated a weighted average cost of \$16 250 per patient to simulate the expected cost of these agents in the aggressive medical management group (Feagan *et al*<sup>[19]</sup> Group 2).

Annual costs were then estimated as a weighted average based on the likelihood of the correct diagnosis multiplied by the expected costs of follow-up care by each patient type. We estimated in-patient hospital costs by examining charge data from the Nationwide Inpatient Sample (NIS) which is one in a family of databases and software tools developed as part of the Healthcare Cost and Utilization Project (HCUP), a Federal-State-Industry partnership sponsored by the Agency for Healthcare Research and Quality (Rockville, MD). This database permits searches of in-patient costs according to ICD-9 disease classifications. The database includes historical information and permits comparisons to inflation-adjusted calculations.

The systematic literature review described above was used to identify mean sensitivity and specificity estimates for the comparator diagnostic techniques (Table 3). These values were used to populate the model as were the inflation-adjusted charges described in the manuscript by Feagan *et al*<sup>[19]</sup>. In addition, a series of sensitivity analyses, including a Monte Carlo analysis, were performed to determine the relative strengths and weaknesses of utilizing CE relative to other diagnostic testing options in patients with suspected CD. Monte Carlo simulation creates a multi-way sensitivity analysis. The base-case decision tree was repeatedly analyzed using inputs with appropriate distributions to determine the proportion

**Table 1** Aggregate charges for each therapeutic grouping used in model

Procedure	Group 1	Group 2	Group 3
Base charge <sup>1</sup>	\$49 074	\$13 258	\$8 295
Monoclonal therapy <sup>2</sup>	-	\$16 250	-
Colon/SBFT <sup>3</sup>	\$50 253	\$30 687	\$9 474
CE <sup>4</sup>	\$50 612	\$31 046	\$9 833

<sup>1</sup>Groups 1, 2, and 3 base annual charges adjusted for inflation from Feagan *et al* by consumer price index (CPI) to estimate 2007 charges; <sup>2</sup>Base annual charge for monoclonal (Infliximab) therapy for Crohn's disease was estimated as \$65 000. We assumed 25% of patients requiring aggressive medical management were treated with this agent and adjusted Group 2 by \$16 250; <sup>3</sup>Colon/SBFT procedure charge \$11 79; <sup>4</sup>CE procedure charge \$1538. SBFT: Small bowel follow-through; CE: Capsule endoscopy.

of runs each strategy was identified as the least costly initial strategy. The following variables were used in the simulation: costs and sensitivity of tests and rates of procedural complications. Each simulation performed included 10 000 trials. Adding the potential cost of complication(s) following possible capsule retention did not change the economic results (data not shown).

## RESULTS

Figure 2 depicts the derived cost model. All patients with suspected CD are initially examined with ileo-colonoscopy followed by SBFT or CE. Overall, patients evaluated and treated by the CE pathway cost \$4641 whereas those evaluated by SBFT cost \$4626, a difference of \$15 per patient slightly in favor of SBFT. Patients with positive small bowel findings on CE who required hospitalization cost \$51 103 and those requiring aggressive medical management cost \$31 537. These values were comparable to the SBFT pathway. In the secondary analysis, patients managed by CE as the initial diagnostic test cost \$4150 or \$15 per patient more than those initially evaluated with SBFT. These results were comparable to the prior results because of the lower cost of omitting an initial ileo-colonoscopy at an estimated cost of \$491.

We conducted a Monte Carlo simulation involving 10 000 patients undergoing evaluation for suspected CD. In evaluating the results shown in Figure 3, there is evidence that the probability of incurring a charge of at least \$1670 is > 97.5%; there is less than a 2.5% probability of incurring a charge of more than \$9965. The mean cost for evaluation of the patient with suspected small bowel CD is \$4601 ± 7467 (SD) for this 10 000 patient model cohort examined by ileo-colonoscopy first, immediately followed by SBFT. Likewise, there is evidence that the probability of incurring a charge of at least \$2029 is > 97.5%; there is less than a 2.5% probability of incurring a charge of more than \$10 324 in those evaluated by ileo-colonoscopy first, immediately followed by CE. The mean cost for evaluating a patient with suspected CD in that group is \$4604 ± 7563 (SD) for the 10 000 patient simulation. The results of the Monte Carlo simulation are essentially identical (\$4604 for SBFT

**Table 2 Procedure charges used for the model according to the 2007 medicare fee schedule**

HCPCS code	Procedure	Reimbursement	Colonoscopy w/SBFT	Capsule endoscopy
45380	Colonoscopy, flexible, proximal to splenic flexure; with biopsy, single or multiple	\$491.15	x	x
74249	Radiological examination, gastrointestinal tract, upper, air contrast, with specific high density barium, effervescent agent, with or without glucagon; with small intestine follow-through	\$177.87	x	
88305	Level IV-surgical pathology, gross and microscopic examination	\$115.38	x	
88323	Consultation and report on referred material requiring preparation of slides	\$150.09	x	
91110	Gastrointestinal tract imaging, intraluminal (e.g. capsule endoscopy), esophagus through ileum, with physician interpretation and report	\$950.00		x
99242	Office (gastroenterologist) consultation (Level 2)	\$97.37	x	x
		Additional Medications <sup>1</sup>	\$50	
		Total	\$1179.00	\$1538.00

<sup>1</sup>Additional medications: Sedatives, anxiolytics, and anti-emetics administered during a routine procedure.

**Table 3 Model inputs**

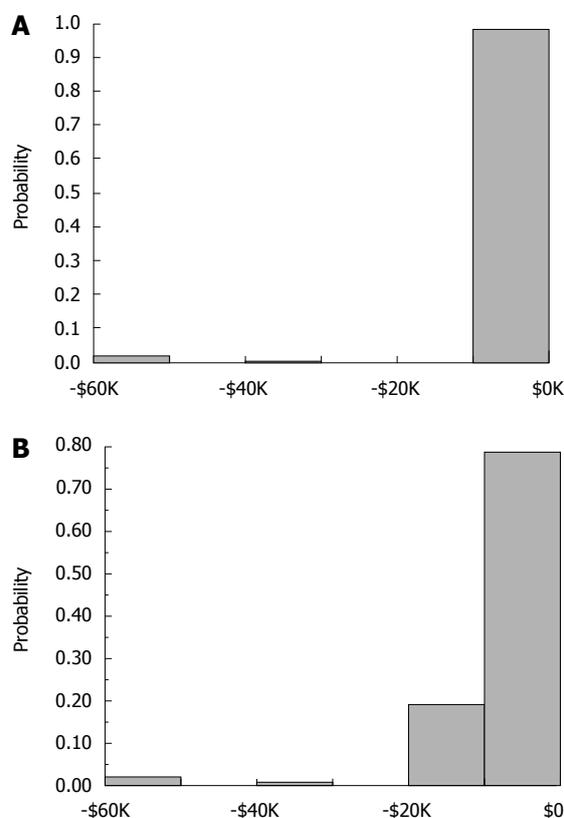
Input	Mean (range)	Ref.
CE sensitivity (sens_CE)	0.96 (0.80-0.99)	[3,14-17,26,27]
CE specificity (spec_CE)	0.87 (0.80-0.99)	[3,14-17,26,27]
SBFT sensitivity (sens_SBFT)	0.96 (0.80-0.99)	[3,14-17,26,27]
SBFT specificity (spec_SBFT)	0.81(0.80-0.99)	[3, 14-17, 26,27]
Disease present/absent	0.10/0.90	[1,2,4]
Hospitalization frequency (Group 1)	0.19	[18]
Aggressive medical management frequency (Group 2)	0.05	[18]
Other management frequency (Group 3)	0.76	[18]

vs \$4601 for CE) for each technique and represent a real-world scenario of relevant costs.

The results of one-way sensitivity analyses on the diagnostic sensitivity and specificity of SBFT and CE are shown in Figure 4 for patients who receive an initial ileo-colonoscopy. The graphs depict how sensitivity (x-axis) and cost (y-axis) (i.e. expected value) interplay. As expected, the cost of performing either test decreases with increasing sensitivity or specificity. The cost of performing SBFT vs CE is essentially equivalent over a wide range of sensitivity values, particularly in the highest ranges of sensitivity and specificity, which might be achieved by an experienced clinician. At a sensitivity > 95.1%, CE becomes less costly than SBFT; at a specificity of > 87.2%, CE is less costly than SBFT. As expected, the results are identical in the secondary analysis (data not shown).

**DISCUSSION**

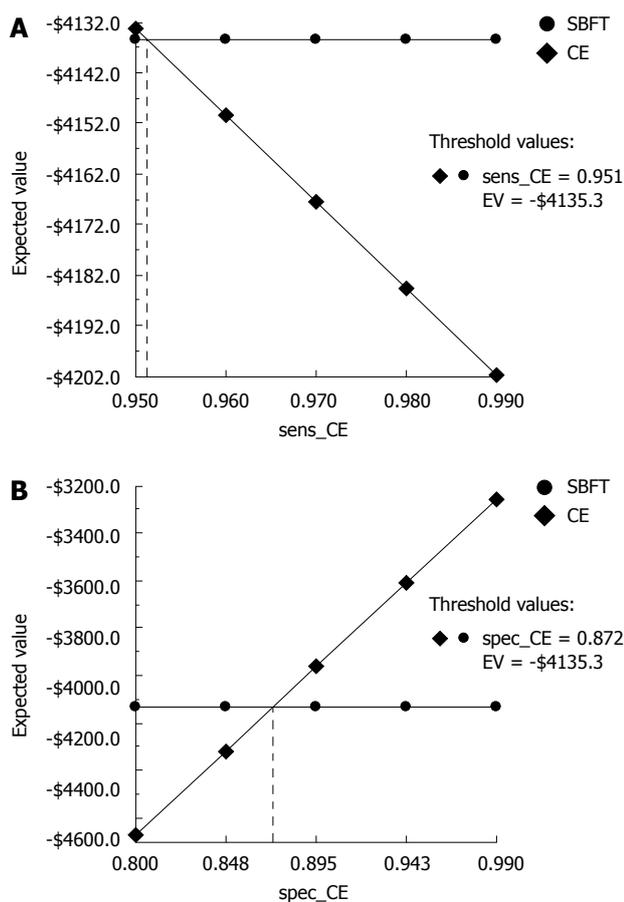
Capsule endoscopy is a valuable tool for the study of patients in whom a clinical suspicion of small bowel CD is raised. We found in this present study, applying a conservative costing methodology, incorporating ileo-colonoscopy with SBFT, and CE, demonstrates that CE is marginally less costly overall than use of SBFT despite CE being a slightly more costly test. Secondary analysis demonstrates the cost advantage of CE is robust over a wide range of diagnostic sensitivity and specificity values. Thus, following ileo-colonoscopy, there appears to be



**Figure 3 Monte carlo simulation at ileo-colonoscopy. A:** Monte carlo simulation at SBFT; **B:** Monte carlo simulation at CE.

evidence and a cost basis to encourage the use of CE initially (e.g. in place of SBFT) in diagnostic algorithms for suspected small bowel CD.

Suspected CD patients often go months and sometimes years without a definitive diagnosis, especially those with more mild or moderate symptoms and with disease that is isolated to the small bowel. For example, ileo-colonoscopy may be normal in such individuals and a functional bowel disorder may be suspected. Symptoms of CD, especially in the early stages, may mimic functional bowel disease, such as irritable bowel syndrome, confounding the differential diagnosis<sup>[19]</sup>. Radiographic diagnostic tests such as SBFT may not be sensitive enough to detect early small bowel mucosal changes (aphthae)



**Figure 4** Sensitivity analysis on CE sensitivity and specificity. A: Sensitivity analysis on sens\_CE; B: Sensitivity analysis on spec\_CE.

and enteroclysis, although a more sensitive test than SBFT is no longer routinely used due to the difficulty of performing the exam, its limited availability, and patient discomfort associated with the procedure. Moreover, exposure to radiation with these tests is a genuine concern among patients and physicians. As a result, these standard radiographic diagnostic testing approaches (SBFT and enteroclysis) can contribute to a delay in diagnosis and hence costs to the payer. While CT enterography may be a superior imaging modality to SBFT and enteroclysis for suspected CD, at present the test has limited clinical availability and as such, SBFT remains the initial study following ileo-colonoscopy in most clinical practices. For those patients with a negative ileo-colonoscopy and negative SBFT in whom obstructive symptoms raise concern for possible capsule retention, CT enterography or the Agile Patency Capsule performed prior to standard small bowel capsule endoscopy may be the next best option to evaluate these patients.

There are a number of limitations to this present study. The model inputs for the test characteristics for diagnostic tests were extracted from the literature based on clinical studies that included selected patients. These patient populations may not reflect the spectrum of patients seen in usual clinical practice. We have attempted however, to temper this limitation by performing sensitivity analyses over a wide range of model estimates,

including a Monte Carlo simulation<sup>[22]</sup>. Another limitation of this study is that there are only a limited number of publications that report findings on the use of CE for CD. Moreover, sample sizes in these trials have invariably been small. However, a meta-analysis of available prospective trials comparing CE with one or more alternate diagnostic modalities for the diagnosis of suspected or established CD, found an incremental yield of 43% (95% CI = 29% to 56%) for CE over SBFT for the diagnosis of small bowel CD, with a number needed to test of only 2 patients for an additional positive finding with CE<sup>[23]</sup>. An updated meta-analysis showed that the yield favors CE compared to SBFT for suspected CD<sup>[24-26]</sup>. Analysis of CE *vs* colonoscopy with ileoscopy (incremental yield with CE = 16%, 95% CI = 3% to 30%) and CT enterography/enteroclysis (incremental yield with CE = 32%, 95% CI = 3% to 61%) also found a significantly improved yield with CE. We utilized data from these meta-analyses to better populate our model estimates.

Our cost-analysis was limited to a third-party payer perspective, although there are additional relevant perspectives. For example, indirect costs to the patient and the cost to the gastroenterologist and their capacity to deliver care (provide CE examination) was not evaluated. In the case of the ability of gastroenterologists to provide care, CE is now a widely accepted diagnostic test that most gastroenterology practices have available and utilize. Moreover, the time horizon for this analysis was limited to one year. However, payers are most interested in models that study short-term (1-2 years) rather than long-term (> 2 years) economic burdens due in part to attrition of members in health plans.

Though CE is a promising technology for the evaluation of patients with CD, there are a number of concerns with its use in this population. The possibility of capsule retention due to known or suspected strictures is a concern in this patient group. The rate of capsule retention in the largest published series of CE for obscure gastrointestinal bleeding was 0.75% (7/934 patients) despite a negative SBFT in 6/7 patients<sup>[27]</sup>. All patients underwent surgical resection, and pathology explaining the reason for each patient's symptoms was found at the site of capsule impaction (a so-called therapeutic complication). Due to the stenosing nature of CD, however, a higher rate of capsule retention may be expected if CE is performed in these patients. Rates of capsule retention in patients with CD range from 0% to 13% despite small bowel imaging performed prior to CE in nearly all patients<sup>[28,29]</sup>. However, the rate of capsule retention is significantly higher in patients with known CD compared to those with suspected CD.

Perhaps the most significant issue facing CE in the evaluation of CD is the lack of specificity and its inability to provide pathologic confirmation of observed findings. In clinical studies, a variety of findings encompassing a broad range of severity have been described as potentially consistent with the diagnosis of CD, and thereby a positive result with CE. As a diagnosis of CD has far-reaching effects on the psychological and financial health

of patients as well as their physical health, caution in making this diagnosis based on subtle CE findings is appropriate. The long-term clinical significance of finding occasional mucosal breaks in the small bowel on CE is presently unclear, and will require prospective studies to clarify the issue.

In summary, this economic model, along with several clinical trials suggest that CE is a valuable tool in the diagnosis and subsequent management of patients with suspected CD. Data from the studies reviewed in this article suggest improved outcomes after CE due to targeted medical therapy for those patients found to have lesions consistent with CD, as well as a change in therapeutic strategy in those patients with normal capsule examinations<sup>[25,30]</sup>. The burden of delay in definitive diagnosis of a debilitating chronic disease is never more apparent than in CD where earlier intervention and treatment makes a significant clinical impact. New technologies and diagnostics, such as CE, require evidence from clinical and payer decision makers who demand rigorous proof of clinical and economic utility. This model provides economic evidence in favor of CE for use immediately following ileo-colonoscopy in the diagnostic pathway for suspected CD.

## COMMENTS

### Background

Using capsule endoscopy (CE) earlier in diagnosis of suspected Crohn's Disease (CD) may reduce direct costs of care due to ability to examine the entire mucosa of the small intestine; currently, no single test establishes this diagnosis.

### Research frontiers

Accurate, early diagnosis of CD is important to patient management decisions. Current therapies may be expensive and their potential inappropriate clinical use due to incorrect diagnosis is troublesome. CE represents a diagnostic technology that may improve overall diagnostic yield therein obviating potentially substantial clinical and economic loss.

### Innovations and breakthroughs

CE is an important breakthrough diagnostic technology that is used in a wide number of clinical settings. The present analysis considers its use in suspected small bowel CD. The findings here extend previous cost analyses. The economic model presented here is the first to combine diagnostic and therapeutic costs associated with this condition.

### Applications

The results of this study may assist in developing cost-effective diagnostic guidelines for evaluation of suspected CD.

### Terminology

Crohn's disease (CD) is a chronic, transmural inflammatory bowel disease that primarily involves the small bowel. Capsule endoscopy (CE) is a technique that directly visualizes the surface of the small bowel where abnormalities due to CD may be identified.

### Peer review

This study presents a valuable economic model that combines the cost-effectiveness of diagnosis and treatment of patients with suspected CD. The results demonstrate the value of capsule endoscopy in the overall management of patients with this condition. These results may be useful in future studies of cost-effective approaches to the diagnosis and treatment of CD as well as to considerations of diagnostic selection in clinical guidelines and practice.

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## Hepatic steatosis in overweight/obese females: New screening method for those at risk

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### Abstract

**AIM:** To identify which parameters could help to distinguish the "metabolically benign obesity", which is not accompanied by insulin resistance (IR) and early atherosclerosis.

**METHODS:** Eighty two of 124 overweight/obese females formed the study population, which was divided into two groups (52 and 30 subjects, respectively) with and without IR according to a HO meostatic Metabolic Assessment (HOMA) cut-off of 2, and were studied in a cross-sectional manner. The main outcome measures were waist circumference, serum uric acid, high-density lipoprotein-cholesterol and triglycerides, alanine amino-transferase, blood pressure and the two imaging parameters, hepatic steatosis and longitudinal diameter of the spleen, which were measured in relation to the presence/absence of IR.

**RESULTS:** A variable grade of visceral obesity was observed in all subjects with the exception of three.

Obesity of a severe grade was represented more in the group of IR individuals ( $P = 0.01$ ). Hepatic steatosis, revealed at ultrasound, was more pronounced in IR than in non-IR subjects ( $P = 0.005$ ). The two groups also demonstrated a clear difference in longitudinal spleen diameter and blood pressure, with raised and significant values in the IR group. Metabolic syndrome was frequent in the IR group, and was not modified when adjusted for menopause ( $P = 0.001$ ). At linear regression, the  $\beta$  values of waist circumference and body mass index predicting HOMA were 0.295,  $P = 0.007$  and 0.41,  $P = 0.0001$ , respectively. Measures of spleen longitudinal diameter were well predicted by body mass index (BMI) values,  $\beta = 0.35$ ,  $P = 0.01$ , and by HOMA,  $\beta = 0.41$ ,  $P = 0.0001$ . Blood pressure was predicted by HOMA values,  $\beta = 0.39$ ,  $P = 0.0001$ . HOMA and hepatic steatosis were highly associated ( $\rho = 0.34$ ,  $P = 0.002$ ). Interestingly, IR patients were almost twice as likely to have hepatic steatosis as non-IR patients. Among the MS criteria, blood pressure was very accurate in identifying the presence of IR (AUROC for systolic blood pressure 0.66, cut-off 125 mm of Hg, sensibility 64%, specificity 75%; AUROC for diastolic blood pressure 0.70, cut-off 85 mm of Hg, sensibility 54.5%, specificity 75%).

**CONCLUSION:** As health care costs are skyrocketing, reliable and mainly inexpensive tools are advisable to better define subjects who really need to lose weight.

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**Key words:** Body mass index; Cardiovascular disease; Fatty liver; Insulin resistance; Metabolic fitness; Obesity

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## INTRODUCTION

The incidence of both Overweight/Obesity (O/O) and Metabolic Syndrome (MS) is dramatically increasing. The generally accepted view is that being overweight causes similar health problems to that of obesity but to a lesser degree. The risk of death from all causes increases throughout the range of moderate and severe increases in body weight for both men and women in all age groups<sup>[1]</sup>. Further co-morbidities<sup>[2-4]</sup> are more frequent in O/O than in normal-weight females. Unless reversed, this trend predicts an epidemic of cardiovascular disease (CVD). Interventions are required to empower adults to increase physical activity and to modify eating habits. Nevertheless, it is mandatory to establish exactly which individuals should be treated and when. In fact, although the negative health consequences of O/O in the general population are well supported by the available published evidence, health outcomes in certain subgroups seem to be improved at an increased body mass index (BMI), a phenomenon known as the obesity survival paradox<sup>[5]</sup>. Furthermore, there is a high prevalence of clustering of cardiometabolic abnormalities among normal-weight individuals and a high prevalence of O/O individuals who are metabolically healthy<sup>[6]</sup>. Consequently, “metabolically benign obesity” which is not accompanied by insulin resistance (IR) and early atherosclerosis has recently been postulated to exist in humans<sup>[7]</sup>.

A way of getting to the grass-roots of the problem could be to tackle the mechanisms underlying O/O. Evidence that this phenomenon can be regarded as a low-grade chronic inflammatory state comes from numerous studies<sup>[8]</sup>. Moreover, inflammation links obesity to early tumor promotion<sup>[9]</sup>. Inflammatory disease could represent a compensatory mechanism for increased adipose tissue turnover<sup>[10]</sup>. Apart from diabetes and atherosclerosis, another associated high-risk condition is non-alcoholic fatty liver disease (NAFLD), the etiologic factors of which include IR<sup>[11]</sup>, high levels of serum interleukin-6 (IL-6)<sup>[12]</sup> and mitochondrial dysfunction<sup>[13]</sup>. The link between NAFLD and CVD has recently been established by the fact that the liver is involved in regulating/secretory numerous CVD risk factors<sup>[14]</sup>, notably a cytokine [tumor necrosis factor-alpha (TNF- $\alpha$ )], an acute-phase protein [C-reactive protein (CRP)], glucose, lipoproteins, coagulation factors (plasminogen activator inhibitor-1<sup>[15]</sup>) and a substance which increases blood pressure (angiotensin II<sup>[16]</sup>).

The NAFLD rate in the general adult population is increasing with large numbers, i.e. 20%<sup>[17]</sup>, in obese non-diabetic adults being much higher<sup>[18]</sup>. Obviously, a clear differentiation between “simple” fatty liver (FL) and non-alcoholic steatohepatitis (NASH) in the spectrum of NAFLD is quite impossible without liver biopsy which is not always performed for ethical and technical issues. For this reason, we generally speak of hepatic steatosis (HS). Ultrasound (US) is widely used to detect HS, without exposing subjects to ionizing radiations. Indeed, more specific imaging techniques<sup>[19]</sup> are costly and not always useful in close follow-up. Tsushima *et al*<sup>[20]</sup> first identified the so-called liver-spleen axis in NAFLD patients. It has recently been proposed that increased spleen volume and

serum levels of high sensibility (hs)-CRP contextually with HS, which “*per se*” unravels an inflammatory status<sup>[14]</sup>, characterize young adult obese subjects<sup>[21]</sup>. Up-to-date findings from a nationally representative sample of adults indicate that the prevalence of MS increases substantially with increasing levels of serum uric acid (UA)<sup>[22]</sup>. Actually, the menopause is associated with the aggravation of total cholesterol, LDL cholesterol and apolipoprotein B<sup>[23]</sup>, representing a physiologic event which should be taken into consideration when evaluating multiple cardiovascular risk factors in O/O females, even though this status is highly correlated with age. Finally, researchers have demonstrated that elevated alanine aminotransferase (ALT) levels are independently associated with increased CVD- or diabetes-related mortality<sup>[24]</sup>.

With numerous studies showing that O/O is a disease requiring long-term commitment to achieve the desired results, and that these results should be achieved as soon as possible<sup>[25]</sup>, we aimed to identify which parameters could help distinguish between healthy and non healthy O/O individuals, by individualizing the boundaries within which is possible to “watch and wait”. As health care costs are skyrocketing, the key point is to utilize tools which are reliable and mainly inexpensive. To achieve this aim we analyzed an anthropometric index, i.e. waist circumference (WC), in addition to the metabolic parameters, serum UA, high-density lipoprotein-cholesterol (HDL) and triglycerides, liver enzyme activity i.e. ALT, a CVD index, i.e. blood pressure, and two imaging parameters which were HS and spleen size, in relation to the presence/absence of IR.

## MATERIALS AND METHODS

This research was performed by screening 124 consecutive female subjects with established (at least 4 years) O/O from February 2008 to April 2009. The study was carried out according to the principles of the Declaration of Helsinki and an informed written consent was obtained from each patient.

### Exclusion criteria

Of the 124 initial subjects, 11 patients, who had undergone steroid therapy (four with bronchial asthma, five with rheumatoid arthritis and two with neuritis), as well as 15, who had received one or more of the following drugs, i.e. aspirin, metformin, statins and fibrates, were excluded from the study because these prior treatments may have caused a change in laboratory data. Three persons were excluded due to the detection of marked intestinal meteorism which made it difficult to perform US. Eight others were excluded due to the presence of co-morbidity (chronic viral hepatitis, alcohol abuse), and five subjects were considered drop-outs, because they avoided undergoing full laboratory-instrumental examinations.

### Inclusion criteria

The remaining 82 subjects whose median age was 46.5 (14-64) years, were divided into two groups on the basis of the presence or absence of IR, and formed the study

population. The degree of overweight or obesity was established on the basis of BMI cut-off points of 25-29.9, 30-34.9, 35-39.9 and  $> 40 \text{ kg/m}^2$ , respectively. Visceral obesity was identified by measuring WC at the midpoint between the lower border of the rib cage and the iliac crest. MS was defined according to the revised Adults Treatment Panel III (2001), and three or more criteria were considered: plasma glucose concentration of at least 100 mg/dL, WC  $> 88 \text{ cm}$ , serum HDL concentration  $< 50 \text{ mg/dL}$ , blood pressure of at least 130/85 mmHg, and serum triglyceride concentration of at least 150 mg/dL. HS was associated with recent US features of "bright liver", with or without aminotransferase increase of unknown origin, in the absence of other chronic liver disease. Women were classified as being menopausal if they had not menstruated in the 12 months before examination. IR status was determined by the HOmeostatic Metabolic Assessment (HOMA), which was assessed using the formula: fasting insulin ( $\mu\text{U/mL}$ )  $\times$  fasting glucose (mg/dL)/405<sup>[26]</sup>. As a stringent measure of IR, a value of HOMA  $> 2$  was introduced.

### Ultrasound evaluation

Sonographic measurements were performed using an ESAOTE *Technos* (Genoa, Italy). Briefly, spleen longitudinal diameter (SLD) was measured by postero-lateral scanning. The maximum length and the cranio-caudal were measured and then averaged. All the indices were measured thrice directly from the frozen images using an electronic caliper. The classification of "bright liver" or HS was based on the following scale of hyperechogenicity: 0 = absent, 1 = light, 2 = moderate, 3 = severe, pointing out the difference between the densities of the liver and the right kidney<sup>[27]</sup>.

### Blood pressure measurements

The systolic/diastolic blood pressure (SBP, DBP) was the average of three consecutive readings taken by the physician during the day, during usual practice hours, and after subjects had rested for five minutes in the sitting position.

### Laboratory data

Serum triglycerides, HDL, basal insulin, ALT, and UA were measured using standard in-house procedures. Hs-CRP was determined using an ELISA Kit, human CRP by BioSupply, UK with a sensibility of 0.03 mg/L and a range of normal values of 0.25-1.5 mg/L.

### Statistical analysis

HDL data, derived from a normally distributed population [Shapiro-Wilk test (S-W),  $P = 0.481$ ], are expressed as mean plus SD. Variables not normally distributed, such as age (S-W,  $P = 0.000$ ), BMI (S-W,  $P = 0.005$ ), SLD (S-W,  $P = 0.002$ ), WC (S-W,  $P = 0.002$ ), HOMA (S-W,  $P = 0.001$ ), DBP (S-W,  $P = 0.001$ ), SBP (S-W,  $P = 0.001$ ), triglycerides (S-W,  $P = 0.003$ ), UA (S-W,  $P = 0.001$ ), hs-CRP (S-W,  $P = 0.001$ ), and ALT (S-W,  $P = 0.001$ ), are expressed as median (range). HS grades were considered ordinals and managed in the same way. The difference

in medians was assessed by the Mann-Whitney test for independent samples. The difference in means of HDL was evaluated by the Two-Sample  $t$  test. The Two-Way Tables cross-tabulated one categorical row variable with one categorical column variable and the significance was set by the Pearson  $\chi^2$  test. Frequencies with a small number of the expected values were evaluated by Fisher's exact test. When cross-tabulation was stratified for another dichotomous variable, the Mantel-Haenszel  $\chi^2$  test was carried out. Tracking the degree of association between single parameters, i.e. HS scores and triglycerides levels, Spearman's rho for non uniform intervals was used. The Pearson's coefficient ( $r$ ) was employed to analyze the correlation between HOMA and HDL. When confronted with the question of how accurate a parameter was in identifying the presence of IR, the discrimination with relative cut-off was evaluated using receiver operating characteristic curve (ROC) analysis, graphically expressed as area under the ROC (AUROC). Sensitivity (true positive rate) and specificity (true negative rate) were also weighted for the same purpose. Optimal cut-off was considered the threshold value with the best specificity/sensitivity. To predict the presence of IR, the logistic regression (Enter Method), with relative odds ratios and 95% confidence intervals (CI), was employed utilizing as independent variables, US values for SLD and HS, BMI and WC measurements, blood pressure determinations and UA, HDL, and triglycerides data. To assess the independent effect of a quantitative variable, i.e. HOMA, on the prediction of spleen size (SLD) values and SBP/DBP determinations, linear regression analysis (least squares) was used, evaluating the standardized coefficient beta ( $\beta$ ). The same tool was used to verify the connection between IR severity and anthropometric measures. Factor analysis was applied to detect the structure in the relationships among variables selecting a subset of variables, which have the highest correlations with the principal component factors. The Cattell's Scree plot, with relative eigenvalues, was performed to screen the real factors. Extraction of the main components amounted to a variance maximizing (varimax) rotation of the original variable space. The critical value was calculated by the formula: doubling the Pearson's correlation coefficient for 1% level of significance (5.152)/square root of subjects minus 2, i.e. 0.576. The concordance correlation coefficient ( $\rho_c$ ), which measures precision and accuracy, was adopted to evaluate the degree of pair observations at US. Statistical analysis was performed operating on Systat 12 (Richmond, CA, USA) and MedCalc Version 10.4.8<sup>®</sup> (Frank Schoonjans) software packages.

## RESULTS

In order to allow readers to gauge the internal validity of this study, we determined the minimum required sample size, with a type 1 error of 0.01 and type 2 error of 0.01 when dividing the population in two groups using HOMA (difference of means = 1.8, SD of non-IR females = 0.4 and SD of IR females = 1.8), which was calculated in 26 subjects. In addition, to assess how well

**Table 1** Anthropometric, clinical, laboratory and ultrasound data of the study population

Variables [females (82)]	Median (Range) mean $\pm$ SD		P-value
	IR present (52)	IR absent (30)	
BMI (kg/m <sup>2</sup> )	33 (25-53.7)	32 (25.6-39.7)	0.78
Overweight (n)	7	6	0.6
Obesity 1 <sup>o</sup> grade (n)	23	15	0.8
Obesity 2 <sup>o</sup> (n)	12	9	0.7
Obesity 3 <sup>o</sup> (n)	10	0	0.01 <sup>a</sup>
HS score at US	1 (0-3)	2 (0-3)	0.005
DBP (mmHg)	90 (65-110)	80 (60-110)	0.03
SBP (mmHg)	120 (100-170)	135 (110-180)	0.0008
Triglycerides (mg/dL)	141 (25-386)	113 (41-249)	0.11
HDL (mg/dL)	48.2 $\pm$ 8.6	50.1 $\pm$ 10.1	0.36
Uric acid (mg/dL)	4.3 (2.7-8.2)	4.4 (2.7-7.6)	0.6
ALT (U/L)	22 (11-117)	22 (12-53)	0.9
hs-CRP (mg/L)	2.4 (0.2-11.7)	1.6 (0.3-6.2)	0.71
SLD at US (mm)	115 (92-150)	102 (90-127)	0.0001
MS (n)	18	6	0.0001
Menopause (n)	20	11	0.9

<sup>a</sup>Fisher's exact test. IR: Insulin resistance; BMI: Body mass index; HS: Hepatic steatosis at US; US: Ultrasonography; DBP: Diastolic blood pressure; SBP: Systolic blood pressure; HDL: High-density lipoprotein-cholesterol; ALT: Alanine aminotransferase; hs-CRP: High sensibility C-reactive protein; SLD: Spleen longitudinal diameter at US; MS: Metabolic syndrome; n: No. of subjects.

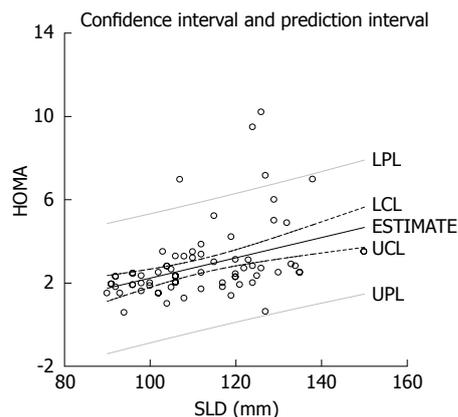
the study findings applied to the patients (external validity) the eighty two subjects divided into two cohorts of 52 and 30, were well balanced for BMI ( $P = 0.78$ ), WC ( $P = 0.45$ ) and age ( $P = 0.79$ ), but different for HOMA ( $P < 0.001$ ), and were studied in a cross-sectional manner.

### Prevalence

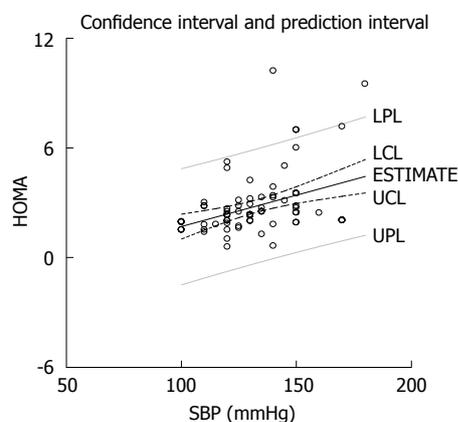
A variable grade of visceral obesity was determined in all subjects with the exception of three (two without IR). Only obesity of the severest grade was represented more in the patients with IR than in the non-IR patients ( $P = 0.01$ ). HS, revealed at US, was more pronounced in subjects with IR than in those without IR ( $P = 0.005$ ). MS was more frequent in IR patients than in non-IR patients. These figures were not modified when adjusted for menopause (Mantel-Haenszel  $\chi^2$  test,  $P = 0.001$ ). The same physiological status was unable to distinguish females with IR from those without IR. No difference in ALT activity was detected between the two cohorts on the basis of IR. The two groups demonstrated no resemblance when analyzing SLD, HS and blood pressure, with raised and significant values in the IR group (Table 1).

### Association & prediction

Anthropometric measurements in the study females showed a similar pattern of reliably predicting IR severity. In fact, the  $\beta$  values of WC and BMI in predicting HOMA were 0.295,  $P = 0.007$  and 0.41,  $P = 0.0001$ , respectively. HDL levels were not associated with HOMA values ( $r = 0.8$ ,  $P = 0.5$ ). SLD measures were well predicted by BMI values,  $\beta = 0.35$ ,  $P = 0.01$ , and by HOMA,  $\beta = 0.41$ ,  $P = 0.0001$  (Figure 1). SBP determinations were predicted by HOMA values,  $\beta = 0.39$ ,  $P = 0.0001$  (Figure 2). HOMA



**Figure 1** Prediction of spleen size by insulin resistance severity. SLD: Spleen longitudinal diameter at ultrasonography (US); HOMA: HOmeostatic metabolic assessment for insulin resistance; LPL: Lower prediction limit; LCL: Lower confidence limit; UCL: Upper confidence limit; UPL: Upper prediction limit.



**Figure 2** Prediction of systolic blood pressure by insulin resistance severity. SBP: Systolic blood pressure.

and HS scores at US were highly associated ( $\rho = 0.34$ ,  $P = 0.002$ ). In IR patients, the grade of HS severity was not correlated with serum triglycerides levels ( $\rho = 0.245$ ,  $P = 0.08$ ). IR patients were almost twice as likely to have HS as those who did not have IR. Further predictions are shown in Table 2.

The complex of WC, HOMA and SLD as well as age, HS and SBP had the highest correlations among the variables, contributing to the loading of the principal components (factor 1 and 2). Triglyceride concentrations (loading factor 3) seemed to play an isolated role (Figure 3).

### Accuracy

Among the MS criteria, blood pressure was very accurate in identifying the presence of IR (AUROC for SBP 0.66, cut-off 125 mm of Hg, sensibility 64%, specificity 75%; AUROC for DBP 0.70, cut-off 85 mm of Hg, sensibility 54.5%, specificity 75%).

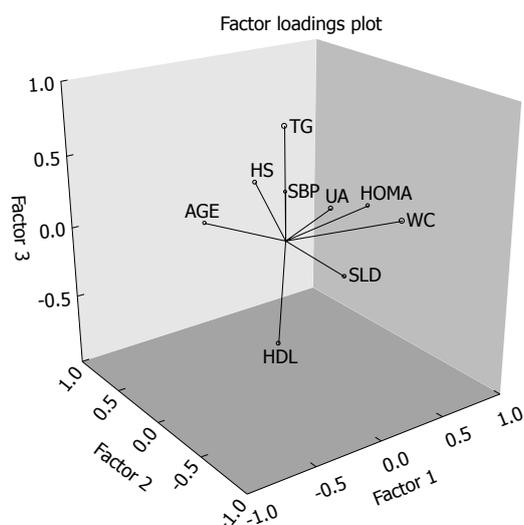
### Precision

The concordance correlation coefficient to evaluate the degree of intra-operator pair observations at US was high,  $\rho_c = 0.91$ .

**Table 2 Estimates in predicting insulin resistance**

Parameter	OR	Lower	Upper	P-value
SLD	1.073	1.027	1.121	0.002
SBP	1.045	1.015	1.075	0.003
DBP	1.046	1.003	1.090	0.036
HDL	1.022	0.974	1.072	0.378
TG	1.006	0.998	1.014	0.143
UA	1.047	0.676	1.623	0.837
BMI	1.083	0.989	1.187	0.086
WC	1.029	0.991	1.069	0.131
HS	1.971	1.234	3.147	0.004

TG: Triglycerides; UA: Uric acid; WC: Waist circumference.



Factor	1	2	3
WC	0.603	-0.424	0.132
AGE	-0.096	0.810	-0.120
HOMA	0.817	0.163	0.005
HDL	0.029	0.118	-0.804
TG	0.163	0.236	0.679
UA	0.440	0.096	0.091
SBP	0.488	0.669	0.026
HS	0.168	0.580	0.192
SLD	0.594	0.126	-0.461

**Figure 3 Unravelling the hidden correlations.** Total variance explained by factor: 1 = 20.9%; 2 = 19.36%; WC: Waist circumference; HOMA: HOmeostatic metabolic assessment for insulin resistance; HDL: High-density lipoprotein-cholesterol; TG: Triglycerides; UA: Uric acid; SBP: Systolic blood pressure; HS: Hepatic steatosis at US; SLD: Spleen longitudinal diameter at ultrasonography (US).

## DISCUSSION

Authorities claim that (1) every subject with a body weight above the established criteria should be treated, stressing this point even to the extent of suggesting that just brief physician-nutritionist support by telephone could be useful in a busy primary care office<sup>[28]</sup>, (2) substantial numbers of overweight/obese individuals remain insulin-sensitive<sup>[29]</sup>. Consequently, the question we pose is: beyond IR, might some other indicators play a role in identifying novel targets, i.e. typology of O/O patients and timing of interventions<sup>[30]</sup>? Accordingly, we propose parameters which may be worth investigating when dealing with the phenotypically O/O and when it is neces-

sary to decide who the metabolically normal subjects are.

The body of pertinent knowledge based on data from the Third National Health and Nutrition Examination Survey testifies that each of the five components of MS, with the possible exception of obesity, independently predicts CVD<sup>[31]</sup>. In contrast, abdominal obesity, measured by WC, is a more accurate predictor of CVD risk than general obesity measured by BMI<sup>[32]</sup>, even though, for a given BMI, women have more subcutaneous adipose tissue and insulin-sensitivity than men<sup>[33]</sup>. However, controversy exists over the type of blood pressure increase. Previous results showed that SBP represents the most prevalent type in obese women<sup>[34]</sup>, being a stronger predictor of CVD than DBP<sup>[35]</sup>. Indeed, these data are challenged by more recent research based on longitudinal studies<sup>[36]</sup>. In addition, according to some other investigators, both blood pressure and plasma aldosterone correlate better with IR in men than in women<sup>[37]</sup>, whereas, there is widespread agreement that NAFLD predicts CVD<sup>[38]</sup>.

In the present study, it can be seen that our data in O/O females supports an association only between the more severe forms of adiposity and the highest grades of IR. Accordingly, MS was characterized by different values in the two groups with and without IR, and HOMA was correlated with severity of HS and to spleen size. The HS score was higher in IR patients, as was another index (hs-CRP), suggesting a possible link between inflammatory status and IR. These findings are consistent with the concept that a partially “benign” obesity, characterized by the absence of IR, lack of HS, or at most the presence of a “light” form of HS, low blood pressure, low expression of a marker of inflammation (SLD) exist in this particular population, at least during the course of this study.

When discussing possible mechanisms for our findings, we pinpoint the following with regard to the liver-spleen axis: (1) peripheral IR determines increased hepatic synthesis of free fatty acids (FFAs) and decreased synthesis of apolipoprotein B, both leading to HS; (2) TNF- $\alpha$ , through JNK-dependent IRS-1 Ser307 phosphorylation, contributes to IR and is responsible for the induction of IL-6 in the liver, both of which aggravate triglyceride accumulation; (3) the overproduction of IL-6, which has “*per se*” a source in visceral and subcutaneous fat, could represent the cause of spleen enlargement in patients with HS *via* uncoupling protein 2<sup>[39]</sup>; (4) a further origin of the increased splenic volume may be found in the accumulation of activated macrophages inside this organ *via* monocyte chemoattractant protein-1 which is over-expressed in adipose tissue<sup>[40]</sup>; (5) once infiltrated, these macrophages produce a large amount of TNF- $\alpha$  which increases the release of FFAs, resulting in HS. With regard to blood pressure, O/O subjects not only exhibit impaired endothelial function in small arteries but also increased arterial stiffness<sup>[41]</sup>. However, IR is associated with greater cardiac reactivity in young women<sup>[42]</sup>. Finally, a recent study performed on a large population showed that patients with HS have increased intima media thickness (IMT<sup>[43]</sup>).

The limitations and strengths of our study deserve

comment. Concerning the limitations, to verify CVD risk, we should have studied a US parameter marker of early atherosclerosis, e.g. IMT in our population, however, this index, as previously emphasized<sup>[43]</sup>, is correlated with HS grade. A further drawback may have been the lack of liver biopsies to assess the severity of NAFLD, even though the canonical difference between FL and NASH has been challenged recently<sup>[44]</sup>. Finally, we did not determine serum levels of folate which are related to elevated homocysteine or blood pressure. In relation to blood pressure, our data showed a new emerging trend, i.e. rather than focusing primarily on body weight, WC, or BMI as a health indicator, a number of scientists have suggested the use of a measure called Metabolic Fitness (MF). Authors define MF as “the absence of biochemical risk factors associated with obesity or elevated blood pressure”. These measures are much better indicators of chronic disease risk, and reductions in these risks factors are not always dependent on weight loss<sup>[45]</sup>.

Most people, who are unhappy with their weight, try to lose weight by changing unhealthy practices. This attitude could result in a dramatic increase in unrealistic goals. On the other hand, some lines of research support the notion that a lifestyle-modification program can reduce the risk of O/O-related comorbid conditions despite minimal or no weight loss. What is more, studies investigating weight loss have methodological limitations that restrict the applicability of findings to O/O patients assessed in clinical practice<sup>[46]</sup>. When we add all this evidence up, it does raise the question of what will our strategy in the near-to-medium term look like. This debate is still ongoing and is mainly related to health-related quality of life<sup>[47]</sup> and the not always favorable balance between costs and treatment outcomes.

## COMMENTS

### Background

The incidence of Overweight/Obesity in the last few years has dramatically increased in highly developed countries. There has also been a simultaneous rise in the frequency of metabolic syndrome. Being overweight causes similar health problems to that of obesity but to a lesser degree. The risk of death from all causes increases throughout the range of moderate and severe increases in body weight for both men and women in all age groups. Unless reversed, this trend predicts an epidemic of cardiovascular disease.

### Research frontiers

It has recently been postulated that “metabolically benign obesity” exists, and is not accompanied by insulin resistance and early atherosclerosis. We sought to identify which parameters could distinguish between healthy and non healthy overweight/obese individuals.

### Innovations and breakthroughs

We claim that “hepatocytes are the last cells to be involved in the progressive chain of fat accumulation and probably the first cells to tell us that something is wrong”. If our proposed parameters are to be adopted more widely, they must be validated in a range of settings and different populations; until then, the most important message is that researchers should identify tools that suit clinicians in their clinical setting, allowing them to screen appropriate cases, in order to improve identification of patients who need to lose weight.

### Applications

Interventions at home, in the office, and in the community are required to empower adults to increase physical activity and to modify eating habits. Nevertheless, before these intervention strategies are set up, it is mandatory to establish exactly which individuals should be treated and when.

### Terminology

The main outcome measures in this study are extremely common, i.e. waist circumference, serum uric acid, high-density lipoprotein-cholesterol and triglycerides, alanine aminotransferase, blood pressure and the two imaging parameters, hepatic steatosis and spleen longitudinal diameter.

### Peer review

This is quite an interesting article. The manuscript reports the studies on the use of simpler parameters in assessing the need for medical intervention with respect to healthy and non healthy overweight/obese individuals. It is suggested that adoption of simpler to perform measurements could not only reduce the cost of medical care but also provide more reliable identification of patients in need of weight loss.

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BRIEF ARTICLE

## Outcome of nonerosive gastro-esophageal reflux disease patients with pathological acid exposure

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### Abstract

**AIM:** To assess the management and outcome of nonerosive gastro-esophageal reflux disease (NERD) patients who were identified retrospectively, after a 5-year follow-up.

**METHODS:** We included patients with gastro-esophageal reflux disease (GERD) symptoms who had a negative endoscopy result and pathological 24-h esophageal pH-monitoring while off therapy. We interviewed them after an average period of 5 years (range 3.5-7 years) by means of a structured questionnaire to assess presence of GERD symptoms, related therapy, updated endoscopic data and other features. We assessed predictors of esophagitis development by means of univariate and multivariate statistical analysis.

**RESULTS:** 260 patients (137 women) were included. Predominant GERD symptoms were heartburn and

regurgitation in 103/260 (40%). 70% received a maintenance treatment, which was proton pump inhibitor (PPI) in 55% of cases. An average number of 1.5 symptomatic relapses per patient/year of follow-up were observed. A progression to erosive gastro-esophageal reflux disease (ERD) was found in 58/193 (30.0%) of patients undergoing repeat endoscopy; 72% of these were Los Angeles grade A-B.

**CONCLUSION:** This study shows that progression to ERD occurs in about 5% of NERD cases per year, despite therapy. Only two factors consistently and independently influence progression: smoking and absence of PPI therapy.

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**Key words:** Esophagus; Nonerosive gastro-esophageal reflux disease; Gastro-esophageal reflux disease; Epidemiology; Acidity

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### INTRODUCTION

Evaluating the natural history of gastro-esophageal reflux disease (GERD) is useful for a number of reasons, as this knowledge may help to: (1) discern the percentage of the population that will progress from non-erosive to erosive disease with its complications, such as stricture, Barrett's oesophagus, and esophageal adenocarcinoma, or from exclusively esophageal to extraesophageal manifestations; (2) define, assess and validate predictivity of risk factors for such disease complications; (3) determine if medical, surgical or endoscopic therapies are able to positively modify the

natural course of the disease; and (4) determine the need for maintenance therapy to prevent complications and persistent symptoms.

Others<sup>[1,2]</sup> have pointed out that many factors make it difficult to study the natural history of GERD, notably the evolving definition of the disease and the lack of a diagnostic standard. As a consequence, few studies in the literature have addressed the issue of defining the natural history of erosive GERD, and even less that of nonerosive gastro-esophageal reflux disease (NERD) or extraesophageal GERD and complications.

Until recently, patients with endoscopic-negative reflux disease were considered to suffer from a milder disease<sup>[3]</sup>, i.e. requiring less intensive/prolonged treatment and possibly characterized by a better long-term prognosis. This concept was subsequently proven to be incorrect; as an example, the impairment in disease-related quality of life appears to be similar in GERD patients with or without endoscopic esophagitis and is related in both instances to symptom severity<sup>[4]</sup>. Also, the symptomatic acute response to proton pump inhibitor (PPI) drugs in patients with or without endoscopic mucosal damage may be worse in NERD<sup>[5,6]</sup>. Finally, after discontinuation of acute treatment, symptomatic relapse within 6 mo appears to affect a similarly high proportion of both GERD groups<sup>[7]</sup>.

Up till now, however, it has not been entirely clear whether and in what proportion GERD patients with symptomatic syndrome but without mucosal damage<sup>[8]</sup> may progress to disease with mucosal damage.

The aim of this study was to assess the outcome of NERD patients with pathological acid reflux at entry after a 5-year follow-up, in three Italian tertiary care centres. Additionally, we investigated which factors are related to development of esophagitis.

## MATERIALS AND METHODS

### Subjects

We retrospectively identified all patients referred to our outpatient departments during the period 1996-2003 for investigation of gastroesophageal (GER) disease symptoms. Subjects were included in the study provided that (1) they were off PPI therapy for at least the preceding 7 d, (2) had a negative upper gastrointestinal (GI) endoscopy, (3) had a 24-h esophageal pH-monitoring result showing pathological GER, i.e. a 24-h esophageal acid exposure greater than 4.4%, corresponding to the 95th percentile of normal Italian subjects and (4) were able to accept a telephone interview<sup>[9]</sup>. We interviewed the subjects after an average period of 5 years (range 3.5-7 years) by means of a structured questionnaire, already used in a previous study<sup>[10]</sup>, to assess presence and type of GERD symptoms (i.e. typical: heartburn or regurgitation, and atypical, i.e. chronic cough, pharyngodynia, hoarseness, chest pain), antireflux drug therapy (presence and type), updated endoscopic data and other features regarding their present clinical status. The questionnaire allowed the patient to identify the most

**Table 1** Clinical and demographic characteristics of patient population (mean  $\pm$  SD)

M/F	121/139
Age (yr)	49.5 $\pm$ 14
BMI	25.24 $\pm$ 3.7
EAE (%)	
24 h	6.8 $\pm$ 5.4
Upright	5.6 $\pm$ 6.6
Supine	6.8 $\pm$ 7.6
No. of smokers	132
Patients with predominant typical symptoms	103 (39.6%)
Patients with predominant atypical symptoms	142 (54.6%)
Patients with both typical & atypical predominant symptoms	15 (5.7%)

EAE: Esophageal acid exposure; BMI: Body mass index.

bothersome GERD symptom, be it typical or atypical, as the predominant one. Collected data at study entry included demographic characteristics, such as age, gender and body mass index (BMI), the reason for undergoing the examination, pattern of GERD symptoms (Table 1), and the results of index upper GI endoscopy and 24-h esophageal pH-metry. Both investigations were carried out while off PPI therapy.

### Statistical analysis

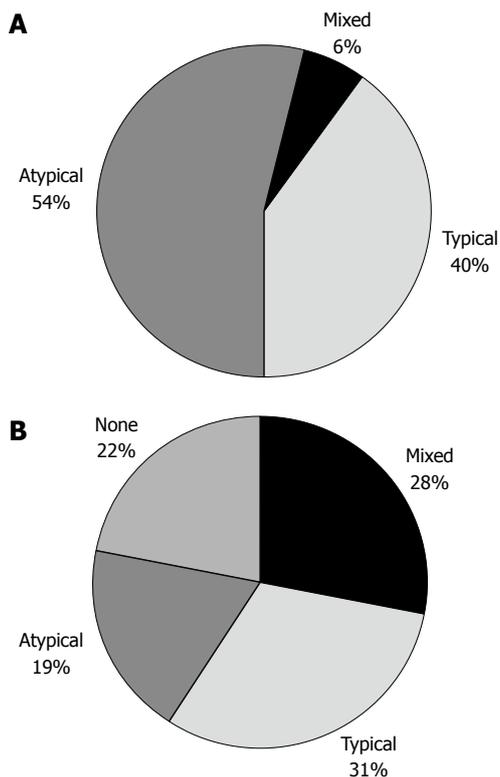
Descriptive statistics consisted of *t* tests for continuous variables and  $\chi^2$  tests for categorical variables. We conducted a univariate logistic regression to evaluate the influence of each risk factor, such as gender, age, BMI, smoking, typical and atypical symptoms, use of therapy, pH-monitoring reflux parameters, on the dependent variable, i.e. development of esophagitis. Significant prognostic factors were then subjected to a multivariate analysis with logistic regression to evaluate the association among the determinants while simultaneously controlling for the effect of other variables. We controlled for several covariates, and only variables with *P* > 0.1 were kept in the model. Statistical significance was defined by a two-sided alpha level of 0.05. A Cox model was ultimately used to construct the survival curves.

All analyses were performed using statistical software (SPSS, Chicago, Illinois, USA).

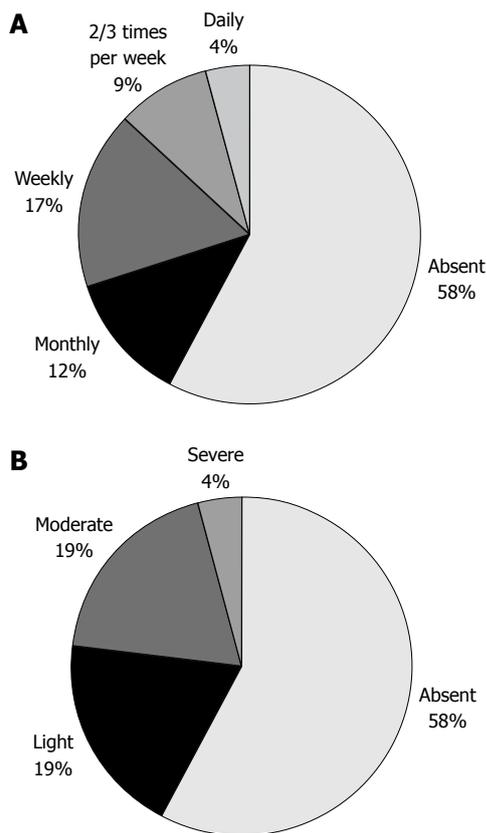
## RESULTS

We were able to identify 995 patients overall who were referred for GERD symptoms. 260 patients (137 women) satisfied the inclusion criteria and were included in the study. Mean age at the time of initial evaluation was 50  $\pm$  14 (SD) years, with a BMI of 25.24  $\pm$  3.72; 50% were smokers. The clinical and demographic characteristics of patients are listed in Table 1.

Predominant GERD symptoms were typical in 103/260 (40%), atypical in 142/260 (54%) and mixed, i.e. similarly dominating the clinical picture, in the remaining 15/260 (6%) (Figure 1A). The mean percentage time with pH < 4 was 7.1%  $\pm$  2.6%. At interview, after a median follow-up time of 5 years, typical symptoms



**Figure 1** Distribution of gastro-esophageal reflux disease (GERD) symptoms at baseline (A) and at follow-up (B).



**Figure 2** Frequency (A) and severity (B) of heartburn at follow-up. Light: Not interfering with day or night activities; Moderate: Interfering with day or night activities, but bearable; Severe: Unbearable.

Risk factor	Odds ratio (95% CI)	P value
Smoking	3.1 (1.6-6.04)	0.0006
Regurgitation > 1/wk	3.5 (1.9-6.6)	0.0001
Severe regurgitation	2.6 (1.35-4.8)	0.005
Nocturnal regurgitation	2.4 (1.2-4.7)	0.01
Supine reflux time > 1.2	5.3 (0.8-38.9)	0.03
Continuing PPI therapy	0.3 (0.1-0.6)	0.001

Note that "continuing PPI therapy" has a negative odds ratio, and can therefore be considered as a protective factor against development of esophagitis. NERD: Nonerosive gastro-esophageal reflux disease; PPI: Proton pump inhibitor.

such as heartburn and regurgitation were still present in 80 patients (31%). The distribution of symptoms at follow-up is presented in Figure 1B.

Frequency and severity of heartburn and regurgitation at follow-up are presented in Figures 2 and 3, respectively. Most patients had received a maintenance treatment during the follow-up period and 181/260 (69.6%) of them were still on therapy; of these, the majority (55%) had been treated with PPIs, 35% had been treated with H2 receptor agonists and the remaining with non-antisecretory agents. Among patients treated with PPIs during the last year (100 subjects), 65% were taking a full dose, i.e. the dose usually used for acute therapy, and the remaining 35% were taking a half dose. In either case, only 44% were using a continuous therapy,

whereas the remaining 48% were using an intermittent one and only 8% used a true on-demand therapy. One hundred and ninety-three out of 260 included patients underwent repeat endoscopy during the follow-up period. An average number of 1.5 symptomatic relapses per patient/year of follow-up were observed with an average of  $1.6 \pm 0.3$  (SD) repeat endoscopies performed during the follow-up. Reason for repeat endoscopy was almost always symptom relapse. Despite active therapy, a progression to erosive gastro-esophageal reflux disease (ERD) was found in 58 patients (30.0%) overall; 72% of these cases were Los Angeles grade A or B.

In the univariate analysis, factors found to be significantly associated with the development of esophagitis were smoking, the presence of severe or nocturnal regurgitation and supine reflux time > 1.2% (but not upright or total reflux time), as well as the absence of continuous use of PPI therapy (Table 2). Interestingly, neither the age of the patient nor the BMI value (and in particular a BMI > 30) were found to influence the development of esophagitis. Table 3 shows the pH-metry parameters and the BMI values at presentation of those patients showing a progression vs. those not showing development of esophagitis.

In multivariate logistic regression analysis, only two factors were found to be independent predictors of development of esophagitis: smoking habit and the use of a continuous PPI therapy (Table 4).

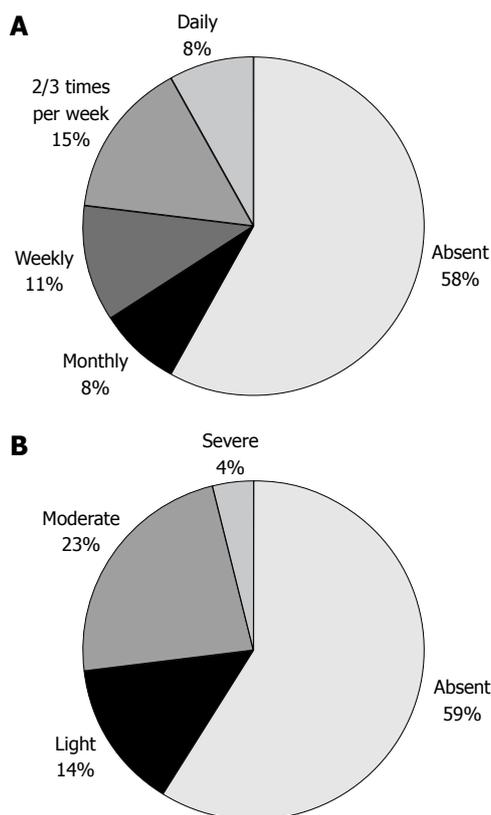


Figure 3 Frequency (A) and severity (B) of regurgitation at follow-up. For explanation of definitions, see legend of Figure 2.

### DISCUSSION

This retrospective study clearly shows that during a relatively long follow-up conducted in a sample of patients with NERD and pathological acid reflux, esophagitis develops in a clinically relevant proportion of patients (roughly one quarter); even in those undergoing a maintenance treatment. Smoking and the lack of a continuous PPI therapy are the only factors independently associated with the development of esophagitis, which, in our study, was predominantly grade A or B according to Los Angeles classification.

Our retrospective study is the largest ever conducted in a population of NERD patients further categorized on the basis of a pathological pH-monitoring.

We preferred to include only patients in whom an abnormal acid exposure could be demonstrated by 24-h esophageal pH monitoring for two main reasons: (1) to replicate the inclusion criteria we used in a previously published paper<sup>[11]</sup> and (2) to deal with a more homogeneous GERD patient population. In fact, as already demonstrated by others, a significant proportion of presumed NERD patients defined on the basis of symptoms alone do not have GERD<sup>[12]</sup>. It has been shown that, on the whole, pH testing is abnormal in about 45% of NERD patients, compared with 75% of patients with erosive esophagitis and 93% of patients with Barrett’s esophagus<sup>[13]</sup>. Thus, our population sample is not representative of the NERD population at large, but reflects a more homogenous subgroup of NERD patients, which can be defined as having a “true” acid-

Table 3 Baseline reflux parameters of pH-metry and BMI in patients showing a progression at follow-up vs those not showing development of esophagitis (mean ± SD)

	% time of esophageal pH < 4			BMI values (kg/m <sup>2</sup> )
	Total	Upright	Supine	
Patients developing esophagitis at follow-up	5.1 ± 1.6	3.2 ± 2.6	6.2 ± 2.4	25.2 ± 3.6
Patients not developing esophagitis at follow-up	7.2 ± 5.6	6.2 ± 7.2	6.9 ± 8.5	25.3 ± 0.5
P value	> 0.5	> 0.5	> 0.5	> 0.5

Table 4 Variables associated with development of esophagitis in NERD patients: results of the multivariate analysis (complete model)

Risk factor	Odds ratio (95% CI)	P value
Smoking	2.9 (1.4-6.2)	0.0055
Ongoing PPI therapy	0.2 (0.09-0.7)	0.04

related NERD. In fact, whereas the majority of NERD patients with abnormal pH test have > 75% of heartburn occurring during an acid reflux event, this happens in only 10% of NERD patients with a negative pH test<sup>[13]</sup>. The particular selection of our patients may be viewed as a limitation as far as the translational capability of the results. On the other hand, the patients have been more precisely defined than solely on the basis of presence of symptoms and negativity of endoscopic features. The natural history of patients such as ours is at present incompletely known; in a previous retrospective study of a small cohort of pH test-positive NERD patients we demonstrated a progression to erosive esophagitis in 5 out of 33 patients treated over a 6 mo period with antacids and/or prokinetics<sup>[11]</sup>. In a subsequent study<sup>[10]</sup>, we extended the observation time of the original patient group up to a median duration of 10 years. We found that almost all the patients we were able to trace (28/29) became affected by GERD symptoms when antisecretory drugs were discontinued, and therefore the majority (75%) were on such a therapy due to GERD symptoms. In addition, esophagitis was found in the vast majority of subjects in whom repeat endoscopy was performed (18/28). Thus, a considerable proportion of the original patient cohort indeed showed a progression from nonerosive to erosive disease within a long enough (> 5 years) time interval.

Other studies, such as that undertaken by Schindlbeck *et al*<sup>[14]</sup> or by McDougall *et al*<sup>[15]</sup>, support the concept that a progression to esophagitis occurs at least in a proportion of NERD patients with a positive pH test at presentation. These results are in keeping with those recently published by Fullard *et al*<sup>[16]</sup>, who performed a systematic review of studies conducted on GERD patients. In their review, they found that the annual progression rate for NERD patients (not further characterized by pH monitoring) ranged between 0% and 30%.

The multivariate analysis in our study found only two factors were independent determinants of a negative outcome, i.e. the development of esophagitis; namely

smoking and the lack of a continuous PPI therapy.

These results seem to indicate that the information obtained with esophageal pH-monitoring in NERD patients is not useful to predict the potential progression of such patients toward erosive esophagitis. This has already been observed in the literature in the past; for example, in our previous study<sup>[11]</sup> conducted on a small and different patient sample, we found that patients who developed endoscopic esophagitis did not have a more severe baseline pattern of GER pH-metrically measured compared with those who did not develop esophageal mucosal damage. Similarly, Cadiot *et al*<sup>[17]</sup>, in a multivariate analysis of factors predicting the development of esophagitis in patients with GERD symptoms, found that three independent factors contributed significantly to differentiating between patients with and without esophagitis; namely basal lower esophageal sphincter pressure, peak acid output and the number of reflux episodes during a 24 h period lasting more than 5 min, but not the total esophageal acid exposure time. Also a stepwise regression analysis, conducted on 66 GERD patients and 16 asymptomatic controls, found that hiatal hernia size and basal lower esophageal sphincter pressure, but not esophageal acid exposure or number of reflux episodes during a 24 h period lasting more than 5 min, significantly predicted presence and severity of esophagitis<sup>[18]</sup>. Moreover, Labenz *et al*<sup>[19]</sup> in a very large prospective study involving more than 6000 GERD patients (The ProGERD Study Initiative) found that no single factor or combination of factors was capable of predicting the presence of esophageal mucosal damage at multivariate analysis. In a case-control study of nearly 5000 patients with and without endoscopic reflux esophagitis investigated with a multivariate regression logistic analysis, Avidan *et al*<sup>[20]</sup> found that the only statistically significant factor associated with development of esophagitis was the presence of hiatal hernia. In both studies, however, no pH esophageal recording was performed. Moreover, all these studies were conducted on a mixed GERD population, e.g. patients with and without erosive esophagitis, and aimed at finding factors capable to discriminate these two categories.

Our study followed a different approach; we followed up a cohort of NERD patients and tried to locate factors potentially related to evolution into erosive esophagitis. The only similar study we were able to find in the literature is a study conducted in Japan, in which 47 patients with symptomatic GERD but without esophagitis (NERD) and 450 control subjects were endoscopically investigated at yearly intervals for 5 years, apparently without receiving any antisecretory therapy during this period<sup>[21]</sup>. The researchers found that among the group of NERD patients, esophagitis developed in 31.9% as compared with 11.3% in the control group ( $P < 0.01$ ), and that the risk of developing esophagitis was significantly related to absence of *Helicobacter pylori* infection ( $P < 0.01$ ), absence of gastric mucosal atrophy ( $P < 0.01$ ), elevated triglycerides during the 5-year follow-up ( $P < 0.05$ ), and an elevated BMI ( $P < 0.05$ ), with a Cox proportional

hazard model<sup>[21]</sup>. This study is however hardly transferable to clinical practice due to exclusion of adequate therapy during the 5-year of follow-up, which in our opinion is an insurmountable ethical drawback of the investigation.

The apparent lack of a relationship between extent of esophageal acid exposure time (and other pathophysiologic parameters of reflux and of esophageal motility) and development of esophagitis has been taken into consideration recently<sup>[22]</sup>, and it has been suggested that the majority of investigations conducted so far have overlooked the resistance of the esophageal mucosa. Although the effect of smoking on GERD symptoms and lesions is not clear-cut, it probably acts on different pathogenetic sites, including mucosal resistance. Smoking may in fact promote GER by several mechanisms including; attenuation of the tone at the lower esophageal sphincter<sup>[23,24]</sup>, a decrease in salivary flow and bicarbonate secretion with a resultant prolongation of acid clearance<sup>[25]</sup>, and a delay in gastric emptying<sup>[26,27]</sup>. Some additional effects of smoking and nicotine that may be relevant to GERD are; an increase in gastric acid and pepsin secretion, augmentation of duodeno-gastric bile reflux and attenuation of the protective mechanisms of the gastric mucosa, such as synthesis of prostaglandins, mucus and epidermal growth factor<sup>[28]</sup>. Finally, in a recent study conducted on a NERD population which included a pathological pH investigation, smoking was found to be an independent predictor of GERD symptoms at multiple linear regression analysis<sup>[29]</sup>.

In conclusion, our study shows that progression to ERD occurs in a considerable proportion of NERD patients (close to 5% per year) despite standard therapy, whereas symptomatic relapse occurs more frequently than once per year. Factors consistently and independently influencing this progression are smoking and the lack of a continuous PPI therapy during the follow-up period.

## COMMENTS

### Background

The natural history of nonerosive gastro-esophageal reflux disease (NERD) is not entirely known. In particular, data are lacking regarding the subgroup of NERD patients with abnormal esophageal pH monitoring. The authors conducted a study to assess the fate of these patients who were followed for a mean of 5 years after the index evaluation.

### Research frontiers

To evaluate by means of a multivariate analysis potential factors leading to a progression of the disease, e.g. the development of mucosal lesions.

### Innovations and breakthroughs

The present study showed that progression to erosive gastro-esophageal reflux disease occurred in about 5% of their NERD patients per year, despite therapy. Only two factors consistently and independently influenced progression: smoking and absence of proton pump inhibitor (PPI) therapy.

### Applications

Due to the clinically relevant number of NERD patients evolving to esophagitis, it is conceivable that the present guidelines for endoscopy in gastro-esophageal reflux disease (GERD) should be changed. In particular, the authors propose the repetition of upper gastrointestinal (GI) endoscopy at least once every 5 years.

### Peer review

The authors have conducted a retrospective study which provides useful information regarding the natural history of nonerosive reflux disease patients and the potential factors leading to esophageal mucosal lesions over 5 years of follow-up.

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BRIEF ARTICLE

## Impact of age-related comorbidity on results of colorectal cancer surgery

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### Abstract

**AIM:** To analyze the correlation between preexisting comorbidity and other clinicopathological features, short-term surgical outcome and long-term survival in elderly patients with colorectal cancer (CRC).

**METHODS:** According to age, 403 patients operated on for CRC in our department were divided into group A (< 70 years old) and group B ( $\geq$  70 years old) and analyzed statistically.

**RESULTS:** Rectal localization prevailed in group A (31.6% vs 19.7%,  $P = 0.027$ ), whereas the percentage of R0 resections was 77% in the two groups. Comorbidity rate was 46.2% and 69.1% for group A and B, respectively ( $P < 0.001$ ), with a huge difference as regards cardiovascular diseases. Overall, postoperative morbidity was 16.9% and 20.8% in group A and B, respectively ( $P = 0.367$ ), whereas mortality was limited to group B (4.5%,  $P = 0.001$ ). In both groups, patients who suffered from postoperative complications had a higher overall comorbidity rate, with preexisting cardiovascular diseases prevailing in group B ( $P = 0.003$ ). Overall 5-year survival rate was significantly better

for group A (75.2% vs 55%,  $P = 0.006$ ), whereas no significant difference was observed considering disease-specific survival (76.3% vs 76.9%,  $P = 0.674$ ).

**CONCLUSION:** In spite of an increase in postoperative mortality and a lower overall long-term survival for patients aged  $\geq$  70 years old, it should be considered that, even in the elderly group, a significant number of patients is alive 5 years after CRC resection.

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**Key words:** Colorectal cancer; Elderly; Post-operative complications; Co-morbidity; Aged

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### INTRODUCTION

Colorectal cancer (CRC) generally is considered a disease of the elderly<sup>[1]</sup> because of its rising incidence with age<sup>[2-6]</sup>. It represents an important cause of morbidity and mortality in the elderly population<sup>[1]</sup>, which mainly undergo emergency surgery because of bowel obstruction or perforation<sup>[1,2,7-9]</sup>. Several authors have reported that elderly patients with CRC can be treated by standard surgical resection and surgery should not be denied on account of chronological age<sup>[1,3,8,10,11]</sup>. Comorbidity, previously different tumors, and poor performance status represent the early and late negative prognostic factors in elderly patients<sup>[1,2,7,10,12-15]</sup>. The purpose of this observational study was to evaluate our experience with CRC, to assess surgical and prognostic features of patients aged  $\geq$  70 years compared with younger patients, with special reference to related comorbidity.

## MATERIALS AND METHODS

### Population under study

Between January 1999 and March 2006, 420 patients with CRC were observed in our department. Among these, seven patients were not operated on because of their poor general status or advanced stage of disease, and 10 patients were lost at follow-up. Thus, 403 patients (229 male and 174 female) with a mean ( $\pm$  SD) age of  $67.2 \pm 11.5$  years (range 23-98 years) were analyzed retrospectively. According to the age at operation, two groups were considered: group A (< 70 years old; 225 patients) and group B ( $\geq$  70 years old; 178 patients).

### Preoperative assessment and staging

Upon admission, all patients were studied with colonoscopy, abdominal ultrasonography and computed tomography (CT) to gain a correct preoperative staging. All comorbidity was noticed and patients underwent clinical and instrumental examinations. Cardiac diseases were assessed as electrocardiographic or echocardiographic abnormalities, or pathology for which the patient was under specific treatment; vascular pathology as cases of hypertension treated with specific drugs; cerebrovascular pathology; pulmonary diseases as abnormal spirometry, or pathology for which the patient was taking medication. Surgical risk was evaluated preoperatively with ASA score by a senior anesthesiologist. Antibiotic and thromboembolic prophylaxis was performed in all patients. The presence of residual tumor (R category) and tumor stage (TNM) were defined on the basis of the criteria established by the Union International Contre Le Cancer (UICC).

### Surgical approach and techniques

The main goal of surgery was the complete removal of the tumour (R0 resection), although palliative resection was performed to treat tumor-related complications. Right and left hemicolectomy, low or very low anterior resection, or transverse colon resection were carried out as standard techniques for CRC, with vessel ligation and respecting oncological criteria. In the event of multiple and synchronous localization, the extent of resection was chosen case by case. Hartmann resection was performed rarely. Definitive loop colostomy or ileostomy was reserved for advanced tumors or patients with very bad performance status in the event of severe bowel obstruction. Temporary covering ileostomy at primary surgery was planned mainly for very low anterior resection for rectal cancer.

### Follow-up and statistical analysis

All clinical and surgical data were collected and stored in a PC database. The data concerning follow-up were collected during outpatient clinical examination and by contacting the family physician. The collected data included demographic and clinical characteristics, co-morbidity (cardiovascular, pulmonary and digestive diseases, diabetes mellitus and other tumors), site of neoplasm (colon or rectum), type of surgery (elective or emergency), presence of residual tumour (R0 or R1-2 resection), histological features, tumour stage (TNM) and Dukes' classification,

as well as postoperative morbidity and mortality. A particular effort was made to analyze comorbidity, postoperative complications and survival rates, to identify specific features of the two groups under study. Cardiovascular diseases included heart disease such as myocardial ischemia or valvular disease, both with hypertension and cerebrovascular disorders; and aortic as well as peripheral arterial disorders were also included. Digestive comorbidity included major gastrointestinal, hepatic or biliary-pancreatic diseases such as inflammatory bowel disease, pancreatitis, hepatic cirrhosis and portal hypertension. We also focused on patients who were affected by tumors other than CRC, including them in the "other tumor" comorbidity group. In assessing postoperative complications and mortality, events that occurred during hospitalization or within 30 d after operation were included in the analysis. Diseases such as pulmonary embolism, myocardial ischemia or acute renal failure, and surgical complications, such as anastomotic leakage or abdominal infection, were considered separately. Statistical differences in clinicopathological characteristics and comorbidity between the groups were assessed by the  $\chi^2$  test for categorical variables and Student's *t* test for continuous variables. Survival curves were calculated according to the Kaplan-Meier model. Survival curves were calculated for the two groups with regard to overall and disease-specific survival; operative mortality was calculated in the analysis. The log-rank test was used to assess the difference between the two groups.  $P < 0.05$  was considered significant.

## RESULTS

### Clinicopathological findings

Clinicopathological features and presence of comorbidity in the two groups are reported in Table 1. Among the 403 patients, we observed 106 tumors that arose from the rectum, 109 from the right colon, 20 from the transverse colon, and 160 from the descending or sigmoid colon. Multiple tumors were observed in eight cases. Rectal tumors prevailed in group A (31.6% *vs* 19.7%,  $P = 0.027$ ); otherwise, topographic distribution of colon cancer was rather homogeneous between the two groups. Elderly patients underwent emergency operation more frequently compared to younger ones, although the difference was not statistically significant (14% *vs* 9.3%,  $P = 0.094$ ). A potentially curative (R0) resection was achieved with a rate of about 77% in both groups ( $P = 0.512$ ). With respect to the Dukes' and TNM classifications, no significant differences were noted between groups A and B. As regards comorbidity, our findings suggest a significant difference between group A and B with an overall rate of 46.2% and 69.1%, respectively ( $P < 0.001$ ). By analyzing different comorbidity in each group, we found that patients aged  $\geq 70$  years had a 51.1% rate of cardiovascular diseases compared to 30.7% in the younger ones ( $P < 0.001$ ). Weak differences were noticed between group A and B with respect to pulmonary diseases ( $P = 0.048$ ) and other tumors ( $P = 0.050$ ). Other comorbidity did not show any significant features in either of the populations studied.

**Table 1** Clinical and pathological data for group A (< 70 years) and B (≥ 70 years) *n* (%)

	Group A ( <i>n</i> = 225)	Group B ( <i>n</i> = 178)	<i>P</i>
Sex			
Males	126 (56)	103 (57.9)	0.392
Females	99 (44)	75 (42.1)	
Localization <sup>1</sup>			
Colon	152 (67.6)	141 (79.2)	0.027
Rectum	71 (31.6)	35 (19.7)	
Type of surgery			
Elective	204 (90.7)	153 (86)	0.094
Emergency	21 (9.3)	25 (14)	
Residual tumor			
R0	174 (77.3)	137 (77)	0.512
R+	51 (22.7)	41 (23)	
Comorbidity			
Overall	104 (46.2)	123 (69.1)	0.0001
Cardiovascular diseases			
Absent	156 (69.3)	87 (48.9)	0.0001
Present	69 (30.7)	91 (51.1)	
Pulmonary diseases			
Absent	217 (96.4)	164 (92.1)	0.048
Present	8 (3.6)	14 (7.9)	
Diabetes mellitus			
Absent	211 (93.8)	160 (89.9)	0.106
Present	14 (6.2)	18 (10.1)	
Gastrointestinal diseases			
Absent	198 (88)	158 (88.8)	0.470
Present	27 (12)	20 (11.2)	
Other tumors			
Absent	216 (96)	163 (91.6)	0.050
Present	9 (4)	15 (8.4)	
Dukes' stage			
A	14 (6.2)	8 (4.5)	0.623
B	96 (42.6)	89 (50)	
C	64 (28.4)	41 (23)	
D	51 (22.7)	40 (22.5)	
T stage			
T1	18 (8)	10 (5.6)	0.485
T2	36 (16)	35 (19.7)	
T3	148 (65.8)	110 (61.8)	
T4	23 (10.2)	23 (12.9)	
N stage			
N0	122 (54.2)	110 (61.8)	0.270
N1	64 (28.4)	45 (25.3)	
N2	39 (17.3)	23 (12.9)	
Metastases			
No	174 (77.3)	138 (77.5)	0.530
Yes	51 (22.7)	40 (22.5)	

<sup>1</sup>Synchronous localizations of colon and rectum are represented in two cases for each group (0.9% vs 1.1%).

### Short-term postoperative results

The distribution of surgical procedures is reported in Table 2. Hartmann resection and palliative stoma were performed rarely, and mostly they were carried out in elderly patients. Concerning the postoperative period, overall morbidity was 16.9% and 20.8% in group A and B, respectively ( $P = 0.367$ ). No significant difference was observed as regards surgical complications, whereas medical adverse events were twofold greater in group B compared to group A (9% vs 4.8%,  $P = 0.122$ ). Among cardiovascular complications, acute heart failure or pulmonary edema was the most common postoperative morbidity. Among surgical complications, the percentage

**Table 2** Surgical procedures and postoperative results *n* (%)

	Group A ( <i>n</i> = 225)	Group B ( <i>n</i> = 178)	<i>P</i>
Postoperative mortality	-	8 (4.5)	0.001
Postoperative complications	38 (16.9)	37 (20.8)	0.367
Surgical complications	27 (12)	21 (11.8)	1
Medical complications	11 (4.9)	16 (9)	0.112
Operation			
Right hemicolectomy	53 (23.6)	61 (34.3)	
Left hemicolectomy	73 (32.4)	50 (28.1)	
Transverse colon resection	5 (2.2)	9 (5.1)	
Abdominoperineal resection	9 (4)	8 (4.5)	
Lower anterior resection	78 (34.7)	43 (24.2)	
Hartmann resection	1 (0.4)	4 (2.2)	
Stoma	2 (0.9)	2 (1.1)	
Miscellaneous	4 (1.8)	1 (0.6)	
Anastomotic leakage rate	10 (4.4)	4 (2.3)	0.282

with anastomosis leakage was 3.5%, with a higher number of cases in group A (4.4% vs 2.3%,  $P = 0.282$ ). Overall postoperative mortality was 2% and it was limited to patients aged ≥ 70 years (4.5%,  $P = 0.001$ ) (Table 2).

### Preexisting comorbidity and short-term results

Table 3 shows the correlation between clinicopathological features, preexisting comorbidity and the occurrence of postoperative complications. The overall comorbidity rate was significantly higher in patients who developed postoperative complications in groups A and B. In group A, male patients experienced complications more frequently than females ( $P = 0.049$ ). In group B, preexisting cardiovascular diseases were significantly associated with postoperative complications ( $P = 0.003$ ). Other features did not influence significantly the postoperative course.

### Survival analysis

The 5-year survival rate for all 403 patients was 62.9%, whereas it was 76.4% for the R0-resected patients. Considering all causes of death, the overall 5-year survival rate was significantly better for group A (75.2% vs 55%,  $P = 0.006$ ) (Figure 1). Conversely, considering disease-specific survival (postoperative and tumor-related deaths only), no difference was observed between groups A and B (76.3% vs 76.9%,  $P = 0.674$ ) (Figure 2).

## DISCUSSION

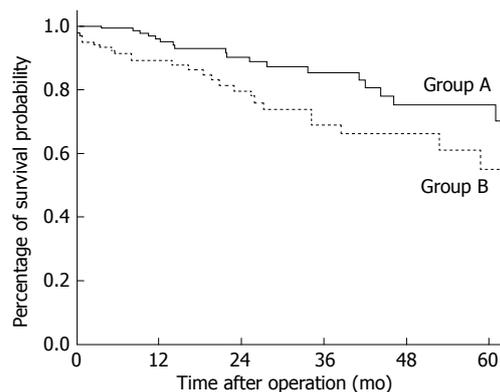
### CRC in the elderly

As for other tumors, the development of CRC is associated with increasing age. The reason for cancer development in the elderly can be explained by a longer duration of exposure to carcinogens, a lower ability to repair damaged DNA, and oncogene amplification or tumor suppressor gene malfunction. Furthermore, the progressive loss of immune surveillance can be considered to occur with aging<sup>[8,16]</sup>. Despite progress in surgical and perioperative care, a lot of physicians evaluate elderly patients by their chronological rather than biological age<sup>[17]</sup>. This attitude may explain why treatment is frequently conditioned in elderly people with malignancies. As a consequence, their therapeutic

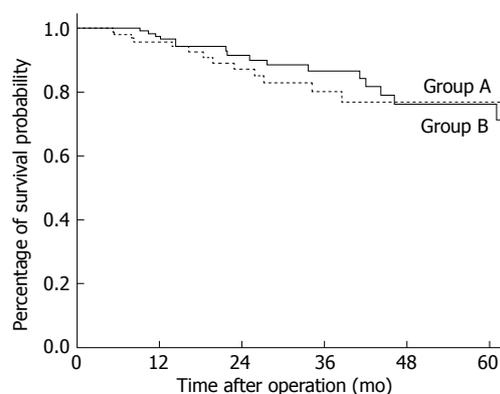
**Table 3** Correlation between clinicopathological features and preexisting comorbidity with postoperative complications in group A (< 70 years) and B (≥ 70 years) *n* (%)

	Postoperative morbidity			
	Group A ( <i>n</i> = 38)	<i>P</i>	Group B ( <i>n</i> = 37)	<i>P</i>
Sex				
Male	27 (71.1)	0.049	23 (62.2)	0.58
Female	11 (28.9)		14 (37.8)	
Localization				
Colon	23 (60.5)	0.344	26 (70.3)	0.171
Rectum	15 (39.5)		11 (29.7)	
Type of surgery				
Elective	32 (84.2)	0.136	30 (81.1)	0.424
Emergency	6 (15.8)		7 (18.9)	
Residual tumor				
R0	27 (71.1)	0.297	29 (78.4)	0.505
R+	11 (28.9)		8 (21.6)	
Comorbidity				
Overall				
Yes	25 (65.8)	0.012	32 (86.5)	0.01
No	13 (34.2)		5 (13.5)	
Cardiovascular diseases				
Absent	22 (57.9)	0.122	10 (27)	0.003
Present	16 (42.1)		27 (73)	
Pulmonary diseases				
Absent	36 (94.7)	0.625	32 (86.5)	0.172
Present	2 (5.3)		5 (13.5)	
Diabetes mellitus				
Absent	34 (89.5)	0.264	34 (91.9)	0.769
Present	4 (10.5)		3 (8.1)	
Gastrointestinal diseases				
Absent	30 (78.9)	0.095	32 (86.5)	0.571
Present	8 (21.1)		5 (13.5)	
Other tumors				
Absent	36 (94.7)	0.65	32 (86.5)	0.314
Present	2 (5.3)		5 (13.5)	
T stage				
T1-T2	5 (13.2)	0.098	12 (32.4)	0.29
T3-T4	33 (86.8)		25 (67.6)	
N stage				
N0	15 (39.5)	0.051	23 (62.2)	0.559
N+	23 (60.5)		14 (37.8)	
Dukes' stage				
A	1 (2.6)	0.385	3 (8.1)	0.669
B	13 (34.2)		17 (45.9)	
C	13 (34.2)		9 (24.3)	
D	11 (28.9)		8 (21.6)	
Metastases				
No	27 (71.1)	0.297	29 (78.4)	0.542
Yes	11 (28.9)		8 (21.6)	

management remains controversial. As a result of their age, elderly people usually suffer from other chronic illnesses in addition to colorectal malignancy. Nonetheless, surgical resection remains the treatment of choice, probably because surgery still represents the mainstay of therapy for CRC, and the most used option to treat bowel obstruction. In a recent study by Lemmens *et al*<sup>[18]</sup>, it has been demonstrated that age > 70 years, a tumor located in the rectum, emergency surgery, and the presence of concomitant chronic obstructive pulmonary disease or deep vein thrombosis increase the risk of developing a surgical complication. On the contrary, CRC patients without comorbidity developed surgical complications in < 30% of the cases.



**Figure 1** Kaplan-Meier estimates of survival probability for R0 population in group A (< 70 yr; 174 pts) and B (≥ 70 yr; 137 pts). Postoperative, tumor-related and -unrelated deaths were included (Log-rank test, *P* = 0.006).



**Figure 2** Kaplan-Meier estimates of disease-specific survival probability for R0 population in group A (< 70 yr; 174 pts) and B (≥ 70 yr; 137 pts). Postoperative and tumor-related deaths were included (Log-rank test, *P* = 0.674).

**Postoperative morbidity**

With reference to our results, we had a comparable postoperative morbidity rate<sup>[19]</sup>, with no statistically significant difference between the two groups. Considering medical complications, elderly patients had a twofold greater, even though not significant, rate of adverse events such as pulmonary embolism and myocardial ischemia. This may be due to a higher prevalence of cardiovascular diseases at the time of cancer diagnosis. Our previous study supports these data that show that cardiac complications are the most frequent in octogenarians affected by gastrointestinal carcinoma<sup>[20,21]</sup>. Based upon our findings, cardiovascular comorbidity has a significant influence on the development of postoperative complications in elderly as well as younger patients. As regards postoperative complications, we observed a low rate of complications with no difference between the two groups. Our data seem to confirm the possibility of achieving a very low percentage of adverse events in aged patients, with curative resection and overall anastomotic leakage rates equally successful in both groups.

**Comorbidity and postoperative complications**

Current literature supports the notion that a higher mortality rate in older patients can be attributed mainly to preexisting comorbid conditions<sup>[10,22]</sup>, such as congestive

heart failure, diabetes mellitus and chronic obstructive pulmonary disease<sup>[23]</sup>. It also emphasizes the role of interactions between disease conditions and CRC, and suggests that multiple comorbidity has a substantial effect on long-term survival. The number of comorbid conditions increases with age and the most frequent diseases are hypertension, other cardiovascular diseases and previous malignancy<sup>[1,12]</sup>. In agreement with other experiences, male patients have a higher prevalence of postoperative complications compared to females<sup>[24]</sup>, and an explanation may be found in the lower number of women suffering from cardiovascular diseases, especially at younger ages. Our data confirmed that elderly patients, in spite of an equal percentage of postoperative complications, have a higher mortality rate compared to younger patients<sup>[7,10,25,26]</sup>. The relationship between preoperative comorbid conditions and postoperative mortality has been demonstrated by previous studies<sup>[10,23,27-30]</sup>. Moreover, we assessed that in most of the eight patients who died, there was more than one comorbid condition. These results suggest that postoperative complications are tolerated poorly by elderly patients, particularly those suffering from comorbidity.

#### Overall and tumor-related survival

In the elderly population with CRC, the relationship between age and long-term outcome after surgery remains not yet completely defined, and the results on survival are still a matter of debate, with alternative conclusions<sup>[3,7]</sup>. Regarding overall survival, it has been demonstrated that it decreases with aging<sup>[1]</sup>. Nonetheless, disease stage influences specific tumor survival independently of age. It has been reported that the decrease in survival is more evident in very old patients, particularly those aged > 85 years. In this regard, our findings clarify that elderly people have a significantly worse overall survival rate compared to younger ones, but, after censoring cancer-unrelated deaths, survival rates become comparable. Thus, strictly considering tumor-related mortality, no difference was observed between the two groups. Other authors have supported our results<sup>[2]</sup> and have emphasized the relationship between early stage of the disease and a similar prognosis in elderly compared to younger patients. In spite of an increase in postoperative mortality and a shorter overall long-term survival for patients aged  $\geq$  70 years, it should be considered that, even among elderly patients, a significant number of patients is alive 5 years after CRC resection.

In conclusion, in agreement with previous studies, our findings suggest that a relevant positive outcome after a potentially curative resection should encourage surgical treatment of elderly patients with CRC. Comorbidity represents a risk factor for developing postoperative complications in younger and older patients. A higher risk of postoperative mortality seems to be a prerogative of elderly patients affected by other comorbid conditions, especially cardiovascular diseases. As a consequence, surgeons should be more cautious when confronted with elderly patients suffering from severe comorbidity. Even if treatment decisions in elderly patients with CRC should be

made on the basis of careful evaluation of cardiovascular and pulmonary parameters, surgical tumor resection clearly is encouraged by high rates of potentially curative resection and satisfactory long-term survival results.

## COMMENTS

### Background

Colorectal cancer (CRC) generally is considered to be a disease of the elderly. Several studies have reported that elderly patients with CRC can be treated by standard surgical resection, although some debate still exists on this topic.

### Research frontiers

The correlation between preexisting comorbidity, clinicopathological features, and short- and long-term outcomes was studied in elderly patients undergoing surgery for CRC.

### Innovations and breakthroughs

A particular effort has been made to analyze preexisting comorbidity, postoperative complications and long-term results, to identify features specific for elderly patients compared to younger ones.

### Applications

In spite of an acceptable increase in postoperative mortality and a shorter overall long-term survival, surgical resection provides encouraging results for patients aged  $\geq$  70 years.

### Peer review

The authors investigated the relationship between preexisting diseases and other clinicopathological features, perioperative surgical outcome, and survival in elderly patients with CRC treated by surgery. The conclusions are definite, and the contents are well-organized and well-written. The results give us some new ideas about the elderly population undergoing surgery for CRC.

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BRIEF ARTICLE

## Effect of ONO-4057 and tacrolimus on ischemia-reperfusion injury of the liver

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### Abstract

**AIM:** To investigate the effects of a novel Leukotriene B<sub>4</sub> receptor antagonist and/or tacrolimus on ischemia-reperfusion in a rat liver model.

**METHODS:** Male Lewis rats were pretreated with ONO-4057 (100 mg/kg) and/or tacrolimus (1 mg/kg) orally, and divided into four experimental groups; group 1 (control), group 2 (ONO-4057), group 3 (tacrolimus), group 4 (ONO-4057 + tacrolimus).

**RESULTS:** There was a tendency for long survival in the groups treated with tacrolimus alone and ONO-4057 plus tacrolimus. Post-reperfusion serum aspartate aminotransferase levels decreased more significantly in ONO-4057 plus tacrolimus group ( $P < 0.01$ ), than in the tacrolimus alone group ( $P < 0.05$ ), compared to controls.

**CONCLUSION:** This study demonstrated that pretreat-

ment with ONO-4057 in combination with tacrolimus produced additive effects in a rat model of liver ischemia-reperfusion injury.

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**Key words:** Ischemia-reperfusion injury; Leukotriene B<sub>4</sub>; Liver; Tacrolimus

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Takeichi T, Uemoto S, Minamiguchi S, Takeyoshi I, Inomata Y, Tanaka K, Kobayashi E. Effect of ONO-4057 and tacrolimus on ischemia-reperfusion injury of the liver. *World J Gastroenterol* 2009; 15(45): 5712-5715 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5712.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5712>

### INTRODUCTION

Neutrophils are mainly responsible for the physiopathological changes that occur after ischemia-reperfusion injury<sup>[1-3]</sup>. These cells are markedly activated when the ischemically damaged liver is transplanted. Experimental efforts have focused on understanding the etiology of ischemic injury and protecting the liver by pharmacological intervention<sup>[4-7]</sup>.

Pretreatment with certain immunosuppressive drugs has been shown to protect against neutrophil-mediated reperfusion injury<sup>[4,5]</sup>. Calcineurin inhibitors are thought to suppress both proinflammatory cytokine production and expression of adhesive molecules of neutrophils<sup>[8]</sup>. Recently, ONO-4057 (5-[2-(2-carboxyethyl)-3-{6-(4-methoxyphenyl)-5E-hexenyl}yphenoxy]valeric acid), an orally administered active leukotriene B<sub>4</sub> (LTB<sub>4</sub>) receptor antagonist, was developed and was shown to have an important effect on LTB<sub>4</sub>-induced neutrophil function<sup>[6,7]</sup>.

In this study, we investigated the effect of pretreatment with ONO-4057 alone and in combination with tacrolimus on ischemia-reperfusion injury of the liver in rats.

### MATERIALS AND METHODS

#### Animals

Male Lewis rats (Charles River, Hamamatsu, Japan),

5-6 wk of age, were used in this study. The animals were housed in a pathogen-free, temperature- and light-controlled environment with free access to food and water. The rats were anesthetized with ether for induction and maintenance. All experimental protocols were approved by the institutional review board of Kyoto University.

### Experimental groups

The animals were divided into four experimental groups: group 1, controls; group 2, ONO-4057; group 3, tacrolimus; and group 4, ONO-4057 and tacrolimus ( $n = 9$  in each group). The control groups received distilled water (DW) and  $\text{NaHCO}_3$  as a vehicle administered orally, and the other groups were pretreated with ONO-4057 (Ono Pharmaceutical Co., Ltd., Osaka, Japan) and tacrolimus (Fujisawa Pharmaceutical Co., Osaka). ONO-4057 was dissolved to a concentration of 30 mg/mL with  $\text{NaHCO}_3$ , and tacrolimus was dissolved to a concentration of 0.3 mg/mL with DW. ONO-4057 was administered orally at a dose of 100 mg/kg and tacrolimus orally at a dose of 1 mg/kg. All agents were administered 1 h before ischemia was induced.

### Ischemia-reperfusion model

**Whole-liver ischemia model:** Normothermic ischemia of the entire liver (with intestinal congestion) was induced as described previously<sup>[8]</sup>. Briefly, a midline incision was made after the animals were anesthetized with ether and the portal vein, hepatic artery, and bile duct were clamped for 40, 50 and 60 min with a vascular micro-clip. The clip was then released and the abdominal wall was closed using a continuous running suture. The animals were returned to their cages for survival analysis for the following 2 wk.

**Partial-liver ischemia model:** To obtain data about more severe normothermic ischemia damage of the liver without intestinal congestion, the left and middle portal vein and hepatic artery were occluded for 60 min according to the procedure of Kawano *et al.*<sup>[9]</sup> with a minor modification. This procedure induced 70% warm-ischemic liver damage, but 30% healthy liver remained to protect the animal's life. After reperfusion, the abdominal wall was closed, and the treated rats were observed. One mL of the peripheral blood were obtained at 3 and 6 h after de-clamping, then the liver was perfused with 10 mL cold saline and immediately taken out. The damaged part of the liver was used for histological assessment and measurement of liver-tissue myeloperoxidase (MPO).

### Serum measurements

Using the peripheral blood obtained from the experimental animals, serum transaminase concentrations [aspartate aminotransferase (AST); alanine aminotransferase (ALT)] were measured by an ultraviolet method using an automatic analyzer (Hitachi 7170, Tokyo, Japan).

### Histological examination

Hepatic samples were fixed in 10% buffered formaldehyde,

Table 1 Survival rates with various pretreatments

Duration of ischemia (min)	Survival rates (%)			
	Controls	ONO-4057	Tacrolimus	ONO-4057 + tacrolimus
40	6/6 (100)	6/6 (100)	NT	NT
50	6/6 (100)	6/6 (100)	NT	NT
60	2/9 (22)	4/9 (44)	6/9 (66)	5/9 (56)

NT: Non-tested.

embedded in paraffin, cut to 3 to 5  $\mu\text{m}$  thick, and stained with hematoxylin and eosin. The specimens were blindly analyzed to evaluate the histological damage according to the modified classification of Suzuki *et al.*<sup>[4]</sup>.

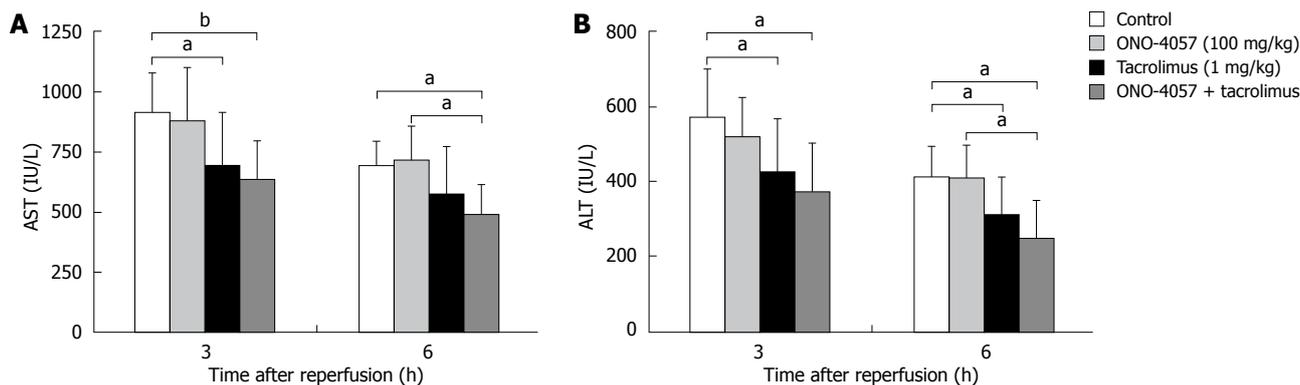
**Liver-tissue MPO activity:** MPO tissue level is considered to be an index of neutrophil infiltration in the liver. Liver samples obtained at 6 h after reperfusion were frozen in liquid nitrogen and stored at  $-70^\circ\text{C}$  until the time of measurement. Tissue samples were then homogenized in 10 mL phosphate-buffered saline and centrifuged at 100 000  $g$  and  $4^\circ\text{C}$  for 30 min. The resultant supernatant was assayed for MPO activity using the modified method of Shindler *et al.*<sup>[10]</sup>. Briefly, the reaction mixture, containing 10:1 of supernatant and 200:1 of 100 mol/L ABTS buffer with  $\text{H}_2\text{O}_2$ , was incubated in 96-well plate for 60 min at  $25^\circ\text{C}$ . The optical density at 414 nm was determined by a plate reader (SPECTRA max<sup>TM</sup> 340; Molecular Devices Co., CA, USA). The MPO activity was calculated using an extinction coefficient of  $(3.6 \times 10^4)/(\text{mol per cm})$  for ABTS and normalized to protein levels ( $\mu\text{mol}/\text{mg protein}$ ). The protein concentration was determined by the method of Lowry *et al.*<sup>[11]</sup>.

### Statistical analysis

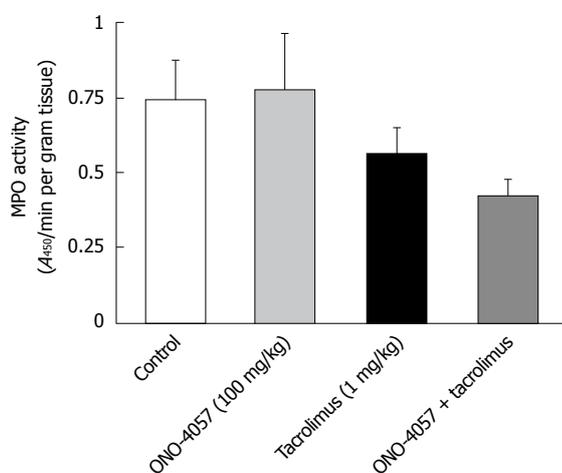
The repeated measurement analysis of variance (ANOVA) was performed among the groups. If repeated measurement ANOVA revealed a significant interaction, the statistical significance among the four groups at each time point was determined using post hoc tests. A  $P$  value of less than 0.05 was considered statistically significant.

## RESULTS

Survival after pretreatment with ONO-4057 and/or tacrolimus on whole-liver ischemia in rats is shown in Table 1. Because 40 to 50 min of whole liver ischemia in adult Lewis rats (about weighting 280-330 g) was sublethal, we initially evaluated the 40 to 50 min ischemic damage in young Lewis rats in this study. However, all rats that had undergone 40 or 50 min of whole-liver ischemia survived indefinitely. When the results of 60 min of ischemia were evaluated, there was no significant difference in survival rate among the groups. However, the survival rate was slightly higher in the groups treated with tacrolimus alone ( $P = 0.053$ ) and ONO-4057 plus tacrolimus. Serum AST values at 3 and 6 h after partial-liver ischemia-reperfusion are shown in Figure 1A. In the group pretreated with tacrolimus alone, the serum



**Figure 1** Serum transaminase levels after reperfusion. A: Aspartate aminotransferase (AST) levels after partial-liver ischemia for 60 min; B: Alanine aminotransferase (ALT) levels after partial-liver ischemia for 60 min. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ .



**Figure 2** Myeloperoxidase (MPO) activity after reperfusion.

AST value was significantly lower than the control group at 3 and 6 h after reperfusion ( $P < 0.05$ ), respectively. In the group pretreated with ONO-4057 plus tacrolimus, the AST level was more significantly lower compared with the control group ( $P < 0.01$ ). Pretreatment with ONO-4057 and tacrolimus produced additive effects, but not when compared with the group treated with tacrolimus alone. ONO-4057 alone could not decrease the transaminase levels. Serum ALT values demonstrated a similar pattern (Figure 1B).

The liver MPO levels after 6 h of reperfusion are shown in Figure 2. There was no significant difference among the groups in MPO activity. However, the MPO level in the group treated with ONO-4057 plus tacrolimus tended to be lower compared with that in the control group, although it was not statistically significant ( $P = 0.059$ ). At 6 h from the reperfusion after 60 min of partial liver ischemia, the liver damage (congestion, vacuolization, and necrosis) that was observed histologically was not significantly different among the groups.

## DISCUSSION

In liver transplantation, ischemia-reperfusion injury is one of the major factors that might influence early graft function and late changes. The importance of

neutrophils in the development of ischemia-reperfusion injury in the liver has been demonstrated<sup>[12,13]</sup>.

LTB<sub>4</sub>, a metabolite formed *via* the 5-lipoxygenase pathway from arachidonic acid, is one of the most potent chemotactic and proinflammatory mediators. LTB<sub>4</sub> causes neutrophil adhesion to vascular endothelial cells and infiltration into vascular endothelial cells. We investigated the effect of the LTB<sub>4</sub> receptor antagonist, ONO-4057, on hepatic ischemia-reperfusion injury to determine whether they affect neutrophil infiltration into the liver and attenuate neutrophil-induced post-ischemic injury. Recent reports have also shown that some immunosuppressive drugs protect or attenuate the neutrophil-mediated ischemia-reperfusion injury in various organs (liver, small intestine, kidney, and heart)<sup>[5,14,15]</sup>. As tacrolimus has variable effects<sup>[16]</sup>, we tested the effect of a combination of ONO-4057 with tacrolimus. The results showed that ischemia-reperfusion injury in the liver was not reduced by pretreatment with ONO-4057 alone. Tacrolimus was necessary for protection from I/R injury. Pretreatment with ONO-4057 had additive effects on the protective function of tacrolimus. Although the I/R damage was significantly prevented with the use of Tac and ONO-4057, the main damage might be related directly to the role of immunosuppressants. The suppression of the elevation of transaminases after I/R in this experiment might be mainly caused by the effect of tacrolimus. The MPO level in the liver specimens and the blood level of the transaminases did not correlate well in this study. MPO levels might be affected sub-significantly by the ONO-4057-mediated modification of neutrophil infiltration, but it did not directly prevent the hepatic damage.

Hepatic ischemia-reperfusion injury is caused by many factors, and this study has limited because it only focused on neutrophils' infiltration. Two main causes have been reported for I/R injury; oxygen free radicals and neutrophil-mediated damage. Recent reports showed a beneficial effect of neutrophil-mediated inhibitors, such as neutrophil elastase inhibitor and tissue factor pathway inhibitor, on hepatic reperfusion injury<sup>[17,18]</sup>. In another study, treatment with leukotriene biosynthesis inhibitor (L664,536) had no effect on hepatic injury, although LTB<sub>4</sub> formation was inhibited<sup>[19]</sup>. We showed the added effect

of a LTB<sub>4</sub> receptor antagonist and immunosuppressive drug. As ONO-4057 has been also reported to have an immunosuppressive effect in allogenic rat liver grafts<sup>[20]</sup>, co-administration of ONO-4057 and tacrolimus might be reasonable for liver transplantation. Before any clinical trials, experiments using the transplantation model might be necessary.

In conclusion, our data showed that pretreatment with ONO-4057 in combination with tacrolimus produced additive effects in liver reperfusion-injury of rat, although treatment with ONO-4057 alone had no effect.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Ischemia-reperfusion injury is a major factor that might influence early graft function in liver transplantation. In this study, we investigated the effect of oral administration, a novel Leukotriene B<sub>4</sub> (LTB<sub>4</sub>) receptor antagonist, and/or Tacrolimus on ischemia-reperfusion of rat liver model.

### Research frontiers

In ischemia-reperfusion injury of the liver, an excessive inflammatory response is considered as a key mechanism. The role of neutrophils in ischemia-reperfusion injury has recently been the focus of several investigations. In this study, we focused the importance of neutrophils.

### Innovations and breakthroughs

We tested the combined effect of a LTB<sub>4</sub> receptor antagonist with Tacrolimus for ischemia-reperfusion injury of the liver.

### Applications

The findings in this study support the hypothesis that co-administration of ONO-4057 and tacrolimus might be reasonable for liver transplantation in the future.

### Peer review

This manuscript is sufficiently written and provides information concerning therapeutic options in I/R injury of the liver.

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BRIEF ARTICLE

## Glutathione-S-transferase (GSTM1, GSTT1) and the risk of gastrointestinal cancer in a Korean population

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### Abstract

**AIM:** To evaluate the association of glutathione S-transferase mu (GSTM1) and glutathione S-transferase theta (GSTT1) null genotypes with the risk of gastric cancer (GC) and colorectal cancer (CRC) in a South Korean population.

**METHODS:** We conducted a population-based, large-scale case-control study including 2213 GCs, 1829 CRCs, and 1699 controls. Null and non-null genotypes

of GSTM1 and GSTT1 were determined using real-time PCR.

**RESULTS:** The null genotypes of GSTM1 and GSTT1 were not significantly associated with elevated risk of gastric (OR = 1.070, 95% CI = 0.935-1.224; OR = 1.101, 95% CI = 0.963-1.259, respectively) or colorectal cancer (OR = 1.065, 95% CI = 0.923-1.228; OR = 1.041, 95% CI = 0.903-1.200, respectively). The frequency of the combined null GST genotype was not different between the two cancer groups and controls. Moreover, smoking, drinking, and age did not modify the association between these genotypes and the risk of gastric or colorectal cancer.

**CONCLUSION:** GSTM1 and GSTT1 null genotypes were not associated with increased risk of GC or CRC in Koreans.

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**Key words:** Glutathione S-transferase mu; Glutathione S-transferase theta; Gastric cancer; Colorectal cancer; South Korean population

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### INTRODUCTION

Gastric cancer (GC) and colorectal cancer (CRC) are the most common malignancies in Korea. Environmental factors, such as diet, infection, and smoking, and genetic factors have been shown to play a role in the development of these malignancies<sup>[1-4]</sup>.

The glutathione S-transferase (GST) enzymes

are involved in detoxification of many potentially carcinogenic compounds. The enzymes are encoded by at least five distantly related gene families (the alpha, mu, pi, sigma, and theta GSTs). In humans, marked interindividual differences exist in the expression of mu (GSTM1), and theta (GSTT1) GSTs<sup>[5]</sup>. The GSTM1 and GSTT1 null genotypes have been linked to increased risk of developing lung, bladder, colon, and skin cancers<sup>[5-7]</sup>, and several studies have shown that GSTM1 and GSTT1 null genotypes were associated with increased risk of GC<sup>[8,9]</sup> and CRC<sup>[10]</sup>. However, some data have suggested that no relationship exists between the GSTM1 or GSTT1 null genotype and the risk of GC<sup>[11,12]</sup> or CRC<sup>[13,14]</sup>. The contradictory results regarding the association between GSTM1 and GSTT1 null genotypes and the risk of GC or CRC may be due to limited sample sizes or differences in the ethnicities or differences in the genetic subtypes studied, or they may also be attributable to differences in exposure to environmental factors. However, the majority of the reports involved small sample sizes, so the association of the GSTM1/GSTT1 null genotype and the risk of GC and CRC need to be confirmed in studies with larger numbers of samples.

The present study aimed to evaluate the association of the GSTM1 and GSTT1 null genotypes with the risk of GC and CRC, and to determine whether smoking, alcohol consumption, and age modify the association between these polymorphisms and GC or CRC risk.

## MATERIALS AND METHODS

### Ethics

This study was approved by the Institutional Review Board of the Chonnam National University Hwasun Hospital in Hwasun, Korea, and all patients provided informed written consent.

### Subjects

The study included 4042 newly diagnosed cancer cases (2213 GC and 1829 CRC) and 1699 controls. The cases were histologically confirmed at the Chonnam National University Hwasun Hospital (Jeollanam-do, Korea), between April 2004 and June 2008. Cases with secondary or recurrent tumors were excluded. The tumor stages were classified according to the TNM classification, including clinical or pathological TNM stages. GC was classified by anatomical site as cardia (C16.0) or non-cardia (C16.1-16.8) and by histological type as intestinal, diffuse, or mixed type.

The control group ( $n = 1699$ ) consisted of participants in the Thyroid Disease Prevalence Study conducted from July 2004 to January 2006 in Yeonggwang and Muan Counties of Jeollanam-do Province and in Namwon City of Jeollabuk-do, Korea<sup>[15]</sup>. At the time of peripheral blood collection, all case and control subjects provided their informed consent to participate in this study.

### Blood samples and DNA isolation

Blood samples were collected in EDTA-containing tubes, and DNA was extracted from the buffy coat for genotyping. Genomic DNA was extracted using a

QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol.

### Genotyping

GSTM1 and GSTT1 genotyping was performed using a TaqMan allelic discrimination assay with previously described primers and modified probes<sup>[16]</sup>. Real-time PCR was performed using a Rotor-Gene 3000 multiplex system (Corbett Research, Sydney, Australia) in a 10- $\mu$ L reaction volume containing 200 nmol/L PCR primer, 10 nmol/L CY5-labeled probe for GSTT1, 100 nmol/L FAM-labeled probe for GSTM1, 0.5 U of *f-Taq* polymerase (Solgent, Daejeon, Korea), and 40 ng of genomic DNA. The primer and probe are as follows: GSTM1, Forward, 5'-GGAGACAGAAGAGGAGAAGATTTC-3', Reverse, 5'-GCCCAGCTGCATATGGTTGT-3', Probe, FAM-CCATGGTCTGGTTCTCCAAAATGTCCA-BHQ1; GSTT1, Forward, 5'-CTTCCAGGAGGCCCATGAG-3' Reverse, 5'-CAGGGCATCAGCTTCTGCTT-3', Probe, CY5-AAGGACTTCCCACCTGCAGACCCC-BHQ3.

### Statistical analysis

Statistical analysis was performed using SPSS for Windows version 17.0. The descriptive data for the major characteristics of study groups are expressed as mean (range) and percent. We used *t*-tests to determine statistical differences in the continuous variables and  $\chi^2$  tests for the categorical variables. Adjusted odds ratios (OR) and their 95% confidence intervals (95% CI) were calculated using logistic regression models with adjustments for age and sex to estimate the association between genotype and GC or CRC. Interactions of genotype with smoking, alcohol consumption, and age were estimated using the logistic regression model, which included an interaction term as well as variables for exposure (smoking and alcohol drinking), genotypes, and potential confounders (sex and age). Subjects with wild-type genotypes were considered to have baseline risk. The subjects for which there was missing data for smoking, drinking, anatomical site, and histological type TNM staging were excluded in interaction analysis related with these variables. All tests were conducted at the  $P = 0.05$  level of significance.

## RESULTS

We included 2213 cases of GC, 1829 cases of CRC, and 1699 cancer-free controls in the present study. The demographic characteristics of subjects are shown in Table 1. The proportion of men in the cancer cases was higher than that in the controls, and cases in both cancer groups tended to be older than controls. The proportion of smoking in GC cases was higher than that in the controls, but in CRC cases was lower than that in the controls. The proportion of drinking in both cancer groups was lower than that in the controls.

The frequency distributions of the GSTM1 null genotype in the control, GC, and CRC groups were 54.3%, 55.4%, and 54.9%, respectively. The frequency distributions of the GSTT1 null genotype in the control, GC, and CRC groups were 50.5%, 53.0%, and 51.9%,

Table 1 Demographic characteristics of subjects *n* (%)

Characteristics	Controls	GC	CRC
No.	1699	2213	1829
Age, mean (yr, range)	52.2 (20-74)	60.2 (21-87) <sup>a</sup>	61.9 (22-88) <sup>c</sup>
≤ 65 yr	1320 (77.7)	1314 (59.4)	985 (53.9)
> 65 yr	379 (22.3)	899 (40.6) <sup>a</sup>	844 (46.1) <sup>c</sup>
Sex			
Male	820 (48.3)	1510 (68.2)	1149 (62.8)
Female	879 (51.7)	703 (31.8) <sup>a</sup>	680 (37.2) <sup>c</sup>
Smoking			
Never-smokers	1000 (58.8)	1127 (50.9)	1137 (62.2)
Ever-smokers	654 (38.5)	997 (45.1) <sup>a</sup>	582 (31.8) <sup>c</sup>
Missing	45 (2.6)	89 (4.0)	110 (6.0)
Drinking			
Never drinkers	825 (48.6)	1198 (54.1)	1084 (59.3)
Current drinkers	832 (49.0)	921 (41.6) <sup>a</sup>	623 (34.1) <sup>c</sup>
Missing	42 (2.5)	94 (4.3)	122 (6.7)
GSTT1			
Null type	858 (50.5)	1172 (53.0)	950 (51.9)
Wild type	841 (49.5)	1041 (47.0)	879 (48.1)
GSTM1			
Null type	923 (54.3)	1225 (55.4)	1004 (54.9)
Wild type	776 (45.7)	988 (44.6)	825 (45.1)
TNM stage			
I		1138 (51.4)	291 (15.9)
II		305 (13.8)	547 (29.9)
III		290 (13.1)	615 (33.6)
IV		386 (17.4)	230 (12.6)
Unspecified stage		94 (4.2)	147 (8.0)
Tumor site			
Cardiac		106 (4.8)	833 (45.5) (colon)
Non-cardiac		2093 (94.6)	996 (54.5) (rectum)
Unspecified site		14 (0.6)	
Histological type			
Intestinal		1286 (58.1)	
Diffuse		561 (25.4)	
Mixed		240 (10.8)	
Unspecified type		126 (5.7)	

<sup>a</sup>GC compared with control,  $P < 0.05$ ; <sup>c</sup>CRC compared with control,  $P < 0.05$ . GC: Gastric cancer; CRC: Colorectal cancer; GSTT1: Glutathione S-transferase theta; GSTM1: Glutathione S-transferase mu.

respectively. No significant differences were observed in the frequencies of the GSTT1 and GSTM1 genotypes between cancer patients and controls (Table 1).

The GSTM1 and GSTT1 null genotypes were not significantly associated with risk of GC (OR = 1.070, 95% CI = 0.935-1.224; OR = 1.101, 95% CI = 0.963-1.259, respectively) or CRC (OR = 1.065, 95% CI = 0.923-1.228; OR = 1.041, 95% CI = 0.903-1.200, respectively). The GSTM1 and GSTT1 null genotypes were not significantly associated with the risk of GC or CRC, classified according to TNM stage, tumor site or histology type (GC) (Tables 2 and 3).

Smoking, alcohol consumption and age did not modify the association between the GSTM1 and GSTT1 null genotypes and the risk of GC or CRC (Tables 2 and 3).

No difference in the frequency of the combined GSTM1 and GSTT1 null genotype was observed between the two cancer groups and controls (Table 4).

## DISCUSSION

The present large-scale study investigated the association

Table 2 GST (M1, T1) genotypes in relation of GC risk stratified by some factors

	GSTM1 (null)		GSTT1 (null)	
	OR (95% CI)	<i>P</i> <sup>a</sup>	OR (95% CI)	<i>P</i> <sup>a</sup>
All case	1.070 (0.935-1.224)		1.101 (0.963-1.259)	
Smoking				
Never smoker	0.994 (0.809-1.222)		1.099 (0.895-1.350)	
Ever smoker	1.127 (0.938-1.355)	0.385	1.082 (0.901-1.299)	0.921
Drinking				
Never drinkers	1.103 (0.916-1.328)		1.116 (0.927-1.344)	
Current drinkers	1.019 (0.829-1.253)	0.559	1.077 (0.877-1.322)	0.748
Age (yr)				
≤ 65 yr	1.069 (0.910-1.256)		1.129 (0.961-1.325)	
> 65 yr	1.053 (0.823-1.348)	0.990	1.056 (0.825-1.352)	0.621
TNM stage				
I + II	1.045 (0.900-1.214)		1.130 (0.974-1.312)	
III+IV	1.050 (0.870-1.267)		1.044 (0.865-1.259)	
Tumor site				
Cardiac	1.200 (0.793-1.817)		1.030 (0.684-1.553)	
Non-cardiac	1.060 (0.925-1.215)		1.100 (0.961-1.260)	
Histological type				
Intestinal	1.050 (0.892-1.235)		1.158 (0.984-1.362)	
Diffuse	1.007 (0.830-1.220)		1.055 (0.871-1.278)	
Mixed	1.210 (0.910-1.609)		0.975 (0.736-1.292)	

Adjusted for age, sex; *P*<sup>a</sup> for *P* interaction.

Table 3 GST (M1, T1) genotypes in relation of CRC risk stratified by some factors

	GSTM1 (null)		GSTT1 (null)	
	OR (95% CI)	<i>P</i> <sup>a</sup>	OR (95% CI)	<i>P</i> <sup>a</sup>
All case	1.065 (0.923-1.228)		1.041 (0.903-1.200)	
Smoking				
Never smoker	1.040 (0.822-1.316)		0.999 (0.790-1.262)	
Ever smoker	1.077 (0.889-1.304)	0.907	1.067 (0.882-1.291)	0.659
Drinking				
Never drinkers	1.025 (0.845-1.243)		1.052 (0.868-1.275)	
Current drinkers	1.170 (0.928-1.475)	0.426	1.084 (0.862-1.364)	0.870
Age (yr)				
≤ 65	1.061 (0.890-1.265)		1.054 (0.885-1.256)	
> 65	1.070 (0.835-1.370)	0.999	1.039 (0.811-1.330)	0.901
TNM stage				
0+ I + II	1.054 (0.882-1.261)		1.106 (0.926-1.323)	
III+IV	1.101 (0.924-1.312)		1.035 (0.870-1.232)	
Tumor site				
Colon	0.980 (0.822-1.169)		1.090 (0.914-1.300)	
Rectum	1.149 (0.970-1.360)		0.996 (0.842-1.178)	

Adjusted for age, sex; *P*<sup>a</sup> for *P* interaction.

between GSTM1 and GSTT1 null genotypes and susceptibility to GC and CRC in a South Korean population. In this study, we observed no significant association between either type of cancer and the GSTM1 and GSTT1 null genotypes. Additionally, no difference was observed in the frequency of the combined GST (M1 and T1) null genotype between the two cancer groups and controls. Moreover, smoking did not modify the association between these polymorphisms and risk of GC or CRC.

The reports examining the GSTM1 and GSTT1 null genotypes and their association with gastric cancer are quite inconsistent. Two reports suggested that the GSTM1

**Table 4** Combined GSTT1/GSTM1 genotype frequencies and their associations with risk GC, CRC *n* (%)

Combined genotypes	GC	CRC	Controls	OR <sup>1a</sup> (95% CI)	OR <sup>1b</sup> (95% CI)
T1(+)/M1(+)	607 (22.1)	478 (21.4)	385 (22.7)	1	1
T1(+)/M1(-)	707 (25.7)	583 (26.1)	456 (26.8)	1.02 (0.84-1.23)	1.19 (0.97-1.47)
T1(-)/M1(+)	632 (23.0)	523 (23.4)	391 (23.0)	1.04 (0.86-1.27)	0.97 (0.79-1.19)
T1(-)/M1(-)	800 (29.1)	648 (29.0)	467 (27.5)	1.17 (0.97-1.41)	1.11 (0.91-1.36)

Adjusted for age, sex; OR<sup>1a</sup> for gastric cancer; OR<sup>1b</sup> for colorectal cancer.

null genotype increased GC risk<sup>[8,10]</sup>, and three reported that the GSTT1 null genotype increased GC risk<sup>[9,17,18]</sup>. However, our results suggest that no associations exist which is in line with the majority of reports<sup>[11,12,19-21]</sup>. In studies of Korean populations, no significant associations were detected between the GSTM1 and GSTT1 null genotypes and GC risk. One study conducted in Iksan, Korea, reported that the GSTM1 and GSTT1 null genotypes had no association with the risk of GC (for GSTM1 null, OR = 0.86 and 95% CI = 0.49-1.51; for GSTT1 null, OR = 0.97 and 95% CI = 0.55-1.71)<sup>[12]</sup>. Hong *et al.*<sup>[20]</sup> reported similar results. Another study suggested that the GSTM1 and GSTT1 null genotypes were not associated with the risk of GC overall, but that in individuals who consumed kimchi, a spicy Korean food made with fermented cabbage, the GSTM1 and GSTT1 non-null genotype increased the risk of GC<sup>[19]</sup>. Two meta-analyses, by La Torre *et al.*<sup>[22]</sup> and Boccia *et al.*<sup>[23]</sup> suggested that the GSTM1 and GSTT1 null genotypes have no effect on the risk of GC per se, but may modulate tobacco-related carcinogenesis of gastric cancer.

Two studies reported that the GSTT1 null genotype increased the risk of CRC<sup>[10,24]</sup>, and three studies reported decreased CRC risk, one for the GSTM1 null<sup>[25]</sup> and two for the GSTT1 null genotype<sup>[26,27]</sup>. However, five studies suggested that the GSTM1 and GSTT1 null genotype are not related to CRC risk<sup>[4,8,13,14,28]</sup>. One Korean study suggested that the genotypes of GSTM1 are associated with cancer occurrence in individuals carrying the hMLH1/hMSH2 mutation who were family members of patients with hereditary nonpolyposis colorectal cancer<sup>[29]</sup>.

The conflicting results regarding the associations between GSTM1 and GSTT1 null genotypes and risks for GC and CRC may be due to limited sample size or differences in the ethnicities or genetic subtypes studied, and they may also be attributable to differences in exposure to environmental factors.

Polycyclic aromatic hydrocarbons (PAHs) are the main carcinogens in tobacco smoke. The ultimate carcinogen (PAH-DE) can be detoxified through conjugation with glutathione by GSTs, which are phase II enzymes<sup>[30]</sup>. Individuals with the null genotype of GSTM1 or GSTT1 would have less capacity for detoxification of PAHs, which would potentially increase their risk of chemical carcinogenesis. However, our data suggested that smoking did not modify the association between the GSTM1 and GSTT1 null genotypes and the risk of GC or CRC.

Four studies examined the interaction between smoking, GSTM1 or GSTT1 polymorphisms, and the risk of GC<sup>[10,11,18,21]</sup>. One study suggested that smoking modi-

fies the association between the GSTM1 null genotype and the risk of GC<sup>[21]</sup>. In contrast, three studies reported that smoking did not modify the association between the GSTM1 and/or GSTT1 null genotype and GC risk<sup>[10,11,18]</sup>, in line with our results. Additionally, six studies evaluated the interaction between smoking, the GSTM1 or GSTT1 null genotype, and the risk of CRC<sup>[13,25,31-34]</sup>. Two studies reported that smoking modifies the association between the GSTM1 and/or GSTT1 null genotype and CRC risk<sup>[13,31]</sup>. However, four studies reported no interaction between the GSTM1 and/or GSTT1 null genotype and smoking in CRC risk<sup>[25,32-34]</sup>, in agreement with our results.

Our study suggests that alcohol consumption did not modify the association of GSTM1 and GSTT1 null genotypes with the risk of GC or CRC, which is consistent with previous studies regarding GC<sup>[9,11,18]</sup>. However, no studies have been reported examining whether alcohol consumption modifies the association of the GSTM1 or GSTT1 null genotype with the risk of CRC.

The detoxification potential of the GSTs was observed to decrease with age<sup>[35]</sup>. Yeh *et al.*<sup>[24]</sup> reported that men aged  $\leq 60$  years with the GSTT1 null genotype were at significantly increased risk of rectal cancer, and one study reported the GSTT1 null genotype was not associated with the risk of GC, classified according to age less than or greater than 60 years<sup>[17]</sup>. However, the present study suggested that age does not modify the association between GSTM1 and GSTT1 null genotypes and the risk of GC or CRC.

In our data, the GSTM1 and GSTT1 null genotypes were not significantly associated with the risk of GC or CRC, classified according to TNM stage, tumor site, and histology type (GC). To the best of our knowledge, no reports have been published regarding the GSTM1 and GSTT1 null genotypes and GC or CRC risk classified according to TNM stage.

Two studies investigated the association between the GSTM1 and GSTT1 null genotypes and the risk of cardia or non-cardia GC, and both studies suggested that no significant association exists<sup>[3,36]</sup>, in line with our result. Seow *et al.*<sup>[37]</sup> reported that the GSTM1 and GSTT1 null genotypes were not associated with the risk of CRC, classified according to location of the tumor in the colon or the rectum. Suzuki *et al.*<sup>[38]</sup> reported finding no association between the GSTM1 null genotype and the risk of intestinal type or diffuse type GC, and Agudo *et al.*<sup>[3]</sup> reported finding no association between the GSTT1 null genotype and the risk of intestinal type or diffuse type GC, consistent with our results.

In our data, smoking was associated with increased risk

of GC, but with decreased risk of CRC. Previous studies reported that smoking increased the risk of GC<sup>[39]</sup>, in line with our results. The association between smoking and CRC has been inconsistent among studies. A recent meta-analysis suggested that smoking is significantly associated with CRC incidence<sup>[40]</sup>. In our data, drinking was associated with decreased risk of both cancer groups. Drinking probably does not affect overall risk of stomach cancer, but there is some evidence that drinking may increase the risk of GC<sup>[41]</sup>. The relationship between drinking and CRC risk has been controversial, but a meta-analysis found that high consumers of alcohol had an elevated CRC risk<sup>[42]</sup>. We could not rule out the possibility that differential misclassification bias may have occurred in our study, because we retrospectively gathered information about smoking and drinking from electronic medical records in both case groups, while cross-sectional surveys were used to gather information about smoking and alcohol in controls.

The major strength of our study was its large sample size. Ours was the first investigation of the risk of GC and CRC according to the GSTM1 and GSTT1 null genotypes in a large Korean population.

The limitations of our study must also be acknowledged. First, the study did not consider genetic polymorphisms of other cancer related genes. Second, the study considered a limited number of environmental factors (smoking and alcohol consumption), and other environmental factors such as dietary intake were not considered.

In conclusion, the results of the present population-based, large-scale case-control study suggest that in a Korean population, the GSTM1/GSTT1 null genotype does not modulate an individual's susceptibility to GC or CRC, and that smoking, alcohol consumption, and age do not modify the association between these genotypes and the risk of GC or CRC.

## COMMENTS

### Background

Gastric cancer (GC) and colorectal cancer (CRC) are the most common malignancies in Korea. Environmental factors and genetic factors have been shown to play a role in the development of these malignancies.

### Research frontiers

The glutathione S-transferase enzymes are involved in detoxification of many potentially carcinogenic compounds. Several studies have shown that glutathione S-transferase mu (GSTM1) and glutathione S-transferase theta (GSTT1) null genotypes were associated with increased risks for GC and CRC. But some data suggested no relationship between GSTM1/GSTT1 null genotype and the risk of GC and CRC.

### Innovations and breakthroughs

This is the first investigation of the risk of GC and CRC according to the GSTM1 and GSTT1 null genotypes in a large Korean population. It also aimed to determine whether smoking, alcohol consumption, and age modify the association between these polymorphisms and GC or CRC risk.

### Applications

This study suggested that GSTM1 and GSTT1 null genotypes were not associated with increased risk of GC or CRC in Koreans. Smoking, drinking, and age did not modify the association between these genotypes and the risk of gastric or colorectal cancer. Future research should focus on other parts of the GST genotype to understand its role and risk of gastric and colorectal cancer in Korean population.

### Terminology

GST: Glutathione-S-transferase is a Phase II detoxification enzyme. The

enzymes are encoded by at least five distantly related gene families (the alpha, mu, pi, sigma, and theta GSTs). In humans, marked interindividual differences exist in the expression of mu (GSTM1), and theta (GSTT1) GSTs.

### Peer review

This is the first investigation of the risk of GC and CRC according to the GSTM1 and GSTT1 null genotypes in a large Korean population. It also determined whether smoking, alcohol consumption, and age modify the association between these polymorphisms and GC or CRC risk. Although results of this study show no association between the null genotypes of GSTM1 and GSTT1 and risk of gastric and colorectal cancer, and no-association results are not as attractive as positive associations, I feel they should be presented to the scientific audience in order not to create a literature bias towards publishing only studies with positive associations.

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BRIEF ARTICLE

## Gastric dysplasia may be an independent risk factor of an advanced colorectal neoplasm

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**CONCLUSION:** The study emphasizes the need for colon surveillance in patients with gastric dysplasia, regardless of *H pylori* infection.

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**Key words:** Gastric adenoma or dysplasia; *Helicobacter pylori*; Colorectal neoplasm

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Bae RC, Jeon SW, Cho HJ, Jung MK, Kweon YO, Kim SK. Gastric dysplasia may be an independent risk factor of an advanced colorectal neoplasm. *World J Gastroenterol* 2009; 15(45): 5722-5726 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5722.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5722>

### Abstract

**AIM:** To evaluate the relationship between gastric dysplasia and *Helicobacter pylori* (*H pylori*) and the occurrence of colorectal adenoma, and to define the necessity for colonoscopy in patients with gastric dysplasia or *H pylori* infection.

**METHODS:** From May 2005 to February 2008, 133 patients with established gastric dysplasia by gastroduodenoscopy (EGD) were additionally investigated by colonoscopy. The authors compared results with those of 213 subjects who underwent both EGD and colonoscopy during the same period at the author's Health Promotion Center as a control group. *H pylori* infection was evaluated in both the gastric dysplasia and control groups.

**RESULTS:** The mean age of all 346 study subjects was  $54.1 \pm 10.5$  years, and there were 258 (73%) men and 87 (27%) women. No significant difference was found between the *H pylori* positive and negative subjects in terms of the prevalence of colorectal adenoma and advanced colorectal adenoma ( $P = 0.261$ ). Patients with gastric dysplasia showed no elevated risk of colorectal adenoma (OR = 0.910, 95% CI: 0.587-1.411,  $P = 0.738$ ), but had a significantly higher risk of having advanced colorectal adenoma (OR = 3.382, 95% CI: 1.700-6.342,  $P = 0.000$ ).

### INTRODUCTION

Colorectal carcinoma is one of the most common cancers and is the second leading cause of cancer-related mortality in the United States<sup>[1]</sup>, and the fourth leading cause of cancer-related mortality in Korea<sup>[2]</sup>. The etiologies and risk factors of colorectal neoplasms have attracted research attention for several decades, especially in association with *Helicobacter pylori* (*H pylori*) or gastric dysplasia. *H pylori* infection is a well-known cause of chronic gastritis, peptic ulcers<sup>[3,4]</sup>, gastric adenocarcinoma<sup>[5]</sup>, and gastric lymphoma<sup>[6]</sup>, and several reports have suggested that *H pylori* infection increases the risk of colorectal adenoma and adenocarcinoma<sup>[7-9]</sup>; however, other reports disagree<sup>[10,11]</sup>. Furthermore, several have concluded that gastric adenoma and carcinoma may be indicators of colorectal adenoma or carcinoma<sup>[12,13]</sup>, and it is accepted that both gastric adenoma and colorectal adenoma have a high potential for malignant transformation<sup>[14-16]</sup>. The aims of this study were to evaluate relations between gastric dysplasia and *H pylori* infection and the occurrence of colorectal adenoma and to define the need for colonoscopy in patients with gastric dysplasia or *H pylori* infection.

## MATERIALS AND METHODS

### Patients

From May 2005 to February 2008, 133 consecutive patients with established gastric dysplasia (the gastric dysplasia group), as determined by gastroduodenoscopy (EGD) at our institute, were additionally subjected to colonoscopy, after obtaining informed consent, within 3 wk of EGD. We defined gastric adenoma as gastric dysplasia. The presence of gastric dysplasia and colorectal adenoma were confirmed histologically using specimens obtained by endoscopic mucosal resection (EMR), endoscopic submucosal dissection (ESD), or polypectomy. We also enrolled 213 consecutive subjects who underwent both EGD and colonoscopy for the purpose of screening at the Health Promotion Center at Kyungpook National University Hospital as a control group during the same period. We reviewed endoscopic findings and pathology specimens retrospectively. The subjects who were included in the control group had no symptoms and no gastric dysplasia. Patients with any kind of malignancy and those with a previous history of colorectal surgery were excluded. Subjects with either family history of colorectal adenocarcinoma or adenoma or gastric cancer were also excluded. This study was approved by our Institutional Review Board.

### Diagnosis of *H pylori* infection

*H pylori* infection was diagnosed by biopsy, CLO test (GASTREX, Warsaw, Poland), or by using an urea breathing test (UBT, Otsuka, Tokyo). The specimens for the CLO test were obtained from both the antrum and the distal body of the stomach during EGD. Cresyl violet staining was used for the pathologic diagnosis of *H pylori* infection. The cut-off value of the UBT was 2.5‰. *H pylori* infection was diagnosed when two of the above three methods were positive.

### Colonoscopic diagnosis

Cecal intubation was successful in all patients. All lesions found during colonoscopy were removed by biopsy, polypectomy, EMR, or ESD. Advanced colorectal adenoma was defined as a size larger than 1 cm, a villous or tubulovillous adenoma histology, or adenoma with high-grade dysplasia.

### Statistical analysis

We compared age and sex distributions, the presence or absence of colorectal adenoma, colorectal adenoma sizes and histology, and *H pylori* infection states in the gastric dysplasia and control groups. Variables were compared using the  $\chi^2$  or independent *t*-tests, depending on the nature of the data. Fisher's exact test was used when numbers were small. Odds ratios (ORs) and 95% CIs were used to describe associations. ORs with corresponding 95% CIs were obtained by conditional logistic regression analysis. *P* values of < 0.05 were considered statistically significant, and statistical calculations were performed using SPSS version 14.0 software for Window (SPSS Inc. Chicago, IL, USA).

Table 1 Baseline characteristics of gastric dysplasia and control group

	Gastric dysplasia ( <i>n</i> = 133)	Control ( <i>n</i> = 213)	<i>P</i> -value
Age (mean $\pm$ SD)	61.2 $\pm$ 9.7	49.6 $\pm$ 8.3	0.0005
Sex [M/F, (%)]	97/36 (73/37)	162/51 (76/24)	0.5200
<i>H pylori</i>			0.2610
(+) <i>n</i> = 204	73	131	
(-) <i>n</i> = 142	60	82	
Colorectal adenoma			0.5220
(+) <i>n</i> = 148	55	93	
(-) <i>n</i> = 198	78	120	
Advanced adenoma			0.0007
(+) <i>n</i> = 44	28	16	
(-) <i>n</i> = 302	105	197	

Advanced adenoma: Advanced colorectal adenoma.

## RESULTS

### Clinical characteristics of the gastric dysplasia and control groups

The mean age of all 346 study subjects was 54.1  $\pm$  10.5 years, and there were 258 men (73%) and 87 women (27%). The gastric dysplasia group was older than the control group (*P* = 0.0005). In the gastric dysplasia group (*n* = 133), colorectal adenoma was diagnosed in 55/133 (41%) and advanced colorectal adenoma in 28/133 (21%). In the control group, colorectal adenoma was diagnosed in 93/213 (44%), and advanced colorectal adenoma in 16/213 (7.5%). No significant difference was observed between the gastric dysplasia and control groups in terms of the prevalence of colorectal adenoma (*P* = 0.5220), and no significant difference was observed between the *H pylori* positive and negative groups in terms of the prevalence of gastric dysplasia, colorectal adenoma, or advanced colorectal adenoma (*P* = 0.2610). Furthermore, no significant difference was observed between the gastric dysplasia and control groups in terms of the sizes of advanced colorectal adenomas (10.50  $\pm$  3.107 mm *vs* 11.92  $\pm$  8.722 mm, respectively, *P* = 0.5860) (Table 1).

### Clinical characteristics of the advanced colorectal adenoma positive and negative groups

Mean age in the advanced colorectal adenoma positive group was greater than in the negative group (*P* = 0.0002). However, group sex distributions (*P* = 0.0600) and *H pylori* (*P* = 0.1060) infection statuses were not different significantly different (Table 2).

### Comparisons of the colorectal adenoma and advanced colorectal adenoma groups

Those with colorectal adenoma or advanced colorectal adenoma were older than those without (Table 3). Moreover, those with gastric dysplasia showed no increased risk of having colorectal adenoma (OR = 0.910, 95% CI: 0.587-1.411, *P* = 0.7380), but they had a significantly higher risk of having advanced colorectal adenoma (adjusted OR = 3.283, CI: 1.700-6.342, *P* = 0.0004) (Table 3). This result was supported by multivariate analysis (Table 4).

**Table 2** Baseline characteristics of advanced adenoma (+) and (-) group

	Advanced adenoma (+) (n = 44)	Advanced adenoma (-) (n = 302)	P-value
Age (mean ± SD)	60.8837 ± 8.55294	53.1419 ± 10.43410	0.0002
Sex [M/F n (%)]	38/6 (86/14)	221/81 (73/27)	0.0600
<i>H pylori</i>			0.1060
(+) n = 204	21	183	
(-) n = 142	23	119	

## DISCUSSION

This study shows that the risk of advanced colorectal adenoma is elevated in patients with gastric dysplasia, and thus, supports the notion that colorectal cancer risk is increased by gastric adenoma, as has been reported previously<sup>[12]</sup>. In addition our study concurs with the findings of a previous study, in which it was found that two of six patients with diffuse gastric adenoma had colon polyps and 40 of 73 patients (54.8%) with gastric polyps had colorectal adenomatous polyps<sup>[17]</sup>. In the present study, 55 of 133 patients (41%) with gastric dysplasia had colorectal adenomatous polyps and 28 of 133 (21%) had advanced colorectal adenomatous polyps. The present study concurs with another study, in which it was found that eight of eleven patients with duodenal adenomas had colonic polyps<sup>[18]</sup>. Furthermore, the present study agrees with other studies concerning the elevated risk of gastric polyps in patients with sporadic colonic polyps<sup>[19]</sup>. Shemesh *et al*<sup>[20]</sup> reported that two of 100 patients with 1-4 colorectal polyps had gastric adenoma, 3 of 80 patients (3.5%) with 5 or more colorectal polyps had gastric adenoma, 3 of 80 patients with colon cancer had gastric adenoma, and one of 100 patients without colorectal adenoma and cancer had gastric adenoma, and these findings are also in line with those of the present study. The progression of colorectal adenoma to colon cancer is known to be associated with an adenoma size larger than 10 mm, more than three lesions, a villous, tubulovillous or high grade dysplastic histology, and the presence of genetic abnormalities<sup>[21-24]</sup>. Genetic abnormalities in colon cancer are very similar to those in gastric cancer<sup>[25,26]</sup>, especially concerning mutations of the *p53*<sup>[27,28]</sup>, *APC*<sup>[29,30]</sup>, *DCC*<sup>[31,32]</sup>, and *K-ras* genes<sup>[33,34]</sup>. Genetic abnormalities are well known to be associated with upper gastrointestinal polyps and with hereditary colonic polyposis syndromes<sup>[25,26]</sup>, which include familial polyposis coli<sup>[25,26]</sup>, Gardner's syndrome, Peutz-Jeghers' syndrome, Cowden's syndrome, Cronkhite-Canada syndrome, hereditary flat adenoma syndrome, and others. In the present study, we deliberately excluded patients with HNPCC, suspected HNPCC, or FAP to avoid the influence of such genetic factors.

Gastric adenoma or dysplasia have high rates of malignant transformation (up to 75%<sup>[14-16,19-21]</sup>). Accordingly, regardless of the existence of colorectal adenoma, treatment of gastric dysplasia by EMR or ESD is inevitable. Moreover, the above-mentioned relations suggest that the pathogenesis of gastric dysplasia and colorectal

**Table 3** Statistics of colorectal adenoma and advanced adenoma groups

	Colorectal adenoma			Advanced adenoma		
	aOR	CI	P-value	aOR	CI	P-value
Age (old age)	1.337	0.872-2.049	0.0020	2.872	1.446-5.704	0.0002
Sex (female)	0.629	0.379-1.042	0.0800	0.431	0.176-1.057	0.0640
<i>H pylori</i> (positive)	1.037	0.672-1.599	0.9120	0.594	0.315-1.120	0.1390
G. dysplasia (presence)	0.910	0.587-1.411	0.7380	3.283	1.700-6.342	0.0004

aOR: Adjusted odds ratio; G. dysplasia: Gastric dysplasia.

adenoma are similar. Several other controversial factors may increase the risk of colorectal cancer development, e.g. an increased serum gastrin level<sup>[35]</sup>, *H pylori* infection<sup>[6-11]</sup> and *Streptococcus bovis* bacteremia<sup>[36]</sup>. However, previous studies have concluded that the presence of *H pylori* infection and an elevated serum gastrin level are not associated with elevated risks of colorectal neoplasm development. Recently, some studies have concluded that increased serum gastrin levels due to the use of proton pump inhibitors do not predispose the development of a colorectal neoplasm. In the present study, we included only gastric dysplasia and *H pylori* infection status to examine the risk of colorectal adenoma development. No significant association was found between *H pylori* infection and colorectal adenoma, which concurs with another study<sup>[10,11]</sup>, and thus, it appears that an agent other than *H pylori* is responsible for co-pathogenesis of colorectal adenoma<sup>[35]</sup>.

The aim of several studies on colorectal adenoma has been to reduce colorectal cancer mortality and morbidity. To achieve this aim, the early diagnoses and treatment of precancerous lesions, like colorectal adenoma and early colorectal cancer, are of the utmost importance, especially, in those deemed to be at high risk group by early surveillance colonoscopy<sup>[37,38]</sup>. Some studies have recommended that gastric cancer patients undergo colonoscopy due to a substantial risk of their developing colon cancer<sup>[13]</sup>. In another study, haemoccult studies were recommended for early detection of colorectal tumors in patients with gastric polyp<sup>[39]</sup>. In addition, it has been reported that the rates of occurrence of colorectal adenoma in gastric adenoma and carcinoma patients are increasing<sup>[12]</sup>. In common with other studies, the present study reveals that gastric dysplasia is an indicator of advanced colorectal adenoma, which is an important risk factor of colorectal cancer. Accordingly, we emphasize the need for colon surveillance in elderly patients with gastric dysplasia because of their elevated risk of developing advanced colorectal adenoma, which is an important risk factor of colorectal cancer, regardless of *H pylori* infection.

Several limitations of the present study should be considered. First, the sample size was small, which introduces the possibility of a type II error, and we could not collect age and sex-matched controls. Second, selection bias is possible due to the retrospective nature of the study. Third, insufficient information was obtained regarding alcohol consumption, smoking status, and

Table 4 Multivariate analysis of colorectal adenoma and advanced adenoma groups

	Colorectal adenoma					Advanced colorectal adenoma				
	B	SE	t	Sig	95% CI	B	SE	t	Sig	95% CI
Age	3.585	1.129	3.176	0.0020	1.365 to 5.805	7.770	1.648	4.714	0.0002	4.529 to 11.01
Sex	-0.085	0.047	-1.810	0.0710	-0.007 to 0.178	-0.132	0.070	1.888	0.0600	-0.269 to -0.006
<i>H pylori</i>	-0.009	0.054	-0.163	0.8710	-0.114 to 0.097	-0.129	0.079	1.623	0.1060	-0.285 to -0.027
G. dysplasia	0.022	0.053	0.421	0.6740	-0.082 to 0.127	0.289	0.077	3.741	0.0004	0.137 to 0.440

serum gastrin, cholesterol, and glucose levels, which were recently proposed as risk factors of gastric dysplasia<sup>[35]</sup>. Fourth, the mean age of the gastric dysplasia group was greater than that of the control group, because the patients who visited our health promotion center were much younger than the gastric dysplasia patients. Accordingly, although age-adjusted odds ratios for advanced colorectal adenoma were statistically significance, we cannot exclude the possibility of an age-related bias.

Even though there were several reports that *H pylori* infection increased the risk of colorectal adenoma and adenocarcinoma<sup>[7-9]</sup>, our report reveals that *H pylori* infection is not a risk factor for advanced colorectal polyps, but that gastric dysplasia and an advanced age are. Thus, we infer that genetic and environmental factors probably importantly contribute to the development of advanced colorectal polyps, because patients are continuously exposed to those factors. However, the environmental factors involved have not been identified as yet. Thus, further diverse complex study models are required to clarify the relationship between gastric dysplasia, *H pylori*, and colorectal adenoma, and identify the environmental factors concerned. Moreover, a large prospective case-controlled study is required to determine whether the presence of gastric dysplasia is an indication for colonoscopy and what intervals would be recommended for patients with gastric dysplasia.

## COMMENTS

### Background

Over the last decades, several studies have been performed on the etiologies and risk factors of colorectal neoplasms, especially on the association with *Helicobacter pylori* (*H pylori*) and gastric dysplasia. This study was performed to define the necessity of screening colonoscopy in patients with gastric dysplasia or *H pylori* infection.

### Research frontiers

Several controversial factors might increase the risk of colorectal cancer development, for example, increased serum gastrin level, *H pylori* infection and *Streptococcus bovis* bacteremia, and so on. In this study, the authors demonstrate the relations between gastric dysplasia and the occurrence of advanced colorectal adenoma.

### Innovations and breakthroughs

Even though there were several reports that *H pylori* infection increased the risk of colorectal adenoma and adenocarcinoma, this report reveals that *H pylori* infection is not a risk factor for advanced colorectal polyps; however, gastric dysplasia and an advanced age are. Thus, genetic and environmental factors probably contribute to the development of advanced colorectal polyps, because of continuous exposure to genetic and environmental factors.

### Applications

This result could emphasize the need for colon surveillance in elderly patients with gastric dysplasia because of their elevated risk of developing advanced

colorectal adenoma, which is an important risk factor of colorectal cancer, regardless of *H pylori* infection.

### Peer review

The authors examined the relation between gastric dysplasia and *H pylori* and advanced colorectal adenoma. However, a large prospective case-controlled study is required to determine whether the presence of gastric dysplasia is an indication for colonoscopy and what intervals would be recommended for patients with gastric dysplasia.

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## Total laparoscopic liver resection in 78 patients

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### Abstract

**AIM:** To summarize the clinical experience of laparoscopic hepatectomy at a single center.

**METHODS:** Between November 2003 and March 2009, 78 patients with hepatocellular carcinoma ( $n = 39$ ), metastatic liver carcinoma ( $n = 10$ ), and benign liver neoplasms ( $n = 29$ ) underwent laparoscopic hepatectomy in our unit. A retrospective analysis was done on the clinical outcomes of the 78 patients.

**RESULTS:** The lesions were located in segments I ( $n = 3$ ), II ( $n = 16$ ), III ( $n = 24$ ), IV ( $n = 11$ ), V ( $n = 11$ ), VI ( $n = 9$ ), and VIII ( $n = 4$ ). The lesion sizes ranged from 0.8 to 15 cm. The number of lesions was three ( $n = 4$ ), two ( $n = 8$ ) and one ( $n = 66$ ) in the study cohort. The surgical procedures included left hemi-hepatectomy ( $n = 7$ ), left lateral lobectomy ( $n = 14$ ), segmentectomy ( $n = 11$ ), local resection ( $n = 39$ ), and resection of metastatic liver lesions during laparoscopic surgery for rectal cancer ( $n = 7$ ). Laparoscopic liver resection was successful in all patients, with no conversion to open procedures. Only four patients received blood transfusion (400-800 mL). There were no perioperative complications, such as bleeding and biliary leakage. The liver function of all patients recovered within 1 wk, and no liver failure occurred.

**CONCLUSION:** Laparoscopic hepatectomy is a safe and feasible operation with minimal surgical trauma. It should be performed by a surgeon with sufficient experience in open hepatic resection and who is proficient in laparoscopy.

### INTRODUCTION

Laparoscopic hepatectomy (LH) was first described in 1995 by Cuesta *et al*<sup>[1]</sup> and it remains an appealing concept: major surgery with a potential for bleeding, carried out using a minimally invasive approach. While the laparoscopic approach has been championed, notably by Cherqui *et al*<sup>[2]</sup> and Buell *et al*<sup>[3]</sup>, it is carried out in only a few centers and has not become a widely accepted surgical technique. In general, laparoscopic liver resection offers some advantages over an open approach. There is less postoperative pain, early mobilization, minimal ileus, earlier resumption of oral intake and shorter hospital stay<sup>[4-7]</sup>. Initial minor hepatectomy for lesions located in superficial segments has been considered possible<sup>[8,9]</sup>, with the development of laparoscopic instruments and parenchymal transection devices, combined with improved understanding of the vascular anatomy of the liver, it is now accepted widely that laparoscopy will be used increasingly in liver surgery<sup>[10]</sup>.

Between November 2003 and March 2009, we performed total laparoscopic hepatectomy in 78 patients with liver neoplasms, the results of which were encouraging.

### MATERIALS AND METHODS

#### Patients

Between November 2003 and March 2009, 78 LHs were performed in the Hepato-biliary Surgery Department at Sun Yat-sen Memorial Hospital of Sun Yat-sen University. Seventy-eight patients with liver neoplasms

(50 men and 28 women), including primary liver carcinoma ( $n = 39$ ), secondary liver carcinoma ( $n = 10$ ), and benign liver neoplasm ( $n = 29$ ). The mean age was 46.6 years (range, 20-82 years). Preoperative liver function was as follows: Child classification A ( $n = 52$ ), B ( $n = 22$ ) and C ( $n = 4$ ). Evidence of cirrhosis, based on clinical and/or morphological abnormalities, was not considered an absolute contraindication in the absence of decompensated cirrhosis. There was no upper limit on neoplasm size, and LH was contraindicated if venous or biliary reconstruction was required.

### Surgical procedure

All procedures were performed under general endotracheal anesthesia and after obtaining informed consent. All resections were performed with the patient in the supine position. The surgeon stood between the patient's spread legs, the first assistant on the left side of the patient and the camera assistant on the right. The pneumoperitoneum was insufflated by umbilicus puncture and controlled electronically at a constant abdominal pressure of 12 mmHg. A 5-mm trocar placed at the umbilical port was used for abdominal exploration with a 30° laparoscope. The trocar insertion sites depended on the location of the hepatic lesion. The surgical technique was as follows. For mobilization of the liver, the falciform and round ligaments were divided by an endoscopic ultrasonic scalpel (LLC, Guaynabo, Puerto Rico). In the case of left hepatectomy, the left liver lobe was mobilized by dividing the left triangular, coronary, and hepato-gastric ligaments. In the case of right segmentectomy, the right coronary ligament was divided. It was not necessary to divide the ligament if the lesion was located at the edge of the liver. Laparoscopic ultrasonography (Esaoite Biomedica, Genoa, Italy) was used for localization of the tumor and the supply vessels, demonstration of satellite nodules, and demarcation of an adequate tumor-free margin. To achieve afferent blood control in anatomical left liver resection, including left segmentectomy, left lateral lobectomy, and left hemihepatectomy, the portal pedicles were dissected outside the liver parenchyma, and the portal venous branch, hepatic arterial branch, and bile duct were separated, clipped and divided. When the branch was too large to apply clips, it was divided with an endoscopic linear stapler (Tyco Healthcare, Norwalk, CT, USA). In right liver resection, the supply vessels of the target segment were found by laparoscopic ultrasonography, and were clipped or divided with an endoscopic linear stapler extraparenchymally. To achieve efferent blood control, the inferior vena cava and left hepatic vein were dissected, and the trunk of the left hepatic vein was clipped with a 12-mm hemoclip, but was not divided before parenchymal division. Liver surface tissue transection was performed using an endoscopic ultrasonic scalpel, while deep parenchyma dissection was performed using LigaSure (Valleylab, Boulder, CO, USA); larger biliary and vascular radicals were clipped and divided. After careful hemostasis, fibrin glue sealant (FibinGluRAAS; Shanghai Raas Blood Products, China) was applied to the raw surface. The specimen was extracted using a plastic bag through an extending port site.

**Table 1** Tumor sites in 78 laparoscopic hepatectomy (LH) procedures

	<i>n</i>
Segment I	3
Segment II	16
Segment III	24
Segment IV	11
Segment V	11
Segment VI	9
Segment VII	4
Total	78

### Assessment of operation

Surgical complications included biliary fistula, hemorrhage and incisional hernia. All patients underwent liver function tests and abdominal computed tomography (CT) before discharge. All patients underwent follow-up examination at 1-mo intervals after operation, and the follow-up examinations included clinical examination, liver function tests, and abdominal ultrasonography. No patient was lost at follow-up.

## RESULTS

### Operative results

Hepatic resection was performed laparoscopically in all 78 patients. There were no operative deaths and no conversions to laparotomy. The mean operation time was 165 min (range, 60-390 min). The mean blood loss was 288 mL (range, 10-1000 mL); only four patients received blood transfusion (400-800 mL). The mean size of the lesions was 6.2 cm (range, 0.8-15 cm) and the mean width of the specimen margins was 1.2 cm (range, 0.5-6 cm). The number of lesions was three ( $n = 4$ ), two ( $n = 8$ ), and one ( $n = 66$ ) in the study cohort. Details of tumor sites are summarized in Table 1; Types of laparoscopic hepatectomy are given in Table 2; Details of histologic results are summarized in Table 3.

### Postoperative recovery

There were no postoperative deaths or complications, such as bleeding and biliary leakage. Liver function returned to preoperative levels within 1 wk. Oral intake was begun on postoperative day 2. The mean postoperative hospital stay was 5.6 d (range, 2-10 d). The patients were discharged without narcotic analgesia. At the first postoperative month, all patients had resumed normal activities.

### Follow-up

The mean follow-up was 30 mo (range, 2-60 mo). No port site metastasis was noted during follow-up in any patient who had hepatectomy for malignant lesions. Recurrence was detected in four patients with primary hepatocellular carcinoma and one with intrahepatic cholangiocarcinoma within 2 years. Two patients with primary hepatocellular carcinoma underwent a second LH for a recurrent lesion, and the other patients had transcatheter arterial chemoembolization and radiofrequency ablation (RFA). New isolated lesions in the liver were detected in two patients with rectal carcinoma within 1 year postoperatively, and were treated with RFA.

Table 2 Types of LH *n* (%)

	<i>n</i>
Anatomical resection	32 (41)
Left hemi-hepatectomy	7
Left lateral hepatic lobectomy	14
Hepatic segmentectomy	11
Local resection	39 (50)
Local liver resection for metastasis and colorectal laparoscopic resection	7 (9)
Total	78

Table 3 Histological results in 78 LH procedures *n* (%)

	<i>n</i>
Malignant tumor	52 (67)
Hepatocellular carcinoma	33
Intrahepatic cholangiocarcinoma	6
Colorectal metastasis	10
Gastric carcinoma metastasis	1
Ovarian carcinoma metastasis	1
Kidney carcinoma metastasis	1
Benign tumor	26 (33)
Haemangioma	21
Focal nodular hyperplasia	3
Granuloma	1
Adenoma	1
Total	78

## DISCUSSION

Laparoscopic liver resection has been viewed with skepticism because of concerns regarding parenchymal transection, bleeding control, bile leakage and incomplete resection<sup>[3,11,12]</sup>. The present study suggests that LH may be safe and feasible in selected patients. The indication for laparoscopic liver resection should be selected with care because of the technical difficulty, and the size and location of the neoplasm must be evaluated before surgery. In patients with neoplasms located in the left liver lobe and the anterior and inferior liver segments (IVb, V and VI), LH has been shown to be safe. On the other hand, neoplasms located in the right lobe and the posterior and superior liver segments (I, IVa, VII and VIII) are technically more demanding and should be approached with caution<sup>[13,14]</sup>. In our cohort, most lesions (91%) were located in segment II, III, IV, V and VI, and no lesion located in segment VII was treated because of the difficulty of exposure. A neoplasm < 6 cm in size is thought to be preferred<sup>[15,16]</sup>. In fact, we believe the size of the neoplasm is not as important as its location for LH. We performed laparoscopic left hemi-hepatectomy in four patients with 10-15-cm neoplasms in the left liver lobe, but without invasion of the inferior vena cava and the root of the hepatic vein. No conversion to laparotomy in our cohort may have been, at least in part, because of the choice of indication before surgery.

One of the main concerns during hepatectomy is minimizing blood loss and avoidance of blood transfusion<sup>[17,18]</sup>. In our experience, laparoscopic surgery may provide better visualization of deep vascular structures and more precise and accurate surgery. To avoid injury



Figure 1 Computed tomography (CT) image before operation, which showed the lesion on the right lobe of the cirrhotic liver.



Figure 2 After laparoscopic hepatectomy (LH), the lesion disappeared and the volume of liver increased clearly.

to the hepatic veins, caution should be exercised during manipulation of secondary hilar structures, and the hepatic veins should be transected in the parenchyma using clips or an endoscopic linear stapler. Conversion to laparotomy is mandatory when a large venous injury occurs, even if initial laparoscopic control of the wound has been achieved<sup>[11]</sup>. In the early stage of LH, bleeding during parenchymal transection due to a lack of effective devices is another important cause of blood loss, but there are now numerous excellent devices for dividing the parenchyma, including an ultrasonic scalpel, microwave tissue coagulator, water jet dissector, LigaSure, Cavitron ultrasonic surgical aspirator, argon beam coagulator, Peng's Multifunction Operative Dissector, and TissueLink<sup>[19-26]</sup>. Based on our experience, using an ultrasonic scalpel and LigaSure to transect the liver facilitates good hemostasis, clear anatomy, less effusion, and minor damage to liver function. The average blood loss was only 200 mL in our 78 cases. Another concern that occurs during LH when resecting a malignant neoplasm involves achieving a disease-free resection margin<sup>[27,28]</sup>. We believe a 1-cm free surgical margin can be obtained using laparoscopic ultrasonography. In our research and a European multicenter study, there was no evidence that the use of a laparoscopic technique increases the risk of local recurrence and port-site metastases<sup>[29,30]</sup>. LH carries the potential risk of gas embolism caused by a pneumoperitoneum during hepatic vein division<sup>[31]</sup>. Although this potential complication

appears to be rare, we utilized several precautions to avoid embolic events, including careful dissection of the hepatic vein, a low-pressure pneumoperitoneum, and preventive obstruction of the left hepatic vein with a 12-mm hemoclip.

LH has the advantage of minimal scarring and fewer adhesions, thereby increasing the feasibility of repeat liver resection. We performed repeat laparoscopic liver resections in two patients for recurrent lesions, and the results were satisfactory. LH may very well become the gold standard for tumor control in patients with hepatocellular carcinoma awaiting liver transplantation<sup>[32]</sup>. The choice of an incision is a dilemma in the colorectal cancer patient complicated by liver metastasis. The present study has shown that simultaneous laparoscopic resection of colorectal cancer and synchronous liver metastases is feasible and safe. Indeed, we performed this operation successfully in seven patients with five or six ports, just one or two more than laparoscopic resection of colorectal cancer, and no complications occurred. LH also has an irreplaceable advantage in patients with poor liver function. We performed LH in four patients with cirrhosis and Child C liver function in whom liver resection *via* laparotomy carried a high risk of complications. They all recovered rapidly without development of ascites and jaundice. In one hepatocellular carcinoma patient with cirrhosis and hepatitis C infection, liver function did not improve following medical treatment, yet returned to normal within 5 d after local laparoscopic tumor resection. LH might improve the postoperative course of patients with cirrhosis for the following reasons: (1) preservation of the abdominal wall and the round ligament avoids interruption of collateral circulation; (2) less mobilization and manipulation of the liver reduces liver trauma; and (3) non-exposure of abdominal viscera restricts fluid requirements and decreases electrolytic and protein losses<sup>[33-35]</sup>. We suggest that local laparoscopic liver tumor resection in patients with cirrhosis not only treats the tumor, but also creates a damaged liver environment with minimal liver load, which provides ideal conditions for regeneration of remnant liver. In fact, with CT scanning, we confirmed that the volume of the liver increased after LH in these patients (Figures 1 and 2).

Lastly, we advocate LH as a complex procedure that requires experience and different skills from those of open surgery because of the 2D representation of the operative site, limited tactile feedback, and the need for eye-hand coordination skills. The surgeons in our study all had sufficient experience with open hepatic resection and proficient skills in laparoscopic procedures, and also had the ability to manage the various complications of liver resection.

## COMMENTS

### Background

With the development of laparoscopic instruments and parenchymal transection devices, combined with the improved understanding of the vascular anatomy of the liver, it is now accepted widely that laparoscopy will be used increasingly in liver surgery.

### Research frontiers

The underlying intent of laparoscopic surgery for liver neoplasms is to provide curative resection while minimizing complications. In this study, the authors

demonstrated that laparoscopic hepatectomy (LH) could be safe and feasible, with minimal trauma.

### Innovations and breakthroughs

This manuscript described the retrospective evaluation of a significant cohort of patients who underwent LH at a single center. The good clinical outcome depended on the reasonable choice of indication, suitable parenchymal transection devices, and sufficient experience in hepatic resection and laparoscopy.

### Applications

LH could be performed in most patients with liver neoplasms, particularly in patients with cirrhosis and those with colorectal cancer and synchronous liver metastases.

### Terminology

Liver resection is always considered a major operation, with blood loss and lengthy hospitalization. LH could benefit patients in need of liver resection, with better cosmetic results, less postoperative pain and shorter hospital stay.

### Peer review

Interesting report about a single centre experience with 78 laparoscopic liver resections of different kind for different indications in different liver diseases.

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BRIEF ARTICLE

## A simple taurocholate-induced model of severe acute pancreatitis in rats

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appeared to be destroyed with loose, shortened microvilli and rupture of the intercellular junction, as shown by electron microscopy.

**CONCLUSION:** Significant gut barrier damage and intestinal bacterial translocation were definitely observed with few potential study confounders in this SAP rat model, suggesting that it may be an appropriate animal model for study of gut barrier damage and bacterial translocation in SAP.

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**Key words:** Acute pancreatitis; Bacterial translocation; Inflammation; Real-time polymerase chain reaction

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### Abstract

**AIM:** To investigate gut barrier damage and intestinal bacteria translocation in severe acute pancreatitis (SAP), a simple rat model of SAP was induced and studied.

**METHODS:** Pancreatitis was induced by uniformly distributed injection of 3.8% Na taurocholate (1 mL/kg) beneath the pancreatic capsule. Rats in the control group were injected with normal saline in the identical location.

**RESULTS:** Serum amylase, plasma endotoxin, intestinal permeability, and pancreatitis pathology scores were all markedly higher in the pancreatitis group than in the control group ( $P < 0.01$ ). The bacterial infection rate was significantly higher in the SAP group than in the control group ( $P < 0.01$ ), observed in parallel by both bacterial culture and real-time polymerase chain reaction. Acute damage of the pancreas was observed histologically in SAP rats, showing interstitial edema, leukocyte infiltration, acinar cell necrosis and hemorrhage. The microstructure of the intestinal mucosa of SAP rats

### INTRODUCTION

A close link has been demonstrated between intestinal bacteria translocation and infection in pancreatic necrosis, which is a main predictor of clinical outcome in patients with severe acute pancreatitis (SAP)<sup>[1]</sup>. Although a multitude of animal models<sup>[2-5]</sup> have been used to study the mechanism of bacterial translocation, its exact origin, route, and mechanism are still unclear. The main reason for this uncertainty is the lack of an "ideal" animal model of acute pancreatitis (AP), especially for the purpose of studying bacterial translocation.

Taurocholate-induced pancreatitis animal models are commonly used nowadays, especially the model induced by perfusion of taurocholate into the biliary or pancreatic duct, which is thought to most closely resemble clinical biliary pancreatitis. However these models have some shortcomings such as prolonged

preparation time, difficult operation, high death rate, and potential infection of the pancreas when puncturing through the gut, which limit their further popularity in study of bacterial translocation in SAP.

To overcome these shortcomings, in this paper we induced a taurocholate pancreatitis rat model by uniformly distributed injection of taurocholate directly beneath the pancreatic capsule. Gut barrier damage and intestinal bacterial translocation were studied and the advantages of the model were characterized.

## MATERIALS AND METHODS

### **Animals and animal procedures**

Thirty two specific pathogen-free rats (half male and half female, obtained from the Experimental Animal Center of Guangdong Province, China), were randomly divided into 2 groups (16 rats in the experimental group, 16 in the control group). Average weight at the time of the experiment was  $250 \pm 20$  g. Anesthesia was performed with intraperitoneal injection of 10% chloral hydrate 3 mL/kg.

The study and all procedures were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University, China.

### **Model induction procedure**

Pancreatitis group: rats were fasted for 12 h before the operation. A 10 mm median laparotomy was made and the pancreas was well exposed. The pancreatic capsule was gently lifted up, and 3.8% Na taurocholate 1 mL/kg was injected beneath the pancreatic capsule and distributed by making a bubble of taurocholate, about 2-3 mm in diameter at every injection point. Several minutes later, the pancreatic tissue would become dark purple, swell, and bleed locally. Then the abdominal organs were gently repositioned, and the peritoneal cavity was doused with about 50 mL of normal saline. Finally the abdominal wall was sutured.

In the control group, rats were injected similarly with normal saline 1 mL/kg in place of Na taurocholate.

### **Post-surgery breeding**

After surgery, rats were housed in a dry and clean room at a temperature of 20-26°C, and 12 h later standard chow and water were freely available. Mortality was observed and recorded every day.

### **Determination of serum amylase and plasma endotoxin level**

Serum amylase and plasma endotoxin were detected by routine clinical chemistry methods. Serum amylase was measured at 3 time points, namely 6, 24 and 48 h. On the 4th and 7th days, plasma endotoxins of every live rat were measured.

### **Determination of gut permeability by measurement of serum fluorescent isothiocyanate dextran (FITC-Dextran) in the superior mesenteric vein (SMV)**

FITC-Dextran (Sigma), a polysaccharide macromolecule with a fluorescent marker, fluorescein isothiocyanate, can-

not be digested and absorbed when in the normal alimentary tract, but can pass through the damaged gut barrier into capillaries of the gut, and finally into the blood of the SMV when intestinal permeability is increased<sup>[6-9]</sup>. The concentration of FITC-Dextran in the SMV depends on the dose of the drug and the severity of gut barrier damage. In this study, rats were perfused with 1 mL of 0.5% FITC-Dextran through a small tube placed in the stomach *via* the mouth, and 12 h later SMV blood samples were taken to measure the FITC-Dextran concentration as follows: blood was centrifuged at 1000 r/min, the supernatant was obtained, and absorbance was detected on a spectrofluorometer (Thermo Ltd., USA) at an excitation wave of 490 nm and transmission wave of 520 nm; the concentration of FITC-Dextran was finally calculated according to the standard curve of FITC-Dextran.

### **Bacterial culture for detection of infection rates and real-time polymerase chain reaction (PCR) to quantify the total number of translocated bacteria**

One millilitre of blood from the inferior vena cava, 0.1 g mesenteric lymph node (MLN) tissue, 0.3 g pancreas tissue and 1 g liver tissue were taken from each rat under sterile conditions by laparotomy. Every sample had 1 mL saline added followed by homogenization by triturating, then uniform smearing on 4 brain heart solid culture medium (Hopebiotechnology Co. Ltd., Qingdao, China), 2 of which were placed in a 37°C thermostat, the other 2 were placed in an anaerobic jar, tightly closed and put in a 37°C thermostat. Results were observed and recorded 48 h later.

The same quantities of samples as above were taken for real-time PCR. Total bacterial genomic DNA was extracted by the DNeasy Blood & Tissue kit (Cat. 69506, Qiagen, USA) according to the manufacturer's instructions. Li *et al.*<sup>[10]</sup> confirmed the appropriateness and effectiveness of this kit for studying gut microbial ecology. The universal PCR primer sets for total bacteria (forward: TCCTACGGGAGGCAGCAGT; reverse: GGACTACCAGGTATCTAATCCTGTT) used in this study were designed for a region in 16S rRNA and had been well demonstrated by other researchers to be uniformly successful in detecting a wide range of bacteria in samples of dentine<sup>[11]</sup> and feces<sup>[12]</sup>. Real-time PCR was performed with the ABI-Prism 7900 Sequence Detection System (Applied Biosystems, USA). The PCR reaction was performed in a total volume of 25  $\mu$ L, containing SYBR Green Real-time PCR Master Mix (TOYOBO Inc., Japan) 12.5  $\mu$ L, with 200 nmol/L of each of the forward and reverse primers and 2 ng of DNA for each reaction. The PCR reaction conditions for amplification of DNA were 95°C for 1 min and 40 cycles of 95°C for 20 s and 60°C for 1 min. Data analysis was performed using Sequence Detection Software supplied by Applied Biosystems.

### **Pancreatic pathology**

The pancreas was cut, formalin-fixed and embedded in paraffin, and 4  $\mu$ m sections were cut and stained with hematoxylin and eosin. The pancreatitis pathology score of every rat at both the 4th and 7th days were

**Table 1 Pancreatitis histopathology: Schmidt Scoring Criteria**

	Score			
	0	1	2	3
Interstitial edema	None	Interlobular	Lobule involved	Isolated island-like acinar cells
Leukocyte infiltration	None	< 20%	20%-50%	> 50%
Acinar cell necrosis	None	< 5%	5%-20%	> 20%
Hemorrhage	None	1-2 points	3-5 points	> 20%

The Schmidt Scoring Criteria of pancreatitis pathology includes 4 aspects, including interstitial edema, inflammatory infiltration, parenchymal necrosis and hemorrhage, all of which were given scores of 0-3 according to the severity of the disease.

determined according to the severity of pancreatic damage by a pathologist using a double-blind method, according to the Schmidt Scoring Criteria<sup>[13]</sup> (Table 1).

### Intestinal mucosa electron microscopy

The 4th day, which was the mid-term course of the illness, was chosen to determine the microstructural changes in the intestinal mucosa. In order to sample from the same intestinal location of all rats, a 2 cm section of the distal ileum (next to the ileocecal valve) was taken and quickly placed in fixative solution, and sent to the Electron Microscopy Center of Sun Yat-sen University for electron microscopy examination.

### Animal mortality

Daily observation of animal deaths was made for 7 d. The cumulative mortality rate was calculated daily.

### Statistical analysis

Data were displayed as the mean  $\pm$  SE. Statistical significance was calculated by one-way analysis of variance or the  $\chi^2$  test, using the Sigma Plot Software Package (Systat Software Inc, Point Richmond, Ca, USA).

## RESULTS

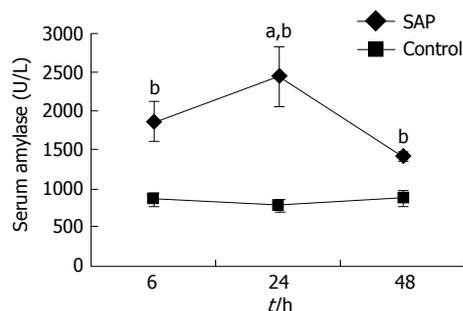
### Abnormal performance of rats

**Pancreatitis group:** Several minutes after injection of taurocholate beneath the pancreatic capsule, the pancreas was found to become swollen, dark purple, with partial subcapsular hemorrhage. The rats took more time to wake after surgery than control rats, and were tired on the first day with reduced activity, no appetite, and slow responses. Conditions became worst on the 2nd and 3rd days, when some rats were lethargic, had conjunctival congestion or petechiae, abdominal distention and hyperventilation and some rats died. However after the 4th day, the surviving rats would always recover gradually.

**Control group:** Rats awoke soon after operation, recovered in 6-8 h with normal characteristics and activity. 24 h later the rats performed as normal, and with no deaths.

### Feasibility of the surgical procedure

Uniformly distributed injections beneath the pancreatic



**Figure 1 Amylase level in serum.** Compared with control animals, serum amylase levels in severe acute pancreatitis (SAP) rats were higher at each time point (6, 24 and 48 h),  $^bP < 0.01$ . At 24 h, the amylase level of the pancreatitis model reached a peak, and was higher than the other 2 time points,  $^aP < 0.05$ .

capsule were successfully achieved in all animals and required an average of  $15 \pm 2$  min per animal when the technique was well mastered. The cumulative death rate at 7 d in the pancreatitis group was about 30%, with most occurring on the 3rd and 4th days (about 80% of all), while no deaths occurred in the control group.

There were 2 main points of note during the operation. First, a puncture too deep into the pancreatic parenchyma could injure the pancreatic veins and cause direct bleeding, but simply pressing with aseptic gauze for a moment would stop this. Second, if the syringe needle pierced through the pancreatic capsule, or taurocholate bubbles under the pancreatic capsule burst, the taurocholate could go into the abdominal cavity and cause biliary peritonitis. However, as a whole, the operation itself was simple and safe, causing few complications.

### Rat autopsy

Autopsies were carried out on dead rats. In all cases, much yellow or bloody exudate with a slight smell would come out of the peritoneal cavity when the abdomen was opened. The intestine would expand to 2-3 times of normal with gas and fluid inside and with a thinner wall. The pancreas was swollen and dark colored with some local hemorrhagic spots. Yellow-white saponified spots were always found in the peripancreatic omentum and retroperitoneum. The lower part of the spleen was always dark black. A pancreatic or hepatic abscess could be observed if the rat died after the 4th day or later.

### Serum level of amylase

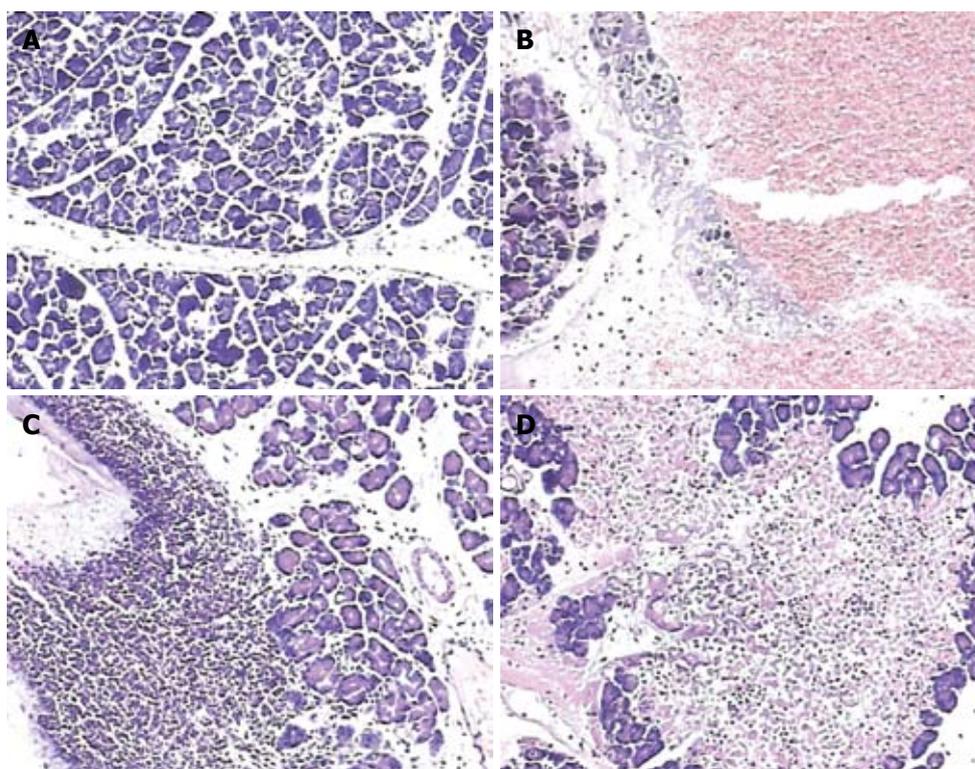
In pancreatitis rats, compared with the control group, serum amylase began increasing 6 h after the operation, and the level reached a peak at 24 h, remaining higher than the control group 48 h later (Figure 1).

### Pancreatic histology

Microscopic pancreatic damage was pronounced in pancreatitis animals in the first 48 h and showed a progressive disease course; 6 h after pancreatitis induction, typical pathological features were edema and hemorrhage (Figure 2B), while apparent leukocyte infiltration was observed 24 h after operation (Figure 2C), and areas of acinar cell necrosis could be observed 48 h after operation

Groups ( <i>n</i> = 8)	Score				Total
	Interstitial edema	Leukocyte infiltration	Acinar cell necrosis	Hemorrhage	
Control					
4 d	0.13 ± 0.33	0.13 ± 0.33	0	0	0.25 ± 0.67
7 d	0.13 ± 0.33	0	0	0	0.13 ± 0.33
SAP					
4 d	1.5 ± 0.76 <sup>a</sup>	2.38 ± 0.74 <sup>a</sup>	1.75 ± 0.71 <sup>a</sup>	0.75 ± 1.17 <sup>a</sup>	6.38 ± 2.20 <sup>a</sup>
7 d	1.25 ± 0.46 <sup>b</sup>	2.13 ± 0.99 <sup>b</sup>	1.5 ± 0.54 <sup>b</sup>	0.50 ± 1.07 <sup>b</sup>	5.38 ± 1.77 <sup>b</sup>

In this table, the pancreatitis pathology scores in severe acute pancreatitis (SAP) rats were significantly higher than in control rats in every single aspect as well as the total score, both on the 4th day (<sup>a</sup>*P* < 0.01) and on the 7th day (<sup>b</sup>*P* < 0.01).



**Figure 2 Pancreatic pathology (HE, × 100).** A: Normal pancreatic histology; B: Pancreatic damage induced 6 h after pancreatitis induction. Typical pathological features were edema and hemorrhage; C: Apparent leukocyte infiltration was observed 24 h after surgery; D: Pancreatic pathology induced 48 h after surgery, showing a confluent area of acinar cell necrosis.

(Figure 2D). The control animals were observed without such abnormalities, and had normal pancreatic gland lobules and cells. (Figure 2A)

On both the 4th and 7th days, pancreatitis pathology scores according to the Schmidt criteria were observed to be significantly higher in the pancreatitis group than in the control group in all 4 single aspects, as well as the total score (*P* < 0.01, Table 2), showing that significant damage had occurred in the pancreas of SAP rats.

### Serum endotoxin

The serum endotoxin level was a sign of intestinal permeability and pancreatitis severity. The plasma endotoxin levels in the pancreatitis group were about 5-7 times higher than that in the control group on both the 4th and 7th days (*P* < 0.01), though the level had decreased almost a half on the 7th day compared to the 4th day (Figure 3).

### Intestinal permeability

FITC-Dextran concentration in the SMV was a sign of intestinal permeability. In this study, FITC-Dextran levels in the SMV of pancreatitis rats were observed to be significantly higher than that of control rats on both the 4th and 7th days (*P* < 0.01, Figure 4), indicating higher intestinal permeability in the pancreatitis rats.

### Ultrastructural changes of the iliac mucosa

Microvilli of iliac mucosa in pancreatitis rats (Figure 5A) were shortened, about half the length of those of the control rats (Figure 5C), and with a loose structure, and some points had disappeared. Tight junctions and intermediate junctions of villous cells in SAP rats were unclear with discontinuities (Figure 5B), while intact and clear junctions between villous cells were seen in control rats (Figure 5D).

**Table 3** Infection determined by the bacterial culture method

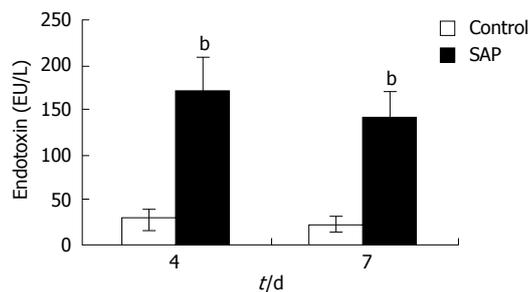
Groups (n = 8)	Infected organs number														
	Blood			MLN			Pancreas			Liver			Total		
	Ae	Ana	T	Ae	Ana	T	Ae	Ana	T	Ae	Ana	T	Ae	Ana	T
Control															
4 d	0	0	0	0	1	1	0	0	0	0	0	0	0	1	1
7 d	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SAP															
4 d	3	2	3	6	6	6	7	7	7	7	7	7	23	22	23 <sup>a</sup>
7 d	3	3	3	5	6	6	7	8	8	5	5	5	20	22	22 <sup>b</sup>
Total	6	5	6 <sup>c</sup>	11	13	13	14	15	15	12	12	12	43	45	46

Infection rates of each organ and overall in the SAP group were higher than that in the control group, both on the 4th day (<sup>a</sup> $P < 0.01$ ) and 7th day (<sup>b</sup> $P < 0.01$ ); Compared with the other 3 types of organ, the infection rate of blood was lowest, <sup>c</sup> $P < 0.05$ . MLN: Mesenteric lymph nodes; Ae: Aerobes; Ana: Anaerobes; T: Total.

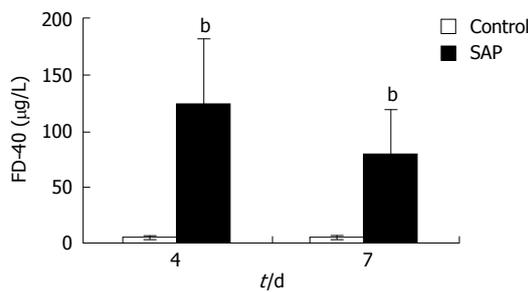
**Table 4** Bacterial translocation detected by real-time polymerase chain reaction

	Samples (n = 16)			
	Blood	MLN	Pancreas	Liver
Infected number in control group	0	1	0	0
Infected number in SAP group	5 <sup>a</sup>	10 <sup>a</sup>	13 <sup>a</sup>	12 <sup>a</sup>
Bacteria number (transformed by log <sub>10</sub> )	5.43 ± 0.847	6.48 ± 1.56	7.70 ± 0.766 <sup>b</sup>	6.57 ± 1.44

<sup>a</sup> $P < 0.01$ , compared with the "Infected number" in the control group; <sup>b</sup> $P < 0.05$ , compared with the "Bacteria number (transformed by log<sub>10</sub>)" in blood, MLN and liver.



**Figure 3** Endotoxin level in serum. On the 4th and 7th days, the serum endotoxin level was markedly higher in the pancreatitis group compared to the control group, <sup>b</sup> $P < 0.01$ .



**Figure 4** FITC-Dextran (FD-40) levels of serum in the superior mesenteric vein (SMV). The serum level of FD-40 in SMV was significantly higher in the pancreatitis group than in the control group on the 4th and 7th days, <sup>b</sup> $P < 0.01$ , though the level had decreased almost a half between days 4 and 7.

### Bacterial culture results

The copies of translocated bacteria differed greatly among pancreatitis rats, sometimes by  $10^3$  times, so it was difficult to define the statistics on bacterial numbers by the bacterial culture method. Therefore we chose the infection rate as a variable to compare the bacterial translocation in the 2 groups by the agar plate culture method. Results showed that infection rates of all the 4 types of organ were higher in the pancreatitis group than in the control group ( $P < 0.01$ ). When comparison was made among the infected rats of the 4 kinds of organs, blood of the inferior vena cava was found to have the lowest rate ( $P < 0.05$ , Table 3).

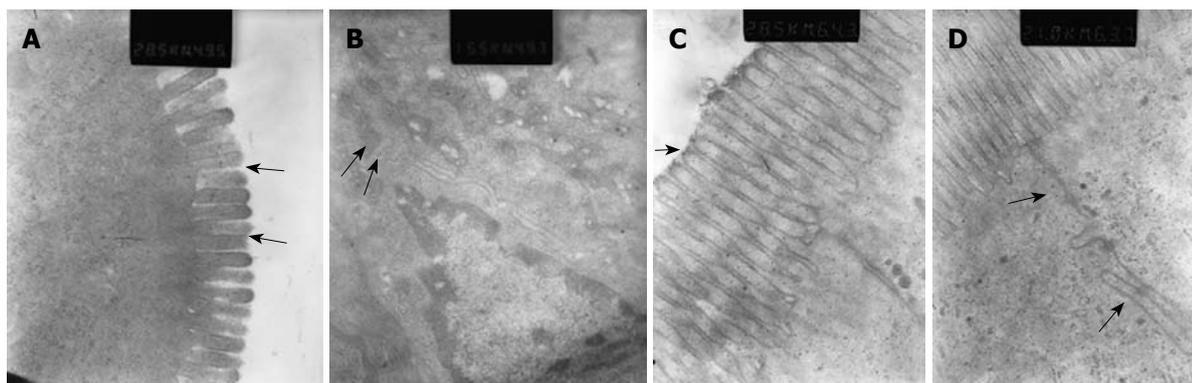
### Real-time PCR results of bacterial translocation

The infection rates detected by real-time PCR were mainly the same as that by bacterial culture. Infection rates by the culture method in MLN and pancreas were a little higher than that by real-time PCR method (MLN 12 to 10,

pancreas 15 to 13), which may result from contamination during culture. An advantage of the real-time PCR method was its accuracy of bacterial enumeration. In this study, the results of bacterial number had been transformed by log<sub>10</sub> for the purpose of better statistics. It was observed that the pancreas had the highest number of bacteria of the 4 kinds of organs ( $P < 0.05$ ), which may relate to necrosis and hemorrhage in pancreas tissue as a good medium for bacteria breeding (Table 4).

## DISCUSSION

Pancreatitis is a distressing disease clinically, especially SAP, which is associated with considerable mortality, as high as 30%-40%. The main features of this condition are pancreatic necrosis and associated sepsis, with both localized and systemic inflammatory response syndromes<sup>[1,14]</sup>. Infection of pancreatic necrosis is regarded to be



**Figure 5 Distal iliac mucosa electron microscopy.** A: Changes of microvilli in a SAP rat, showing loose, shortened microvilli, with some points lost (arrows) (Original magnification,  $\times 28.5$  K); B: Changes of the intermediate junction in a SAP rat, which was unclear with breakage in some parts (arrows) ( $\times 15.5$  K); C ( $\times 28.5$  K) and D ( $\times 21.0$  K) are representative electron microscopy images of the iliac mucosa in control rats, showing long, tight microvilli (arrow) and intact intermediate junctions (arrows).

a main predictor of outcome during SAP, and bacterial translocation of intestinal flora is considered to be the cause of pancreatic infection<sup>[15]</sup>.

Since Bernard first introduced an AP model of rabbits by injection of bile and olive oil into the pancreatic duct 150 years ago, animal experiments have become an irreplaceable method to study the pathophysiology, diagnosis and treatment of AP which, compared with clinical trials, have many advantages such as accessible subjects, standardization of the degree of the lesion, practicability of invasive inspection, adequate tissue samples, practicability of prophylactic treatment.

Nowadays there are many kinds of experimental models used to study bacterial translocation in AP. An ideal model of AP should encompass several features including easy reproducibility, with the ability to vary the severity of AP in a standardized manner according to the experimental aim, and to mimic the morphology and pathophysiology of the human situation<sup>[16]</sup>. At present, several commonly used experimental models were developed to imitate the human biliary pancreatitis, including duodenal loop, biliopancreatic duct ligation, and biliopancreatic duct perfusion.

A duodenal loop in rats can lead to AP of varying severity<sup>[17]</sup>. However, block of the gastrointestinal tract leads to mucosal atrophy and functional changes to the mucosal barrier<sup>[18]</sup>. Obstruction of bile flow into the intestine was shown to reduce intestinal motility, causing small bowel bacterial overgrowth and increased bacterial translocation<sup>[19-21]</sup>. Furthermore, the reflux of duodenal contents, including bacteria, into the biliopancreatic duct will confound the bacterial infection of the pancreas. All these limit its popularity.

The duct ligation model, in which the common biliopancreatic duct is surgically clipped or tied at the sphincter of Oddi complex, could produce moderate pancreatitis with minimal acinar cell necrosis only in American opossum inducing SAP. There are several factors that limit its use. First, obstruction of bile flow into the intestine causes small bowel bacterial overgrowth and bacterial translocation<sup>[20]</sup>. Second, induction of jaundice would impair the immune system to an uncertain degree and make the research result inaccurate. Third,

exclusion of pancreatic proteases in the gut lumen alters intestinal permeability, which does not truly occur in human pancreatitis<sup>[22,23]</sup>.

The duct perfusion model, usually made by perfusion of taurocholate after puncturing the duodenum and cannulating the papilla of Vater, was the most popular model of AP, with etiology resembling human biliary pancreatitis and with the advantage of quick induction of AP and reproducibility of results. However several potential confounders exist in it for study of bacterial translocation. The introduction of duodenal bacteria, through the papilla of Vater into the biliopancreatic duct could potentially affect the result of pancreatic infection; duodenal puncture and intestinal handling during surgery may also potentially affect the mucosal barrier function<sup>[24]</sup>. A complicated surgical procedure and prolonged time are other shortcomings of it.

In 2002 Wu *et al.*<sup>[20]</sup> first reported that pancreatic subcapsular injection of 3.8% Na taurocholate could establish an SAP animal model with multiple organ dysfunction in rats, but with a high mortality rate (90% in a week), which may be related to excessive use of 3.8% Na taurocholate (1 mL/rat) and of small rats (body weight about 100 g). This study used pancreatic subcapsular injection of 3.8% Na taurocholate (1 mL/kg) in rats of about 250 g body weight, and successfully established a model of AP. AP rats showed abnormal performance resembling human disease and the autopsy was in line with the performance of human SAP as well. The serum amylase level and pancreatic tissue pathology gave a clear diagnosis of AP. Changes of serum amylase levels in the AP group were similar to that in human disease, and increased significantly 6 h after induction, reaching a peak in 24 h and remaining at a higher level 48 h later. Pancreatitis pathology showed interstitial edema of the pancreas 6 h after model induction, with local hemorrhaging and slight inflammatory infiltration. Lesions developed progressively to moderate necrosis with obvious inflammatory infiltration 48 h later. The pancreatic pathology score on the 4th and 7th days showed a clear phenomenon of necrotic pancreatitis, including interstitial edema, leukocyte infiltration, acinar cell necrosis and hemorrhage. The scores of edema and hemorrhage in

the pancreatitis group were less than that of necrosis and inflammatory infiltration, which may be related to edema absorption and hematoma organization after several days. Some other organs were involved, peripancreatic calcified plaques formed, with liver and spleen ischemia, intestinal expansion, peritoneal exudates, and even death. Severity reached a peak in the medium term of the disease (3rd and 4th days), with the highest mortality, and although some indices were still high on the 7th day, few deaths occurred after 7 d, which may be related to the adaptation of the body or a sign of recovery. The cumulative mortality rate in 7 d was moderate, about 30%.

Agar plate culture is nowadays the gold standard method for bacterial measurement, however it has some shortcomings such as prolonged time and inaccuracy that limit early detection of bacterial translocation in the clinic. Recently, a real-time PCR method has been demonstrated successfully in the fast and accurate detection of bacteria in samples of blood, feces, and dentine; however few results are available on its use to detect bacteria in parenchymal organs such as lymphoid tissue, spleen, *etc.* Our study confirmed its use in enumeration of bacteria in this AP model in samples of not only blood but also parenchymal organs. Infection rates of all the 4 types of organ in the SAP group were significantly higher than that of the control group with quite similar results detected by bacterial culture or real-time PCR. Infection rate and bacterial number of inferior vena cava blood in the SAP group were both much lower than that of the other organs, while the MLN had a similar infection rate and bacterial number to that of the liver, suggesting a lymphatic route of bacterial translocation may exist in this SAP model.

A damaged intestinal barrier is thought to be a key factor for bacterial translocation in SAP<sup>[25-28]</sup>. In this study, obvious gut barrier damage was observed, with the FITC-Dextran in the SMV and endotoxin levels both significantly increased in AP rats compared to control rats. Electron microscopy of the intestinal mucosa directly showed the damage in the mucosa cells, with microvillus atrophy and intercellular junction disruption.

This rat model requires an invasive laparotomy, with some inevitable potential confounding factors, such as anesthesia, trauma, stress and so on, but few other potential confounding factors existed in it. The surgical procedures were so simple that they could be finished in 15 min once the skills were mastered. No other organs except the pancreas were affected during the surgery, therefore avoiding some potential factors that could affect the measurement of bacterial translocation.

In conclusion, in this study we induced a simple taurocholate pancreatitis model in rats to study the situation of intestinal barrier damage and bacterial translocation in SAP. The results confirmed the situation, as observed clinically, that intestinal barrier damage and bacterial translocation exist in SAP with endotoxemia. However the exact pathophysiologic mechanism of bacterial translocation in SAP is still unknown and needs more intense research for the purpose of determining better preventative measures and treatment of SAP in the clinic.

## COMMENTS

### Background

Severe acute pancreatitis is associated with considerable mortality, as high as 30%-40%. Infection of pancreatic necrosis is regarded to be a main predictor of outcome during severe acute pancreatitis (SAP), and bacterial translocation of intestinal flora is considered to be the cause. Although a multitude of animal models have been used to study the mechanism of bacterial translocation, the exact origin, route, and mechanism of bacterial translocation causing infection of the necrotic pancreas are still unclear.

### Research frontiers

There are now many kinds of experimental models used to study bacterial translocation in acute pancreatitis (AP). However, an "ideal" animal model of SAP was lacking, and should encompass several features including easy reproducibility, ability to vary the severity of AP in a standardized manner, and similar morphology and pathophysiology to that of the human situation.

### Innovations and breakthroughs

A new simple model of SAP in the rat was induced by Na taurocholate, and results showed that significant gut barrier damage and intestinal bacterial translocation occurred in this SAP rat model, with few potential study confounders and with a shorter induction time compared to other commonly used animal models of SAP, suggesting that it may be an appropriate animal model for the study of gut barrier damage and bacterial translocation in SAP.

### Applications

In future, this simple taurocholate-induced rat model may be used in the study of SAP, especially to determine the exact origin, route, and mechanism of bacterial translocation causing infection of the necrotic pancreas.

### Terminology

Intestinal bacterial translocation: normally, intestinal bacteria cannot go through the gut barrier and into the blood or other organs. In the situation of SAP or other severe disease such as shock or major burns, the gut barrier would be damaged and the intestinal permeability increased, causing the intestinal bacteria to translocate from the gut to blood or even other distant organs and induce sepsis, septicemia or even death.

### Peer review

This is a well performed research trying to induce a new simple rat model of SAP, and results definitely showed it was an appropriate animal model for study of bacterial translocation in SAP, with significant gut barrier damage and intestinal bacterial translocation observed but with few potential confounders.

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BRIEF ARTICLE

## Single-center experience of 309 consecutive patients with obscure gastrointestinal bleeding

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### Abstract

**AIM:** To investigate the diagnostic yield of capsule endoscopy (CE) in patients with obscure gastrointestinal bleeding (OGIB), and to determine whether the yield was affected by different bleeding status.

**METHODS:** Three hundred and nine consecutive patients (all with recent negative gastric and colonic endoscopy results) were investigated with CE; 49 cases with massive bleeding and 260 cases with chronic recurrent overt bleeding. Data regarding OGIB were obtained by retrospective chart review and review of an internal database of CE findings.

**RESULTS:** Visualization of the entire small intestine was achieved in 81.88% (253/309) of cases. Clinically positive findings occurred in 53.72% (166/309) of cases. The positivity of the massive bleeding group was slightly higher than that of the chronic recurrent overt bleeding group but there was no significant difference (59.18% vs 52.69%,  $P > 0.05$ ) between the two groups. Small intestinal tumors were the most common finding in the entire cohort, these accounted for 30% of clinically significant lesions. In the chronic recurrent overt bleeding group angioectasia incidence reached more than 29%, while in the massive bleeding

group, small intestinal tumors were the most common finding at an incidence of over 51%. Increasing patient age was associated with positive diagnostic yield of CE and the findings of OGIB were different according to age range. Four cases were compromised due to the capsule remaining in the stomach during the entire test, and another patient underwent emergency surgery for massive bleeding. Therefore, the complication rate was 1.3%.

**CONCLUSION:** In this study CE was proven to be a safe, comfortable, and effective procedure, with a high rate of accuracy for diagnosing OGIB.

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**Key words:** Obscure gastrointestinal bleeding; Capsule endoscopy; Massive bleeding; Chronic recurrent overt occult bleeding

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Zhang BL, Fang YH, Chen CX, Li YM, Xiang Z. Single-center experience of 309 consecutive patients with obscure gastrointestinal bleeding. *World J Gastroenterol* 2009; 15(45): 5740-5745 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5740.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5740>

### INTRODUCTION

Obscure gastrointestinal bleeding (OGIB) is defined as recurrent or persistent bleeding or iron deficiency anemia after a negative result from gastric and colonic endoscopy<sup>[1]</sup>. OGIB accounts for approximately 5% of all gastrointestinal (GI) bleeding and lesions in the small bowel<sup>[2]</sup>. Previous capsule endoscopy (CE) investigations of small intestine showed the value in finding small intestinal diseases, especially inflammatory bowel disease and OGIB. CE is a tool that allows for visualization of mucosa throughout the entire small bowel and it has already gained widespread clinical acceptance. CE has been shown to be superior to push enteroscopy<sup>[3,4]</sup>, small bowel follow-through<sup>[5]</sup> and computed tomography

(CT) scan<sup>[6]</sup> in detecting bleeding lesions in the small intestine. Early reports indicated that 45%-66% of patients with OGIB were detected by CE<sup>[3,5,7]</sup>. Despite promising preliminary results, published studies were limited to small groups of patients. In this study a large group of OGIB patients were investigated by CE examination, including obscure-overt and a few obscure-occult patients. We divided these patients into a massive bleeding group (bloody stool or circulatory failure, serious anemia) and a chronic recurrent overt occult bleeding group (melena). Therefore, we investigated the outcome of CE in a large group of patients with OGIB from a single institution and determined whether the results of CE for OGIB patients were related to bleeding status. Also, the selection of OGIB candidates for successful CE evaluation was discussed in our study.

## MATERIALS AND METHODS

### Patients

Three hundred and nine cases underwent CE for the indication of OGIB at our institution from May, 2003 to April, 2008. All patients suffering at least two episodes of bleeding prior to CE had undergone endoscopy without localization of a bleeding lesion. Data of the 309 cases were obtained by retrospective chart review. Data included: age, gender, inpatient or outpatient status, status of GI bleeding (massive bleeding *vs* chronic recurrent overt occult bleeding), median units of red blood cells (RBCs) transfused, mean hemoglobin value prior to endoscopic evaluation, prior radiographic evaluation, computed tomography (CT) evaluation, and lesion diagnosis confirmed by surgery. CE data collected included: whether the entire small intestine was visualized, CE findings in the small intestine and whether complications occurred. Pregnant women, patients with pace-makers or intestinal obstruction were excluded from the study. The study was approved by the local ethics committees.

### Examination with CE

The Given Diagnostic Imaging System (Yoqneam, Israel) was used in our study. This system is composed of three main subunits: an ingestible capsule endoscope, a data recorder, and a workstation. On the day before the examination, patients were allowed to be on a semi-liquid diet and received complete colonic irrigation (polyethylene glycol 4000, PEG4000) 12 h before the examination. Patients were deprived of water for 4 h and defoamer (simethicone) was administered orally 30 min prior to the examination, respectively. Informed consent was obtained. Four hours after swallowing the capsules, subjects were allowed to take light snacks such as milk. During the examination, subjects were allowed to move freely but exposure to any strong electromagnetic field would be avoided. Eight hours later, data recorded would be downloaded to a RAPID workstation for graphic analysis. All capsules were for single use only.

### Data analysis

Findings on CE were classified as diagnostic and non-

**Table 1** Baseline characteristics of patients with OGIB (*n* = 309)

Item	All ( <i>n</i> = 309)	Massive bleeding ( <i>n</i> = 49)	Chronic recurrent overt bleeding ( <i>n</i> = 260)
Mean age ± SD (yr)	55.5 ± 16.6	52 ± 19.1	56.1 ± 16.1
Years (range, yr)	13-87	13-87	15-86
Gender <i>n</i> (%)	150 M (48.5), 159 F (51.5)	27 M (55.1), 22 F (44.9)	123 M (47.3), 137 F (52.7)
Patient status ( <i>n</i> )			
Outpatient	141	0	141
Inpatient	168	49	119
Median units of RBCs transfused (U/mL)	4.63 ± 4.23	8.00 ± 6.46	4.00 ± 6.85
Mean Hb value (range, g/dL)	8.73 (4.52-12.10)	6.20 (4.52-9.85)	9.21 (6.56-12.10)

RBCs: Red blood cells; Hb: Hemoglobin; M: Male; F: Female.

diagnostic lesions. Examples of non-diagnostic lesions included red spots, white spots, erythema, scatter lymphangiectasia, active bleeding, blood clots, or small polyps. Examples of diagnostic lesions included angioectasias, tumors, or ulcers, obvious small bowel inflammation, and roundworms. A diagnostic lesion had to definitely or probably account for the patient's bleeding and was diagnosed by two experienced endoscopists.

### Statistical analysis

Statistical methods included  $\chi^2$  analysis for comparison of the positive diagnostic yield of CE between patients with acute large bleeding and chronic recurrent overt occult GI bleeding. SPSS 16.0 (SPSS Inc. Chicago, IL) was used in our study, and *P* < 0.05 was considered a statistical significance.

## RESULTS

### Patient baseline characteristics

The 309 cases with OGIB in this study are listed in Table 1 with regard to their baseline characteristics. Prior to CE, for all patients, the cause of OGIB could not be identified with either gastroscopy or colonoscopy. Among them, 39.16% (121/309) underwent a CT scan to detect the source of GI bleeding at the same time, and the percentage of patients who finally underwent surgical operation was 11% (34/309).

### Results of CE

**Completion of CE:** Of all CE procedures performed, 81.88% (253/309) extended through the entire small intestine. However, the rate of going right through the intestine in the chronic recurrent overt bleeding group was higher than that of the massive bleeding group (83.85% *vs* 69.39%, *P* = 0.017, respectively). Four cases with gastric motility disorder resulting in the capsule remaining in the stomach during the entire test were compromised, and another case resulted in emergency surgery because of the large amount of bleeding.

**The diagnostic ability of CE:** The yield of CE, defined

**Table 2** The yield of small bowel diagnostic findings on capsule endoscopy *n* (%)

	All ( <i>n</i> = 309)	Massive bleeding ( <i>n</i> = 49)	Chronic recurrent overt bleeding ( <i>n</i> = 260)
Diagnostic	166 (53.7)	29 (59.2)	137 (52.7)
Non-diagnostic/negative	143 (46.3)	20 (40.8)	123 (47.3)

**Table 3** CE diagnostic yields of massive bleeding and chronic recurrent bleeding *n* (%)

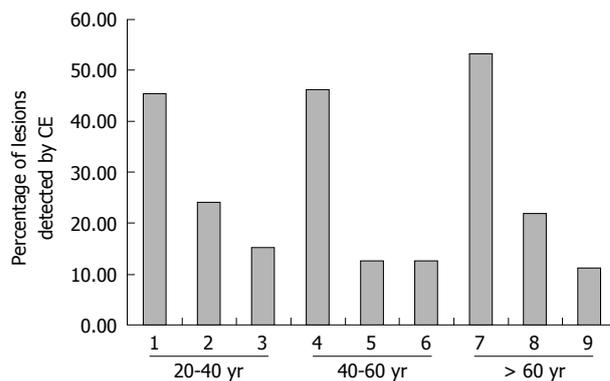
Type of lesion	All	Massive bleeding	Chronic recurrent overt bleeding
Angioectasias (Figure 2)	49 (28.3)	6 (22.2)	43 (29.9)
SB tumor	53 (30.6)	15 (51.7)	38 (26.0)
Mesenchymoma (Figure 3)	15 (8.7)	5 (17.2)	10 (6.9)
Polyps	13 (7.5)	2 (6.9)	11 (7.6)
Hemangioma (Figure 4)	6 (3.5)	2 (6.9)	4 (2.8)
Lipoma	4 (2.3)	0 (0)	4 (2.8)
Lymphoma (Figure 5)	4 (2.3)	2 (6.9)	2 (1.4)
BRBNS	2 (1.2)	2 (6.9)	0 (0)
Adenoma	1 (0.6)	1 (3.5)	0 (0)
Metastatic melanoma	1 (0.6)	0 (0)	1 (1.4)
Mass	7 (4.0)	1 (3.5)	6 (4.2)
Crohn's disease (Figure 6)	25 (14.5)	2 (6.9)	23 (16.0)
Ulcer	15 (8.7)	2 (6.9)	13 (9.0)
Multiple ulcer	11 (6.4)	1 (3.5)	10 (7.6)
Isolated ulcer	4 (2.3)	1 (3.5)	3 (2.1)
SB verminosis	12 (6.9)	2 (6.9)	10 (6.9)
Non-specific enteritis	10 (5.8)	0 (0)	10 (6.9)
SB diverticulum	7 (4.0)	1 (3.5)	6 (4.2)
SAME	1 (0.6)	1 (3.5)	0 (0)
Portal hypertension SB disease	1 (0.6)	0 (0)	1 (1.4)
Total	173	29	144

SAME: Superior mesenteric artery embolus; SB: Small bowel; BRBNS: Blue rubber bleb nevus syndrome.

as diagnostic lesions as the source of OGIB, was 53.72% (166/309) in the entire cohort and slightly higher in those patients with massive bleeding compared to those in the chronic recurrent overt bleeding group, with no significant statistical difference between groups (59.18% *vs* 52.69%, *P* > 0.05, respectively) (Table 2).

Small bowel angioectasias and small bowel tumors (masses) were common and accounted for over 28% of lesions in the entire cohort (Table 3). In the chronic recurrent overt bleeding group, angioectasia incidence was over 29% (Table 3), while in the massive bleeding group small bowel tumors were a common finding at over 51%. Angioectasias were the second most common lesion and accounted for 22.2%, while Crohn's disease was in third place (Table 3).

**Age and diagnostic yield of CE:** Increasing patient age was associated with positive diagnostic yield of CE and the findings of OGIB were different according to age range. In the 20-40 years age group, Crohn's disease was found in almost 45.5% (15/33) of cases, and in the 40-60 years age group, tumor incidence was 46.2% (26/56). Angioectasias occurred in over 50% (39/73) of cases when the patients' age was over 60 years (Figure 1).

**Figure 1** Lesions detected by capsule endoscopy (CE) for different age ranges. 1: Crohn's disease; 2: SB tumor; 3: Non-specific enteritis; 4: SB tumor; 5: Crohn's disease; 6: Angioectasias; 7: Angioectasias; 8: SB tumor; 9: Ucler.

**Failed diagnoses of CE:** Among the non-diagnostic studies, 15 cases were diagnosed by further examinations, such as CT scan, angiography, and surgery. In the massive bleeding group, two cases were negative and three cases were actively bleeding with unavailable diagnostic lesions. In the chronic recurrent overt bleeding group, four cases were actively bleeding but without diagnostic lesions. One case was diagnosed as tumor but finally confirmed as Meckel's diverticulum by surgical operation. One case was diagnosed by CE as ulcer and was confirmed as tumor by surgical operation. Four cases were negative.

## DISCUSSION

OGIB is the main indicator of the appropriateness of CE as a diagnostic procedure. Many studies have demonstrated a high degree of usefulness of CE as a diagnostic method. The common findings of OGIB reported are angioectasias, small bowel tumor or mass, inflammation, Meckel's diverticulum, *etc.* Barium small bowel examination, angiography and push enteroscopy were the common evaluations for OGIB before the development of CE. Barium showed a diagnostic rate of 3%. Angiography was an invasive method, with a diagnostic rate of 27%-77%, requiring a blood flow > 0.5 mL/min, while push enteroscopy could not reach the lower part of the small bowel and had a diagnostic rate of 19%-32%. Recently, double-balloon enteroscopy (DBE) has been introduced for the diagnosis of OGIB, as reported by a meta analysis<sup>[8]</sup>. The diagnostic yield from CE was significantly higher than that of DBE without the combination of oral and anal insertion approaches [137/219 *vs* 110/219, OR 1.67 (95% CI: 1.14-2.44), *P* < 0.01, respectively], but not superior to the yield of DBE with combination of those two insertion approaches [26/48 *vs* 37/48, OR 0.33 (95% CI: 0.05-2.21), *P* > 0.05, respectively]. However, the DBE is invasive and patients are not as tolerant of this method. CE is the only non-invasive method which can examine the whole small intestine.

All cases in our study could tolerate well the CE examination. Five cases were compromised, one in the chronic recurrent overt bleeding group because of capsule



**Figure 2 Angioectasias.** The red spot is the site of angioectasias (arrow).



**Figure 4 Hemangioma.** The red-blue bubble is the site of hemangioma (arrow).



**Figure 3 Mesenchymoma.**

retained in the stomach, and four in the massive bleeding group. Among these four cases, three were compromised because of somatostatin; the capsules were all retained in the stomach due to disorders of gastric mobility. In one Crohn's patient CE was obstructed. No other complication occurred. Therefore, the complication rate was 1.3%. Technical problems and complications of CE are infrequent. Former studies showed a rate of CE complications ranging from 0.75%-5%<sup>[9-11]</sup> and also that the existence of patients who had difficulty swallowing the capsule was a rare event. In patients with known altered gastric mobility, the problem could be easily solved by endoscopic placement of the capsule into the duodenum<sup>[12,13]</sup>.

In our study, CE progressed through the whole small bowel in 81.55% (253/309) of patients, and complete progression rate in the chronic recurrent overt bleeding group was higher than that of the massive bleeding group. CE did not reach the ileocecum because of retention in the intestine due to motility disorder or stricture of the intestinal lumen. Capsule retention occurred almost universally at the site of lesions. In these patients, surgical intervention to retrieve the trapped capsule also allowed for resection of the small bowel involved in the lesion. CE retention by the lesions in our study was found in small bowel Crohn's disease cases and small bowel tumors.

The bowel preparation with PEG and simethicone ensured a good quality of pictures. The 309 patient cohort in our study is the largest series in published studies. The diagnostic rate in our study was 53.72%, which was similar to previous published studies. The diagnostic accuracy yield of CE in published reports ranges from a rate of 30% to 92%, mainly depending on the definition of positive findings and the status of bleeding investigated<sup>[3,5,9,14-16]</sup>. The

yield in a series of 260 patients previously reported was 53%. In patients with ongoing obscure overt GI bleeding the yield was 87%, and 46% in patients with obscure occult bleeding<sup>[11]</sup>. In this present study we included the presence of active blood as a positive finding. We discovered that the diagnostic rates in both the massive bleeding group and the chronic recurrent overt bleeding group were not statistically significantly different; 59.18% (29/49) and 52.69% (137/260), respectively. These results demonstrate that CE is the first diagnostic option for patients with massive bleeding, since the diagnostic rate was not affected by blood in the small bowel lumen. The relationship between the timing of the procedure and the diagnostic yield of CE remains a controversial issue. Pennazio *et al*<sup>[9]</sup> found the highest yield in patients with ongoing GI bleeding, and therefore argued for ordering a CE procedure earlier in the setting of obscure overt bleeding. One would presume that blood in the GI tract would mask visualization of the mucosa and that lesions could easily be missed. Delaying CE until after an acute bleed may allow for clearance of residual blood and better visualization of the mucosa and suspicious lesions. However, our study demonstrated that acute large bleeding did not affect the diagnostic rate.

The definition of a positive finding on CE is an important idea not addressed in early reports<sup>[17]</sup>. Although experts have begun to address this issue, final consensus has not yet been reached<sup>[17-19]</sup>. For the purposes of this study, nonspecific mucosal changes such as red spots, erythema, scatter lymphangiectasia and thickened folds were considered to be clinically insignificant. Lesions such as angioectasias, tumors, masses or ulcers were included as positive findings in this series if they could completely or partially account for the GI bleeding. In our study, active bleeding but without definite lesion was described as a non-diagnostic finding. Figures 2-6 show the typical lesions found in our study.

There were 15 cases confirmed as being a pseudo-negative result surgically. Five were in the massive bleeding group; 2 of these on CE were negative, and 3 of them found only active bleeding but without diagnostic lesions. Ten cases were in the massive bleeding group, 4 of them on CE were negative, and 4 of them found only active bleeding but without diagnostic lesions. One case was diagnosed as tumor but finally confirmed as Meckel's diverticulum by surgery, while another case indicating an ulcer was confirmed as small tumor by surgery.



Figure 5 Lymphoma.



Figure 6 Crohn's disease.

CE can completely cover the whole GI tract and the procedure is well tolerated. It may provide false-positive and false-negative findings due to the possibility of uncontrollable movement and low-resolution pictures taken<sup>[20]</sup>. It was recently reported that CE missed an advanced small intestinal cancer that was later diagnosed with push enteroscopy<sup>[21]</sup>, suggesting that CE is not an exclusive procedure but one complementary to other diagnostic tools in the assessment of small intestinal lesions. In Mehdizadeh's series<sup>[22]</sup>, CE found a potential bleeding source in 63 patients, but 34.9% (22/63) of them received negative results in the following DBE procedure. In our study, failed or negative findings of CE may have resulted from four main reasons: (1) growth pattern of lesions: transmural and extra-mural lesions could not be detected by CE; some angioectasias could only be detected by microscope; (2) technical factors of CE: the visual field of CE was only 140°, which perhaps leads to a blind spot; some lesions could be omitted due to movement of CE which could not be controlled. One case with Meckel's diverticulum was diagnosed as small bowel tumor because of only partial views of the lesion; (3) cleanness of small bowel: in our study all cases were prepared with PEG and simethicone prior to examination and the visualization of CE was better than without any preparation, but in some cases, bubbles and food debris still affected the visualization. Delay of gastric motility caused by disease or drugs such as somatostatin would retain the capsule in the stomach, which could not go through the whole small bowel during the working time. The large number of false positives may result from the definition of clinically significant *vs* clinically insignificant findings.

In this study, common findings of lesions with OGIB were small bowel tumor (mass), angioectasias and

Crohn's disease. In the massive bleeding group, common findings of OGIB were small bowel tumor or mass and angioectasias, while in the chronic recurrent overt bleeding group common findings of OGIB were angioectasias, small bowel tumor (mass), and Crohn's disease. A higher positive percentage of small intestine tumors was found compared with all previous studies published due to the definition of diagnostic lesion. Some of the angioectasias discovered in our study cannot be assumed to contribute to the bleeding, and these cases were not included in the positive findings.

In this study, younger patients were more often found to have Crohn's disease, and older patients (older than 60 years) were prone to have angioectasias, while between 40 and 60 years, tumors accounted for a bigger proportion. According to the American Gastroenterological Association (AGA)<sup>[23]</sup>, younger patients are likely to have small intestinal tumors, Meckel's diverticulum, Dieulafoy's lesion, and Crohn's disease, while older patients (older than 40 years) are prone to bleeding from vascular lesions, which comprise of up to 40% of all causes, and nonsteroidal anti-inflammatory drug-induced small bowel disease. Our finding was somewhat different from the AGA. Thus, in clinical work, patients who are suspected of having Crohn's disease should be examined by CE as early as possible.

In summary, tolerance of administration, higher diagnostic yield, improvement in patient outcome and lower complication rate make CE an important advance in the diagnosis of OGIB. The AGA strongly recommended the CE as the first choice for OGIB. In our large cohort undergoing clinical investigation, we demonstrate that diagnostic rate of OGIB is determined by the definition of a clinically significant lesion. In addition, the massive bleeding status did not affect the positive detection rate.

## COMMENTS

### Background

Capsule endoscopy (CE) is a tool for visualization of mucosa throughout the entire small bowel. It has already gained a wide clinical acceptance in the evaluation of obscure gastrointestinal bleeding (OGIB). Despite promising preliminary results, published studies were limited to small groups of patients with heterogeneous indications.

### Research frontiers

CE is now commonly performed for gastrointestinal bleeding of obscure origin. However, reports about large groups of patients with OGIB receiving CE are rare in the literature up till now.

### Innovations and breakthroughs

Published studies of CE in the diagnosis of OGIB were limited to small groups of patients. The study analyzed a large group of OGIB patients, so the finding will be more objective. The authors concluded that the clinically important findings by CE in patients with OGIB were not affected by the different bleeding status, but disease diagnosis by CE is related to different bleeding status and ages. Small bowel tumor was the most common finding in general and also in the massive bleeding group. Angioectasias accounted for the majority of cases in the chronic recurrent overt bleeding group.

### Applications

CE is now widely used to diagnose small bowel diseases, especially in patients with OGIB. More and more patients who are suspected of having small bowel diseases are diagnosed using CE with less discomfort, less time taken and also fewer financial costs.

**Terminology**

CE is a device that can be easily swallowed by patients. It can progress through the whole digestive tract and take two photos every second. These photos can be studied on a workstation.

**Peer review**

The study is well constructed. It is a retrospective review of all the OBIG cases from a single referral centre and is, admittedly, one of the biggest series of its kind. Other than that though, it simply confirms findings of previous reports in the field.

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CASE REPORT

## Complete remission of gastric Burkitt's lymphoma after eradication of *Helicobacter pylori*

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the question of the potential role of *H pylori* in the pathogenesis of some gastric Burkitt's lymphomas, and show the importance of searching for and eradicating the bacteria in combination with conventional chemotherapy regimens.

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**Key words:** Burkitt's lymphoma; Gastric lymphoma; *Helicobacter pylori*; Therapy

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Baumgaertner I, Copie-Bergman C, Levy M, Haioun C, Charachon A, Baia M, Sobhani I, Delchier JC. Complete remission of gastric Burkitt's lymphoma after eradication of *Helicobacter pylori*. *World J Gastroenterol* 2009; 15(45): 5746-5750 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5746.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5746>

### Abstract

Burkitt's lymphoma is a highly aggressive non-Hodgkin lymphoma, often presenting in extra-nodal sites. It generally has a poor spontaneous outcome and needs aggressive treatment with systemic and intrathecal chemotherapy. Occurrence at the gastric site is rare. We report the case of a 39-year old woman who presented with a prominent ulcerated lesion of the antrum corresponding histologically to a Burkitt's lymphoma associated with *Helicobacter pylori* (*H pylori*) infection. Interphase fluorescence *in situ* hybridization (FISH) demonstrated *c-MYC* gene rearrangement in tumour cells without *BCL2* or *BCL6* gene translocations. Ulcer healing and tumour regression with a complete histological response were obtained 8 wk after *H pylori* eradication. In spite of this complete remission, taking into account the high risk of recurrence, the patient received systemic and intrathecal chemotherapy. Two years later, the patient remained in complete remission. This is the first report of a gastric Burkitt's lymphoma responding to *H pylori* eradication. These findings raise

### INTRODUCTION

The stomach is the most common site of extranodal malignant lymphomas, constituting 30%-45% of all extranodal malignant lymphoma and 1%-7% of all gastric malignancies<sup>[1]</sup>. The most common gastric lymphomas are extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT), characterized by an infiltration of the mucosa by centrocyte-like B-cells associated with lymphoepithelial lesions, and diffuse large B cell lymphomas (DLBCL)<sup>[2]</sup>. This last terminology is commonly used to describe *de novo* DLBCL or DLBCL associated with a low grade component. Burkitt's lymphoma (BL) is a highly aggressive non-Hodgkin lymphoma, often presenting in extranodal sites or as acute leukaemia. The incidence of gastric BL is low compared to other types of gastric lymphomas, although its true incidence is not yet defined<sup>[3]</sup>.

There is a close link between *Helicobacter pylori* (*H pylori*) and gastric MALT lymphomas and probably also with gastric DLBCL<sup>[4,5]</sup>. But the possibility of an association between *H pylori* and gastric BL has only been suggested

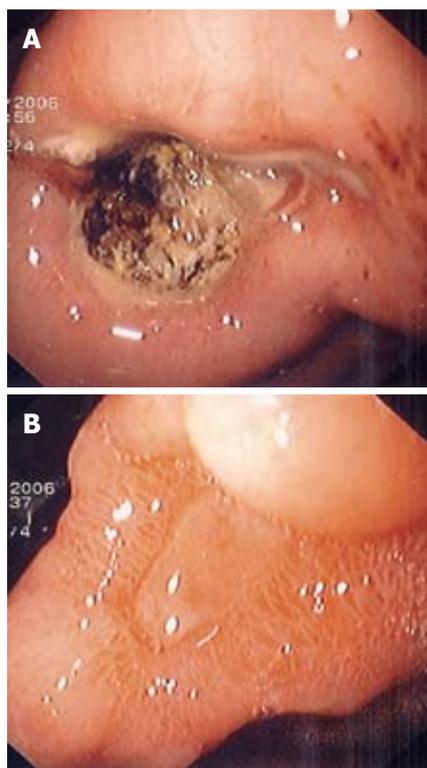
in occasional cases in children<sup>[6,7]</sup>. Eradication of *H pylori* is now well established as the first line treatment of gastric MALT lymphoma<sup>[8]</sup> and reports in the literature show 60%-80% remission rates<sup>[9-11]</sup>. In addition, several cases of complete remission of gastric DLBCL after *H pylori* eradication have been reported<sup>[12-16]</sup>. On the contrary, BL is known to require aggressive chemotherapy in order to induce remission, with systemic and intrathecal chemotherapy, and the impact of *H pylori* eradication on gastric BL has never been evaluated. We describe the first case of complete remission of a gastric BL in an adult patient following *H pylori* eradication.

## CASE REPORT

A 39-year old woman underwent a first gastroscopy in January 2006. She had complained of abdominal pain for 3 mo. She had no previous history of peptic ulcer disease. Examination revealed no palpable mass in the abdomen, ascites or superficial lymphadenopathy. A barium contrast radiography of the stomach showed an important ulcerated lesion of the antrum on the lesser curvature, suggesting malignancy. The patient underwent a gastroscopy that confirmed the ulcer at the angularis, measuring 5 cm × 5 cm (Figure 1A).

Histologic examination of gastric biopsies showed a diffuse infiltration of the mucosa by atypical lymphoid cells with a CD20+, CD3- immunophenotype and a diagnosis of “*de novo* DLBCL” was made initially by the local pathologist. *H pylori* corpuscles were observed histologically using modified Giemsa stain, and the patient was treated for *H pylori* eradication with pantoprazole 40 mg twice daily, amoxicillin 1 g twice daily and clarithromycin 500 mg twice daily for one week. Then she continued pantoprazole 40 mg once daily for three weeks and was referred to our gastroenterology unit four weeks later for further management of her disease. Initial gastric biopsies were reviewed by an expert hematopathologist (CCB) and additional immunostains and molecular studies were performed. The ulcerated mucosa was diffusely infiltrated by monotonous medium-sized lymphoid cells with round nuclei, scarce cytoplasm and starry sky pattern at low magnification (Figure 2A and B). Numerous mitoses were observed. *H pylori* were detected in biopsy specimens. Immunohistochemistry showed that the lymphoid cells were CD20+, CD5-, CD10+, BCL2-, BCL6+, MUM1-, P53+ and 100% of tumour cells were Ki67 positive (Figure 2C and D, and Figure 3A-C). *In situ* hybridization using EBERS probes was performed and EBV transcripts were not detected in tumour cells. Interphase fluorescence *in situ* hybridization (FISH) analysis was performed on formalin-fixed paraffin-embedded (FFPE) tissue sections using split signal FISH DNA probes for *c-MYC*, *BCL2* and *BCL6* (Dako AS, Glostrup, Denmark) according to the manufacturer's recommendations (www.euro.fish.org). *C-MYC* gene rearrangement was observed in tumour cells without *BCL2* or *BCL6* gene rearrangement (Figure 3D). All these findings were in accordance with the diagnosis of BL.

Follow up endoscopy performed 6 wk after initiation

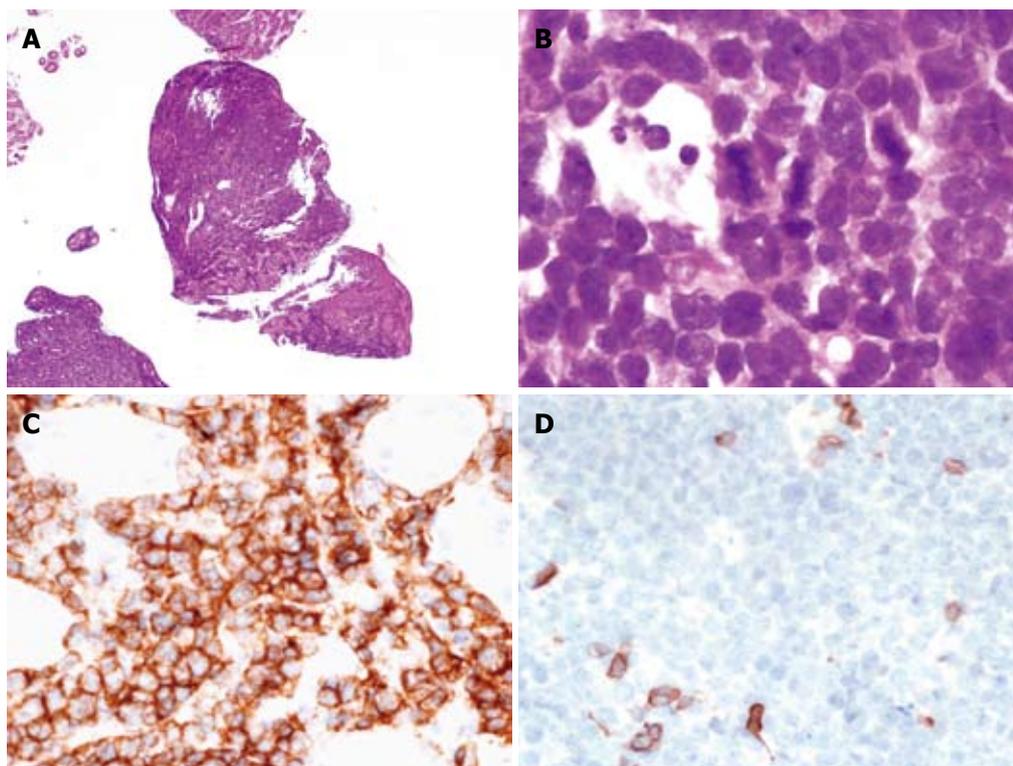


**Figure 1** Endoscopic aspect of the gastric lesion before and after *Helicobacter pylori* (*H pylori*) eradication. A: Oesophagogastroduodenoscopy shows a large ulcer at the angularis; B: Ulcer healing 3 mo after *H pylori* eradication.

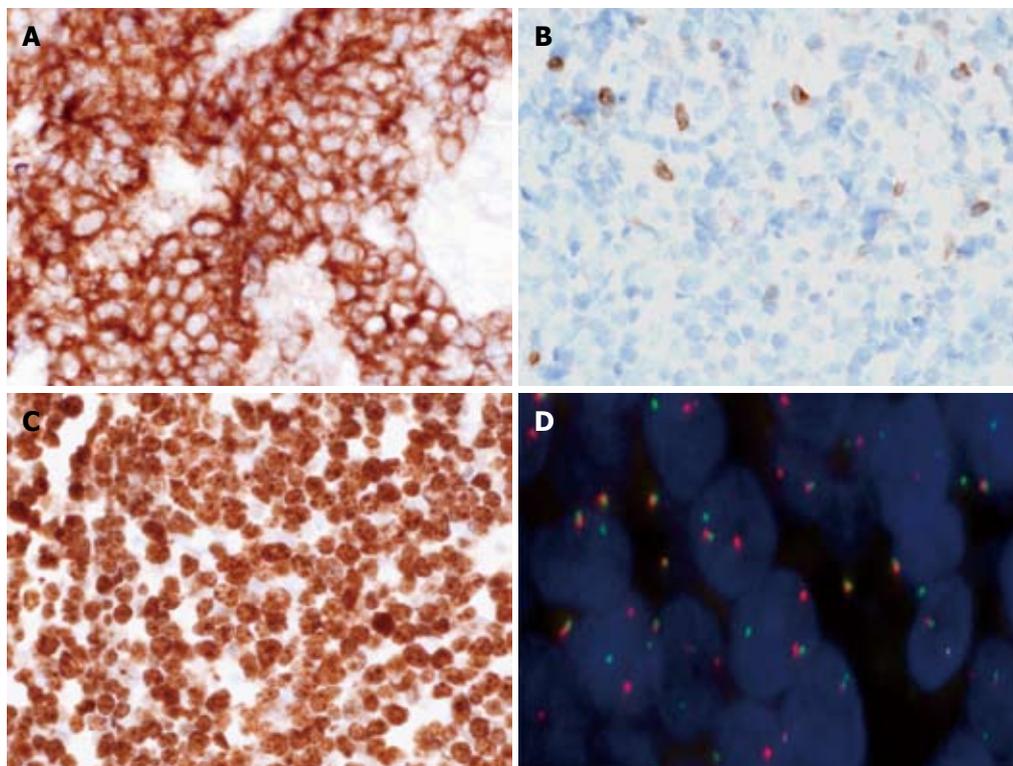
of *H pylori* eradication therapy showed the ulcer size had decreased, measuring 3 cm in diameter, and histological analysis of gastric biopsies showed complete remission of the disease and absence of *H pylori*. Endoscopic ultrasonography showed a markedly increased thickness of the gastric wall (10 mm) with disappearance of the layer structure on the lesser curvature of the antrum and with a perigastric lymphadenopathy measuring 7 mm in diameter.

Appropriate investigations were performed to stage the lymphoma. Blood cell counts and biochemical parameters, including LDH and  $\beta$ 2microglobulin level, and hepatic biology were normal. An EBV serology test showed IgG positive and IgM negative viral capsid antigen (VCA) and Epstein-Barr nuclear antigen (EBNA), reminiscent of “an old infection”. Human immunodeficiency virus serology was negative. Bone marrow biopsy and lumbar puncture were normal. Abdominal computed tomography showed an important increase in gastric wall thickness. As a perigastric lymphadenopathy was present at endoscopic ultrasonography, the patient was classified as stage IIE according to the Ann Arbor classification. The International Prognostic Index, including age (older or not than 60 years), LDH level (normal or elevated), tumour stage (I, II/III, IV), number of extranodal sites involved by disease (0 or 1/more than 1), patient performance status (less or more than 2), was scored 1 (low-risk group).

A new endoscopy with biopsy was performed one month later and showed complete healing of the ulcer (Figure 1B), and persistent histological remission of the disease. In spite of the absence of residual disease, taking



**Figure 2 Morphological and immunohistochemical features of the disease.** A: At low magnification, the gastric mucosa is diffusely infiltrated by medium sized lymphoid cells (HE,  $\times 25$ ); B: Tumour cells are medium sized with scarce cytoplasm, round nuclei and fine chromatin; numerous mitoses are observed; tangible body macrophages are present (HE,  $\times 1000$ ); C: Immunohistochemistry shows that the tumour cells are CD20+,  $\times 400$ ; D: CD5-,  $\times 400$ .



**Figure 3 Immunohistochemical and cytogenetic features of the disease.** A: Tumour cells are CD10+,  $\times 400$ ; B: BCL2-,  $\times 400$ ; C: 100% of tumour cells are Ki67+,  $\times 400$ ; D: Fluorescence *in situ* hybridization (FISH) performed with split signal FISH DNA probes for *c-MYC* revealed distinct red and green split signals in many nuclei, consistent with *c-MYC* gene rearrangement, beside yellow fusion signals for the non-rearranged alleles,  $\times 1000$ .

into account the high risk of lymphoma recurrence, she then received systemic chemotherapy, which consisted of

cyclophosphamide, vincristine, prednisone, doxorubicin and methotrexate, associated with intrathecal injection of

methotrexate, until August 2006. The gastric endoscopic aspect with systematic biopsies was checked every 6 mo. The last outcome visit, in March 2008, with a new scanography and a new endoscopy and endoscopic ultrasonography confirmed the complete remission of the disease.

## DISCUSSION

BL is a mature aggressive B-cell lymphoma, characterized by a high degree of proliferation of the neoplastic cells and deregulation of the *c-MYC* gene<sup>[17,18]</sup>. It is a distinct entity that occurs sporadically worldwide, sometimes associated with immunodeficiency or immunosuppression, and is endemic in central Africa (the lymphoma belt of Africa). Cells in both sporadic and endemic forms of BL are B lymphocytes that have clonal immunoglobulin (*IG*) gene rearrangement and 8q24/*MYC* translocation to the *IG* heavy chain region, 14q32 or less commonly at the lambda 22q11 or kappa 2p12 light chain loci. Epstein Barr virus and malaria are recognised as important cofactors of endemic BL. A combination of these two diseases seems to boost the incidence of endemic BL in the lymphoma belt by a factor of 100-150. But this disease can occur in the absence of both of these infections and arboviruses and plant tumour promoters are other possible local cofactors. There are probably other cofactors which are unknown.

The stomach is the most common site of extranodal malignant lymphomas. The incidence of gastric BL is low as compared to other types of gastric lymphomas, although its true incidence is not yet defined. Like other types of BL, gastric BL is known to have a highly aggressive clinical course. However, one study including 21 patients with a gastric BL, showed a better outcome for these patients as compared to 26 non-gastric BL with a long-term survival of 90%. An especially good outcome was observed when the disease was localized to the stomach and the authors concluded that, in this case, prognosis of BL and DLBCL could be similar. This observation suggested a common pathogenesis<sup>[3]</sup>.

There is a close association between *H pylori* and gastric lymphomas<sup>[4]</sup>. But the link between *H pylori* and BL has not been demonstrated yet. *H pylori* plays an important role in the development and growth of MALT lymphoma and its eradication has been shown to achieve durable regression in 56% to 100% of patients with early stage low-grade gastric MALT lymphoma<sup>[8-11]</sup>. *In vitro* co-culture of *H pylori* with tumour B cells in the presence of non-neoplastic T cells proved that the bacteria were able to induce proliferative responses of MALT lymphocytes but not large B cells<sup>[19]</sup>. Therefore, DLBCL of the stomach were generally believed to be *H pylori*-independent, and unlikely to respond to antibiotic therapy. However, in addition to sporadic cases, one prospective study of 24 patients with DLBCL has shown that antibiotic therapy could also result in complete remission in patients with early stage gastric DLBCL with low grade components<sup>[15]</sup>. The same authors compared two prospective studies of the efficacy of *H pylori* eradication

in the treatment of early-stage MALT lymphoma and DLBCL of the stomach and showed that the therapeutic efficacy of antibiotics for stage IE gastric high grade transformed MALT lymphoma was similar to their efficacy in stage IE low-grade lymphoma. It was suggested that the response was possible only in DLBCL with low grade component and more frequent in cases of superficial gastric wall involvement and in the absence of BCL10 protein nuclear expression<sup>[16-20]</sup>.

Histologically, the distinction between diffuse large B cell lymphoma and BL is sometimes difficult, especially in adult patients. It is well known that these two entities may show overlapping morphological and immunohistochemical features<sup>[21,22]</sup>. Whether this case represents "a B cell lymphoma, unclassifiable, with features intermediate between diffuse large B cell lymphoma and Burkitt lymphoma", a provisional entity defined in the recent 2008 World Health Organization (WHO) classification of lymphoid neoplasms may be discussed<sup>[2]</sup>. However, this case met all the characteristic morphological (starry sky appearance, monotonous medium sized tumour cells with round nuclei and scarce cytoplasm), immunohistochemical (CD10+, BCL2-, 100% Ki67 positivity) and cytogenetic (*MYC* translocation without *BCL2* or *BCL6* translocation) diagnosis criteria of BL according to the 2008 WHO classification, therefore excluding the hypothesis of *de novo* DLBCL.

In the present case, eradication of *H pylori* was performed before the definitive histological results were available. So, we had the opportunity to observe the first case of BL remission following *H pylori* eradication in an adult patient. This observation highlights the potential role of an infectious agent, notably *H pylori*, as a cofactor in the development of BL and suggests that *H pylori* eradication should be systematic in the context of *H pylori* positive gastric BL in association with conventional chemotherapy regimens.

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## A case of asymptomatic fungal and bacterial colonization of an intragastric balloon

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### Abstract

Intragastric balloon therapy, as a part of a multidisciplinary weight management program, is an effective short-term intervention for weight loss. Although the insertion procedure is easy and generally well tolerated by patients, a few complications can occur. We report here a heavy smoker with intragastric balloon insertion complicated by colonization with opportunistic organisms. The 27-year-old female, body mass index 35.5 kg/m<sup>2</sup>, had a BioEnterics® Intragastric Balloon inserted under conscious sedation without any peri-operative complications. Six months later, when the standard removal time arrived, the balloon was seen to be covered with a necrotic white-gray material. Microbiological examination revealed *Enterobacter cloacae* and *Candida* species yeast colonies. We recommend that asymptomatic fungal and/or bacterial colonization should be considered among the complications of the intragastric balloon procedure, despite its rarity.

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**Key words:** Gastric balloon; Obesity; Complications; Infection

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Coskun H, Bozkurt S. A case of asymptomatic fungal and bacterial colonization of an intragastric balloon. *World J Gastroenterol* 2009; 15(45): 5751-5753 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5751.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5751>

### INTRODUCTION

Obesity will be one of the 21st century's most important problems and will become a worldwide epidemic. In 2015 approximately 2.3 billion people will be overweight and 700 million people will be reported to be obese<sup>[1]</sup>. Obesity treatment options consist of a calorie-restricted diet, lifestyle modification, medical treatment, endoscopic intragastric balloon application and bariatric surgery<sup>[2]</sup>. In 1982, the intragastric balloon was used for the first time by Nieben *et al*<sup>[3]</sup>, who observed that long-lasting and well-tolerated gastric bezoars can result in significant weight loss.

Intragastric balloon therapy, as a part of a multidisciplinary weight management program is an effective short-term intervention for weight loss. In experienced hands the application and removal of the intragastric balloon is easy and has relatively low morbidity and mortality compared to bariatric surgery<sup>[4,5]</sup>.

The major complications of the intragastric balloon are intolerance, gastric ulcers, gastric erosion and esophagitis, spontaneous deflation of the balloon, ongoing vomiting for 1-3 wk or more, abdominal pain and gastroesophageal reflux. There are also a few reported cases of gastric perforation, dilatation and small intestinal obstruction<sup>[6,7]</sup>.

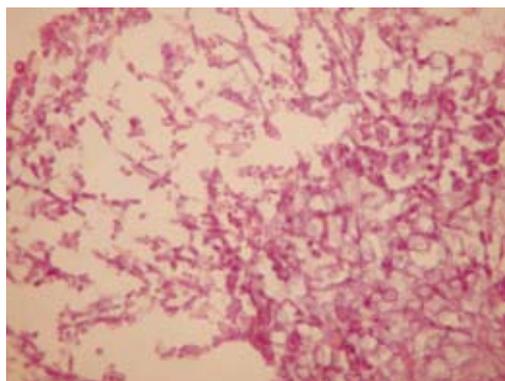
We present here an asymptomatic intragastric balloon infected by *Candida* and *Enterobacter* species which has not been encountered previously in the literature, and we discuss possible and probable causes.

### CASE REPORT

A 27-year-old female, heavily smoking patient, 91 kg in weight and body mass index (BMI) 35.5 kg/m<sup>2</sup> had a BioEnterics® Intragastric Balloon (BIB; BioEnterics Corporation, California, USA, S/N: 123456789) inserted. The patient was premedicated with 0.05 mg/kg iv midazolam (Dormicum Roche, Turkey) for 10 min before the insertion and an additional 0.5-0.75 mg/kg



**Figure 1** Removed silicon balloon covered with a necrotic white-gray material.



**Figure 2** Necrotic material with mycotic hyphae and spores (HE, x 600).

propofol (Diprivan, Astra Zeneca, Turkey) was given iv during the application to attain deeper sedation<sup>[8]</sup>. Under video-endoscopic view, the balloon was positioned in the stomach and inflated with 550 mL NaCl 0.09% mixed with 10 mL methylene blue solution. The upper gastrointestinal endoscopic findings were normal. As is common, the patient experienced intense nausea, vomiting and cramping abdominal pain in the first 48 h. With appropriate medical support these standard complaints subsided significantly by the fifth day after the procedure. In the following days the patient was given a 1200 kcal/d diet. The balloon was removed according to protocol on the 180th d after placement. On the 180th d, the patient's weight was 69 kg, with BMI 26.9 kg/m<sup>2</sup>, excess weight loss 62.8%, and excess BMI loss 81.4%. During the removal of the balloon, endoscopy revealed normal esophageal and gastric mucosal surfaces except slight gastritis. However, the surface of the silicon balloon was covered with a necrotic white-gray layer (Figure 1). This necrotic material covered almost 80% of the total surface of the balloon. The balloon had been removed endoscopically with no problems. Histopathologic examination revealed a few fungal hyphae, polymorphonuclear infiltration of leukocytes and mononuclear cells (Figure 2), with active and chronic inflammation. Gram staining showed epithelial cells, leukocytes, gram-negative bacilli and yeast cells. With Ziehl-Nielsen staining, acid-resistant bacilli were not seen. Microbiologic examination revealed *Enterobacter cloacae* and *Candida* species yeast colonies. The patient was discharged with appropriate non-specific supportive treatment without any clinical problems. The patient was evaluated endoscopically after a 3-mo clinical follow-up and no pathology was found.

## DISCUSSION

Intra-gastric balloon application has relatively low morbidity and mortality compared to other bariatric procedures. In particular it is the primary choice for excessively morbidly obese patients who are prepared to undergo surgical bariatric procedures.

The complication rate is extremely variable between studies. This is because minor complications can easily be managed by phone calls or during scheduled visits. The major complications of the intra-gastric balloon procedure are balloon intolerance (7%), gastric ulcer (0.4%),

gastric erosion and esophagitis (18.2%), spontaneous deflation of the balloon (3%-23%), ongoing vomiting for 1-3 wk or more (0%-15.9%), abdominal pain (5%), and gastroesophageal reflux (1.8%). There are also a few reported cases of gastric perforation (0.1%-0.21%), dilatation and small intestinal obstructions (0.17%-0.8%)<sup>[5-7]</sup>. Infection arising from intra-gastric balloon insertion is not a common issue. There is no such published case in the literature.

The isolated microorganism, *Enterobacter cloacae* is a gram-negative bacillus. Frequently, it causes nosocomial infections in patients in whom respiratory support instruments or catheterizations are applied, or it appears as opportunistic infections in patients with immunodeficient and debilitating diseases<sup>[9]</sup>.

*Candida* is a yeast and the most common cause of opportunistic mycoses worldwide. It is also a frequent colonizer of human skin and mucous membranes. *Candida* is a constituent of the normal flora of the skin, mouth, vagina, and stool<sup>[10]</sup>.

Colonization of soft lining materials with microorganisms, particularly *Candida* species, is a common clinical problem. The intra-gastric balloon has a silicon elastomer structure and da Silveira *et al*<sup>[11]</sup> mentioned in their paper that silicone-based soft lining materials are more susceptible to *Candida* adhesion.

Candidiasis of the gastrointestinal system mostly affects the esophagus and the infection is seen as patch plaques on the mucosa<sup>[12]</sup>. Although a typical fungal infection was not detected in the esophagus of our patient, such contamination can not be excluded and may have occurred during endoscopic intervention, i.e. the balloon passage through the oral cavity or during its application.

Of the predisposing factors causing gastric candidiasis, delayed gastric emptying and gastric stasis have been proposed. It is a well known issue that one of the mechanisms of action of the intra-gastric balloon is to delay gastric emptying<sup>[13]</sup>. Thus the slowing down of normal peristalsis of the stomach by the intra-gastric balloon may allow opportunistic organisms to colonize more readily.

The patients in whom an intra-gastric balloon is inserted should take proton pump inhibitors during application of the balloon because of gastric hyperacidity. These proton

pump inhibitors result in a hypochlorhydric gastric medium which makes opportunistic infections more likely<sup>[14-17]</sup>.

The important feature identified in the present case was that she was a heavy smoker. It is known that cigarette smoke has many effects on the gastric mucosa. These can result in progression from gastritis to gastric ulcers and even carcinoma. Smokers appear to be at higher risk of becoming infected with *Helicobacter pylori* (*H. pylori*) and this increased risk may result from the adverse effects of smoking on antioxidants or the immune system which may interfere with normal protection against *H. pylori*<sup>[18-20]</sup>.

Because the infection detected on the balloon was a colonization rather than a systemic infection we recommended appropriate non-specific supportive treatment for the patient. However, we recommend systemic antifungal and antibacterial treatment for patients in whom the gastrointestinal integrity is damaged, who are immunocompromised or who are scheduled for bariatric surgery.

We present here a heavy smoker with an intragastric balloon infection. In our series of 201 endoscopic removals of 263 intragastric balloons, this case was the only one with such an infection. The rare incidence of colonization leads us to believe that this kind of infection may be multifactorial, and no single cause can be highlighted. In patients who receive an intragastric balloon, predisposing factors for opportunistic infections such as gastric stasis, smoking and antacid medications should be taken into consideration and patients monitored for these infections. Any asymptomatic balloon infection, especially opportunistic pathogens, should be treated with appropriate medications if the integrity of the gastrointestinal system is damaged.

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## Spontaneous bacterial peritonitis: Few additional points

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### Abstract

Spontaneous bacterial peritonitis (SBP) is a treatable complication of decompensated cirrhosis. Coagulopathy with evidence of hyperfibrinolysis or clinically evident disseminated intravascular coagulation precludes paracentesis. Alcoholic hepatitis with fever, leucocytosis and abdominal pain should be evaluated for SBP. Oral ofloxacin is as effective as parenteral cefotaxime in treatment of SBP except for inpatients with vomiting, encephalopathy, or renal failure. Albumin is superior to hydroxyethyl starch in treatment of SBP.

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**Key words:** Spontaneous bacterial peritonitis; Albumin; Antibiotics

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### TO THE EDITOR

We read with great interest the article "Spontaneous bacterial peritonitis" by Koulaouzidis *et al*<sup>[1]</sup> in March 7, 2009 issue of *World Journal of Gastroenterology*. The review was extensive. However, some points may be useful for the management of patients with spontaneous bacterial peritonitis (SBP).

Serious complications unusually occur in paracentesis, but a subgroup of patients with renal failure should be carefully monitored. Pache *et al*<sup>[2]</sup> observed 9 bleeding complications in their study of 4729 paracentesis patients, which were probably due to qualitative platelet abnormality in 8 patients with renal failure.

Coagulopathy precludes paracentesis only when there is clinically evident hyperfibrinolysis or disseminated intravascular coagulation<sup>[3]</sup>. Ascitic fluid leak post paracentesis can be prevented by inserting a needle along the Z-track and keeping the patient in the right lateral position for a few hours.

Biochemical tests are required for total protein and albumin in each ascitic fluid sample whereas optional tests are required for glucose and lactate dehydrogenase levels<sup>[3]</sup>.

Ascitic fluid culture should be performed before antibiotics are used because even a single dose of antibiotics causes the culture to produce no growth of bacteria in 86% of cases<sup>[4]</sup>.

Patients with alcoholic hepatitis present with fever, leukocytosis and abdominal pain mimicking SBP. An elevated ascitic fluid polymorphonuclear count in these patients is not due to peripheral leukocytosis<sup>[5]</sup> but may represent SBP. Empiric treatment with antibiotics can be discontinued after 48 h if ascitic fluid, blood and urine cultures are negative.

Oral ofloxacin (400 mg, twice a day for an average of 8 d) is as effective as parenteral cefotaxime against SBP when the patients do not have vomiting, shock, grade II (or higher) hepatic encephalopathy or serum creatinine > 3 mg/dL<sup>[6]</sup>.

Sigal *et al*<sup>[7]</sup> have shown in their study that albumin should be used in SBP patients with their serum creatinine > 1 mg/dL, blood urea nitrogen > 30 mg/dL, or total bilirubin > 4 mg/dL, and not used in patients without such indications. Albumin is superior to hydroxyethyl starch in treatment of SBP<sup>[8]</sup>.

Fernández *et al*<sup>[9]</sup> in their randomized trial have shown that daily norfloxacin can prevent SBP and hepatorenal syndrome and has a survival advantage in patients with their ascitic fluid protein < 1.5 gm/dL and at least with one of the following indications, namely serum creatinine  $\geq$  1.2 mg/dL, blood urea nitrogen  $\geq$  25 mg/dL, serum sodium  $\leq$  130 mg/L, Child-Pugh  $\geq$  9 points, and bilirubin  $\geq$  3 mg/dL.

Intermittent dosing of double, enforced trimethoprim-sulfamethoxazole (5 doses per week) or ciprofloxacin (single oral dose of 750 mg per week) may be ineffective against resistant flora<sup>[10]</sup>.

In conclusion, proper selection of patients for paracentesis, high index for suspected SBP in alcoholic hepatitis patients and albumin treatment can help the management of SBP.

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## Meetings

### Events Calendar 2009

January 12-15, 2009  
Hyatt Regency San Francisco, San Francisco, CA  
Mouse Models of Cancer

January 21-24, 2009  
Westin San Diego Hotel, San Diego, CA  
Advances in Prostate Cancer Research

February 3-6, 2009  
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)  
Second AACR Conference  
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009  
Hyatt Regency Boston, Boston, MA  
Translation of the Cancer Genome

February 8-11, 2009  
Westin New Orleans Canal Place, New Orleans, LA  
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009  
Hong Kong Convention and Exhibition Centre, Hong Kong, China  
19th Conference of the APASL  
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009  
Orlando, Florida  
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009  
Vienna, Austria  
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease  
[www.easl.ch/vienna2009](http://www.easl.ch/vienna2009)

March 13-14, 2009  
Phoenix, Arizona  
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009  
Marriott Wardman Park Hotel  
Washington, DC  
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009  
Glasgow, Scotland  
British Society of Gastroenterology (BSG) Annual Meeting  
Email: [bsg@mailbox.ulcc.ac.uk](mailto:bsg@mailbox.ulcc.ac.uk)

April 8-9, 2009  
Silver Spring, Maryland  
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009  
Colorado Convention Center, Denver, CO  
AACR 100th Annual Meeting 2009

April 22-26, 2009  
Copenhagen, Denmark  
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)  
<http://www.easl.ch/>

May 17-20, 2009  
Denver, Colorado, USA  
Digestive Disease Week 2009

May 29-June 2, 2009  
Orange County Convention Center  
Orlando, Florida  
45th ASCO Annual Meeting  
[www.asco.org/annualmeeting](http://www.asco.org/annualmeeting)

May 30, 2009  
Chicago, Illinois  
Endpoints Workshop: NASH

May 30-June 4, 2009  
McCormick Place, Chicago, IL  
DDW 2009  
<http://www.ddw.org>

June 17-19, 2009  
North Bethesda, MD  
Accelerating Anticancer Agent Development

June 20-26, 2009  
Flims, Switzerland  
Methods in Clinical Cancer Research (Europe)

June 24-27 2009  
Barcelona, Spain  
ESMO Conference: 11th World Congress on Gastrointestinal Cancer  
[www.worldgicancer.com](http://www.worldgicancer.com)

June 25-28, 2009  
Beijing International Convention Center (BICC), Beijing, China  
World Conference on Interventional Oncology  
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009  
Snowmass, CO, United States  
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009  
Aspen, CO, United States  
Molecular Biology in Clinical Oncology

August 1-7, 2009  
Vail Marriott Mountain Resort, Vail, CO, United States  
Methods in Clinical Cancer Research

August 14-16, 2009  
Bell Harbor Conference Center, Seattle, Washington, United States  
Practical Solutions for Successful Management  
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009  
Beijing International Convention Center (BICC), Beijing, China  
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists (IASGO)  
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009  
Taipei, China  
Asian Pacific Digestive Week  
<http://www.apdwcongress.org/2009/index.shtml>

October 7-11, 2009  
Boston Park Plaza Hotel and Towers, Boston, MA, United States  
Frontiers in Basic Cancer Research

October 13-16, 2009  
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States  
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009  
Versailles, France  
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009  
Boston, MA, United States  
The Liver Meeting

November 15-19, 2009  
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States  
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009  
London, UK  
Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

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*English journal article (list all authors and include the PMID where applicable)*

- 1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

*Chinese journal article (list all authors and include the PMID where applicable)*

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

*In press*

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of

balancing selection in *Arabidopsis*. *Proc Natl Acad Sci USA* 2006; In press

#### Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

#### Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

#### No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

#### Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

#### Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

### Books

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

#### Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wicczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

#### Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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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

#### Electronic journal (list all authors)

- 15 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>

#### Patent (list all authors)

- 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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Write as mean  $\pm$  SD or mean  $\pm$  SE.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as  $\chi^2$  (in Greek), related coefficient as *r* (in italics), degree of freedom as  $\nu$  (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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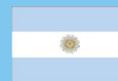
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## Clinical relevance and public health significance of hepatitis B virus genomic variations

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### Abstract

Ten hepatitis B virus (HBV) genotypes (A-J) and 34 HBV subgenotypes have been identified so far. HBV genotypes and subgenotypes have distinct geographical distributions, and have been shown to differ with regard to clinical outcome, prognosis, and response to interferon treatment. Infection with subgenotype A2 is frequently associated with high viral load, resulting in acute infection *via* horizontal transmission. Genotypes A and B are more sensitive to interferon treatment than genotypes D and C, respectively. Genotype B is more frequent in acute hepatitis than genotype C, whereas genotype C (C2) is more frequently associated with an increased risk of hepatocellular carcinoma (HCC), mostly cirrhotic, as compared with genotype B (B2). Genotype mixture is associated with high viral load and worse outcome of HBV infection. HBV mutations in the S genes, especially amino acids substitution at position 145 (G145R), are associated with immune escape, whereas mutations in the PreS or S genes which impair HBsAg secretion could present a risk to blood safety. HBV variants harboring mutations in the viral polymerase gene that confer resistance to nucleoside analogs may be selected during antiviral therapy. Different genotypes have distinct mutation patterns in the PreS and Enh II/BCP/Precore regions. PreS deletions, C1653T, T1753V, and A1762T/G1764A are associated with an increased risk of HCC. HCC-associated HBV mutants may not transmit *via* mother-to-child transmission, and are likely generated during HBV-induced pathogenesis. Examination of HBV mutations alone or in combination and host genetic suscep-

tibility will be helpful in classifying the HBV-infected subjects who will develop HCC and need active antiviral treatments.

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**Key words:** Hepatitis B virus; Genotype; Subgenotype; Mutation; Clinical; Public health; Evolution

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### INTRODUCTION

Hepatitis B virus (HBV) belongs to the hepadnaviridae, a family of enveloped viruses with an incomplete double-stranded DNA genome of 3.2 kb. The double-stranded DNA genome of HBV contains four overlapping open reading frames that encode the surface protein, the core protein, a polymerase, and a multifunctional nonstructural protein called X. The PreS region, which consists of PreS1 (nucleotides 2848-3204) and PreS2 (nucleotides 3205-154) domains, overlaps a region encoding the polymerase gene. The enhancer II (Enh II; nucleotides 1636-1744) and basic core promoter (BCP; nucleotides 1751-1769) regions overlap with the X gene (nucleotides 1374-1835).

Infection with HBV is a major public health problem. Approximately 45% of the world's population lives in regions where HBV infection is endemic. Approximately 2 billion people have been exposed to HBV, and more than 300 million are chronically infected with HBV<sup>[1]</sup>. In Asia and most of Africa, chronic HBV infection is common and usually acquired perinatally or in childhood<sup>[2]</sup>. Chronic HBV infection is one of the most important determinants of the occurrence of liver cirrhosis (LC) and hepatocellular carcinoma (HCC). Most HCC

cases (> 80%) occur in either Eastern Asia or in sub-Saharan Africa where HBV is endemic<sup>[3]</sup>. HBV infection contributes to more than 50% of HCC cases worldwide and 70%-80% of HCC cases in highly HBV endemic regions. The relative risks of HCC among people infected with HBV ranges from 5 to 49 in case-control studies and from 7 to 98 in cohort studies<sup>[4]</sup>. The incidence of HCC (per 100 000 person/year) among people with chronic HBV infection ranges from 400 to 800 in males and from 120 to 180 in females<sup>[4]</sup>. Standard HBV vaccination dramatically decreases HCC prevalence among vaccinees aged 6-19 years<sup>[5]</sup>. HBV genomic variations, including genotypes, subgenotypes, and HBV mutations in the PreS region and the Enh II/BCP/Precore region are associated with the development of LC and HCC in different HBV replication and hepatitis B e antigen (HBeAg) status.

## HBV GENOTYPES/SUBGENOTYPES AND THEIR CLINICAL RELEVANCE

### *Distribution of HBV genotypes and subgenotypes*

Eight genotypes (genotypes A-H) have been identified by a sequence divergence greater than 8% in the entire HBV genome or a sequence divergence greater than 4% in the S region<sup>[6]</sup>. HBV isolated in Vietnam and Laos has been suggested to form a ninth genotype I<sup>[7,8]</sup>. The designation has been questioned due to complex recombination. Recently, a HBV strain isolated from a Japanese patient has been provisionally designated HBV genotype J. HBV genotype J is closer to gibbon/orangutan genotypes than to human genotypes in the P and large S genes and closest to Australian aboriginal strains and orangutan-derived strains in the S gene, whereas it is closer to human than ape genotypes in the C gene<sup>[9]</sup>. Genotypes have further been separated into subgenotypes if the divergence in whole nucleotide sequence is between 4% and 8%<sup>[10]</sup>. Currently, subgenotypes 1-5 of genotype A, subgenotypes 1-8 of genotype B, subgenotypes 1-8 of genotype C, and subgenotypes 1-7 of genotype D have been identified<sup>[11-37]</sup>. HBV genotypes and subgenotypes have distinct geographical distributions (Table 1), and often present demographic characteristics. HBV genotype A1, A3, A4, and A5 are endemic in Africa, especially in West Africa, whereas genotype A2 is endemic in Europe<sup>[11-15]</sup>. Genotypes B and C are predominant in Asian and Pacific islanders<sup>[6,10,16-22,28]</sup>. Of HBV genotypes B and C, subgenotypes B2 and C2 are endemic in most parts of Asia. Subgenotype B1 is endemic in Japan<sup>[16]</sup>. Subgenotype C4 is encountered in Aborigines from Australia, and frequently termed as the Australian aboriginal strain<sup>[9,19]</sup>. Subgenotypes B3-B8, C1, C3, and C5-C8 have been isolated in South Asia, especially in Indonesia and the Philippines<sup>[17-22,28]</sup>. Genotype D is endemic in the entire Old World including Africa, Northern and South Eastern Asia, the Mediterranean area, and most European countries<sup>[13,23-26,32-34,37]</sup>. Subgenotype D1 is predominant in Moslem ethnicity. Subgenotype D2 is endemic in Russia and the Baltic region<sup>[26,32]</sup>. Subgenotypes D2, D3, and D5 have been found in In-

dia<sup>[32]</sup>. Subgenotypes D4 and D6 are endemic in Oceania and Indonesia, respectively<sup>[19,33]</sup>. A new subgenotype D7 has been found in Tunisia<sup>[34]</sup>. HBV genotype E is endemic in Western and Central Africa<sup>[13,27,35]</sup>. HBV genotypes F, G, and H are endemic in America<sup>[29-31,36]</sup>. More subgenotypes have been found in South Asia, Oceania, and Africa than other areas in the world, probably indicating evolutionary history of HBV. The distribution of HBV genotypes often provides clues about human migration. HBV genotype B is more frequent than genotype C in Taiwan<sup>[38,39]</sup>. Although genotype C is more frequent than genotype B in Mainland China<sup>[40]</sup>, HBV genotype B accounts for more than 80% in Zhangzhou (our unpublished data), a city at the coastal line of Fujian Province of Mainland China where early Taiwan residents had migrated from. Subgenotype C1 is endemic in Southern Guangdong Province of Mainland China<sup>[41]</sup>, this subgenotype is also endemic in Hong Kong<sup>[42]</sup>. Hong Kong residents are mostly from Southern Guangdong Province of Mainland China. HBV genotype E is endemic in Africa. As genotype E is essentially absent from the Americas despite the Afro-American slave trade until at least the beginning of the 19th century, genotype E strains may have been introduced into the general African population only within the past 200 years<sup>[14,35]</sup>. The genetic diversity of HBV and the geographical distribution of its subgenotypes provide a tool to reconstruct the evolutionary history of HBV and may help to complement genetic data in the understanding of the evolution and past migrations of man<sup>[19]</sup>.

### *Genotype mixture and its clinical relevance*

In an HBV endemic area, infection with more than one HBV genotype often results in genotype recombination and genotype mixture (co-infection or super-infection with multiple HBV genotype in an infected person). With the use of multiplex PCR, a genotype mixture has been frequently identified in the HBV-infected subjects<sup>[43,44]</sup>. HBV genotype mixture has been associated with high viral load in patients with chronic hepatitis B (CHB) as compared with patients with a single genotype, also associated with increased *in vitro* HBV replication<sup>[45]</sup>. In our recent study, the prevalence of genotype mixture in asymptomatic hepatitis B surface antigen (HBsAg) carriers (ASC), patients with HCC, and patients with CHB is 5.4%, 10.6%, and 13.7%, respectively. Genotype mixture (mostly mixed genotype B with genotype C) is associated with higher viral load and more severe course of the disease than HBV genotype C alone<sup>[40]</sup>. These results indicate that co-infection or superinfection with multiple genotypes is associated with worse prognosis of HBV infection.

### *Clinical relevance of HBV genotypes/subgenotypes*

HBV genotypes and subgenotypes have been shown to differ with regard to clinical outcome, prognosis, and response to antiviral treatment. Infection with HBV genotype A is associated with high viral load which facilitates viral transmission. High replication rates of genotype A

Table 1 Geographic distribution and important clinical relevance of HBV genotypes and subgenotypes

Genotype	Subgenotype	Geographic distribution	Important clinical relevance	Ref.
A	A1	Africa	ND	[13-15]
	A2	Europe	Acute infection, chronicificaton, more sensitive to interferon treatment than genotype D	[11,12,47,48]
	A3	West Africa	ND	[13-15]
	A4	West Africa	ND	[15]
	A5	West Africa	ND	[14,15]
B	B1	Japan	Fulminant hepatitis	[16]
	B2	Most of Asia, except Korea	Acute hepatitis, HCC in mostly those younger than 50 years	[6,10]
	B3	Indonesia	ND	[17,18]
	B4	Indonesia, Vietnam	ND	[18,19]
	B5	Indonesia, Philippines	ND	[17,18,22]
	B6	Indonesia	ND	[28]
	B7	Indonesia	ND	[17,18]
	B8	Indonesia	ND	[17]
C	C1	South Asia, Southern China	HCC and LC	[18]
	C2	Northeast Asia, China	HCC and LC mostly in those older than 50 years	[6,10]
	C3	Indonesia, Oceania	ND	[18,19]
	C4	Australia	ND	[19]
	C5	Indonesia, Philippines	ND	[17,22]
	C6	Indonesia, Philippines	ND	[17,21]
	C7	Indonesia	ND	[17]
	C8	Philippines	ND	[20,21]
	C9	Tibet, China	ND	Unpublished [13,23-25, 37,81]
D	D1 (mainly D)	Middle East, the Mediterranean area	Chronic liver disease, HCC	[26,32]
	D2	Russia, the Baltic region, India	No apparent clinical relevance	[17,32]
	D3	Indonesia, India	Occult HBV infection	[19]
	D4	Oceania	ND	[32]
	D5	India	No apparent clinical relevance	[33]
	D6	Indonesia	ND	[34]
	D7	Tunisia	ND	[13,27,35]
E	ND	Western and Central Africa	ND	[29,36,62]
F	F I a	Central America Chile, Alaska	HCC	[30,31]
	F I b	Argentina, Japan, Venezuela, USA		
	F II	Brazil, Venezuela, Nicaragua		
	F III	Venezuela, Panama, Columbia		
	F IV	Argentina, Bolivia, France		
G	ND	Mexico, Canada	ND	[30]
H	ND	Mexico	ND	[7,8]
I	ND	Vietnam, Laos	ND	[9]
J	ND	Japan (might be from Borneo)	HCC	

HBV: Hepatitis B virus; ND: Not determined; HCC: Hepatocellular carcinoma.

in adults lead to an increased risk of horizontal transmission of HBV by sexual activity because a high concentration of HBV DNA in serum is associated with high concentrations in semen and other body fluids of HBV carriers<sup>[46]</sup>. Genotype A also tends to cause chronic infection following an acute course. This has been demonstrated in Japan where genotype A introduced from Europe has started to increase sharply in patients with acute infection since 1991, and gradually in those with chronic infection<sup>[47]</sup>. As compared with HBV genotype D endemic in Europe, genotype A is more sensitive to interferon  $\alpha$  treatment<sup>[48]</sup>. Spontaneous HBeAg seroclearance was significantly higher in genotype A carriers than in carriers of genotypes A, B, D, and F. After losing HBeAg, those with genotypes C and F were more likely to revert to the HBeAg-positive state<sup>[49]</sup>. Infection with subgenotype B2 is associated with HCC or HCC recurrence in young, mostly noncirrhotic, patients in Mainland China and Taiwan<sup>[39,40,50]</sup>, whereas infection with subgenotype

B1 is frequently associated with fulminant hepatitis B in Japan<sup>[16]</sup>. Infection with HBV genotype C is associated with increased risks of LC and HCC at an older age as compared with infection with the HBV genotype B<sup>[38,51,52]</sup>. Although HBV subgenotypes C1 and C2 are associated with the risk of HCC, only HBV subgenotype C2 is independently associated with an increased risk of HCC<sup>[53]</sup>. Genotype B has recently been shown by us to be more likely to cause acute hepatitis B, while the serum viral load of ASCs with genotype B is significantly higher than that of ASCs infected with genotype C<sup>[54]</sup>. As compared with genotype C, HBV genotype B has been shown to be associated with earlier HBeAg seroconversion, and associated with better response to interferon therapy in HBeAg-positive chronic hepatitis<sup>[55,56]</sup>. Early HBeAg seroconversion typically confers a favorable outcome<sup>[57]</sup>. In HBeAg-negative patients, detectable HBV DNA and HBV genotype C are associated with more severe liver damage<sup>[58]</sup>. Thus, infection with HBV genotype C is as-

sociated with worse clinical outcome as compared with genotype B. Data from India have shown that HBV sub-genotype D1 is significantly associated with chronic liver disease, whereas HBV subgenotype D3 is significantly associated with occult HBV infection. No apparent clinical relevance was observed in those infected with HBV subgenotypes D2 and D5<sup>[32]</sup>. There are very limited data on the association of genotype E with its clinical relevance. Population-based prospective cohort studies have found that HBV genotypes C and F are associated with the highest risk for HCC or LC<sup>[59]</sup>. HBV genotypes E, F, and H appear to be sensitive to IFN- $\alpha$  treatment<sup>[60]</sup>. The recombination of two genotypes is frequent in the area where the two genotypes are endemic<sup>[6,15,61]</sup>, probably due to the selection of viral growth advantage. Lower rates of response to IFN- $\alpha$  treatment in patients with HBV genotype G might be related to the frequent occurrence of double infection<sup>[60]</sup>.

## CLINICAL AND PUBLIC HEALTH SIGNIFICANCE OF HBV MUTATIONS

### **Association of HBV PreS and S region mutations with immune escape, occult infection, and the development of HCC**

HBV genomic variations in the PreS and S regions which are selected during the infection course are of clinical and public health importance. The HBV envelope is composed of 3 forms of HBsAg, the so-called large (L, coded for by the PreS1/S2/S gene), middle (M, the PreS2/S gene), and small (S, the S gene) proteins. The small or major peptide is 226 amino acids in length, and the M and L proteins are assembled by amino-terminal extension of 55 amino acids at the PreS2 domain and of 108-119 amino acids of the PreS1 domain. HBsAg is the main target for viral neutralization, either by natural or vaccine-induced anti-HBs. A central major hydrophilic region (MHR, approximately residues 103-173) exposed at the surface of viral particles. The MHR itself is structured into five regions, including three central loops held together by disulphide bonds. The immunodominant "a" determinant (residues 124-147), against which most neutralizing antibodies are directed and which is the major target of HBsAg detection tests, is formed by loops 2 and 3<sup>[62]</sup>. HBV with mutations in the portion of the S gene coding the "a" determinant of hepatitis surface antigen, including a glycine to arginine substitution at position 145 (G145R) and other S gene mutations in the region of amino acids 120-147, can potentially evade neutralizing anti-HBs antibody and infect vaccinated people. G145R is by far the most common immune escape mutant, whereas the most important immune escape mutants with substitutions outside of the "a" determinant is P120S/T. Mutations in the S genes within "a" determinant (but not G145R) are partially responsible for occult HBV infection, which are characterized by the presence of HBV DNA in serum in the absence of detectable HBsAg, and could present a risk to blood safety<sup>[63]</sup>. Mutations in PreS are also associated with occult HBV infection<sup>[64]</sup>, probably due

to the inactivation of the overlapping PreS2/S promoter which causes impaired HBsAg secretion.

The 5' flanking region of the S gene coding the PreS1 and PreS2 domains is overlapped by the region of the P gene coding the spacer domain of the viral polymerase. Genotype D and non-human primate isolates inherently have a 33 nucleotide deletion at or near the beginning of the PreS1 open reading frame<sup>[10,62]</sup>. The PreS1 protein contains the hepatocyte binding site (amino acids 21-47) and is known to be essential for virion assembly and for the transporting of virions out of the hepatocyte<sup>[65]</sup>. The PreS1 and PreS2 regions play an essential role in the interaction with immune responses because they contain several epitopes for T or B cells<sup>[66]</sup>. There is little evidence supporting the idea that PreS mutants are transmissible, therefore, the PreS mutation might generate during the pathological process following the infection. The PreS mutations emerge in chronic infections, often in patients treated with interferon, and seem to represent desperate attempts to escape from host immune surveillance<sup>[62]</sup>. Many of the mutations affecting the PreS domains of the envelope proteins are deletions. More recently, PreS deletions are frequently associated with an increased risk of HCC, especially in those infected with HBV genotype C<sup>[66-69]</sup>. Our recent meta-analysis showed that the frequencies of the PreS deletion mutation consecutively increased during the progression of chronic HBV infection from ASC states to LC or HCC ( $P_{trend} < 0.001$ ), while the frequencies of mutations at the promoter sites of PreS1 and PreS2 were significantly higher in the patients with HCC than in the patients without HCC ( $P < 0.001$ ,  $P = 0.032$ , respectively)<sup>[70]</sup>. It is suggested that PreS deletion and nucleotide substitution mutations at the promoter sites of PreS1 and PreS2 may serve as useful biomarkers for predicting the clinical outcomes of HBV-infected patients, especially for predicting HCC.

### **Polymerase mutations associated with drug resistance**

HBV variants harboring mutations in the viral polymerase gene that confer resistance to antiviral drugs may be gradually selected during long-term antiviral therapy with nucleoside analogs. The main polymerase gene mutations conferring resistance to nucleoside analogs have been well characterized. Lamivudine resistance mutants harbor a M204V or I substitution in the YMDD motif of the C domain of the polymerase/reverse transcriptase. Adefovir resistance mutants harbor a N236T and/or A181V amino acid substitution in the D and B domains of viral polymerase, respectively<sup>[71,72]</sup>. Entecavir resistance mutations occurred on a background of lamivudine resistance, as these patients received entecavir for lamivudine failure, with a combination of substitutions I169T and M250V, or T184G and S202I. These additional mutations clearly conferred an increased level of entecavir resistance compared to the initial lamivudine resistant strain<sup>[62]</sup>. Resistance to telbivudine has been associated with a M204I mutation in the viral polymerase<sup>[73]</sup>. Table 2 summarizes drug-resistance-associated amino acid substitutions in the 5 domains of HBV polymerase. On the other hand, the reappearance of wild-type virus

Table 2 HBV mutations associated with drug resistance

Nucleoside analogs	Mutations in the polymerase domains (amino acids)				
	A	B	C (YMDD)	D	E
Lamivudine/Emtricitabine	-	V173L L180M	M204I/V	-	-
Adefovir	-	A181V	I233V	N236T	-
Entecavir	-	I169T T184G	S202I	M250V	-
Telbivudine	-	-	M204I	-	-
Famciclovir	-	L180M	-	-	-

-: Not reported.

as the major viral population after cessation of drug treatment is probably due to persistence of non-mutated cccDNA molecules in hepatocytes even after long-term drug treatment<sup>[62,74]</sup>. Naïve patients infected with adefovir resistance mutants have been reported<sup>[71]</sup>. In this case, drug resistant mutants transmitted to a naïve subject may be stable since there will be no competition with wild-type virus.

#### The association of viral mutations in the Enh II/BCP/Precore region with hepatocarcinogenesis

The core promoter, positively and negatively regulated by Enhancer II and to some extent by Enhancer I, controls the transcription of precore mRNA and pregenomic RNA that can be the mRNA for both core protein and the viral polymerase and is the template for viral replication. HBeAg expression indicates active viral replication. There are two classes of mutants that affect HBeAg expression, BCP mutants and precore mutants. Although viral loads are generally several logs lower in HBeAg-negative patients than in HBeAg-positive patients and children born to HBeAg-positive mothers have a much higher risk of contracting chronic HBV infection than children born to HBeAg-negative mothers<sup>[62]</sup>, some combined mutations in the Enh II/BCP/Precore region like 1766/1768, 1762/1764/1766, 1753/1762/1764, and 1753/1762/1764/1766 mutations have been associated with high HBV DNA production in the *in vitro* transfection studies<sup>[75,76]</sup>. HBV core promoter mutations other than those at 1762/1764 appear to upregulate viral DNA replication and, at the same time, greatly reduce HBeAg production. Although expression of HBeAg has been associated with an increased risk of HCC in a prospective study<sup>[77]</sup>, high viral load in HBeAg-negative patients is often associated with worse outcome of chronic HBV infection, especially in those with HBV carrying mutations at the PreS and Enh II/BCP/Precore regions<sup>[66,78-82]</sup>.

Several mutations at the Enh II/BCP/Precore region have been recently associated with an increased risk of HCC. These mutations include C1653T, T1753V, T1766/A1768, and A1762T/G1764A<sup>[81-92]</sup>. Our recent meta-analysis using published data up to August 31, 2008 has shown that C1653T, T1753V, and A1762T/G1764A are each associated with an increased risk of HCC, whereas precore mutations G1896A and C1858T are not associ-

ated with the risk of HCC, regardless of HBeAg status and HBV genotype<sup>[70]</sup>. A1762T/G1764A has been shown to be a valuable biomarker for identifying a subset of male HBsAg carriers who are at extremely high risk of HCC in a prospective study<sup>[92]</sup>. In a community-based prospective study, A1762T/G1764A and genotype C have been associated with an increased risk of HCC, whereas G1896A in the precore region has been associated with decreased risk of HCC<sup>[84]</sup>. G1896A has been associated with fulminant hepatitis in Japan<sup>[93]</sup>. Since the Enh II/BCP/Precore region overlaps with X gene in the HBV genome, mutations in the Enh II/BCP/Precore region should be included in evaluating the role of HBV X protein on the development of HCC. That is to say, the mutated X protein might be more carcinogenic than the wild-type X protein in HBV-induced hepatocarcinogenesis.

C1653T, T1753V, and A1762T/G1764A are increasingly more prevalent as chronic HBV infection progresses from the asymptomatic HBsAg carrier state to liver cirrhosis or HCC, indicating that these mutations accumulate before the diagnosis of HCC<sup>[70]</sup>. This finding suggests that these HBV mutations may serve as useful biomarkers for predicting clinical outcomes of the patients with CHB, especially with regard to predicting whether they will develop HCC. Like the PreS mutants, HCC-associated HBV mutants in the Enh II/BCP/Precore region, e.g. A1762T/G1764A mutants, may not transmit *via* mother-to-child vertical transmission because the children whose mothers carrying HBV mutants were mostly found to be infected with wild-type form of the same viruses<sup>[94,95]</sup>. These HBV mutations are likely generated during HBV-induced pathogenesis. A1762T/G1764A is frequently detected approximately 10 years before the diagnosis of HCC<sup>[70]</sup>. It is therefore necessary to set up likely checkpoints in the life time for the examination of the HCC-associated HBV mutations in HBV-infected subjects. Recent epidemiological studies demonstrated that male sex, old age, high HBV DNA (> 10000 copies/mL), viral mutations in the PreS and the Enh II/BCP/Precore regions, HBV genotypes (C and F), cirrhosis, and family history were associated with an increased risk of HCC<sup>[50-52,65,79-92,96,97]</sup>. Further study should focus on systemic evaluation of these risk factors for the prediction of HCC.

Combined HBV mutations in the PreS and the Enh II/BCP/Precore regions are becoming important in evaluating HCC risk of HBV-infected subjects<sup>[66,69,98]</sup>. In a meta-analysis, we have demonstrated that the frequencies of A1762T/G1764A+C1653T (8.6%), A1762T/G1764A+T1753V (14.6%), A1762T/G1764A+PreS mutation (2.2%), and A1762T/G1764A+C1653T+T1753V (3.2%) are low in ASCs, whereas the frequencies of A1762T/G1764A-based combined mutations are statistically significantly higher in patients with HCC than in patients without HCC<sup>[70]</sup>. For the prediction of HCC in HBV-infected subjects, A1762T/G1764A alone has a sensitivity and specificity of 70.6% (95% CI = 68.7% to 72.5%) and 60.6% (95% CI = 68.7% to 62.0%), respectively, whereas C1653T+T1753V and A1762T/G1764A+C1653T+T1753V has high specificity [92.6% (95% CI = 89.2% to 96.0%) and 93.9% (95%

CI = 90.5% to 97.2%), respectively] but low sensitivity [20.6% (95% CI = 14.9% to 26.3%) and 24.3% (95% CI = 17.5% to 31.1%), respectively]<sup>[70]</sup>. These mutations, alone or in combination, might be reasonably arranged as predictive markers for the prediction of HCC.

## INTERACTIONS BETWEEN HBV AND HOST SUSCEPTIBLE GENES

HBV genetic variations are necessary but insufficient for HBV-induced hepatocarcinogenesis. HBV genotype-associated mutations might be selected by the host immune system and in turn promote host hepatocarcinogenesis. It is possible that the mutated X protein could transactivate host oncogenes responsible for the development of HCC or that transactivators encoded by some oncogenes select the specific HBV mutations during HBV-induced hepatocarcinogenesis. There are many important trans-activating nuclear factors binding sites located in the PreS and the Enh II /BCP/Precore regions<sup>[66,69,70]</sup>. Mutations in the PreS and the Enh II /BCP/Precore regions might alter the binding ability of some potential trans-activating factors and therefore alter viral replication and/or change expression profiling of some related host genes. The genetic predisposition of some host genes like *MDM2* and *p53* gene polymorphisms, cytokine and TGF- $\beta$ 1 gene polymorphisms, and DNA repair gene polymorphisms have been associated with HBV-induced hepatocarcinogenesis<sup>[99-102]</sup>. Our recent study demonstrated that nuclear factor  $\kappa$ B1 gene promoter *NFKB1*-94ATTG2 allelic carriage,  $\kappa$ B $\alpha$  gene promoter *NFKBLA*-826T and *NFKBLA*-881AG allelic carriage, and HBV genotype C are independently associated with an increased risk of HCC, while the estimated haplotype frequency of *NFKBLA* promoter -881G-826T-519C is significantly higher in the patients with HCC than in the HBV-infected subjects without HCC<sup>[103]</sup>. Even so, it is largely unknown so far how HBV variations interact with host genetic susceptibility. Understanding the interactions between HBV genetic variations and host genetic susceptibility is undoubtedly helpful in classifying the HBV-infected subjects who will develop HCC in future and need active anti-viral treatments and extensive surveillance of HCC.

## CONCLUSION

Ten HBV genotypes (A-J) and 34 subgenotypes have been identified so far. HBV genotypes and subgenotypes have distinct geographical distributions, and have been shown to differ with regard to clinical outcome, prognosis, and response to interferon treatment. Infection with subgenotype A2 is frequently associated with high viral load, resulting in acute infection *via* horizontal transmission. Genotypes A and B are more sensitive to interferon treatment than genotypes D and C, respectively. Genotype B is more common in acute hepatitis than genotype C, whereas genotype C (C2) is more frequently associated with an increased risk of HCC, mostly cirrhotic, as compared

with genotype B (B2). Genotypes C and F are frequently associated with the development of HCC. Viral load of the patients with genotype mixture is usually higher than that of those infected with unique genotype. HBV mutations in the S genes, especially amino acid substitutions at position 145 (G145R), are associated with immune escape, whereas the mutations in the PreS or S genes which cause impaired HBsAg secretion could present a risk to blood safety. HBV variants harboring mutations in the viral polymerase gene that confer resistance to antiviral drugs may be selected during antiviral therapy with nucleoside analogs. Genetic diversity of HBV is partly due to virus/host interactions and partly due to parallel evolution in geographically distinct areas. Different genotypes have a distinct pattern of mutations in the PreS and Enh II /BCP/Precore regions. PreS deletions, C1653T, T1753V, and A1762T/G1764A are associated with an increased risk of HCC. HCC-associated HBV mutants may not transmit *via* mother-to-child vertical transmission, and are likely generated during HBV-induced pathogenesis. Frequent examination of HBV mutation alone or in combination as well as genetic susceptibility will be helpful in classifying the HBV-infected subjects who will develop HCC and need active anti-viral treatments.

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## TOPIC HIGHLIGHT

Giovanni D De Palma, Professor, Series Editor

# Confocal laser endomicroscopy in the “*in vivo*” histological diagnosis of the gastrointestinal tract

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## INTRODUCTION

In recent years, endoscopic image quality has improved as the devices have advanced technologically. Although techniques such as chromoendoscopy, high resolution and magnification endoscopy, narrow band imaging, and auto-fluorescence imaging improve the visualization and detection of mucosal lesions, biopsy of the targeted lesion must still be performed for a formal histological diagnosis of cellular and architectural atypia.

Suspicious areas identified during endoscopy are targeted and biopsied or removed endoscopically. However, there are several disadvantages that may be associated with biopsies or endoscopic resection, including bleeding or perforation. Non-representative biopsies may miss relevant portions of tissue, leading to underestimation of the diagnosis. Random biopsy sampling or endoscopic resection for non-neoplastic lesions can also be time-consuming. It would be ideal if a definite diagnosis could be made during endoscopy without a biopsy.

Recent technological advances in miniaturization have allowed for a confocal scanning microscope to be integrated into a conventional flexible endoscope, or into trans-endoscopic probes, a technique now known as confocal endomicroscopy (CEM) or confocal laser endomicroscopy (CLE). This newly-developed technology has enabled endoscopists to collect real-time *in vivo* histological images or “virtual biopsies” of the gastrointestinal (GI) mucosa during endoscopy, and has stimulated significant interest in the application of this technique in clinical gastroenterology<sup>[1-8]</sup>.

This report aims to evaluate the current data on the utility of this new technology in clinical gastroenterology and its potential impact in the future, particularly in the screening or surveillance of GI neoplasia.

## PRINCIPLES OF CONFOCAL MICROSCOPY

Confocal microscopy has been used in the biological sciences since 1961 when the concept of optical sectioning of a biological specimen was introduced.

## Abstract

Recent technological advances in miniaturization have allowed for a confocal scanning microscope to be integrated into a conventional flexible endoscope, or into trans-endoscopic probes, a technique now known as confocal endomicroscopy or confocal laser endomicroscopy. This newly-developed technology has enabled endoscopists to collect real-time *in vivo* histological images or “virtual biopsies” of the gastrointestinal mucosa during endoscopy, and has stimulated significant interest in the application of this technique in clinical gastroenterology. This review aims to evaluate the current data on the technical aspects and the utility of this new technology in clinical gastroenterology and its potential impact in the future, particularly in the screening or surveillance of gastrointestinal neoplasia.

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**Key words:** Confocal microscopy; Diagnostic imaging; Gastrointestinal neoplasms; Precancerous conditions; Endoscopy; Virtual histology

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To create confocal images, a low-powered laser (an argon-ion laser that generates an excitation wavelength of 488 nm, blue laser light) is focused by an objective lens into a single point, within a fluorescent specimen. The same lens is used as both the condenser and objective folding. The point of illumination thus coincides with the point of detection within the specimen. Light emanating from that point is focused through a pinhole to a detector, and light emanating from outside the illuminated spot is rejected. The illumination and detection systems are in the same focal plane and are termed “confocal” (Figure 1).

After passing the pinhole, the fluorescent light is detected by a photodetection device (a photomultiplier tube or avalanche photodiode), transforming the light signal into an electrical one that is recorded by a computer. All detected signals from the illuminated spot are captured and measured.

As the laser scans over the plane of interest, a whole image is obtained pixel-by-pixel and line-by-line, whereas the brightness of a resulting image pixel corresponds to the relative intensity of detected fluorescent light.

The gray-scale image created is an optical section representing one focal plane within the examined specimen.

Confocal microscopy provides the capacity for direct, non-invasive, serial optical sectioning of intact, thick, living specimens with a minimum of sample preparation as well as a marginal improvement in lateral resolution. Because confocal images depend on fluorescence, a fluorescent dye (contrast agent) is required to make objects visible.

## CONTRAST AGENTS

A fluorescent contrast agent is used and is needed to achieve high contrast images using CEM. Potentially suitable agents in humans are fluorescein, acriflavine, tetracycline or cresyl violet. The contrast agents can be applied systemically (fluorescein, tetracycline) or topically (acriflavine, cresyl violet) by using a spraying catheter. Of these, intravenous fluorescein sodium (10%) and topically applied acriflavine (0.2%) have been most commonly used in humans. No data is so far available on the use of tetracycline and cresyl violet.

Fluorescein is an agent used for diagnostic fluorescein angiography or angioscopy of the retina and iris vasculature.

After intravenous injection, fluorescein binds extensively to serum albumin in the bloodstream. The unbound contrast diffuses across capillaries, entering the tissue and staining the extracellular matrix of the surface epithelium and the lamina propria for up to 30 min<sup>[9]</sup>. Cell nuclei and mucin are not stained by fluorescein and therefore appear dark. The mucosal structures that can be identified after fluorescein administration include enterocytes, cellular infiltrate, surface epithelial cells, blood vessels, and red blood cells. Fluorescein is a highly safe agent whose major side effects are short term (1-2 h) and include yellowish skin discoloration and 1-2 d

of bright yellow-colored urine. Nausea and vomiting were reported during angiography and were transient and minor. Serious side effects, such as anaphylaxis or cardiac or respiratory effects, are extremely rare, and to date, have not been recorded in CEM.

Topical acriflavine is highly specific for labeling acidic constituents, and stains the nuclei of superficial layers of the mucosa. The staining provides clear visualization of cell nuclei in the uppermost mucosa and may allow better differentiation between intra-epithelial neoplasia and cancer of the GI tract. Although there has been a hypothetical concern about the risk of mutagenesis, no severe adverse reactions have been reported after the topical use of acriflavine. However, concerns about DNA damage by acriflavine stain have reduced its use in humans.

Fluorescein and acriflavine can also be used simultaneously which adds to the labeling properties.

## EQUIPMENT

CLE can be performed currently with 2 devices: (1) integrated into an endoscope (Pentax, Tokio, Japan, herein termed eCLE); and (2) as a stand-alone probe (herein termed pCLE) capable of passage through the accessory channel of most endoscopes (Cellvizio, Mauna Kea Technologies, Paris, France)<sup>[10-14]</sup>.

### ***The Pentax EG-3870CIK (upper endoscope) and EC-3870CILK (colonoscope)***

The components of the confocal laser endoscope are based on the integration of a confocal laser microscope in the distal tip of a conventional video endoscope, which enables confocal microscopy in addition to standard video endoscopy (Figure 2). The diameter of both the distal tip and the insertion tube is 12.8 mm. The distal tip contains an air and water jet nozzle, 2 light guides, an auxiliary water jet channel (used for topical application of the contrast agent) and a 2.8 mm working channel. This imaging system provides confocal imaging using an incident 488 nm wavelength laser, and enables the detection of fluorescence of 505-585 nm wavelength, with reduced image noise compared with the reflectance confocal systems. CLE imaging data are collected at a scan rate of rate of 1.6 frames per second (1024 × 512 pixels) or 0.8 frames per second (1024 × 1024 pixels) with an adjustable depth of scanning ranging from 0 to 250 μm, a field of view of 475 μm × 475 μm, a lateral resolution of 0.7 μm, and an axial resolution of 7 μm. Confocal images are generated simultaneously with the endoscopic images and the endoscope working channel can still be used.

### ***The Cellvizio® Endomicroscopy System***

The Cellvizio® Endomicroscopy System (Figure 3) is based on a different catheter probe with a semiconductor laser that oscillates at 488 nm. The latest model of Cellvizio confocal miniprobes created for GI tract applications include CholangioFlex, GastroFlex, ColoFlex, GastroFlex-UHD, and ColoFlex-UHD. CholangioFlex probes designed for use during endoscopic retrograde

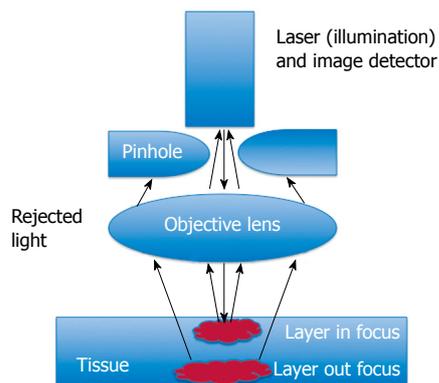


Figure 1 Schematic of confocal endomicroscopy principles.

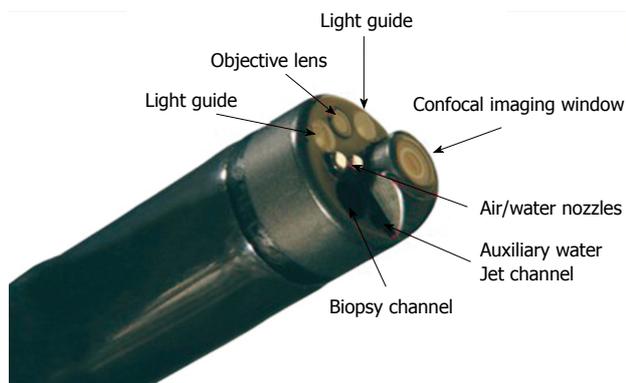


Figure 2 The endoscope-based confocal laser endomicroscopy (eCLE) imaging system: the distal tip.

cholangiopancreatography require an endoscope accessory channel of at least 1.2 mm, whereas the other probes, which are designed for use in esophagogastroduodenoscopy and colonoscopy, require a channel larger than 2.8 mm. All probes generate dynamic (12 frames per second) images with a scanning field of 30 000 pixels.

This system has a field of view of 240-600  $\mu\text{m}$  (CholangioFlex probes: 325  $\mu\text{m}$ ; GastroFlex and ColoFlex: 600  $\mu\text{m}$ ; GastroFlex-UHD and ColoFlex-UHD: 240  $\mu\text{m}$ ) with a lateral resolution of 1-3.5  $\mu\text{m}$  (the lateral resolution for CholangioFlex and for GastroFlex and ColoFlex probes is 3.5  $\mu\text{m}$ ; the lateral resolution for GastroFlex-UHD and ColoFlex-UHD is 1  $\mu\text{m}$ ). This system has a fixed imaging plane depth, and different confocal miniprobes are required to vary the depth of imaging. The depth of imaging for CholangioFlex probes is 40-70  $\mu\text{m}$ , 70-130  $\mu\text{m}$  for GastroFlex and ColoFlex), and 55-65  $\mu\text{m}$  for GastroFlex-UHD and ColoFlex-UHD.

Single video frames are reconstructed by a special computer algorithm ("mosaicing") in an image with an enlarged field of view (4 mm  $\times$  2 mm).

## PROCEDURE

Once a suspicious area of the mucosa is identified, contrast agents (fluorescein and/or acriflavine) can be given, and a CEM examination of the targeted area is performed by placing the distal tip of the endoscope or

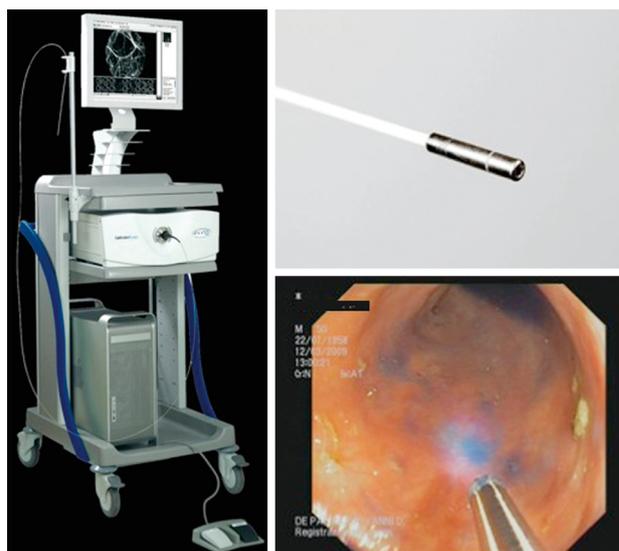


Figure 3 The probe-based CLE (pCLE) imaging system.

the distal tip of the catheter probe against the targeted mucosa. Gentle suction and/or an endoscopic cap can be used to stabilize the equipment and minimize excessive movement, which is important to reduce movement artifacts during imaging. At the area of interest, images can be obtained using an image-capture foot pedal and stored digitally.

## INTERPRETATION OF CLE IMAGES

The orientation of the "cut" plane of confocal *in vivo* histological images is horizontal whereas in conventional histological specimens it is longitudinal. CLE images cannot display a simultaneous overview of the mucosal and submucosal structures, unlike conventional histology. CLE images provide a thorough view of the mucosal architecture and allow rapid differentiation between normal, regenerative, and neoplastic mucosa of the GI tract. Differentiation between grades of intra-epithelial neoplasm by CEM, however, is not yet possible with the currently available staining techniques.

Endoscopists who perform CEM require a fundamental knowledge of the normal and disease micro-architecture of the GI tract. An onsite pathologist or at least a review of the stored images by a pathologist and correlation with biopsy histology is recommended during the learning process.

Some studies of the learning curve for CLE performed by endoscopists demonstrates that highly accurate, efficient *in vivo* prediction of Barrett's esophagus can be achieved after approximately 20-30 independently performed CLE procedures.

Normal and pathological aspects of CLE imaging of the upper and lower GI tract are shown in Tables 1-3 and Figures 3-5.

## CLINICAL DATA

The current potential indications for CLE imaging are

Table 1 Confocal criteria for squamous cell epithelium and carcinoma

	Squamous cell epithelium	Squamous cell neoplasia
Cellular criteria	Dark, homogeneous epithelial cells; regular architecture and clearly visible borders	Dark cells with different sizes; no clearly visible borders; irregular architecture
Vascular criteria	Capillaries directed to luminal epithelium without leakage of fluorescein	Twisted and irregular vessels; elongated capillaries; capillary leakage

Table 2 Confocal laser endomicroscopy (CLE) classification of Barrett's esophagus

Confocal diagnosis	Vessel architecture	Crypt architecture
Gastric-type epithelium	Capillaries with a regular shape only visible in the deeper parts of the mucosal layer	Regular columnar-lined epithelium with round glandular openings and typical cobblestone appearance
Barrett's epithelium	Subepithelial capillaries with a regular shape underneath columnar-lined epithelium visible in the upper and deeper parts of the mucosal layer	Columnar-lined epithelium with intermittent dark mucin in goblet cells in the upper parts of the mucosal layer. In the deeper parts, villous, dark, regular cylindrical Barrett's epithelial cells are present
Neoplasia	Irregular capillaries visible in the upper and deeper parts of the mucosal layer. Leakage of vessels leads to a heterogeneous and brighter signal intensity within the lamina propria	Black cells with irregular apical and distal borders and shapes, with strong dark contrast against the surrounding tissue

Table 3 CLE classification of patterns in colorectal lesions

Grading	Vessel architecture	Crypt architecture
Normal	Hexagonal, honeycomb appearance that presents a network of capillaries outlining the stroma surrounding the luminal openings of the crypts	Regular luminal openings and distribution of crypts covered by a homogeneous layer of epithelial cells, including goblet cells
Regeneration	Hexagonal, honeycomb appearance with no increase or only a slight increase in the number of capillaries	Star-shaped luminal crypt openings or focal aggregation of regular-shaped crypts with a regular or reduced amount of goblet cells
Neoplasia	Dilated and distorted vessels with increased leakage; irregular architecture, with little or no orientation to the adjoining tissue	Ridge-lined irregular epithelial layer with loss of crypts and goblet cells; irregular cell architecture, with little or no mucin

broad and include almost all current applications of endoscopic biopsy.

Unequivocally, this technology is best used in conjunction with other "red-flag" techniques because of its minute scanning area, and thus is only appropriate for classification of tissue at a site already detected by standard or optically enhanced endoscopy. Ideally, the no-dye "red-flag" techniques such as narrow band imaging or auto-fluorescence imaging, should be used to screen the mucosa for "areas of interest", which can then be interrogated by CEM for a "histological" diagnosis. An example would be use of narrow-band imaging to detect regions of suspicion in Barrett's esophagus, followed by CLE to confirm intraepithelial neoplasia, and guide immediate therapy.

The confocal laser endoscope can be used routinely for screening and surveillance. Suspicious lesions can be examined in a targeted fashion by placing the endomicroscopy window onto the lesion. Confocal images can be graduated according to cellular and vascular changes. The images correlate well with conventional histology after targeted biopsies.

Numerous studies have addressed the clinical applications of CLE, in particular in the study of precancerous

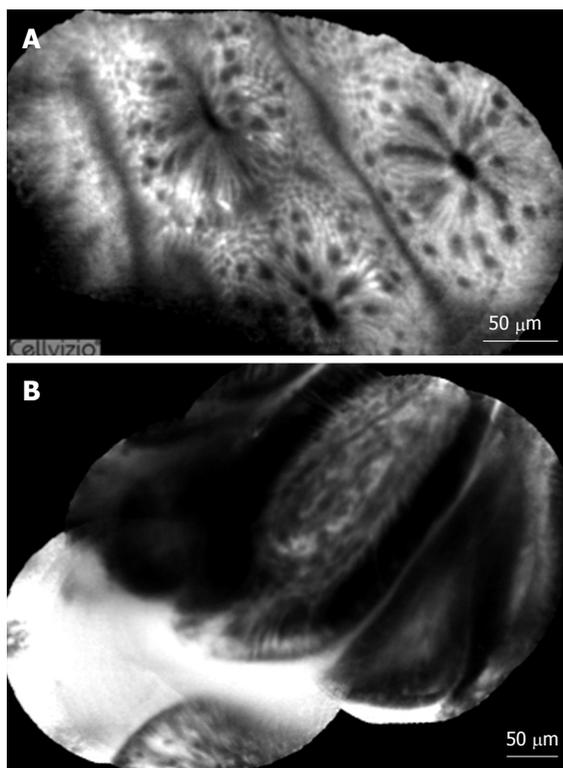
lesions of the upper and lower GI tract<sup>[15,16]</sup>.

In patients with Barrett's esophagus, CLE can diagnose Barrett's epithelium and Barrett's-associated neoplastic changes with an accuracy > 90% for both e-CLE or p-CLE<sup>[17-23]</sup>.

CLE in the stomach allows good visualization of normal and pathologic gastric pit patterns, making it a potentially useful tool for diagnosis of gastric cancer and precancerous conditions. Direct *in vivo* identification of *Helicobacter pylori* infection can be obtained<sup>[24-27]</sup>.

In patients with suspected celiac disease, confocal endomicroscopy can demonstrate villous atrophy and an increased number of intraepithelial lymphocytes, enabling immediate *in vivo* diagnosis of celiac disease<sup>[28]</sup>.

The presence of neoplastic changes in a colonic mucosa can be predict with high accuracy (> 95%). CLE has several potential roles in polyp management. The best-studied application is to distinguish between hyperplastic and adenomatous polyps, thus negating the need to remove hyperplastic polyps. In patients with long-term ulcerative colitis, chromoscopy with supplemental CEM, has recently been shown to further increase the yield for intraepithelial neoplasia above and beyond methylene blue. pCLE also has the capacity



**Figure 4** pCLE fluorescein sodium 10% imaging of the normal colon (A) and normal duodenum (B).

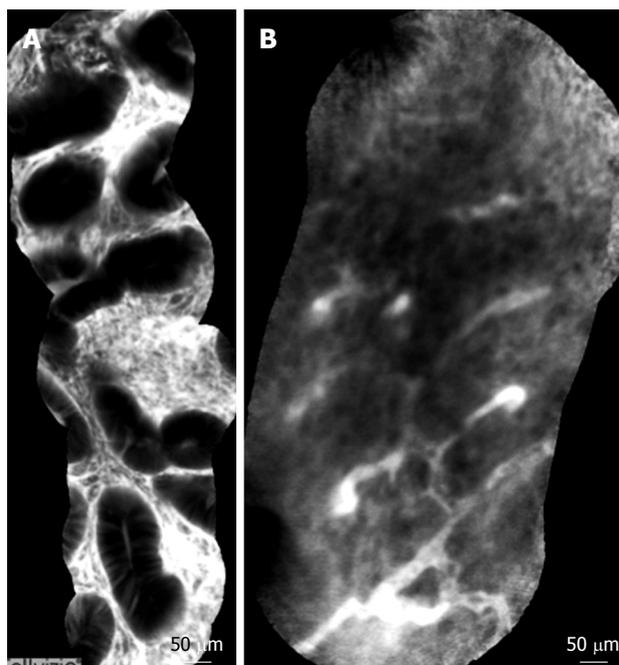
to differentiate normal from inflamed tissue, and thus target biopsies for the purpose of grading and mapping the extent of colitis<sup>[29-34]</sup>.

CEM can also be helpful for the diagnosis of microscopic colitis in patients with chronic diarrhea. In patients with collagenous colitis, it allows direct *in vivo* visualization of collagenous bands under the epithelial layer of the colon, and in patients with lymphocytic colitis, it can demonstrate crypt distortion and an increased distance between the colonic crypt. Thus, CLE has the potential to replace or direct a large number of random biopsies in patients with chronic diarrhea, where the confocal image is normal<sup>[35-37]</sup>.

Pancreato-biliary applications are under way using the probe-based CLE. The major role of pCLE in the bile duct is likely to detect cancer in indeterminate bile duct and pancreatic strictures<sup>[38,39]</sup>.

## CONCLUSION

CLE is a rapidly emerging field of gastroenterology that bridges the interface between endoscopy and histology. It further expands our ability to image living tissue in real time and to provide therapy in the same setting. The immediate impact will be the ability to target biopsies much more precisely, and eliminate a large number of random biopsies. Currently available devices for CEM have a very narrow field of view and allow only visualization of the superficial mucosal layer of the GI tract. Further technological developments are needed to enlarge the field of view, which will facilitate the use of CEM for cancer screening and surveillance. Increased



**Figure 5** pCLE fluorescein sodium 10% imaging of an adenomatous colonic polyp (A) and colonic adenocarcinoma (B).

depth of penetration is also needed to assess depth of invasion during cancer staging.

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REVIEW

## Signal molecule-mediated hepatic cell communication during liver regeneration

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### Abstract

Liver regeneration is a complex and well-orchestrated process, during which hepatic cells are activated to produce large signal molecules in response to liver injury or mass reduction. These signal molecules, in turn, set up the connections and cross-talk among liver cells to promote hepatic recovery. In this review, we endeavor to summarize the network of signal molecules that mediates hepatic cell communication in the regulation of liver regeneration.

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**Key words:** Signal molecule; Hepatic cells; Cellular cross-talk; Signal communication; Liver regeneration

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### INTRODUCTION

The liver is a vital organ. It has a wide range of functions, which include synthesis, metabolism, storage and

redistribution of amino acids, proteins (e.g. albumin and acute-phase proteins, enzymes and cofactors), carbohydrates, fats and vitamins. It also is involved in detoxification through: (1) removal of waste and xenobiotics, by breakdown of insulin and other hormones, hemoglobin, toxic substances, and medical products of drugs; (2) conversion of ammonia to urea; and (3) production and excretion of bile. The parenchymal cells (hepatocytes), which make up 70%-80% of hepatic cells, carry out most of these functions. The other 20% comprise the non-parenchymal cells, which include Kupffer cells, stellate cells, sinusoidal endothelial cells (SECs), biliary epithelial cells, lymphocytes, and oval cells<sup>[1]</sup>.

The liver is the only organ that can regenerate fully after injury in mammals<sup>[2]</sup>. When the liver is subjected to surgery, toxic substances, or viral infection, it has an amazing regenerative ability to restore functional hepatic mass. Injury induces the priming of recovery mechanisms with large changes in hepatic composition, such as activation of non-parenchymal cells, production and activation of multiple factors. These factors further lead to proliferation of hepatocytes and non-parenchymal cells, recovery and re-establishment of tissue architecture. When the liver recovers its normal volume and function, the regenerative response is terminated. Liver regeneration is actually compensatory hyperplasia, which is mediated typically by the proliferation of surviving hepatocytes<sup>[3]</sup>. When hepatocyte proliferation is inhibited, oval cell proliferation occurs<sup>[4]</sup>. This review endeavors to summarize the roles of hepatocytes and non-parenchymal cells, as well as signal communication among the hepatic cells during liver regeneration.

### FUNCTION OF VARIOUS TYPES OF LIVER CELL REGULATED BY DIFFERENT SIGNAL MOLECULES

#### Hepatocytes

Hepatocytes are organized into single-cell plates in mammals separated by vascular channels (sinusoids). Hepatocytes perform most liver functions such as synthesis, storage, metabolism and transformation of carbohydrates, amino acids, proteins, fats and vitamins, and detoxification, modification and excretion of exogenous and endogenous substances. The hepatocyte also initiates the formation and secretion of bile. During

liver regeneration, hepatocytes are primed to proliferate, maintain metabolic function, secrete interleukin (IL)-6, proteases and protease inhibitors, and hepatocyte growth factor (HGF)<sup>[5]</sup>.

### **Kupffer cells**

Kupffer cells are liver-resident macrophages with a pronounced phagocytic and endocytic capacity mainly located within the sinusoids<sup>[6]</sup>. Kupffer cells play an important role in the clearance of senescent and damaged erythrocytes. Kupffer cells also secrete potent mediators of the inflammatory response<sup>[7]</sup>. During the priming phase of liver regeneration, Kupffer cells are activated and secrete pro-inflammatory cytokines, most prominently tumor necrosis factor (TNF)- $\alpha$ , IL-6, and IL-1 $\beta$ , which can initiate the acute phase response in hepatocytes<sup>[8]</sup>. Small-for-size orthotopic liver transplantation may often cause graft failure. In a mouse partial 30% liver transplantation model, interruption of TNF- $\alpha$  signaling by Kupffer cell inactivation through administration of pentoxifylline and GdCl<sub>3</sub>, or the use of *Tnfr-1*<sup>-/-</sup> mice improves animal survival and enhances liver regeneration. These results are partly due to the fact that inhibition of TNF- $\alpha$  reduces leukocyte adherence, and improves portal flow and microcirculation<sup>[9]</sup>.

### **Hepatic stellate cells (HSCs)**

HSCs, also known as Ito cells, fat-storing cells or liver fibroblasts, are located in the space of Disse between the hepatocytes and the hepatic sinusoidal endothelial lining. In normal liver, HSCs sustain a quiescent state. Quiescent HSCs represent 5%-8% of liver cells, store 80% of total body retinol (vitamin A) as cytoplasmic lipid droplets, control turnover of extracellular matrix (ECM), and regulate the contractility of sinusoids<sup>[5-7]</sup>. Following hepatic damage, HSCs trans-differentiate into ECM-secreting myofibroblasts (activated HSCs)<sup>[10]</sup>, with the loss of retinol-rich droplets<sup>[11]</sup>. HSCs can secrete ECM proteins, including laminins, collagens and proteoglycans, growth factors such as HGF, fibroblast growth factor (FGF), transforming growth factor (TGF)- $\beta$  and cytokines such as IL-6, and produce some matrix metalloproteinases and tissue inhibitors of metalloproteinases<sup>[5]</sup>. The HSC is the major cell type involved in liver fibrosis. HSCs also behave as professional liver-resident antigen-presenting cells (APCs)<sup>[6,11]</sup>. A recent study has suggested that quiescent HSCs of rats retained within the space of Disse express stem/progenitor cell markers (e.g. CD133 and Oct4) and possess a differentiation potential. The space of Disse acts as a HSC niche, which is similar to the stem cell niche. These characteristics of quiescent HSCs are determined by the special microenvironment in the space of Disse, which is composed of basal lamina proteins (laminin and collagen type IV), sympathetic innervation and the adjacent cells. The adjacent cells include SECs, which release stromal cell-derived factor-1 to attract HSCs *via* the cysteine-X-cysteine receptor 4, and hepatocytes, which synthesize  $\beta$ -catenin-dependent Wnt ligands and Jagged-1 to attract HSCs through Wnt receptor frizzled, and to have direct physical interactions with HSCs through

Jagged-1 receptor notch<sup>[11-13]</sup>.

Neurotrophin receptor p75<sup>NTR</sup> is a low-affinity pan-neurotrophin receptor that belongs to the TNF receptor superfamily. In the diseased liver, p75<sup>NTR</sup> is expressed in HSCs. When p75<sup>NTR</sup> is activated, it activates the Rho/Rho kinase pathway, which enhances actin filament formation and phosphorylation of cofilin in a ligand-independent manner. This pathway contributes to the transition of quiescent stellate cells into myofibroblast-like cells that are important cellular sources of HGF. HGF drives healthy hepatocytes into proliferation. During perpetuation of trans-differentiation, secretion of pro-nerve growth factor (NGF) or NGF by neighboring regenerating hepatocytes leads to ligand-induced activation of apoptotic pathways, including the neurotrophin receptor interacting factor/TNF receptor-associated factor/Ras-related C3 botulinum toxin substrate/c-Jun N-terminal kinase/caspase pathway in stellate cells. In mice, depletion of p75<sup>NTR</sup> exacerbates liver pathology and inhibits hepatocyte proliferation *in vivo*. p75<sup>NTR</sup><sup>-/-</sup> HSCs fail to differentiate into myofibroblasts and do not support hepatocyte proliferation<sup>[14,15]</sup>.

### **SECs and biliary epithelial cells**

Liver SECs constitute the wall of the hepatic sinusoid and separate hepatocytes from the sinusoidal blood. They perform an important filtration function due to the presence of open fenestrations, with an average diameter of 120 nm, which allow free diffusion of many substances, but not of particles of the size of chylomicrons, between the blood and the hepatocyte surface. SECs have large endocytic and metabolic capacity for many ligands including glycoproteins, lipoproteins, ECM components (e.g. hyaluronate, collagen fragments, fibronectin, or chondroitin sulfate proteoglycan), immune complexes, transferrin and ceruloplasmin. SECs may function as APCs in the context of both major histocompatibility complex (MHC)-I and MHC-II restriction, with the resulting development of antigen-specific T cell tolerance. They are also active in the secretion of cytokines (IL-6), HGF, TGF- $\beta$ , eicosanoids (i.e. prostanoids and leukotrienes), endothelin-1, nitric oxide, and some ECM components<sup>[5,7]</sup>.

Biliary epithelial cells (cholangiocytes) constitute the bile ducts in hepatic portal triads. Cholangiocytes may transport water, ions and solutes; secrete growth factors and peptides that mediate cross-talk with other cells of the liver in a paracrine mode; secrete chemokines (e.g. monocyte chemoattractant protein-1) and cytokines (e.g. IL-6) and express adhesion molecules that attract effector leukocytes and promote the clearance of infected cells; and promote fibrogenesis by attraction of HSCs<sup>[5,16]</sup>.

Notch-1 and Jagged-1 are expressed in bile duct cells and hepatocytes in normal rat liver. Moreover, Notch-1 is also expressed in endothelial cells of the sinusoids and small vessels. After partial hepatectomy (PH) in rats, both Notch-1 and Jagged-1 proteins are upregulated and mainly exist in periportal hepatocytes. Notch receptor expressed in endothelial cells may be stimulated by its ligand Jagged, which is highly expressed in proliferating hepatocytes. Such interactions between ligands/receptors cause a decrease in endothelial cell proliferation and

promote formation of mature sinusoids. The Jagged/Notch signal is required to maintain a differentiated phenotype of bile duct cells, but more functions of Jagged/Notch signaling in bile duct cell proliferation and duct assembly remain vague<sup>[17]</sup>.

### **Dendritic cells (DCs), natural killer (NK) cells and NKT cells**

Interstitial liver DCs play important roles in innate and adaptive immunity. The outcome of liver DCs interacting with the antigen-specific T cells determines the balance between tolerance and immunity. Systemic and local environmental factors influence hepatic DC migration, maturation, and function<sup>[18]</sup>.

After 75% PH in male C57BL/6 mice, CD11c<sup>+</sup> (DC marker) liver (L)DCs increased significantly within 6 h and maintained an inherent, immature phenotype in both PBS- and Flt3L (fms-like tyrosine-3 ligand, a hematopoietic growth factor that expands dramatically the number of DCs in lymphoid and non-lymphoid tissues, including the liver, without changing their maturation state)-pretreated mice<sup>[19]</sup>. The increase was more notable in mice pretreated with Flt3L compared with PBS. The numbers of CD11c<sup>+</sup> LDCs returned to pre-hepatectomy levels by 24 h. The expanded LDC population showed increased IL-10 and reduced interferon (IFN)- $\gamma$  gene transcription 6 h after PH. The concomitant increase in expression of the anti-inflammatory cytokine IL-10 suggests that LDCs are involved actively in promoting a state of local immunosuppression. The decrease in IFN- $\gamma$  is associated with inhibition of hepatic NK cell lytic activity. LDCs isolated from the liver 6 h after 75% PH exhibit enhanced estrogen receptor expression, concomitant with increased serum 17- $\beta$ -estradiol levels. Flt3L-treated mice showed a significant increase in proliferating cell nuclear antigen labeling index compared with PBS-treated mice at 12, 24, 48 and 72 h after 40% PH, with a peak at 48 h. These results indicate that the increased numbers of estrogen-exposed DCs may play a key role in local immune suppression and promote progression of liver regeneration, by altering the balance toward a Th2-like microenvironment<sup>[19]</sup>.

NK cells are cytotoxic large granular lymphocytes derived from CD34<sup>+</sup> hematopoietic stem cells. NKT cells have three categories: I type (classical, V $\alpha$ 14-J $\alpha$ 18<sup>+</sup>TCR/CD1-dependent); II type (non-classical, all other CD1d-dependent T cells); and NKT-like cells (CD1d-independent NK1.1<sup>+</sup> T cells). NK and NKT cells are components of the innate immune system and participate in the inflammatory processes during hepatic injury. NK and NKT cells also contribute to adaptive immune responses by interacting with APCs<sup>[20]</sup>. NK and NKT cells accelerate liver injury through production of pro-inflammatory cytokines and killing hepatocytes. NKs inhibit liver fibrosis *via* killing early-activated and senescent-activated stellate cells and producing IFN- $\gamma$ . For the regulation of liver fibrosis, NKT cells appear to be less important than NK cells as a result of hepatic NKT cell tolerance<sup>[21]</sup>.

Treatment of mice with murine cytomegalovirus

(MCMV) infection and toll-like receptor (TLR)3 ligand poly I:C results in the activation of NK cells to produce IFN- $\gamma$  and attenuates liver regeneration after PH. NKT cells may only play a minor role in the negative suppressive effects of MCMV and poly I:C on liver regeneration<sup>[22]</sup>. However, in HBV transgenic (HBV-tg) mice, PH-induced liver regeneration is delayed. The impaired liver regeneration is related to the increased activation and number of NKT cells and their enhanced IFN- $\gamma$  production. NKT cells usually are activated by antigen-loading CD1d on APCs and soluble cytokines, such as IL-12 that is produced by Kupffer cells. Elevated CD1d on hepatocytes contributes to NKT cell activation and subsequent impairment of liver regeneration in HBV-tg mice. The impairment of liver regeneration in HBV-tg mice is largely ameliorated by NKT cell depletion, but not by NK cell depletion<sup>[23]</sup>.

Pretreatment of V $\alpha$ 14 NKT/J $\alpha$ 281<sup>+/+</sup> mice with IL-12 or  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer) 5 d before PH induced activation of NKT cells. The activated NKT cells expressed increased mRNA levels for TNF- $\alpha$  and IFN- $\gamma$  and enhanced liver injury at 24 h after PH. Hepatic NKT cells rather than Kupffer cells might produce TNF- $\alpha$  after the administration of IL-12 or  $\alpha$ -GalCer. TNF- $\alpha$  produced by activated V $\alpha$ 14 NKT cells was more than sufficient to enhance liver damage during the early phase of liver regeneration. The regenerating hepatocytes were destroyed specifically through the TNF receptor 1 (TNFR1) mediated TNF- $\alpha$ /TNFR1 pathway, which led eventually to impaired liver regeneration<sup>[24]</sup>. However, in another study, in mice injected with  $\alpha$ -GalCer 36 h after 70% PH, the induced activation of NKT cells greatly enhanced hepatocyte mitosis 44 h after surgery, *via* the TNF- $\alpha$ /TNFR1 and Fas/FasL-mediated pathways, accompanied by increased expression of TNFR1 and FasL in liver NKT cells<sup>[25]</sup>.

### **Stem cells and progenitor cells**

After injury, liver regeneration occurs typically through replication of existing hepatocytes, however, when hepatocyte proliferation is attenuated or blocked, the liver is repopulated by induction, proliferation, and differentiation of the progenitor cell compartment. Hepatic progenitor cells are rare quiescent cells that are thought to reside in the canals of Hering. The oval cells are a type of liver progenitor cells that express markers in common with cholangiocytes and embryonic hepatocytes in rodents<sup>[4,26-28]</sup>. Oval cells are characterized by expressing phenotypic markers such as A6<sup>[27,28]</sup> and thymus cell antigen 1, and  $\alpha$ -fetoprotein (AFP)<sup>[29]</sup>.

Oval cells are much less sensitive to TGF- $\beta$ -induced growth inhibition than hepatocytes *in vivo* and *in vitro*. These results are partly due to Mothers against decapentaplegic homolog (Smad)6 intervention. Smad6 is present in much higher amounts in oval cells (LE-2 and LE-6 cells) compared with hepatocytes (AML-12 cells or primary hepatocytes). The significant levels of Smad6 in oval cells inhibit TGF- $\beta$  signaling by associating with the type I receptor, thereby interfering with Smad2 phosphorylation by the activated receptor complex, which

prevents translocation of Smad2 to the nucleus, and subsequent target gene transcription<sup>[4]</sup>. The combination of IFN- $\gamma$  with lipopolysaccharide (LPS) or TNF- $\alpha$  causes a reversible cell cycle arrest in cultured hepatocytes (AML-12 cells), but stimulates DNA replication in oval cells (LE-6 cells). Hepatocyte cell cycle arrest is caused, at least in part, through NO release produced by inducible NO synthase after IFN- $\gamma$ /LPS or IFN- $\gamma$ /TNF- $\alpha$  administration<sup>[26]</sup>.

The hepatic expression of *lymphotoxin- $\beta$*  (*Lt- $\beta$*  or *Tnf- $\beta$* ) and *Ifn- $\gamma$*  produced by oval cells is upregulated and promotes oval cell-mediated liver regeneration in a choline-deficient, ethionine-supplemented (CDE) diet/PH mouse model. In *Lt- $\beta$*  knock-out (KO), *Lt- $\beta$*  KO, and *Ifn- $\gamma$*  KO mice, oval cell-mediated liver regeneration is impaired, which confirms a role for LT- $\beta$ /LT- $\beta$ R and IFN- $\gamma$  in oval cell-mediated liver regeneration<sup>[27]</sup>.

Gp130-mediated IL-6 signaling may play a role in oval cell proliferation *in vivo*. In livers of *IL-6*<sup>-/-</sup> mice fed with a CDE diet, the numbers of oval cells were reduced compared with *IL-6*<sup>+/+</sup> control mice. The hyperactive signal transducer and activator of transcription (STAT)3 signaling *Gp130*<sup>Y757F</sup> mouse model was derived when the tyrosine 757 residue of gp130 was mutated to a phenylalanine that prevented suppressor of cytokine signaling proteins (SOCS)3 binding and Src homology 2 (SH2) domain-containing protein-tyrosine phosphatase (SHP)2/RAS/extracellular signal-regulated kinases (ERK) signaling upon gp130 activation. Hyperactive STAT3 signaling in *Gp130*<sup>Y757F</sup> and *Socs3*<sup>-/-</sup> *LAAb* (*Socs3*<sup>-/-</sup>) mice fed a CDE diet results in increased oval cell proliferation compared with wild-type and gp130-mediated hyperactive ERK1/2 signaling (*Gp130*<sup>ΔST3</sup>) mice. However, SOCS3 overexpression or ERK1/2 activation inhibits oval cell proliferation in oval cell lines<sup>[28]</sup>. Proliferation of oval cells is associated with activation of nuclear factor (NF)- $\kappa$ B and STAT3 during oval cell-mediated liver regeneration in a 2-acetylaminofluorene (AAF)/PH rat model. Sustained NF- $\kappa$ B signaling has a critical role in protecting oval cells against apoptosis during stem cell-mediated liver regeneration. STAT3 plays a important role in driving proliferation and regulating differentiation of the hepatic stem cell progenies<sup>[30]</sup>.

Connective tissue growth factor (CTGF or CCN2) is a secreted matricellular protein that belongs to the CCN family. This protein comprises four mosaic conserved modules. CTGF normally is expressed at a very low level in the liver. However, when the liver suffers from chronic or acute injury, CTGF level is upregulated in diverse repair processes<sup>[29]</sup>. Recruitment and proliferation of Thy-1<sup>+</sup> oval cells is a hallmark of liver regeneration in rats after 2-AAF/PH. Sorted Thy-1<sup>+</sup> oval cells in rats after 2-AAF/PH express a high level of *Ctgf* gene accompanied by upregulated CTGF protein expression. Blocking CTGF induction by iloprost (a known inhibitor of CTGF synthesis) significantly decreased the oval cell proliferation and lowered the level of AFP expression as compared with control animals. These results suggest that CTGF induction is important for robust oval cell proliferation

after 2-AAF/PH treatment in rats<sup>[31]</sup>. Yeast two hybrid experiments have identified that fibronectin (FN) is a CTGF-binding protein. FN that binds to modules I and IV of CTGF and co-localizes with CTGF on the provisional hepatic ECM around the periportal regions promotes oval cell adhesion and migration, thereby facilitating oval cell activation<sup>[29]</sup>.

In 2-AAF/PH-treated rats, hepatocytes strongly express multidrug resistance protein 1b, but oval cells express high levels of active multidrug resistance associated protein (Mrp)1 and Mrp3. Mrp1 functions mainly as a cellular efflux pump of cysteinyl-leukotrienes and glutathione-S-conjugates, and Mrp3 as a pump for glucuronides and mono- and divalent bile salts, thus Mrp1 and Mrp3 may have a role in removing exogenous and endogenous toxic drugs/metabolites from oval cells, and facilitate oval cell proliferation in conditions of severe hepatotoxicity<sup>[32]</sup>.

Sympathetic nervous system inhibition using prazosin (PRZ, an  $\alpha$ -1 adrenoceptor antagonist) or 6-hydroxydopamine (6-OHDA, an agent that induces chemical sympathectomy) significantly enhanced hepatic accumulation of oval cells and reduced liver damage in mice fed antioxidant-depleted diets to induce liver injury. Neither PRZ nor 6-OHDA affects the expression of cytokines, growth factors, or growth factor receptors that are known to regulate progenitor cells<sup>[33]</sup>.

The plant lectin concanavalin A (Con A) may induce T cell-mediated hepatitis in mice. Following PH, ConA-treated mice show significantly impaired early regenerative responses, such as decreased cyclin D1 and E expression and STAT3 activation within hepatocytes, in conjunction with reduced IL-6 production, increased IFN- $\gamma$ , TGF- $\beta$  and p21<sup>waf</sup> expression, and increased TGF- $\beta$ -induced Smad2 phosphorylation. However, Con A may induce an increase in the number of NK cell-sensitive oval cells (CD117, AFP, albumin, and cytokeratin-positive cells) and hematopoietic-like cells (Sca-1<sup>+</sup> cells) in these mice<sup>[34]</sup>. Furthermore, much more accumulated lipid was seen in the liver of mice with Con A-induced hepatitis, by Oil Red O staining<sup>[35]</sup>.

The IL-6/gp80/gp130 signaling system contributed to rapid expansion of the progenitor cell populations including liver hematopoietic progenitor cells and liver epithelial progenitor cells in a Con A/PH-mediated mouse liver injury model<sup>[36]</sup>.

## SIGNAL COMMUNICATION THAT OCCURS IN THE PROGRESSIVE PHASES OF LIVER REGENERATION

Liver injury causes significant changes in the expression and activity of a variety of signal mediators produced by hepatic cells, endocrine glands and platelets. These molecules include complement components C3 and C5; cytokines (TNF- $\alpha$  and ILs); growth factors [TGF, epidermal growth factor (EGF), platelet-derived growth factor, vascular endothelial growth factor (VEGF), FGF,

insulin-like growth factor (IGF)-I, and HGF]; hepatic ECM; extracellular proteases and protease inhibitors; hormones [insulin, growth hormone (GH), thyroid hormone, vasopressin, prostanoids, and endothelin-1] and neurotransmitters (serotonin); metabolites [bile acids, reactive oxygen species (ROS), NO, lipids, glutathione, S-adenosylmethionine, and sphingosine-1-phosphate]; and chemokines<sup>[1,5,7,37-42]</sup>. Liver regeneration progression is highly coordinated by the signal communication between hepatocytes and non-parenchymal cells, and is also influenced by endocrine glands, sympathetic innervation, and blood circulation. The progression of liver regeneration is segmented into several phases. Here, we describe the mechanisms of liver regeneration in each phase.

### Priming phase

In models of liver injury induced by toxins, such as CCl<sub>4</sub>, or Fas ligand, the hepatocytes are damaged and undergo necrosis, for which, the growth factor- and cytokine-mediated pathways are similar to those in PH models<sup>[1]</sup>. Liver injury causes the release of ROS and LPS, which trigger the activation of the complement system. After complement activation, cleavage of C3 or C5 leads to the generation of the potent anaphylatoxins C3a and C5a. LPS, C3a and C5a in turn activate the non-parenchymal cells such as Kupffer cells, through the cell surface receptor TLR4 and G protein-coupled receptors C3aR and C5aR, which causes activation of the NF- $\kappa$ B signaling pathway and the production of cytokines such as TNF- $\alpha$  and IL-6. TNF- $\alpha$  then interacts with TNFR on Kupffer cells, which stimulates intensive synthesis of TNF- $\alpha$  and IL-6. Furthermore, SECs, HSCs, biliary epithelial cells and hepatocytes may also produce IL-6<sup>[5,43,44]</sup>. The cytokines TNF- $\alpha$  and IL-6 are responsible for priming the quiescent hepatocytes into the cell cycle (G0 to G1) through binding to their receptors TNFR1 and IL-6R; activating the NF- $\kappa$ B, JAK/STAT3 and MAPK signal pathway; initiating the transcription of immediate early genes; and sensitizing hepatocytes to the proliferative effects of growth factors<sup>[37]</sup>.

### Proliferative phase

In rat liver PH models, the rate of DNA synthesis in hepatocytes begins to increase after about 12 h and peaks around 24 h. However, induction of DNA synthesis occurs later in the non-parenchymal cells (at about 48 h for Kupffer, biliary epithelial and stellate cells, and at about 96 h for endothelial cells). Subsequent levels of DNA synthesis in hepatocytes are lower, as complete restoration of liver mass requires an average of about 1.66 cycles of replication in all cells. By comparison, the peak in DNA synthesis in mice occurs later (36-40 h after PH) and varies between strains<sup>[1,45]</sup>. Many growth factors and growth factor-binding proteins are produced to promote the progression of liver regeneration.

The bulk of IGF-I is synthesized by hepatocytes, but is also produced by other types of non-parenchymal liver cells. Hepatic IGF-I synthesis is not only regulated

by growth hormone, insulin, and IGF-I, but also by cytokines released from activated Kupffer (IL-1, TNF- $\alpha$  and TGF- $\beta$ ) or stellate (TGF- $\alpha$  and TGF- $\beta$ ) cells<sup>[7]</sup>. The biological actions of IGF-I are mediated through its physiologic receptor IGF-1R and insulin receptor. The activity of IGFs is modulated by a family of high-affinity binding proteins (IGFBP-1-6) and IGFBP proteases<sup>[46]</sup>. HGF is produced mainly by HSCs, but also by hepatocytes, SECs, and performs its functions through interacting with its receptor c-met<sup>[5]</sup>. The EGF family consists of several members, including EGF, TGF- $\alpha$ , heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR),  $\beta$  cellulin, and epiregulin<sup>[47]</sup>. HB-EGF is expressed mainly in Kupffer and endothelial cells<sup>[48]</sup>; TGF- $\alpha$  is synthesized mainly by hepatocytes<sup>[49]</sup>; expression of AR is induced by hepatocytes, Kupffer cells and HSCs<sup>[50,51]</sup>; and these factors transmit their signal through EGF receptor.

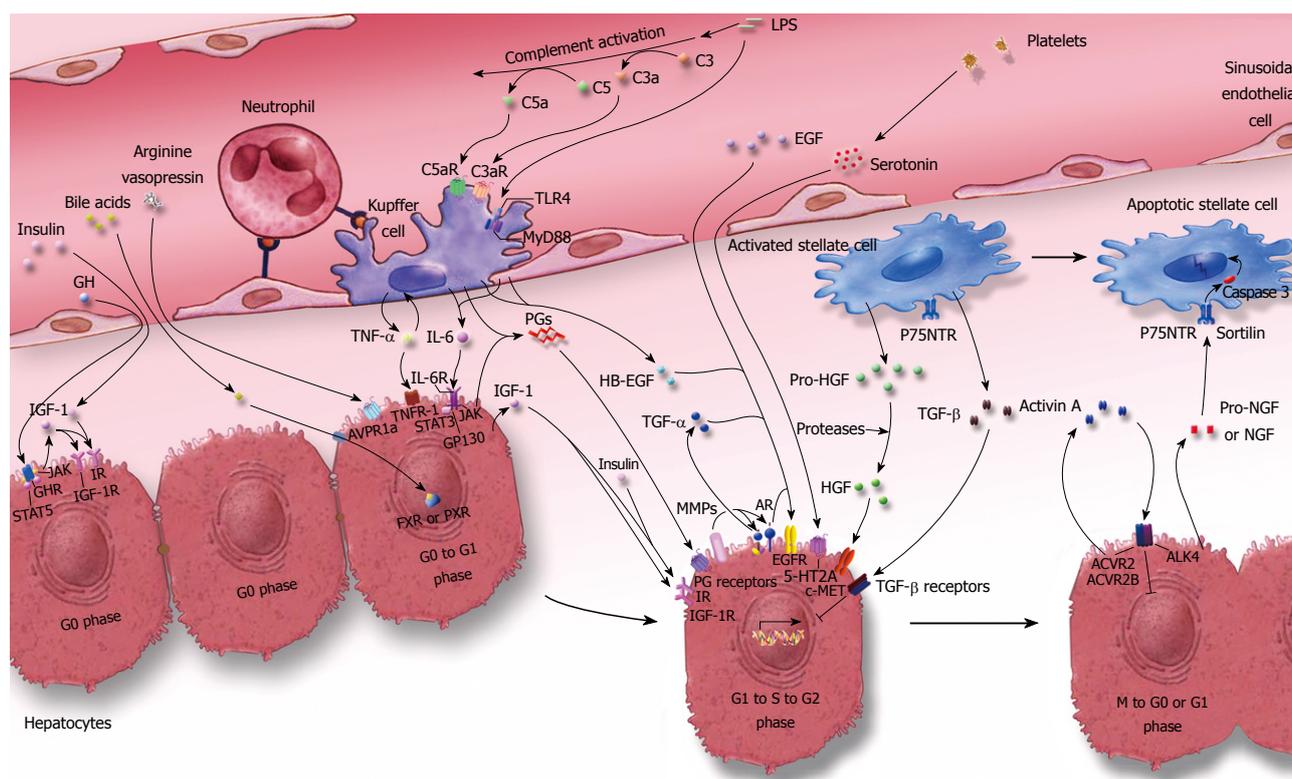
Most growth factors are usually formed in an inactive precursor bound with ECM or integral to membrane. During liver regeneration, extracellular protease activation and ECM degradation occur in the first few hours before hepatocyte DNA synthesis and division, and growth factors are released and activated from the bound ECM or cell membrane by the extracellular protease<sup>[5]</sup>. These activated growth factors binding to their corresponding receptors drive hepatic cells into DNA replication and mitosis. Furthermore, factors such as insulin from the pancreas, EGF from the duodenum or salivary gland, norepinephrine from the adrenal gland, triiodothyronine from the thyroid gland, GH from the anterior pituitary gland, arginine vasopressin (AVP) from the posterior pituitary, serotonin from platelets, and prostaglandins (PGs) from Kupffer cells and hepatocytes are also involved in the process of liver regeneration<sup>[1,38,52-54]</sup>.

### Remodeling phase

After cell division, hepatocytes are formed in clusters that no longer associate with sinusoids. The nascent endothelial cells travel among cell clusters to form sinusoids that will line hepatocyte plates. VEGF, angiopoietin and their receptors Flt-1 (VEGF-R1), Flk-1/KDR (VEGF-R2), Flt-4 (VEGF-R3), and Flt3/Flk2, as well as tyrosine kinase containing immunoglobulin and epidermal growth factor homology domain (Tie)-1 and Tie-2 might be involved in this process<sup>[5,55]</sup>. Meanwhile, biliary epithelial cells rebuild the biliary tree in hepatic portal triads. Synthesis of new ECM, vasculature and biliary tree then reestablishes tissue architecture.

### Terminating phase

TGF- $\beta$  and activin A belong to the TGF- $\beta$  superfamily of cytokines. TGF- $\beta$  is produced mainly in HSCs, but is also expressed in SECs and Kupffer cells<sup>[4,5]</sup>. In the liver, activin A is synthesized predominantly in hepatocytes, but is also expressed in non-parenchymal cells under pathological conditions. Activin A is an autocrine growth inhibitor that is produced in hepatocytes, and is involved in inhibiting the proliferation of hepatocytes,



**Figure 1** Scheme depicting the network of signaling among hepatic cells and blood during liver regeneration. After liver injury such as PH, gut-derived factors such as LPS reach the liver through the portal blood supply. LPS activate the complement system, which releases the anaphylatoxins C3a and C5a. LPS, C3a and C5a activate Kupffer cells through TLR4, C3aR and C5aR, and further lead to production of TNF- $\alpha$  and IL-6. These factors are involved in priming the hepatocytes from G0 to G1 phase. Insulin, GH, bile acids, AVP, platelet-derived serotonin, and EGF from the blood, cooperating with PGs, HB-EGF, HGF, as well as IGF-1, TGF- $\alpha$  and AR from different hepatic cells, promote hepatocyte transition from G1, through S and G2, to the M phase of the cell cycle. TGF- $\beta$  produced mainly by HSCs inhibits G1 to S phase transition of hepatocytes, and TGF- $\beta$  signalling is blocked during the proliferative phase. Pro-NGF or NGF produced by the neighboring regenerating hepatocytes promotes the termination of the activated state of HSCs by pro-NGF- or NGF-induced apoptotic pathways. When the liver mass is restored to its normal volume, the increased signaling by activin A, apoptosis and other factors may promote termination of liver regeneration. The prototype of this figure is originated from reference<sup>[43]</sup>.

inducing the differentiation of hepatocytes, augmenting the tubulogenesis of SECs, and stimulating collagen production in HSCs<sup>[56]</sup>.

TGF- $\beta$  and activin A may bind to their high-affinity cell surface type II receptor TGFBR2/T $\beta$ R II and ACVR2/ActR II or ACVR2B/ActR II b, respectively, either directly or *via* co-receptors, and recruit and activate their cell surface type I receptors TGFBR1/ALK5 (T $\beta$ R I), ACVRL1/ALK1, ACVR1/ALK2 and ACVRL1/ALK1, ACVR1/ALK2, ACVR1B/ALK4, respectively, which leads to activation of their downstream Smad signal pathway<sup>[57,58]</sup>. TGF- $\beta$  inhibits G1 to S phase transition in hepatocytes, but TGF- $\beta$  signaling is blocked during the proliferative phase; furthermore, intact TGF- $\beta$  signaling is not required for the termination of liver regeneration<sup>[1,59]</sup>. When the liver mass restores its normal volume, the increased signaling by activin A, apoptosis and other factors<sup>[59]</sup>, and the decreased expression and activation of promoting proliferation factors due to the restored ECM and tissue architecture may promote termination of liver regeneration. Figure 1 depicts the main signal communication network that occurs in the progression of liver regeneration.

Following liver injury, the expression and activity of signal molecules produced by activated hepatic cells

are controlled in a time- and micromilieu-dependent mode. The direct interactions among hepatic cells and the indirect interactions mediated by secreted signal molecules with hepatic cells constitute the dynamic recovery process of liver regeneration. Many more signal molecules and pathways are involved in the regulation of liver regeneration than those mentioned above. Further study will reveal more facts about the mechanisms of liver regeneration.

## CONCLUSION

Liver regeneration is a very complex process, which is accompanied by a highly regulated intercellular and intracellular signal communication network. The extracellular signal molecules that regulate the progression of liver regeneration are produced by hepatic cells, endocrine glands and platelets in autocrine, paracrine, juxtacrine and endocrine modes. Most of these signal molecules are inactive precursors that need to be further processed into a mature form by activated proteases in ECM or on membrane of adjacent hepatic cells. The extracellular signaling interfaces with intracellular signals through their specific receptors. Liver regeneration is highly coordinated by the cross-talk between these signal

molecules and hepatic cells. Liver has a regenerative ability to restore functional hepatic mass after liver injury, but under some pathological conditions, the recovery of liver is not autonomous, so the study of the pathogeny of the diseased liver is more significant, to provide methods for treating patients with liver damage.

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REVIEW

## Potential role of Th17 cells in the pathogenesis of inflammatory bowel disease

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### Abstract

The etiopathology of inflammatory bowel disease (IBD) remains elusive. Accumulating evidence suggests that the abnormality of innate and adaptive immunity responses plays an important role in intestinal inflammation. IBD including Crohn's disease (CD) and ulcerative colitis (UC) is a chronic inflammatory disease of the gastrointestinal tract, which is implicated in an inappropriate and overactive mucosal immune response to luminal flora. Traditionally, CD is regarded as a Th1-mediated inflammatory disorder while UC is regarded as a Th2-like disease. Recently, Th17 cells were identified as a new subset of T helper cells unrelated to Th1 or Th2 cells, and several cytokines [e.g. interleukin (IL)-21, IL-23] are involved in regulating their activation and differentiation. They not only play an important role in host defense against extracellular pathogens, but are also associated with the development of autoimmunity and inflammatory response such as IBD. The identification of Th17 cells helps us to explain some of the anomalies seen in the Th1/Th2 axis and has broadened our understanding of the immunopathological effects of Th17 cells in the development of IBD.

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**Key words:** Crohn's disease; Inflammatory bowel disease; Interleukin-17; Interleukin-23; Th17 cells; Ulcerative colitis

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### INTRODUCTION

Current evidence strongly suggests that inflammatory bowel disease (IBD) arises from a disruption of mucosal immune homeostasis in genetically susceptible individuals, resulting in altered processing of enteric antigens, pathogenic T cell activation, and chronic inflammation<sup>[1-3]</sup>. Although the etiology of IBD remains unclear, accumulating evidence has indicated that dysfunction of the mucosal immune system plays an important role in the pathogenesis of IBD. Among a variety of inflammatory cells in the gut, mucosal CD4<sup>+</sup>T cells are thought to play a central role in both the induction and persistence of chronic inflammation by producing proinflammatory cytokines. Studies have indicated that Th1-related cytokines [e.g. tumor necrosis factor (TNF), interferon (IFN)- $\gamma$ , interleukin (IL)-12] as well as Th17-associated cytokines (e.g. IL-17A, IL-21, IL-23) are markedly increased in inflamed mucosa of CD, whereas the cytokine profiles in inflamed areas of UC seem to exhibit increased production of the Th2 cytokines such as IL-5 and IL-13<sup>[1-3]</sup>. These proinflammatory cytokines are potent *in vitro* stimulators of intestinal mucosal effector functions including T cell and macrophage proliferation, adhesion molecule expression, chemokine expression, and secretion of other proinflammatory cytokines.

### Th17 CELLS AND THE DIFFERENTIATION REGULATION

CD4<sup>+</sup>T cells play an important role in the initiation of immune responses by providing help to other cells and by taking on a variety of effector functions during immune reactions. Upon antigenic stimulation, naive CD4<sup>+</sup>T cells are activated, expand and differentiate into different effector subsets such as Th1 and Th2 cells

characteristic of the production of distinct cytokines and effector functions<sup>[4,5]</sup>. Th1 cells produce IFN- $\gamma$  and lymphotoxin and can mobilize the cellular arm of the immune system to combat intracellular pathogens. Th2 cells secrete IL-4, IL-13, and IL-25, which are essential for the generation of appropriate classes of antibodies and for the elimination of extracellular pathogens<sup>[4,5]</sup>.

The identification of the IL-17 family of cytokines as well as the IL-23-mediated expansion of IL-17-producing T cells uncovered a new subset of Th cells, designated as Th17 cells<sup>[6,7]</sup>. Th17 cells require specific cytokines and transcription factors for their differentiation. Although the function of this cell subtype is not completely elucidated, emerging data suggest that Th17 cells may play an important role in host defense against extracellular pathogens, which are not efficiently cleared by Th1-type and Th2-type immunity. The first pathogen implicated in a Th17 response was observed in human Lyme arthritis caused by *Borrelia burgdorferi*, in which *B. burgdorferi*-derived lipopeptides could stimulate the production of IL-17A by T cells from synovial fluid, leading to a Th17 lineage differentiation<sup>[8]</sup>. Previous work has demonstrated that Th17 cells with specificity for self-antigens lead to severe autoimmunity in various animal models. In the murine model of psoriasis, evidence has shown that Th17 cells along with their upstream cytokines (e.g. IL-23) and their downstream effector cytokines (e.g. IL-22) might play a critical role in the pathogenesis of psoriasis<sup>[9,10]</sup>. Moreover, increased levels of IL-17 produced by Th17 cells have been observed in murine models of rheumatoid arthritis and correlate with more severe joint damage<sup>[11]</sup>.

The IL-17 cytokine family is a recently discovered group of cytokines, which includes six members, IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (or IL-25) and IL-17F, and act *in vitro* and *in vivo* as potent proinflammatory cytokines<sup>[6]</sup>. IL-17 can induce the expression of proinflammatory cytokines (such as IL-6 and TNF), chemokines (such as KC, MCP-1 and MIP-2) and matrix metalloproteases, which mediate tissue infiltration and tissue destruction<sup>[12]</sup>. It is also involved in the proliferation, maturation and chemotaxis of neutrophils<sup>[13]</sup>. In agreement with this point, mice deficient in the IL-17 receptor (IL-17R) are more sensitive to lung bacterial infection because of reduced recruitment of neutrophils to the lung<sup>[14]</sup>. In contrast, overproduction of IL-17 in the lungs leads to chemokine expression and tissue inflammation infiltrated by large amounts of leukocytes<sup>[15]</sup>. Moreover, IL-17 is able to costimulate T cells and enhance the maturation of dendritic cells<sup>[16]</sup>. Taken together, these data indicate that IL-17 has pleiotropic activities, functions through the adaptive and innate immune system to promote immune response, and plays an important role in immune responses.

Several cytokines have been reported to be associated with the development and/or proliferation of Th17 cells. Neutralization of IFN- $\gamma$  and IFN- $\alpha$  *in vitro* increases the number of IL-17-producing cells generated by IL-23 stimulation. The number of Th17 cells is further increased by the addition of an IL-4-neutralizing antibody, indicating that IL-4 and IFN- $\gamma$  could inhibit the IL-23-driven

expansion of Th17 cells<sup>[17]</sup>. IL-2, a cytokine important for growth and survival of Th1 and Th2 subsets, is also involved in the long-term expansion and survival of Th17 cells, since restimulation of differentiated Th17 cells with IL-2 could abolish IL-17 production and induce IFN- $\gamma$  production<sup>[18]</sup>. In addition, the differentiation of Th17 cells might also require costimulatory signals distinct from those involved in the differentiation of Th1 and Th2 cells. These studies of the costimulatory requirements of Th17 cells were undertaken to determine which costimulatory molecules are important for IL-6- and TGF- $\beta$ -mediated differentiation of Th17 cells. However, recent work has also demonstrated that Th1-derived IFN- $\gamma$  could trigger antigen-presenting cells to produce IL-23 and then induce memory Th17 cell expansion in a B7-H1-independent manner<sup>[19]</sup>. These data indicate that this complex differentiation of Th17 cells may be dependent on various cytokine milieu.

IL-23 is a heterodimeric protein, which is a member of the IL-12 family of cytokines. It is composed of a p19 subunit in addition to a p40 subunit, which is also a component of IL-12<sup>[20]</sup>. IL-23 functions through the receptor-signaling complex, which is composed of its distinct receptor (namely IL-23R) and IL-12R $\beta$ 1<sup>[21]</sup>. Because IL-23 and IL-12 share a common p40 subunit and IL-12R $\beta$ 1, IL-23 may function like IL-12 to trigger Th1 response. However, p19-deficient mice could contribute to normal Th1 response but could not promote the production of IL-17 cells<sup>[22]</sup>. Other work has demonstrated that Th17 cells are absent in IL-23<sup>-/-</sup> mice, and could not be amplified and survive albeit in the presence of normal Th17 cells *in vivo*<sup>[23]</sup>. Studies have shown that IL-23R is not expressed on naïve T cells, therefore IL-23 could not induce naïve T cells to differentiate into Th17 cells, but could promote Th17 cells amplification<sup>[24]</sup>. These data imply that IL-23 could provide survival signaling to induce the differentiation of Th17 cells. Recently, increasing evidence has shown that IL-1 $\beta$  and IL-23 are required for the generation of Th17 cells and differentiation<sup>[25]</sup>. Naïve T cells stimulated with TGF- $\beta$  plus IL-6 could secrete large amounts of IL-17, whereas IL-23 could trigger the proliferation of Th17 cells from activated memory T cells, only the combination of IL-6 plus TGF- $\beta$  is sufficient to induce differentiation of Th17 cells from naïve T cells<sup>[25]</sup>. Moreover, IL-1 $\beta$  and TNF could increase the number of Th17 cells generated *in vitro* in the presence of IL-6 plus TGF- $\beta$ <sup>[26]</sup>. These data suggest that an inflammatory milieu could regulate the expression of IL-23R on Th17 cells and thereby allow IL-23 to sustain and strengthen the Th17 phenotype.

IL-27 is another IL-12 family member and has been found to downregulate Th17 cell development<sup>[27,28]</sup>. IL-27 is a heterodimeric cytokine composed of Epstein-Barr virus-induced gene 3 (*EBI-3*) and p28 chains. Activation of T cells in the presence of IL-27 induces T-bet, a transcription factor critical for the differentiation of naïve CD4<sup>+</sup> T cells into Th1 cells. However, IL-27R-deficient mice develop severe immunopathology resulting from a general dysregulation of effector T cell responses not restricted to any particular Th cell subtype<sup>[29,30]</sup>. The absence of IL-27-mediated signaling exacerbates

neuroinflammation, enhances the generation of Th17 cells and increases the number of IL-17-expressing T cells in inflamed tissue. The transcription of the two subunits of IL-27 is differentially regulated, leading to the immunosuppressive effects of IL-27. *EBI-3* is strongly induced by Toll-like receptors (TLRs) and the stimulation of TLR results in the binding of NF- $\kappa$ B complexes to a promoter region of the *EBI-3* gene, while activation of TRIF downstream of TLR3 or TLR4 is critical in the induction of p28<sup>[28]</sup>. IL-27, independently of IFN- $\gamma$ R and IL-6R signaling, can inhibit the differentiation of Th17 cells triggered by IL-6 and TGF- $\beta$ . Previous work has demonstrated that T-bet and the suppressor protein SOCS3 are not involved in the IL-27-mediated inhibition of Th17 cells, but that the transcription factor STAT1 seems to be required for the suppressive effect of IL-27 on the development of Th17 cells<sup>[27,28]</sup>.

ROR $\gamma$ t, an orphan nuclear hormone receptor, is expressed by fetal lymphocyte tissue-inducer cells and participates in the formation of lymph nodes and Peyer's patches, intestinal lymphocyte tissue-inducer-like cells and immature thymocytes<sup>[31]</sup>. Interestingly, ROR $\gamma$ t is also found to be expressed by differentiated Th17 cells and IL-17-producing T cells present in the intestinal lamina propria (LP). Consistent with this, Th17 cells are observed to be absent in ROR $\gamma$ t-deficient mice, whereas transduction of naive T cells with a ROR $\gamma$ t-encoding retrovirus could induce IL-17 production. These data indicate the importance of ROR $\gamma$ t in the differentiation of Th17 cells<sup>[32]</sup>. In addition, a recent study has observed that ROR $\gamma$ t-deficiency could not abolish Th17 cell generation and that Th17 cells also express high levels of another related nuclear receptor ROR $\alpha$ <sup>[33]</sup>. Importantly, ROR $\alpha$  deficiency results in reduced IL-17 expression, while coexpression of ROR $\alpha$  and ROR $\gamma$  synergistically leads to Th17 cell differentiation. Thus, these data indicate that Th17 differentiation is directed by two lineage-specific nuclear receptors, ROR $\alpha$  and ROR $\gamma$ <sup>[33]</sup>.

## PATHOGENIC ROLE OF Th17 CELLS IN IBD

It was widely believed that the chronic intestinal inflammation characteristic of human IBD is the consequence of pathogenic Th1 CD4<sup>+</sup> cell responses against the luminal flora, especially in CD, which in turn is driven by proinflammatory cytokines such as IL-12 and TNF<sup>[1-3]</sup>. Animal models of IBD support this hypothesis as intestinal inflammation could be blocked by treatment with monoclonal antibodies specific for IL-12 or TNF<sup>[34,35]</sup>. In recent years, studies on the Th17 cell subset has highlighted our understanding of the formation of human inflammatory diseases, which helps us to explain some of Th1/Th2 balance of abnormal phenomena, particularly in human IBD.

Evidence has shown that high numbers of CD4<sup>+</sup> Th17 cells are found in the colonic LP of the ileum and colon but not the duodenum, jejunum, mesenteric lymph nodes or spleen in conventionally-raised mice, and that these cells are highly infiltrated in inflamed areas of colitic

mice<sup>[36,37]</sup>. Further analysis confirmed that commensal gut flora contribute to the expansion of these CD4<sup>+</sup> Th17 cells, leading to intestinal mucosal inflammation. In terms of mucosal immunity, the IL-23/IL-17 axis has been observed to play an important role in normal intestinal homeostasis, although the precise actions of these cytokines in the gut remain to be fully delineated. To date, IL-17 and other Th17-associated cytokines (e.g. IL-22, IL-23) have been found to have protective effects or pathogenic effects dependent on other effective factors in local tissue. Previous study has demonstrated that IL-23 is mainly expressed by LP dendritic cells in the terminal ileum of normal mice and the frequency of Th17 cells in the intestinal LP is markedly higher than their frequency in peripheral lymphoid tissues<sup>[38]</sup>. Evidence has shown that IL-23 may have important immune protective effects in the gut and that IL-23<sup>-/-</sup> mice exhibited enhanced susceptibility and mortality following infection with the intestinal bacterial pathogen *Citrobacter rodentium*<sup>[38]</sup>. Interestingly, *C. rodentium*-infected IL-23<sup>-/-</sup> mice still generate potent mucosal Th17 responses, suggesting that IL-23-mediated protective responses need not necessarily involve IL-17 production<sup>[25]</sup>. Similarly, although studies in various murine colitis models have implied that IL-23-driven intestinal pathology is associated with increased IL-17 production, a plethora of other inflammatory cytokines have also been found to be elevated in the inflamed colon, including IL-1 $\beta$ , IL-6, IFN- $\gamma$  and TNF<sup>[39]</sup>. In many of the T cell-dependent IBD murine models, Th1 cells clearly predominated in inflamed mucosa and inhibition of Th1 responses could attenuate disease<sup>[1-3]</sup>. Moreover, the observations that IL-23 also drives chronic colitis mediated by cells of the innate immune system are also consistent with the hypothesis that IL-23-mediated intestinal inflammation need not necessarily involve Th17 cells<sup>[39,40]</sup>.

IL-17 mRNA has been found to be highly expressed in inflamed mucosa from both UC and CD patients, and immunohistochemistry revealed that CD68-positive cells express IL-17<sup>[41]</sup>. Recent work<sup>[42]</sup> has also demonstrated that most of the transcripts for Th17-related cytokines were increased in both UC and CD compared to normal controls, but more abundant in UC than in CD. In contrast, up-regulation of IFN- $\gamma$  mRNA was marked in CD LP CD4<sup>+</sup> T cells. Up-regulation of IL-23p19 mRNA was detected in colonic mucosa from both UC and CD patients. The significance of Th17 immunity in UC was further supported by the finding that recombinant IL-23 actually enhanced IL-17 production by LP CD4<sup>+</sup> T cells in UC, but had a lesser effect on LP CD4<sup>+</sup> T cells in CD. Since the Th1 pathway has been reported to antagonize the Th17 pathway *via* various mechanisms, IFN- $\gamma$  or IL-12 could actually suppress IL-17 production by human LP CD4<sup>+</sup> T cells. Therefore, we can hypothesize that excess IFN- $\gamma$  production by Th1 cells in CD patients may negatively affect the IL-17 production by Th17 cells in CD, despite the fact that Th17 cells are present in CD mucosa.

A previous report has shown that IL-23 can enhance IFN- $\gamma$  production by LPMCs from CD patients and that the mucosal IL-23p19 expression levels were correlated with IL-17 in UC and IFN- $\gamma$  in CD<sup>[43]</sup>. These results

suggest that IL-23 may enhance the production of distinct cytokines between UC and CD patients, thereby contributing to the local Th1/Th17 balance in IBD. Additionally, IL-21, belonging to the IL-2 family, has been described to play an important part in the differentiation and maintenance of Th17 cells<sup>[44,45]</sup>. Comparing the Th1, Th2, and Th17 subsets, the largest amounts of IL-21 are produced by Th17 cells. IL-21 produced by differentiating Th17 cells may act in a positive feedback loop, which amplifies the precursor frequency of Th17 cells<sup>[44,45]</sup>. Recently, our study found that IL-21 facilitated IBD CD4<sup>+</sup> T cells to differentiate into Th17 cells, characterized by increased expression of IL-17A and ROR $\gamma$ t. Thus, we proposed that IL-21 might be involved in the pathogenesis of IBD and blockage of IL-21R signaling may have therapeutic potential in IBD<sup>[46]</sup>.

The exact role of IL-17 and Th17 cells in intestinal pathology and homeostasis is currently not well understood. IL-17 may have some protective functions in the epithelial layer, as it has been shown to fortify tight junction formation between epithelial cells *in vitro*<sup>[47]</sup>, and treatment of mice with anti-IL-17 neutralizing antibody actually enhanced the severity of colitis induced by administration of dextran sodium sulphate<sup>[48]</sup>. In contrast, a recent study comparing the ability of Th1 and Th17 cells to induce colitis in mice has proven that Th17 cells are significantly more pathogenic than their Th1 counterparts<sup>[49]</sup>.

So far, there have been few studies that have employed selective blockade of IL-17 during intestinal inflammation. However, in IL-10<sup>-/-</sup> mice, treatment with anti-IL-17 specific antibody had little impact on colitis unless anti-IL-6 antibody was also co-administered<sup>[48]</sup>, suggesting that IL-17 may synergize with other inflammatory mediators in the gut. Recent studies have highlighted further potential heterogeneity within Th17 cell populations by demonstrating that some may even secrete IL-10<sup>[50]</sup>, a factor known to inhibit intestinal inflammation. Thus, it is possible that the actions of Th17 cells may differ dependently on other factors that may be present in the local environment. In the normal intestine, the primary function of Th17 cells may be like sentinels which contribute to maintaining epithelial barrier function, whereas in sites of chronic intestinal inflammation, high levels of IL-23 may activate their full pathogenic and anti-bacterial functions.

## CONCLUSION

Through the potential role of Th17 cells in IBD animal models of chronic intestinal inflammation as well as in human IBD, target therapy directed against the Th17/IL-17 axis may have a therapeutic role in the treatment of intestinal mucosal inflammation. However, the precise mechanisms of the Th17/IL-17 axis in intestinal homeostasis should be further elucidated in murine models and human IBD patients.

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## Role of the receptor for advanced glycation end products in hepatic fibrosis

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### Abstract

**AIM:** To study the role of advanced glycation end products (AGE) and their specific receptor (RAGE) in the pathogenesis of liver fibrogenesis.

**METHODS:** *In vitro* RAGE expression and extracellular matrix-related gene expression in both rat and human hepatic stellate cells (HSC) were measured after stimulation with the two RAGE ligands, advanced glycation end product-bovine serum albumin (AGE-BSA) and N<sup>ε</sup>-(carboxymethyl) lysine (CML)-BSA, or with tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). *In vivo* RAGE expression was examined in models of hepatic fibrosis induced by bile duct ligation or thioacetamide. The effects of AGE-BSA and CML-BSA on HSC proliferation, signal transduction and profibrogenic gene expression were studied *in vitro*.

**RESULTS:** In hepatic fibrosis, RAGE expression was enhanced in activated HSC, and also in endothelial cells, inflammatory cells and activated bile duct epithelia. HSC expressed RAGE which was upregulated after stimulation with AGE-BSA, CML-BSA, and TNF- $\alpha$ .

RAGE stimulation with AGE-BSA and CML-BSA did not alter HSC proliferation, apoptosis, fibrogenic signal transduction and fibrosis- or fibrolysis-related gene expression, except for marginal upregulation of procollagen  $\alpha 1(I)$  mRNA by AGE-BSA.

**CONCLUSION:** Despite upregulation of RAGE in activated HSC, RAGE stimulation by AGE does not alter their fibrogenic activation. Therefore, RAGE does not contribute directly to hepatic fibrogenesis.

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**Key words:** Advanced glycation end product; Extracellular matrix; Hepatic stellate cell; Matrix metalloproteinase; Myofibroblast; Receptor for advanced glycation end products; Transforming growth factor  $\beta$ ; Tissue inhibitor of metalloproteinase; Tumor necrosis factor  $\alpha$

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### INTRODUCTION

Advanced glycation end products (AGE) are formed *in vitro* and *in vivo* from non-enzymatic glycation of the amino groups of proteins with reducing sugars such as glucose. Although serum levels of AGE are usually low due to constant turnover, they can be detected *in vivo* once levels of reducing sugars are elevated, as occurs in diabetes<sup>[1]</sup>. Thus, glycated hemoglobin in the serum of diabetic patients was the first described physiologically relevant AGE<sup>[2]</sup>. Interest in AGE has increased since several studies suggested that AGE may be responsible for pathological features associated with diabetes. For example, in endothelial cells, AGE were shown to increase the expression of pro-coagulant activity,

induce expression of vascular cell adhesion molecule-1, and promote nuclear translocation of nuclear factor- $\kappa$ B (NF- $\kappa$ B). In mononuclear phagocytes, AGE induce the production of platelet-derived growth factor, increase migration, and drive NF- $\kappa$ B activation<sup>[3,4]</sup>.

AGE interact with several receptors, such as the receptor for advanced glycation end products (RAGE), 80K-H phosphoprotein, galectin-3, lactoferrin, scavenger receptors such as SRA or SRB I, and CD36<sup>[5,6]</sup>. RAGE, a member of the immunoglobulin superfamily of cell surface receptors, is expressed in a variety of tissues and interacts with several AGE ligands, especially with N<sup>ε</sup>-(carboxymethyl) lysine (CML)<sup>[4]</sup>.

However, while a role for RAGE in the progression of diabetic vasculopathy and kidney failure has been established<sup>[7-9]</sup>, its role in hepatic fibrosis is poorly understood. This is important due to the emerging epidemic of nonalcoholic steatohepatitis (NASH) related to obesity and the metabolic syndrome, conditions that are associated with increased AGE and RAGE and hepatic fibrosis<sup>[10-12]</sup>. RAGE expression has been described in inflammatory cells<sup>[13]</sup> and in activated hepatic stellate cells (HSCs)<sup>[14]</sup>, the major fibrogenic effector cells that can undergo activation to myofibroblasts producing the excess extracellular matrix in hepatic fibrosis<sup>[15]</sup>. Many features of this activation process are mimicked by spontaneously occurring activation on tissue culture plastic *in vitro*<sup>[16,17]</sup>. While certain cytokines, growth factors and culture conditions and, as recently demonstrated, AGE<sup>[18]</sup>, can modulate HSC activation and extracellular matrix (ECM) synthesis, the functional contribution of AGE and RAGE expression to fibrogenic activation of HSC and to hepatic fibrosis remains to be elucidated.

We have therefore studied whether physiological AGE concentrations occur in the serum of patients with diabetes<sup>[11,19]</sup>, and whether AGE-RAGE interactions are involved in the progression of liver fibrosis. To this end we investigated RAGE expression in HSC *in vitro*, and in normal and cirrhotic livers *in vivo*. Furthermore, the effects of AGE and the key proinflammatory cytokine tumor necrosis factor (TNF)- $\alpha$  on HSC RAGE expression and on the proliferation, kinase activation and profibrogenic and fibrolytic gene expression of HSC were determined.

## MATERIALS AND METHODS

### Synthesis of AGE and CML-modified BSA

For preparation of AGE-modified bovine serum albumin (BSA), 0.6 mmol/L BSA and 0.16 mmol/L D-glucose were dissolved in 20 mL PBS, sterile filtered, incubated for 60 d at 37°C and dialyzed against PBS under sterile conditions to remove unreacted D-glucose. Control BSA was incubated in parallel in the absence of D-glucose. Preparation of CML-modified BSA was carried out as previously described<sup>[20]</sup>.

Glycation of AGE-BSA and CML-BSA was determined using the 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay<sup>[21]</sup>, resulting in a 45.6% and 36.5% glycation of lysines for AGE-BSA and CML-BSA, respectively.

After endotoxin removal with the Detoxi-Gel™ (Pierce, Rockford, IL), the final endotoxin concentration determined with the E-toxate® endotoxin detection kit (Sigma, Taufkirchen, Germany) was below 0.04 and 0.02 ng/mL for AGE-BSA and CML-BSA, respectively.

### Cell lines

Cell lines were cultured as previously published<sup>[20]</sup>. The fully activated rat HSC line HSC-T6 (kind donation of Dr. SL Friedman, NY)<sup>[22]</sup>, the moderately activated rat HSC line CFSC-2G (kindly provided by Dr. M Rojkind, Washington, D.C.)<sup>[23]</sup> and human HSC of passage 3 to 5 (kind gift of Dr. M Pinzani, Florence, Italy)<sup>[24]</sup> were maintained as previously described (references see below).

**Cell culture and animal experimentation:** Animals were treated according to the Council of International Organizations of Medical Sciences for the Care and Use of Laboratory Animals in Research. The experimental protocol was approved by the Animal Care Committee of the University of Erlangen-Nuremberg.

**Isolation of rat primary hepatocytes:** Hepatocytes were freshly isolated from male Wistar rats (190-200 g, Charles River, Sulzfeld, Germany) according to a modified two-step collagenase perfusion method<sup>[25]</sup> as previously described in detail<sup>[26]</sup>. Experiments were performed 6 h after plating with cell viabilities  $\geq$  85% as determined by Trypan Blue exclusion.

**Isolation of rat primary hepatic stellate cells:** HSC were isolated from male Wistar rats (retired breeders, 400-500 g) as described<sup>[27]</sup>. Cell viability was usually between 95%-98%. The purity of HSC was confirmed by their stellate shape, and autofluorescence of the cytoplasmic lipid-droplets at 390 nm. Freshly isolated HSC were activated by culture on plastic in the presence of 10% fetal calf serum (FCS) for 1, 5, and 10 d prior to lysis and RNA extraction. HSC plated for 1 d were designated as quiescent, those cultured for 5 and 10 d as partially and fully activated, respectively.

**Experimental liver fibrosis:** Experimental liver fibrosis was induced in groups of four adult male Wistar rats weighing about 400 g as follows: (1) bile duct ligation (BDL) for 6 wk, (2) thioacetamide (TAA) treatment, 200 mg/g body weight thrice weekly for 12 wk, as previously published<sup>[27]</sup>. Sham-operated rats served as controls. After sacrifice of all animals, pieces of the right and left liver lobes were removed, fixed in 4% formalin and paraffin embedded, or snap frozen in liquid nitrogen for further analysis.

For RNA analysis 150-200 mg of tissue were homogenized in 1 mL RNAPure for 30 s and an aliquot representing 10 mg of tissue was mixed with 900  $\mu$ L fresh RNAPure. Morphology of connective tissue was evaluated with hematoxylin-eosin and Sirius Red staining.

**Immunohistochemistry:** For immunohistochemical analysis specimens from two different liver segments were

studied. Sequential deparaffinized sections were blocked with avidin and biotin. After antigen retrieval in a decloaking chamber for 30 s at 120°C in Target Retrieval Solution, pH 6.0 (Dako), sections were incubated overnight at room temperature with monoclonal antibodies to RAGE (1:30, kindly provided by Dr. B Weigle (Dresden, Germany), CD3 (1:10, Serotec MCA 772), CD45 (1:50, Serotec MCA 43R), CD68 (1:30, Serotec MCA 341R), and  $\alpha$ -smooth muscle actin (SMA) (1:30, DAKO M 0851), followed by biotinylated horse anti-mouse IgG and streptavidin-biotin alkaline phosphatase<sup>[29]</sup>. Sections were developed using Fast Red and nuclei counterstained with hematoxylin. RAGE was additionally detected with the catalyzed signal amplification system as previously described<sup>[29,20]</sup>. For double staining, the slides were treated with a Double Staining Enhancer (Zytomed 50-056) for 30 min before application of the secondary antibody. RAGE-antibody was first applied, followed by the other antibodies developed with Fast Red (RAGE) and with Fast Blue. The co-expressions of RAGE and of CD3, CD68 and of  $\alpha$ -SMA inside the liver (portal and lobular areas) were counted in ten randomly selected high-power fields (400  $\times$  magnification) using ImageAccess Enterprise 5 software (Imagic Bildverarbeitung, Glattbrugg, Switzerland). The number of immunohistochemically positive cells are given as the percentage of all cells (RAGE) and the respective cell population (CD3, CD68 and of  $\alpha$ -SMA) in the studied areas.

### SDS-PAGE and Western blotting

Preconfluent (80%) HSC lines, HSC-T6 and CFSC-2G, were seeded at 20 000 cells per well in 24-well plates, washed and incubated with 10-100  $\mu$ g/mL BSA, AGE-BSA, or CML-BSA, or with 0-10 ng/mL TNF- $\alpha$  (Sigma) in serum-free medium. Protein extraction and Western blotting were performed as previously described<sup>[20]</sup>.

### Quantitative real time PCR

Total RNA was isolated using peqGOLD RNApure reagent (PeqLab Biotechnologie, Erlangen, Germany) and reverse transcribed, followed by real time RT-PCR using a LightCycler instrument (Roche, Mannheim, Germany), as described in detail elsewhere<sup>[27,30,31]</sup>, and the TaqMan principle<sup>[32]</sup>. Results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or  $\beta$ 2-microglobulin amplified in a parallel reaction. The specific sense and antisense oligonucleotide primers and probes have been published<sup>[31,20]</sup>.

### Cell proliferation

Cell proliferation of CFSC-2G and HSC-T6 cells was determined using BrdU incorporation according to the manufacturer's protocol (Roche, Mannheim, Germany) as recently described<sup>[33]</sup>.

### Determination of p44/42 and p38 MAPK activity

These enzyme activities were determined as previously described<sup>[27]</sup> using Western Blotting with antibodies to phosphorylated and total ERK1/2 MAPK (Thr202/Tyr204, 1:2000) and anti-phospho-p38 MAPK (Thr180/Tyr182, 1:1000) (from Cell Signaling Technology,

Frankfurt, Germany). Phospho-specific signals were normalized to unphosphorylated kinase signals.

### Statistical analysis

Statistical analysis was performed with SPSS v. 16.0 (SPSS GmbH Software, Munich, Germany). Student's *t* test and univariate ANOVA (analysis of variance) was used to test for differences between two and more groups, respectively ( $P < 0.05$  was significant). All graphs represent the mean  $\pm$  SD and were performed at least in triplicate.

## RESULTS

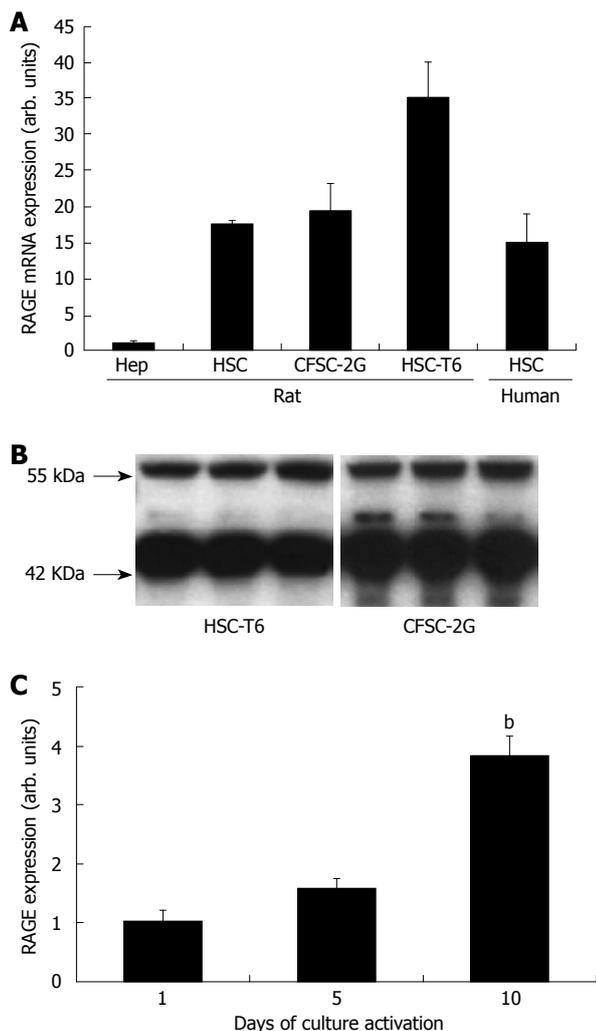
### Expression of RAGE by hepatic stellate cells

Freshly isolated rat HSC, and the rat HSC lines HSC-T6 and CFSC-2G, as well as human HSC, expressed significant RAGE transcripts and protein (Figure 1A and B). During culture, activation of freshly isolated rat HSC RAGE transcripts was upregulated 1.6- and 3.8-fold, respectively, on days 5 and 10 of primary culture (Figure 1C). Culture activation of HSC was associated with highly increased expression of procollagen- $\alpha$ 1(I) and  $\alpha$ -SMA mRNA which were upregulated  $> 100$ -fold and  $> 50$ -fold after 5 and 10 d of activation (data not shown).

### Upregulation of RAGE mRNA in rat models of cirrhosis

In cirrhotic livers of rats subjected to BDL, RAGE transcripts were upregulated 4-fold as compared to healthy controls ( $P < 0.01$ ), whereas in thioacetamide (TAA)-induced cirrhosis RAGE mRNA expression remained unchanged. Fibrosis-related transcripts such as  $\alpha$ -SMA, procollagen- $\alpha$ 1(I), matrix metalloproteinase (MMP)-13, and tissue inhibitor of metalloproteinase (TIMP)-1 mRNA were highly upregulated in both fibrosis models (Table 1).

Immunohistochemistry of normal livers showed a significantly lower expression of RAGE protein compared to the fibrotic/cirrhotic livers ( $P < 0.001$ , Table 2 and Figure 2A), with predominant expression in portal vein and arterial endothelial cells and sparse expression in lymphocytes and myofibroblasts in the hepatic lobule. Hepatocytes did not express RAGE. In BDL livers RAGE protein was highly expressed in bile duct proliferating epithelia, in periductular  $\alpha$ -SMA positive myofibroblasts and in inflammatory cells that were identified as CD3-positive T-lymphocytes and CD68-positive macrophages by use of double staining immunohistochemistry (Table 2, Figure 2A and B). In TAA cirrhosis the number of RAGE-expressing cells was less pronounced than in biliary cirrhosis, in concert with lower RAGE gene expression and fewer inflammatory cell infiltrates, being primarily expressed by macrophages, as opposed to mostly T-lymphocytes and proliferating bile duct epithelia in BDL-cirrhosis (Table 2 and Figure 2B). Only a few  $\alpha$ -SMA- and RAGE-positive myofibroblasts were detected in portal areas and septa of both models. The expression of RAGE by endothelial cells in the cirrhotic livers was slightly enhanced in areas of neo-capillarization compared to normal controls (Figure 2A).



**Figure 1** RAGE expression in hepatic stellate cells (A, B) and upregulation of RAGE expression during activation of HSC (C). A: Bars represent RAGE mRNA expression, as determined by real time quantitative PCR relative to GAPDH mRNA, by freshly isolated rat hepatocytes (Hep), 10 d culture-activated primary rat HSC, CFSC-2G and HSC-T6 HSC lines, and human HSC; B: RAGE protein is detected as a 57 kDa band after SDS-PAGE of cell lysates and Western blotting with a monoclonal anti-RAGE IgG. Experiments were repeated at least three times with similar results; C: Freshly isolated rat HSC were culture-activated for 1, 5, and 10 d and RAGE mRNA expression was quantified by real-time PCR. Bars represent mean RAGE expression  $\pm$  SD in arbitrary units relative to GAPDH from at least three individual experiments. Values were normalized to 50 ng of extracted RNA transcribed into cDNA. <sup>b</sup> $P < 0.01$  vs 1 d activation. RAGE: Receptor for advanced glycation end products; HSC: Hepatic stellate cells; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

### Regulation of RAGE expression in HSC: induction by AGE and TNF- $\alpha$

Incubation of CFSC-2G and HSC-T6 HSC with 50  $\mu$ g/mL AGE-BSA significantly ( $P < 0.001$ ) upregulated RAGE protein expression by 2-3 fold (Figure 3A). While CFSC-2G cells were more sensitive to AGE-BSA, the highest concentration, 100  $\mu$ g/mL, was not effective in either cell line. Addition of 50  $\mu$ g/mL CML-BSA increased RAGE protein expression about 2 fold in both cell lines ( $P < 0.05$ ) (Figure 3B).

Similarly, TNF- $\alpha$  upregulated RAGE protein expression significantly ( $P < 0.01$ ) in both CFSC-2G and HSC-T6 HSC (Figure 3C). Again, a greater RAGE induction was

**Table 1** Expression of RAGE and ECM-related genes in rats cirrhotic due to BDL and TAA treatment

	Control	BDL	TAA
RAGE	1.00 $\pm$ 0.31	3.89 $\pm$ 0.92 <sup>b</sup>	1.44 $\pm$ 0.47
$\alpha$ -SMA	1.00 $\pm$ 0.49	5.60 $\pm$ 1.59 <sup>a</sup>	10.39 $\pm$ 6.78
Procollagen- $\alpha$ 1(I)	1.00 $\pm$ 0.99	26.45 $\pm$ 3.37 <sup>d</sup>	21.92 $\pm$ 8.48
MMP-13	1.00 $\pm$ 0.38	3.68 $\pm$ 2.50	52.88 $\pm$ 25.34 <sup>a</sup>
TIMP-1	1.00 $\pm$ 0.46	6.50 $\pm$ 1.55 <sup>b</sup>	25.47 $\pm$ 15.02

RAGE,  $\alpha$ -SMA, procollagen- $\alpha$ 1(I), MMP-13, and TIMP-1 transcript levels relative to  $\beta$ 2-microglobulin  $\pm$  SD of four animals per group. Data are expressed as n-fold increase compared to sham-operated and normal rats, respectively. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>d</sup> $P < 0.001$  vs normal controls. RAGE: Receptor for advanced glycation end products; ECM: Extracellular matrix; BDL: Bile duct ligation; TAA: Thioacetamide; SMA: Smooth muscle actin; MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinase.

**Table 2** Cell-specific expression of RAGE as determined by immunohistochemistry

	RAGE	CD3	CD68	$\alpha$ -SMA
Normal liver				
P	2.8 $\pm$ 0.7 <sup>1</sup>	3.3 $\pm$ 0.7	0.3 $\pm$ 0.5	0.8 $\pm$ 0.7 <sup>2</sup>
L	1.0 $\pm$ 0.7	1.5 $\pm$ 0.6	0.7 $\pm$ 0.6	0.2 $\pm$ 0.4
BDL				
P	11.1 $\pm$ 1.5 <sup>3</sup>	13.8 $\pm$ 1.2	15.2 $\pm$ 1.7	8.1 $\pm$ 1.2 <sup>4</sup>
L	8.1 $\pm$ 1.7	13.2 $\pm$ 1.9	13.8 $\pm$ 1.6	4.8 $\pm$ 1.0
TAA				
P	7.3 $\pm$ 1.9	8.4 $\pm$ 1.1	8.5 $\pm$ 1.1	6.8 $\pm$ 1.2 <sup>5</sup>
L	5.7 $\pm$ 1.0	6.6 $\pm$ 1.1	6.0 $\pm$ 1.1	1.9 $\pm$ 0.7

Quantitative analysis of the immunohistochemical co-expression of RAGE with CD3, CD68 and  $\alpha$ -SMA in normal controls and livers with cirrhosis due to BDL or TAA-intoxication using double staining immunohistochemistry and image analysis. Mean of cells [%] ( $\pm$  SD) per 10 high power fields. Quantitative assessment as described in material and methods. <sup>1</sup>Mainly endothelial cells in the portal areas; <sup>2</sup>Vascular smooth muscle cells, subset of activated HSC; <sup>3</sup>Focally highly expressed by proliferating bile duct epithelia; <sup>4</sup>Mainly around proliferating bile ducts; <sup>5</sup>Mainly at the portal interface and septa. P: Portal; L: Lobular.

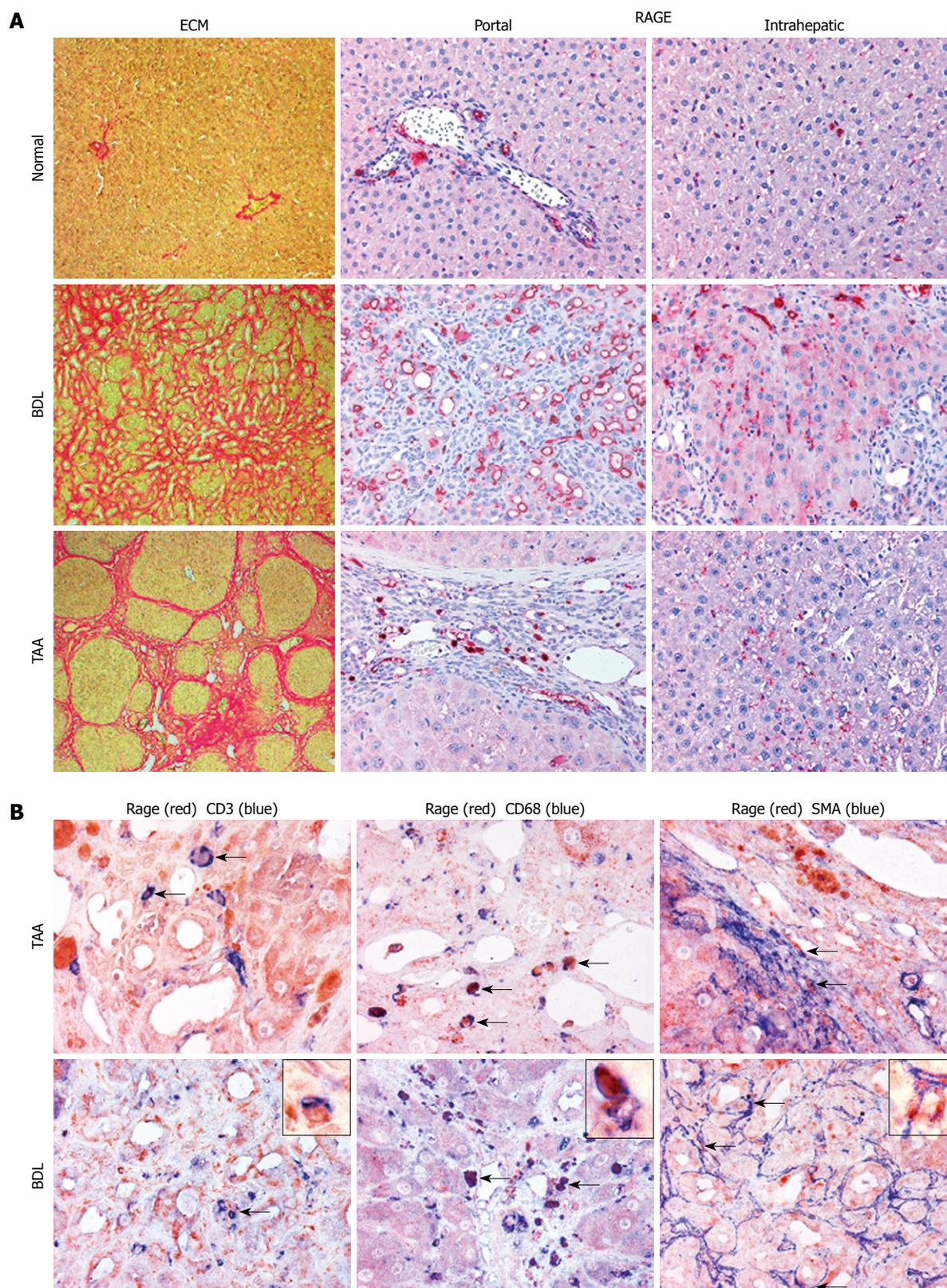
observed in CFSC-2G cells, where 0.1 ng/mL TNF- $\alpha$  resulted in a nearly 3-fold ( $P < 0.001$ ) upregulation of RAGE expression.

### AGE do not modulate extracellular matrix-related gene expression in HSC

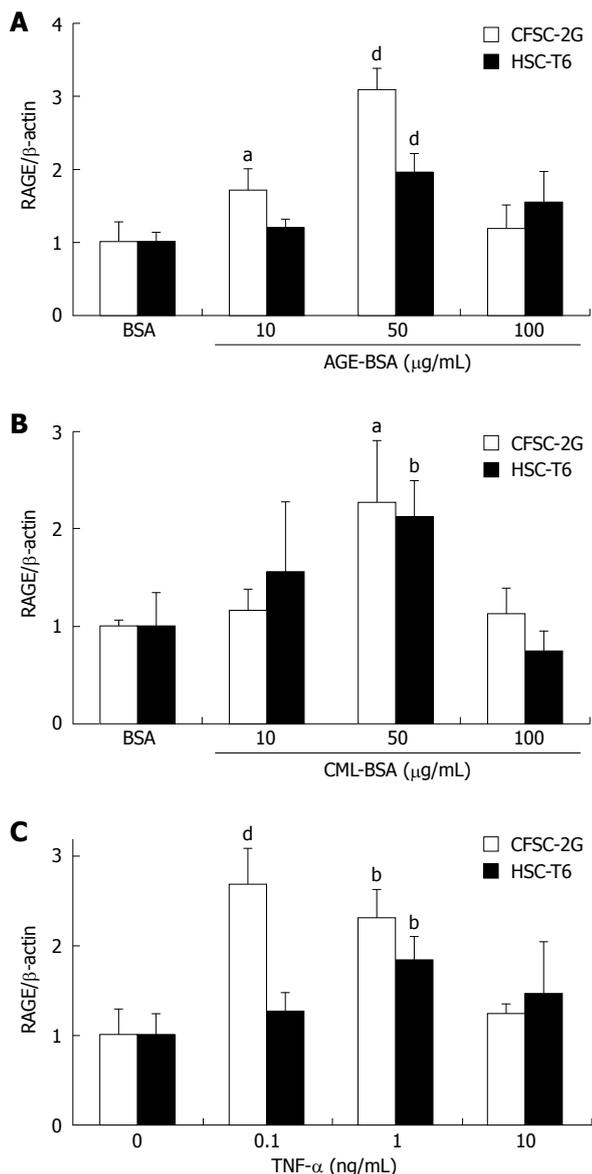
Incubation of CFSC-2G HSC with 50  $\mu$ g/mL AGE-BSA or CML-BSA did not significantly modify transcript levels of transforming growth factor (TGF)- $\beta$ 1,  $\alpha$ -SMA, and MMP-13, or RAGE itself, except for a marginal (23%,  $P < 0.05$ ) but reproducible upregulation of procollagen- $\alpha$ 1(I) mRNA by AGE-BSA (Table 3). These results could be confirmed in HSC-T6 and human HSC (data not shown).

### AGE do not modify hepatic stellate cell proliferation or p42/44 and p38 MAPK activation

CFSC-2G and HSC-T6 cells were exposed to 1-1000  $\mu$ g/mL AGE-BSA for 24 h, and DNA synthesis was assessed by BrdU incorporation. As opposed to the mitogen FCS, AGE-BSA did not induce DNA synthesis



**Figure 2** Cell-specific expression patterns of RAGE in normal and cirrhotic rat livers. **A:** Portal and lobular expression patterns of RAGE in normal liver and in livers of rats with cirrhosis due to BDL or TAA treatment (magnification,  $\times 200$ ). Parallel sections were stained for collagen with picrosirius red. In normal liver, minor RAGE expression was found in endothelial cells of portal veins and arteries, in bile duct epithelia as well as in lymphocytes, macrophages and (myo-) fibroblasts in the hepatic lobular areas. No RAGE expression was found in hepatocytes. RAGE was clearly upregulated in cirrhosis induced by BDL and TAA, with higher numbers of RAGE-expressing cells in BDL, mainly attributable to a prominent contribution by proliferating bile duct epithelial cells. In areas of lobular fibrosis, perisinusoidal endothelia started to express RAGE, apparently in parallel with sinusoidal capillarization; **B:** Double labeling immunohistochemistry for RAGE (in red) in combination with CD3 (T-lymphocytes), CD68 (macrophages), and  $\alpha$ -SMA (myofibroblasts) (in blue), magnification  $\times 400$  being exemplarily highlighted with insets (magnification,  $\times 1000$ ). In both cirrhosis models, mainly CD3-positive T-lymphocytes and CD68-positive macrophages expressed RAGE, independently of their microanatomic locations (portal, interface, septal or intrahepatic). Additionally, RAGE was more colocalized with macrophages than with T-lymphocytes independent of the cirrhosis model. RAGE expression was also found on  $\alpha$ -SMA-positive myofibroblasts, either localized around periportal bile duct proliferations in BDL or in the interface and septal area in TAA-induced cirrhosis (co-localization indicated by arrows). BDL: Bile duct ligation; TAA: Thioacetamide; SMA: Smooth muscle actin; ECM: Extracellular matrix.



**Figure 3** Advanced glycation end product-bovine serum albumin (AGE-BSA) (A), N<sup>ε</sup>-(carboxymethyl) lysine (CML)-BSA (B) and tumor necrosis factor (TNF)-α (C) upregulate RAGE protein expression in hepatic stellate cells. Mean RAGE protein expression as determined by quantitative Western blotting from extracts of rat HSC lines relative to β-actin. Cells were incubated for 24 h and results are derived from at least three independent experiments and expressed as mean ± SD. Data are shown as x-fold increase compared to cells incubated with BSA alone. <sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01, <sup>d</sup>P < 0.001 vs BSA.

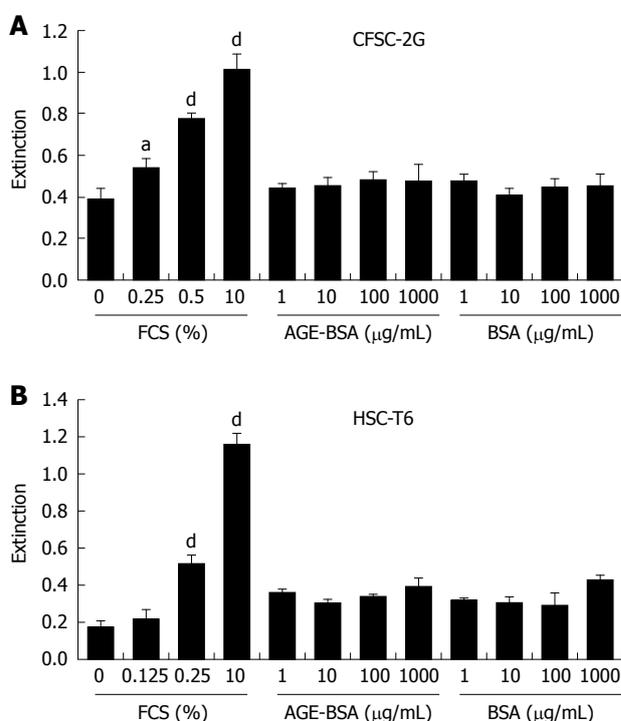
in these cells (Figure 4). In addition, cell numbers remained unchanged after addition of AGE-BSA (data not shown). In line with the proliferation data, AGE-BSA did not induce p44/42 (Figure 5A) or p38 (Figure 5B) MAPK activation when compared to 10% FCS as positive control.

## DISCUSSION

Our findings show that RAGE, a prominent receptor for AGE, is expressed in hepatic stellate cells (HSC) derived from various species. Our data are in line with previous studies showing the RAGE upregulation in single culture-activated HSC either of rat or human origin<sup>[14,18]</sup>.

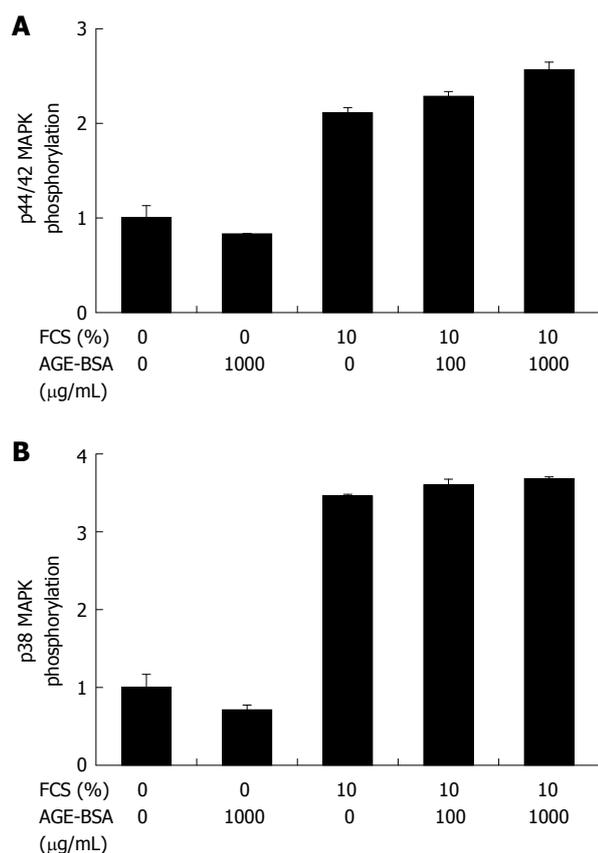
	BSA	AGE-BSA	CML-BSA
RAGE	1.00 ± 0.14	1.20 ± 0.11	1.26 ± 0.07
TGF-β1	1.00 ± 0.06	1.12 ± 0.21	0.99 ± 0.07
Procollagen-α1(I)	1.00 ± 0.08	1.23 ± 0.06 <sup>a</sup>	1.12 ± 0.19
α-SMA	1.00 ± 0.14	1.20 ± 0.20	1.16 ± 0.04
MMP-13	1.00 ± 0.05	1.09 ± 0.14	0.97 ± 0.09

CFSC-2G HSC were incubated with 50 µg/mL BSA, AGE-BSA, or CML-BSA for 24 h. RAGE, TGF-β1, procollagen-α1(I), α-SMA, and MMP-13 transcript levels are expressed relative to GAPDH and as n-fold increase compared to cells treated with BSA alone (means ± SD of at least three individual experiments). <sup>a</sup>P < 0.05 vs BSA. ECM: Extracellular matrix; HSC: Hepatic stellate cells; AGE: Advanced glycation end product; BSA: Bovine serum albumin; CML: N<sup>ε</sup>-(carboxymethyl) lysine; TGF: Transforming growth factor.



**Figure 4** AGE do not stimulate DNA synthesis in hepatic stellate cells. DNA synthesis as a surrogate of proliferation was determined in CFSC-2G (A) and HSC-T6 (B) HSC after a 24 h incubation with increasing concentrations of FCS, AGE-BSA or CML-BSA in 0% FCS. Data are results of six independent experiments and expressed as mean ± SD. <sup>a</sup>P < 0.05, <sup>d</sup>P < 0.001 vs 0% FCS.

Using different experimental hepatic fibrosis models in rats we found that transcript levels of RAGE correlate well with RAGE protein expression in agreement with our immunohistochemical co-localization studies. Compared with earlier studies of RAGE expression in other organs<sup>[4]</sup>, we could detect RAGE not only in myofibroblasts (HSC) and endothelial cells, but also in lymphocytes, macrophages/small Kupffer cells and proliferating bile duct epithelial cells. These results are in contrast to prior studies that either identified RAGE expression in bovine hepatocytes *in vivo*<sup>[34]</sup>, or exclusively in HSC and myofibroblasts, but not in hepatocytes, sinusoidal endothelial or Kupffer cells<sup>[14]</sup>. The reasons for these discrepancies may be the use of different



**Figure 5** AGE-BSA does not activate p44/42 (A) and p38 (B) MAPK signaling in hepatic stellate cells. Kinase phosphorylation was determined in CFSC-2G and HSC-T6 HSC using quantitative western blotting with phosphospecific antibodies relative to total kinase antibodies after a 10 min incubation with increasing concentrations of AGE-BSA or CML-BSA in 0% to 10% FCS. Bars represent n-fold phosphorylation compared to controls with 0% FCS and results are derived from at least three individual experiments.

antibody reagents with different specificities. We used an antibody that has been characterized thoroughly and found to react specifically with cells on tissue sections<sup>[28]</sup>. In addition, we could partly confirm our *in vivo* data with our *in vitro* studies using various types of HSC.

During culture-activation of HSC, RAGE expression was increased significantly. This increase was paralleled by the known upregulation of major transcripts related to fibrosis progression, i.e. TGF- $\beta$ 1, the most prominent profibrogenic cytokine; procollagen  $\alpha$ 1(I), a precursor of the major fibrillar collagen; TIMP-1, the central inhibitor of matrix metalloproteinases (MMP); and  $\alpha$ -SMA, a marker for HSC activation<sup>[16,17]</sup>.

Enhanced RAGE expression in hepatic fibrogenesis was further shown in rats with cirrhosis induced by BDL and TAA treatment, which was in line with prior findings in CCl<sub>4</sub>-induced hepatic fibrosis where RAGE transcript and protein levels were upregulated until 6 wk after the completion of CCl<sub>4</sub> treatment<sup>[55]</sup>. Differences in RAGE expression may be due to the lack of inflammation in TAA-treated animals, since TAA treatment was stopped one week prior to tissue removal. Progressive injury, however, was still present in bile duct-ligated animals at the time of sacrifice, with enhanced numbers of CD68-positive macrophages/small Kupffer cells and CD3-

positive T-lymphocytes as compared to the TAA-cirrhosis model. Furthermore, we showed that  $\alpha$ -SMA-positive HSC/myofibroblasts of the septal or portal interface, representing the prominent fibrogenic effectors, expressed RAGE in both fibrosis models.

In studies of diabetes-associated cardiovascular and renal disease, an upregulation of RAGE has been linked to enhanced levels of AGE<sup>[7,9]</sup>, in association with epithelial-myofibroblast transdifferentiation<sup>[36]</sup> and induction of fibrogenesis<sup>[37-39]</sup>. Additionally, inhibition of the interaction of AGE-RAGE with neutralizing monoclonal anti-RAGE antibodies or the AGE cross-link disrupting agent ALT-711 prevented pathological effects of hyperglycemia in blood vessels and kidneys<sup>[8,40]</sup>.

Our results show that AGE-BSA, as well as the specific RAGE ligand CML-BSA, upregulated the expression of the receptor itself in HSC. This phenomenon was reported previously in human vascular and umbilical vein endothelial cells<sup>[41]</sup>, where 50  $\mu$ g/mL AGE-BSA induced a 2- and 2.5-fold increased RAGE protein expression, respectively, compared to untreated cells. Other studies showed that certain vascular domains, renal glomeruli or intima media and adventitia of the aorta exhibit increased RAGE expression<sup>[42]</sup> and that interactions of AGE with RAGE resulted in autoinduction of RAGE expression<sup>[43]</sup>. Our observed reduction of response using higher concentrations of AGE-BSA could reflect receptor saturation, downregulation, or an antagonist effect on RAGE expression.

Addition of the proinflammatory cytokine TNF- $\alpha$  to HSC upregulated RAGE protein and mRNA expression up to 3-fold, in accordance with previous studies showing that RAGE is upregulated in inflamed tissues<sup>[44]</sup>. Interestingly, the blockade of RAGE by murine-soluble RAGE as decoy could decrease acetaminophen-induced hepatotoxicity<sup>[45]</sup> and liver injury in an ischemia and reperfusion model in mice<sup>[46]</sup>. Furthermore, an approximately 2-fold increase of RAGE protein expression after incubation of human microvascular endothelial cells with up to 100 ng/mL TNF- $\alpha$  has been reported<sup>[41]</sup>. In the present study, however, RAGE expression peaked at 0.1 and 1 ng/mL TNF- $\alpha$  and decreased to baseline levels at 10 ng/mL TNF- $\alpha$ , apparently due to receptor downregulation at high concentrations and this indicated a greater sensitivity of HSC than that of human microvascular endothelial cells to TNF- $\alpha$ . Increased RAGE expression under inflammatory conditions, such as those triggered by TNF- $\alpha$ , may result in enhanced binding of AGEs to RAGE, further increasing RAGE expression and expression of proinflammatory cytokines. This could be relevant for patients with insulin resistance, overt diabetes, and obesity, who frequently present with hepatic inflammation and fibrosis, i.e. patients with NASH.

The upregulated RAGE expression in HSC may lead to the conclusion that AGE-RAGE interactions play a role in hepatic fibrogenesis. However, in contrast to upregulation of RAGE by AGE/CML-AGE and TNF- $\alpha$ , we clearly showed that AGE-BSA and CML-BSA were unable to induce expression of fibrosis or

fibrolysis-related genes in CFSC-2G or HSC-T6 HSC. Using another experimental approach Xia *et al*<sup>[35]</sup> showed that targeting of RAGE by specific siRNA downregulated fibrogenesis-related transcripts *in vitro* and *in vivo*. Of note, we took great care to synthesize AGE-BSA and CML-BSA in a sterile environment and to remove any remaining endotoxin contamination in the products. Possible discrepancies between previously published cellular effects of AGE and the present lack of induction may be explained by the presence of endotoxin in previously synthesized AGE preparations.

Another pathogenic feature of HSC activation, besides migration, apoptosis, ECM synthesis, or contractility, is increased proliferation<sup>[47]</sup>. According to our experimental setup, we can conclude that AGE do not alter hepatic fibrogenesis through induction of HSC proliferation.

Previous studies of endothelial cells and monocytes, especially mononuclear phagocyte-derived dendritic cells of the liver after massive liver injury<sup>[13]</sup>, showed that interactions of AGE with RAGE induce, besides RAGE, the expression of proinflammatory cytokines, such as TNF- $\alpha$  and interleukin-1 and -6<sup>[41]</sup>. These events are mediated by activation of redox-sensitive signaling pathways involving NADPH oxidase, or mitogenic pathways involving the small G-protein Ras that lead to activation of mitogen-activated protein kinases (MAPK), or involving nuclear factor- $\kappa$ B (NF- $\kappa$ B). Since no data on AGE-induced MAPK activation in HSCs exist, we aimed to investigate whether RAGE upregulation by AGE may be due to stimulation of MAPK signal transduction pathways. We could not find any stimulatory effect of endotoxin-free AGE-BSA or CML-BSA on activation of p44/42 MAPK, which mediates cellular growth and differentiation, and p38 MAPK, which regulates cytokine expression and controls cellular responses to cytokines and stress. Again, this contrasts with previous reports showing that interaction of AGE with RAGE induced intracellular signaling pathways involved in inflammatory responses including MAPK or NF- $\kappa$ B activation<sup>[48]</sup>. Moreover, downregulation of RAGE by specific siRNAs was associated with NF- $\kappa$ B degradation supporting the linkage of RAGE to the NF- $\kappa$ B pathway<sup>[35]</sup>. Only a single study indicated that AGE may not uniformly play a role in cellular activation<sup>[49]</sup>.

In summary, the present data do not support a direct role of AGE and AGE-RAGE axis in the fibrogenic activation of HSC, such as profibrogenic ECM-related gene expression, signal transduction, or proliferation. However, the finding that RAGE in HSC is upregulated during their activation *in vitro* and in HSC/myofibroblasts, macrophages/small Kupffer cells, endothelia of neo-capillarization and proliferating bile duct epithelia during fibrogenesis *in vivo* does not exclude the possibility that RAGE may drive fibrogenesis indirectly, e.g. *via* soluble factors that are released from these non-HSC cell types. This could still have relevance for patients with insulin resistance and NASH who display elevated serum and tissue levels of AGE<sup>[1,50]</sup>. Whether the observed RAGE upregulation may contribute to fibrosis *via* these indirect pathways needs to be investigated in further studies using

either co-culture experiments, gene modified animals, or the administration of AGEs to animals with experimental liver fibrosis.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Advanced glycation end products (AGE) and their specific receptor (RAGE) play an important role in the pathogenesis of inflammation and fibrosis in diabetes mellitus. While RAGE has been detected in numerous tissues, its role in organs such as the liver which are also exposed to (circulating) AGE remains largely unexplored.

### Research frontiers

The study of liver fibrosis is a challenging research area due to the enormous socio-epidemiologic and medical-therapeutical impact of chronic liver diseases which frequently progress to cirrhosis. We have made tremendous progress in our understanding of the pathomechanisms underlying liver fibrosis progression, including the structural components of the hepatic scar tissue (extracellular matrix), the molecules that are central to its excess deposition and to its removal, and the direct and indirect effector cells that drive fibrosis progression, i.e. fibrogenesis (Friedman, Gastro 08; Schuppan and Afdhal, Lancet 08). Recent studies suggested an association of an activated AGE-RAGE-axis with fibrogenesis, but clear functional data were lacking.

### Innovations and breakthroughs

The findings suggest a more indirect than direct effect of the AGE-RAGE-axis on liver fibrogenesis and rule out a direct effect of AGE on the fibrogenic effector cells, i.e. hepatic stellate cells.

### Applications

Future applications depend on additional studies that would explore the putative indirect effects of RAGE on fibrogenesis, e.g. *via* cytokines produced by AGE-activated macrophages, biliary duct epithelia or endothelial cells. This could facilitate the development of specific AGE-RAGE-inhibitors that could generate a novel class of therapeutics, especially in conditions where AGE play a prominent role, such as non-alcoholic fatty liver disease.

### Terminology

AGE are formed *in vitro* and *in vivo* from non-enzymatic glycation of the amino groups of proteins with reducing sugars such as glucose. AGE can be detected *in vivo* once levels of reducing sugars are elevated as occurs in diabetes. AGE interact with several receptors, most specifically with the receptor for advanced glycation end products (RAGE). RAGE is a member of the immunoglobulin superfamily of cell surface receptors which are expressed in a variety of tissues.

### Peer review

The originality of this study resides in the broad and exhaustive study of RAGE expression in two well-defined and complementary rodent models of liver fibrosis, the use of different cells and cell lines, and extensive *in vitro* stimulation studies of hepatic stellate cells with AGE to assess their effect on the expression of extracellular matrix component and matrix dissolving metalloproteinases. This broad approach is novel and has for the first time provided clear results as to the effect of stimulation of the AGE-RAGE axis in hepatic stellate cells and the putative fibrogenic role it plays in fibrogenic activation of macrophages/Kupffer cells, endothelia or biliary duct epithelia.

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## Identification of TRPM7 channels in human intestinal interstitial cells of Cajal

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### Abstract

**AIM:** To investigate the characteristics of slow electrical waves and the presence of transient receptor potential melastatin-type 7 (TRPM7) in the human gastrointestinal (GI) tract.

**METHODS:** Conventional microelectrode techniques were used to record intracellular electrical responses from human GI smooth muscle tissue. Immunohistochemistry was used to identify TRPM7 channels in interstitial cells of Cajal (ICCs).

**RESULTS:** The human GI tract generated slow electrical waves and had ICCs which functioned as pacemaker cells. Flufenamic acid, a nonselective cation channel blocker, and 2-APB (2-aminoethoxydiphenyl borate) and  $La^{3+}$ , TRPM7 channel blockers, inhibited the slow

waves. Also, TRPM7 channels were expressed in ICCs in human tissue.

**CONCLUSION:** These results suggest that the human GI tract generates slow waves and that TRPM7 channels expressed in the ICCs may be involved in the generation of the slow waves.

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**Key words:** Electrophysiology; Interstitial cells of Cajal; TRPM cation channels; Transient receptor potential melastatin-type 7 protein; Human; Gastrointestinal tract

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### INTRODUCTION

Smooth muscles in the gastrointestinal (GI) tract are spontaneously active with rhythmic generation of slow electrical waves<sup>[1]</sup>. The slow waves determine the frequency and amplitude of the phasic contractions of GI muscles<sup>[2]</sup>. Slow waves originate in specialized regions such as the border between the circular and longitudinal muscle layers in the stomach and small intestine and along the submucosal surface of the circular muscle layer in the colon<sup>[3]</sup>. Each pacemaker region is populated by interstitial cells of Cajal (ICCs), and many studies have demonstrated the pacemaker role of these cells<sup>[4-9]</sup>. ICCs generate spontaneous inward currents and slow wave-like depolarizations<sup>[10,11]</sup>. Slow waves propagate within the ICC networks, conduct into smooth muscle cells *via* gap junctions, and initiate phasic contractions by activating  $Ca^{2+}$  entry through L-type  $Ca^{2+}$  channels. The pacemaker currents of the murine small intestine are due mainly to periodic activation of non-selective cation channels

(NSCCs)<sup>[12]</sup>.

In *Caenorhabditis elegans*, the melastatin-type transient receptor potential (TRPM) channel, particularly TRPM7, was suggested as being involved in the defecation process<sup>[13]</sup>. Also recently, we suggested that TRPM7 was a good candidate for the NSCC in ICCs of the murine small intestine<sup>[14]</sup>. TRP channels were first cloned from *Drosophila* species and constitute a superfamily of proteins that encode a diverse group of Ca<sup>2+</sup>-permeable NSCCs<sup>[15]</sup>. The TRP family is divided into 7 subfamilies: classical TRPs (TRPC), which display the greatest similarity to *Drosophila* TRP; vanilloid TRPs (TRPVs); TRPMs; mucolipin TRPs; polycystin TRPs; NOMPC (no mechanoreceptor potential C) TRP; ankyrin 1 TRPs. TRPC channels mediate cation entry in response to phospholipase C activation, whereas TRPV proteins respond to physical and chemical stimuli, such as changes in temperature, pH, and mechanical stress. The TRPM family members differ significantly from other TRP channels in terms of domain structure, cation selectivity, and activation mechanisms<sup>[15]</sup>.

The characteristics of the slow wave and the presence of TRPM7 in human GI tract have not yet been investigated. Therefore, we undertook to investigate the characteristics of the slow wave in the human GI tract and the involvement of TRPM7 in ICCs.

## MATERIALS AND METHODS

### Human tissue preparation

The segments of human colon or small intestine used in this study were obtained from cancer patients of either sex ranging in age from 46 to 59 years as discarded surgical tissue during operations. The protocol was approved by the human subjects research committees at Seoul National University College of Medicine. A segment of colon or jejunum was opened along the mesenteric border, and the mucosal layers, the serosal layers and a part of the longitudinal layers were carefully peeled away under a dissecting microscope. A tissue segment (about 0.5 mm wide and 0.5 mm long) was pinned out on a silicone rubber plate with the serosal side uppermost, and the plate was fixed at the bottom of an organ bath. The preparation was continuously perfused with CO<sub>2</sub>/bicarbonate-buffered Tyrode solution at 36-37°C and equilibrated for 2 h before the experiment, at a constant flow rate of about 2 mL/min.

### Intracellular recording of electrical activity

Conventional microelectrode techniques were used to record intracellular electrical responses from smooth muscle tissues, and the glass capillary microelectrodes, filled with 3 mol/L KCl, had tip resistances of 40-80 MΩ. Electrical responses recorded *via* a high input impedance amplifier (Axoclamp-2B, Axon Instruments, USA) were displayed on a cathode ray oscilloscope (SS-7602, Iwatsu, Osaka, Japan) and also stored on a personal computer for later analysis.

### Solutions and drugs

The ionic composition of the CO<sub>2</sub>/bicarbonate buffered-

Tyrode solution was as follows (mmol/L): NaCl 116, KCl 5.4, CaCl<sub>2</sub> 1.5, MgCl<sub>2</sub> 1, NaHCO<sub>3</sub> 24, glucose 5. The solutions were aerated with O<sub>2</sub> containing 5% CO<sub>2</sub>, and the pH of the solutions was maintained at 7.3-7.4. Drugs used were flufenamic acid, 2-aminoethoxydiphenyl borate (2-APB), lanthanum ion (La<sup>3+</sup>) and nifedipine (all from Sigma, USA). Drugs were dissolved in distilled water, and added to CO<sub>2</sub>/bicarbonate buffered-Tyrode solution to the desired concentrations, immediately prior to use. Addition of these chemicals to the Krebs solution did not alter the pH of the solution.

### Immunohistochemistry

Whole-mount preparations from the colon or small intestine of human were used for immunohistochemistry. Experimental protocols approved by Seoul National University were followed. For whole-mount preparations, the mucosa was removed by sharp dissection, and the remaining muscle layer was stretched before fixation. Whole-mount preparations were fixed in cold acetone (4°C) for 5 min. After fixation, they were washed in phosphate-buffered saline (PBS; 0.01 mol/L; pH 7.4) and immersed in 0.3% Triton X-100 in PBS. After blocking with 1% bovine serum albumin (Sigma) in 0.01 mol/L PBS for 1 h at room temperature, they were incubated with a rat monoclonal antibody raised against c-kit (Ack2; eBioscience) at 0.5 μg/mL or goat polyclonal antibody against TRPM7 (Abcam, Cambridgeshire, UK) in PBS for 24 h (4°C). After a rinse in PBS at 4°C, they were labeled with the fluorescein isothiocyanate-coupled donkey anti-goat immunoglobulin G secondary antibody (1:100; Jackson ImmunoResearch Laboratories, Baltimore, MD, USA) or Texas red-conjugated donkey anti-rat immunoglobulin G (1:100; Jackson ImmunoResearch Laboratories) for 1 h at room temperature. Control tissues and sections were prepared by omitting either the primary or secondary antibodies from the incubation solutions.

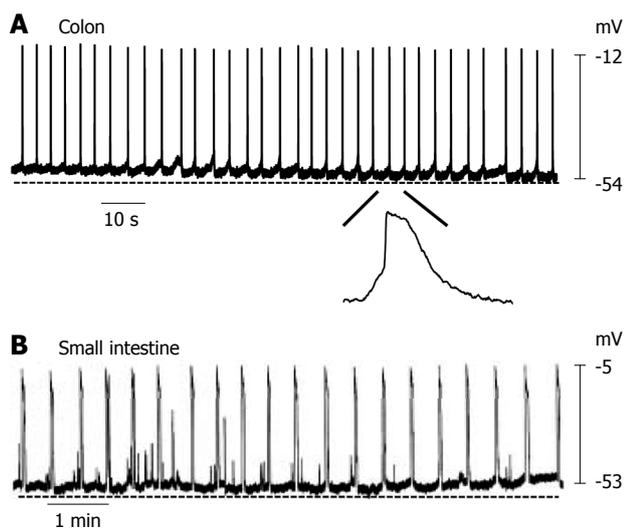
### Statistics analysis

All data are expressed as mean ± SE. The Student *t*-test for unpaired data was used to compare control and experimental groups. A *P*-value < 0.05 was considered statistically significant.

## RESULTS

### Spontaneous electrical activities recorded from intact tissue preparations of human colon and small intestine

In intact human colon preparations, most of the tissues generated slow electrical waves (Figure 1A). The membrane potential of cells generating slow waves with the most negative value (equal to the resting membrane potential) ranged between -54.7 mV and -68.7 mV (mean -61.9 ± 1.9 mV, *n* = 6; each *n* value represents the number of human tissues used). Slow waves had a frequency of 18.1 ± 2.1/min (*n* = 6). In intact human small intestine preparations, most of the tissues generated slow waves (Figure 1B). The membrane potential of cells generating slow waves ranged between -51.2 mV and -63.5 mV (mean -57.3 ± 5.2 mV, *n* = 5). Slow waves had a frequency of 3.1



**Figure 1** Spontaneous electrical activity of smooth muscle cells in human colon and small intestine. A: Colonic smooth muscle cells produced slow waves with a frequency of  $18.1 \pm 2.1/\text{min}$ ; B: Small intestinal smooth muscle cells also produced slow waves with a frequency of  $3.1 \pm 0.5/\text{min}$ .

$\pm 0.5/\text{min}$  ( $n = 5$ ). These results suggest that the GI tract in humans can generate slow waves and have ICCs which function as pacemaker cells.

### Pharmacological properties of slow waves in human colon

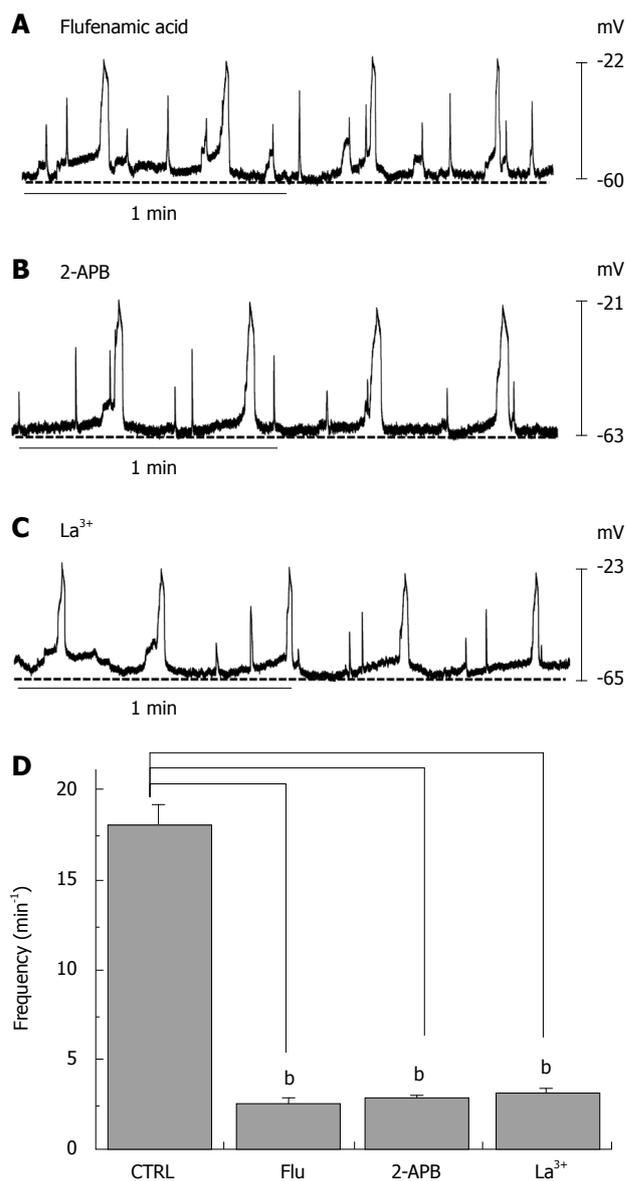
We investigated the effect of a nonselective cation channel blocker and several TRPM7 channel blockers on the slow waves recorded from human colon tissue. First, we investigated the effects of flufenamic acid, a nonselective cation channel blocker. When flufenamic acid was applied in the bath solution, the slow waves were inhibited ( $2.6 \pm 0.3/\text{min}$ ) (Figure 2A and D). As indicated previously, 2-APB is known to inhibit TRPM7 channels<sup>[16]</sup>. When 2-APB was applied in the bath solution, the slow waves were inhibited in a concentration-dependent manner ( $2.8 \pm 0.2/\text{min}$ ) (Figure 2B and D). Again, as described previously, TRPM7 has been shown to be blocked by trivalent ions such as  $\text{La}^{3+}$ <sup>[17]</sup>. When  $\text{La}^{3+}$  was applied in the bath solution, the slow waves were inhibited ( $3.2 \pm 0.2/\text{min}$ ) (Figure 2C and D). These results indicate that NSCCs and TRPM7 in ICCs may be involved in the generation of the slow wave in the human GI tract.

### Expression of TRPM7 protein in ICC of native tissues

To test this possibility more directly, we examined the expression of TRPM7 in ICCs in human tissues. Expression of TRPM7 proteins was investigated by immunohistochemistry. Double staining with anti-c-kit (a marker of ICCs) and anti-TRPM7 antibodies showed TRPM7 immunoreactivity in c-kit-immunopositive ICCs in the human colon (Figure 3), and human small intestine (Figure 4).

## DISCUSSION

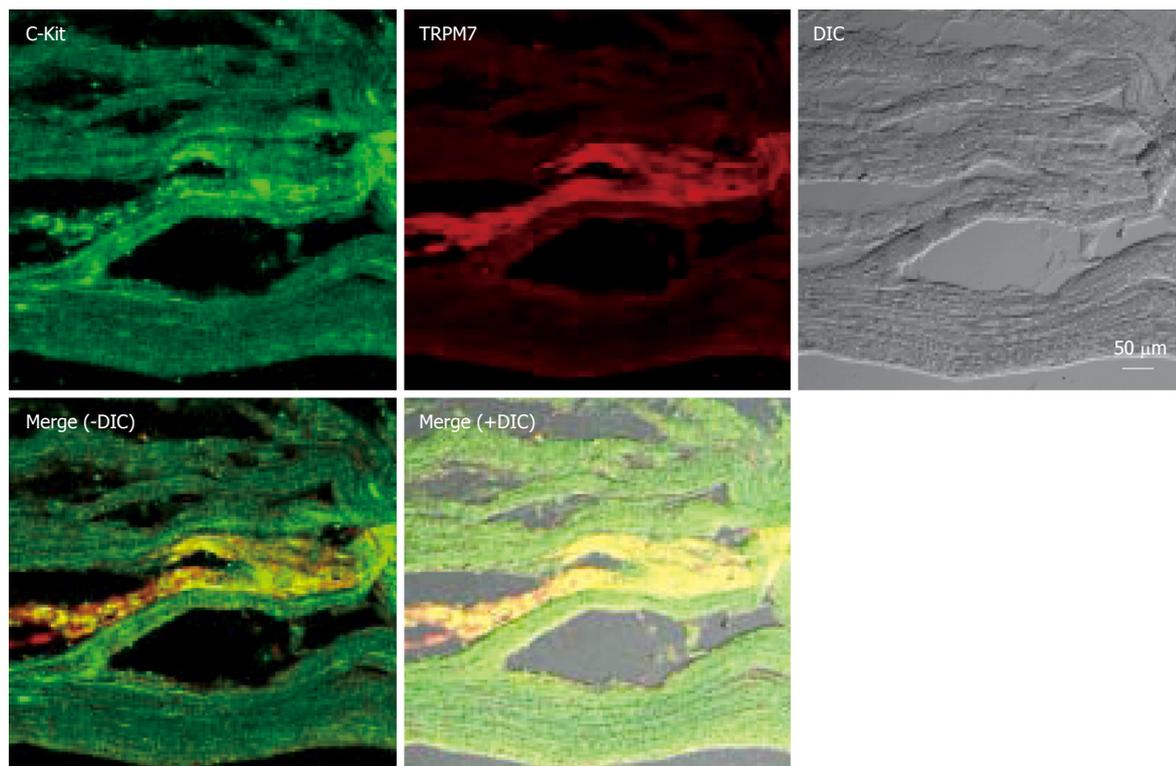
GI smooth muscles are spontaneously active, and



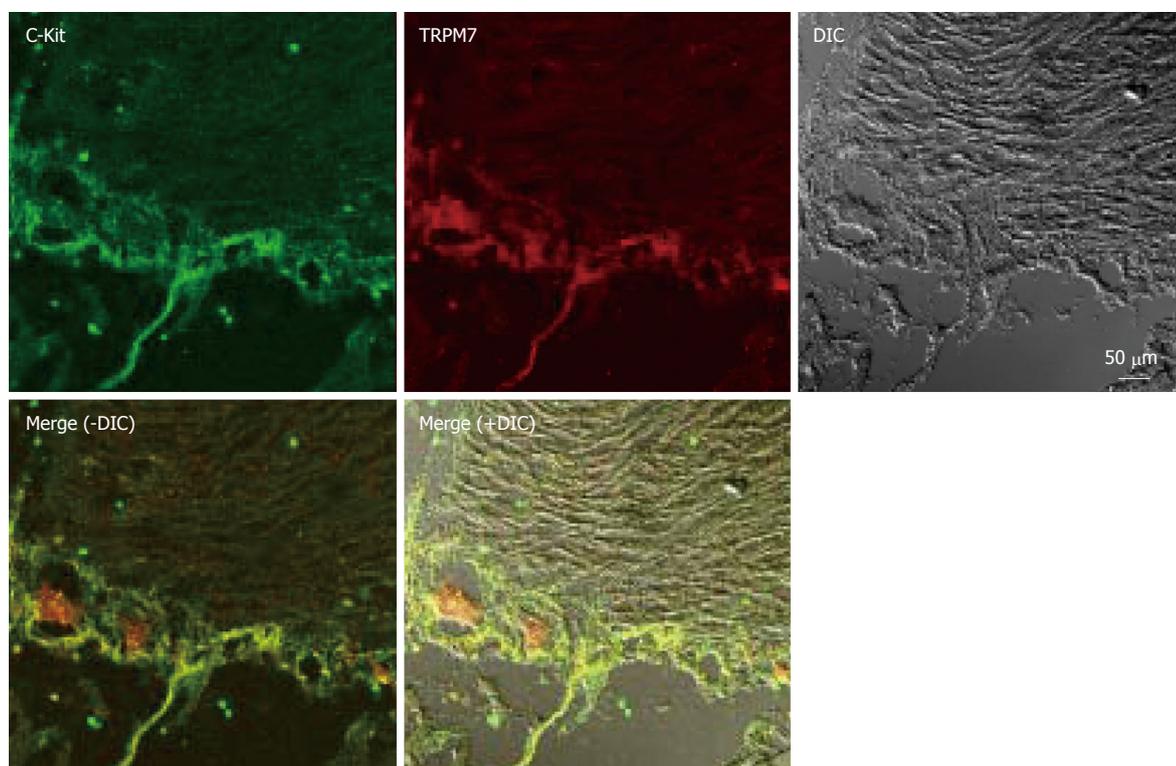
**Figure 2** Effects of flufenamic acid, 2-aminoethoxydiphenyl borate (2-APB) and  $\text{La}^{3+}$  on electrical responses. Flufenamic acid ( $50 \mu\text{mol/L}$ , A), 2-APB ( $50 \mu\text{mol/L}$ , B) or  $\text{La}^{3+}$  ( $50 \mu\text{mol/L}$ , C) were applied while recording electrical activity of isolated smooth muscles of the human colon. All drugs inhibited the spontaneous electrical activity; D: The histograms summarize the frequency of spontaneous electrical activities in human colon with flufenamic acid, 2-APB, and  $\text{La}^{3+}$ . <sup>b</sup> $P < 0.01$ .

generate rhythmic slow electrical waves<sup>[1]</sup>. The slow waves originate in the ICCs distributed in the GI tract<sup>[4-9]</sup>. ICCs express c-kit immunoreactivity and form gap junctional connections with ICCs and with smooth muscle cells<sup>[18-20]</sup>. Many types of ICC with different immunohistochemical and electrical properties, such as myenteric ICC (ICC-MY), intramuscular ICC, deep muscular plexus ICC and submucosal ICC, are distributed in the GI tract<sup>[21]</sup>. In animal models lacking ICC-MY, the slow waves in the small intestine are strongly attenuated, indicating that these cells are indeed essential for pacemaking activity in the GI tract<sup>[6,22]</sup>.

Research into the distribution and function of ICCs was greatly stimulated by discovering that ICCs express



**Figure 3** Expression of transient receptor potential melastatin-type 7 (TRPM7) protein in human colon. Double labeling of TRPM7-like immunoreactivity (red) and c-kit-like immunoreactivity (green) within smooth muscle layers of the human colon. The mixed color yellow indicates the colocalization of both TRPM7-like and c-kit-like immunoreactivity (bar = 50 μm). DIC: differential interference contrast.



**Figure 4** Expression of TRPM7 protein in human small intestine. Double labeling of TRPM7-like immunoreactivity (red) and c-kit-like immunoreactivity (green) within smooth muscle layers of human small intestine. The mixed color yellow indicates the colocalization of both TRPM7-like and c-kit-like immunoreactivity (bar = 50 μm).

c-kit, and signaling *via* kit protein is necessary for development and maintenance of the ICC phenotype<sup>[7,23]</sup>. ICCs are involved in physiological GI motility, therefore

have clinical importance in many bowel disorders, including inflammatory bowel disease, chronic idiopathic intestinal pseudo-obstruction, intestinal obstruction with

hypertrophy, achalasia, Hirschsprung disease, juvenile pyloric stenosis, juvenile intestinal obstruction, and anorectal malformation<sup>[21]</sup>.

Discovering the molecules involved in the generation of pacemaker activity in ICCs may lead to dramatic new therapies for chronic GI diseases that result in lifelong suffering.

Strege *et al.*<sup>[24]</sup> suggested that a mechanosensitive Na<sup>+</sup> channel current is present in human intestinal ICC and appears to play a role in the control of intestinal motor function. Also in human jejunum, each ICC-MY generates spontaneous pacemaker activity that actively propagates through the ICC network, and the pacemaker activity was dependent on inositol-1,4,5-triphosphate receptor-operated stores and mitochondrial function<sup>[25]</sup>. Although some papers have identified the ion channels in ICCs<sup>[10,11,26,27]</sup>, there is little known about the ionic basis of its pacemaker activity. Recently, we used electrophysiological, molecular biological, and immunohistochemical techniques to establish the close relationship between NSCCs in ICCs and a mammalian TRP homologue, TRPM7 and found that TRPM7 is required for murine intestinal pacemaking<sup>[14]</sup>.

Little is known about the involvement of TRPM7 channels in ICCs in the human GI tract. In this study, we found that the human GI tract generated slow waves and various pharmacological properties of the slow waves were the same as those of TRPM7. Also, immunohistochemistry showed abundant and localized expression of TRPM7 protein in the human GI tract. TRPM7 has been suggested to have a central role in cellular Mg<sup>2+</sup> homeostasis<sup>[28]</sup>, in central nervous system ischemic injury<sup>[29]</sup>, in skeletogenesis in zebrafish<sup>[30]</sup>, in the defecation rhythm in *C. elegans*<sup>[31]</sup>, in cholinergic vesicle fusion with the plasma membrane<sup>[31]</sup>, in phosphoinositide-3-kinase signaling in lymphocytes<sup>[32]</sup>, in cell death in gastric cancer<sup>[33]</sup>, in osteoblast proliferation<sup>[34]</sup>, and in breast cancer cell proliferation<sup>[35]</sup>.

The physiological role of TRPM7 channels in ICCs in human GI tract requires more investigation. As a primary molecular candidate for the NSCC responsible for pacemaking activity in ICCs, TRPM7 may be a new target for pharmacological treatment of GI motility disorders.

## COMMENTS

### Background

Previously, transient receptor potential melastatin-type 7 (TRPM7) was found to be required for intestinal pacemaking activity in mice. However, in the human gastrointestinal (GI) tract, the characteristics of slow electrical waves and the presence of TRPM7 has not been investigated.

### Research frontiers

The human GI tract generates slow waves and TRPM7 channels are expressed in interstitial cells of Cajal (ICCs).

### Innovations and breakthroughs

The human GI tract generates slow waves and has ICCs functioning as pacemaker cells. Flufenamic acid, a nonselective cation channel blocker, and 2-APB (2-aminoethoxydiphenyl borate) and La<sup>3+</sup>, TRPM7 channel blockers, inhibited the slow waves. Also, TRPM7 channels were expressed in ICCs in human tissues.

### Applications

TRPM7 protein may be a new target for pharmacological treatment of GI motility disorders.

## Peer review

The authors well demonstrated the involvement of TRPM7 in ICCs and characterized the pharmacological properties of slow waves in the human GI tract. The finding will form the basis for future treatments of GI motility disorders and is a useful addition to the literature.

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## Role of diffusion-weighted magnetic resonance imaging in the differential diagnosis of focal hepatic lesions

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### Abstract

**AIM:** To evaluate the utility of diffusion-weighted imaging (DWI) in screening and differential diagnosis of benign and malignant focal hepatic lesions.

**METHODS:** Magnetic resonance imaging (MRI) examinations were performed using the Signa Excite XI Twin Speed 1.5T system (GE Healthcare, Milwaukee, WI, USA). Seventy patients who had undergone MRI of the liver [29 hepatocellular carcinomas (HCC), four cholangiocarcinomas, 34 metastatic liver cancers, 10 hemangiomas, and eight cysts] between April 2004 and August 2008 were retrospectively evaluated. Visualization of lesions, relative contrast ratio (RCR), and apparent diffusion coefficient (ADC) were compared between benign and malignant lesions on DWI. Superparamagnetic iron oxide (SPIO) was administered to 59 patients, and RCR was compared pre- and post-administration.

**RESULTS:** DWI showed higher contrast between malignant lesions (especially in multiple small metastatic cancers) and surrounding liver parenchyma than did contrast-enhanced computed tomography. ADCs (mean  $\pm$  SD  $\times 10^{-3}$  mm<sup>2</sup>/s) were significantly lower ( $P < 0.05$ ) in malignant lesions (HCC:  $1.31 \pm 0.28$  and liver metastasis:  $1.11 \pm 0.22$ ) and were significantly higher in benign lesions (hemangioma:  $1.84 \pm 0.37$  and cyst:  $2.61 \pm 0.45$ ) than in the surrounding hepatic tissues. RCR between malignant lesions and surrounding hepatic tissues significantly improved after SPIO administration, but RCRs in benign lesions were not improved.

**CONCLUSION:** DWI is a simple and sensitive method for screening focal hepatic lesions and is useful for differential diagnosis.

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**Key words:** Hepatic tumor; Liver imaging; Magnetic resonance imaging; Diffusion-weighted imaging; Apparent diffusion coefficient

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### INTRODUCTION

Diffusion is the thermally induced motion of water molecules, which is also referred to as Brownian motion<sup>[1]</sup>. Diffusion-weighted imaging (DWI) is a new magnetic resonance imaging (MRI) technique that provides imaging of diffusion in biological tissues.

DWI has been reported to be useful in evaluating the early stages of brain ischemia<sup>[2-4]</sup>. Recent technical developments have reduced the image distortion associated with this technique and have increased the signal-to-noise ratio, thus making DWI of the body feasible<sup>[5,6]</sup>. Screening, accurate detection, and characterization of focal hepatic lesions are important for planning

treatment of malignant hepatic lesions. The differential diagnosis of malignant and benign focal hepatic lesions remains a diagnostic challenge; however, to improve the diagnosis of such lesions, new methods for existing modalities, such as MRI, computed tomography (CT), angiography, and ultrasonography are being developed.

Recently, some studies have reported that the apparent diffusion coefficient (ADC), which is one of calculated parameters of DWI, might be useful for differential diagnosis of benign and malignant lesions in the liver<sup>[7]</sup>. Superparamagnetic iron oxide (SPIO)-enhanced MRI was reported to be as useful as CT during arteriography and CT during hepatic arteriography for diagnosing metastatic liver tumors<sup>[8,9]</sup>. SPIO improves the contrast-to-noise ratio (CNR) between focal hepatic lesion and the surrounding liver parenchyma on T2-weighted imaging<sup>[9]</sup>. SPIO has also been reported to further improve the performance of DWI of the liver because SPIO reduces the signal in normal liver parenchyma<sup>[10]</sup>.

The purpose of the present study was to retrospectively evaluate the utility of DWI in screening, accurate detection, and differential diagnosis of benign and malignant focal hepatic lesions.

## MATERIALS AND METHODS

### Patients

We retrospectively evaluated 70 patients (52 men and 18 women; age, 39-86 years; mean age, 65.3 years) with 85 lesions [29 hepatocellular carcinomas (HCC), four cholangiocarcinomas, 34 metastatic liver cancers, 10 hemangiomas, and eight cysts] who had been examined between April 2004 and August 2008. The locations of each focal hepatic lesion were: posterior segment in 32, anterior segment in 33, median segment in 15, and lateral segment in five patients. These lesions measured 1.0 to 10.0 cm (mean 3.1 cm) along their long axes on CT images.

The criteria for selecting patients to evaluate in the present study were as follows: (1) the diagnoses of HCC were pathology confirmed in 25 lesions and other lesions were confirmed by measurement of the serum  $\alpha$ -fetoprotein (AFP) level, clinical data, ultrasonography, angiography and CT or MRI or both; (2) hepatic metastasis of primary lesions was pathologically confirmed; (3) the diagnosis of cholangiocarcinomas was pathologically confirmed in three lesions and the other lesion was confirmed by clinical data, ultrasonography, CT and MRI; (4) the diagnosis of cavernous hemangioma and hepatic cysts was confirmed by clinical data, ultrasonography, CT or MRI or both, and follow-up observation.

### Imaging protocol

MR examinations were performed using the Signa Excite XI Twin Speed 1.5T system (GE Healthcare, Milwaukee, WI, USA) with a four-channel torso-array coil. Diffusion weighted single-shot echo-planar imaging was performed

in individual patients using the following parameters: repetition time/echo time (TR/TE) = 6000/73.1 ms, 7-8 mm thickness, water selective excitation for fat suppression, matrix size = 128 × 128, field of view = 36 cm × 36 cm, number of excitations = 6.0, slice thickness/gap = 8 mm/0 mm, 20 axial slices, scan time = 2 min 24 s, *b* value = 0 and 1000 s/mm<sup>2</sup>, under free breathing. A parallel imaging technique, array spatial sensitivity encoding technique (ASSET) was used. Motion-probing gradient pulses were placed along three orthogonal oblique directions. Additional post-contrast-enhanced DW images were obtained after intravenous administration of SPIO (Resovist; Bayer Schering Pharma AG, Berlin, Germany). The dose of SPIO was 0.016 mL/kg, corresponding to 0.45 mg/kg of Fe.

Contrast enhanced CT was conducted using the LightSpeed ultra 16-MDCT scanner (GE Healthcare, Milwaukee, WI, USA) with pre- and postcontrast triple-phase (arterial, portal venous, and equilibrium phase) scans after injection of 80 to 100 mL of Iopamidol (Iopamiro; Bayer Schering Pharma AG, Berlin, Germany) at an injection rate of 1.5 to 3.0 mL/s.

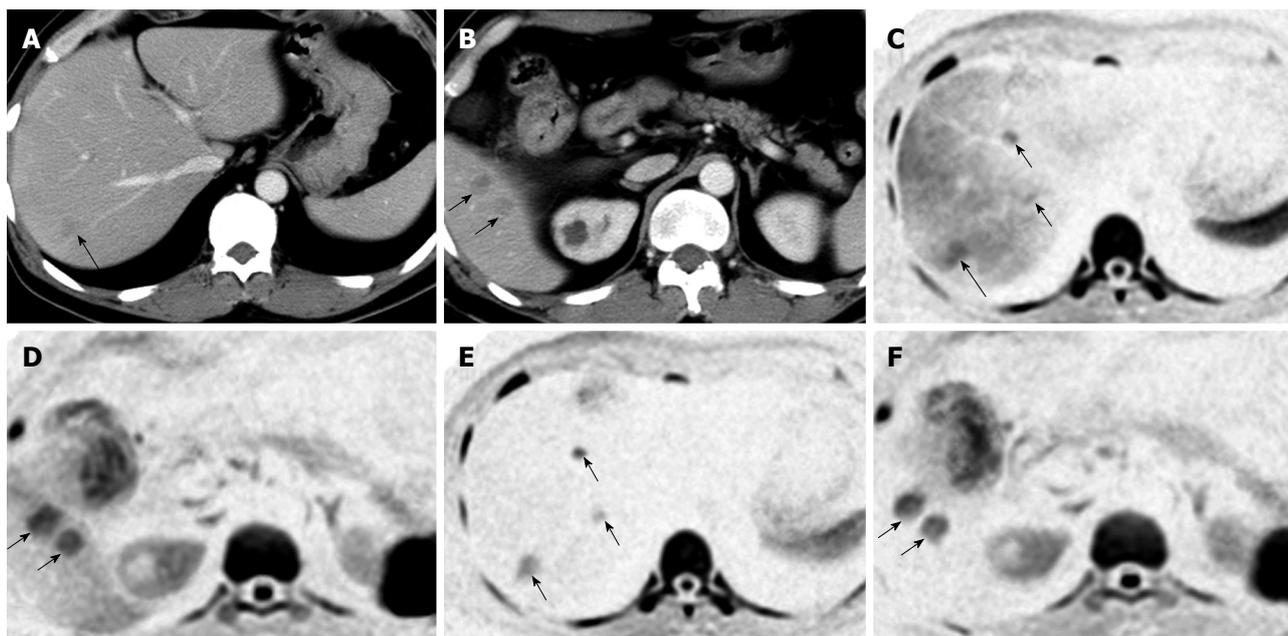
Visualization of lesions, relative contrast ratio between the lesion and surrounding liver parenchyma (RCR), and ADC values were compared between benign and malignant lesions on DWI. Analysis and measurements of DWI data was performed using the GE FUNCTOOL software. All regions of interest (ROI) were created as large as possible in each lesion. In cases with multiple lesions, only the most conspicuous lesion was selected for quantitative measurements. If different types of lesions were mixed (for example, hepatocellular carcinomas and cysts), each lesion was measured.

### Visualization of lesions

All but two lesions (CT examinations were not performed in one case of metastatic liver cancer and hemangioma.) were evaluated. Two diagnostic doctors performed a visual evaluation of each selected CT image (best phase for CT examinations in individual lesions) and DWI (only before SPIO administration) based on mutual agreement. They classified the visualization of lesions on CT and DWI into three categories according to the following criteria. Grade 1: no or slight visualization and unclear margin. Grade 2: moderate visualization and clear margin. Grade 3: marked visualization and very clear margin.

### RCR measurements

SPIO was administered to 59 patients (18 HCC, 26 metastatic liver cancers, 10 hemangiomas, seven cysts) and the RCR was compared before and after SPIO administration to differentiate between benign and malignant lesions. The RCR was calculated by the following equation:  $RCR = SI_{\text{lesion}}/SI_{\text{liver}}$ , where  $SI$  is signal intensity of the lesion and  $SI_{\text{liver}}$  was evaluated from hepatic tissue surrounding the lesion. In this paper, the conventional contrast-to-noise ratio analysis was not employed because the standard deviation of the



**Figure 1** A case of hepatic metastases of colon cancer. A, B: Dynamic computed tomography of the liver in the portal phase showing multiple metastatic lesions, which are indicated as low-density masses (arrows); C, D: Diffusion-weighted imaging (DWI) of the same locations as in (A) and (B), clearly showing metastatic lesions as high signal intensities; E, F: After SPIO administration, the background signal intensity of the liver parenchyma was reduced and the signal intensities of the metastatic lesions were seen more clearly.

**Table 1** Visual evaluation of focal hepatic lesions

Lesions	Modalities	Grade 1	Grade 2	Grade 3
Meta <sup>a</sup> (n = 33)	CT	9	17	7
	DWI	1	8	24
HCC <sup>a</sup> (n = 29)	CT	4	19	6
	DWI	6	11	12
Cholangiocarcinoma (n = 4)	CT	1	1	2
	DWI	0	0	4
Cyst <sup>a</sup> (n = 8)	CT	0	1	7
	DWI	8	0	0
Hemangioma <sup>a</sup> (n = 9)	CT	0	8	1
	DWI	5	2	2

Meta: Metastatic liver cancer; HCC: Hepatocellular carcinoma; DWI: Diffusion-weighted imaging; CT: Computed tomography. <sup>a</sup>*P* < 0.05.

background could not be measured easily in the images obtained using ASSET<sup>[11]</sup>.

### ADC measurements

ADCs of both focal hepatic lesions and surrounding liver parenchymas (26 HCC, 32 metastatic liver cancers, four cholangiocarcinomas, 10 hemangiomas, eight cysts) were measured in 65 patients. Lesions in the lateral segments were excluded for ADC measurement because their ADC could not be correctly measured in the DWI sequences without simultaneous cardiac gating<sup>[12]</sup>.

### Statistical analysis

ADC and SI were measured twice and averaged. All data are expressed as mean ± SD. The visualization data was statistically analyzed by Wilcoxon's signed rank test, and RCR and ADC were analyzed by Student's *t* test using Prism 4.0 software (GraphPad Software, Inc., San Diego,

CA, USA). A *P* value of < 0.05 was considered statistically significant.

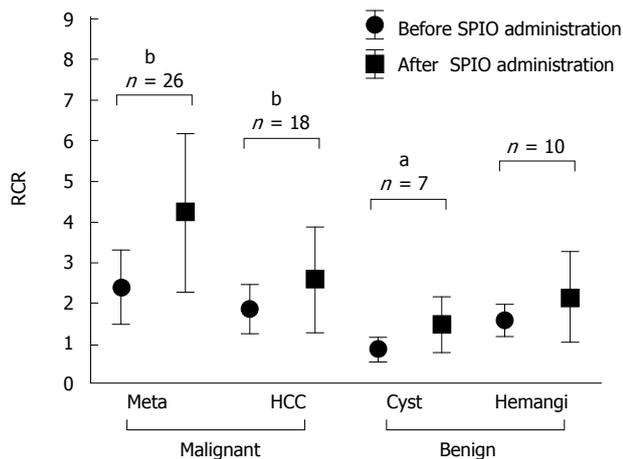
## RESULTS

### Visualization of lesions

Most malignant lesions were significantly more clearly visualized on DWI than on CT (Table 1). In particular, multiple small focal hepatic lesions were visualized clearly on DWI (Figure 1). Two malignant lesions were not detected on DWI, even though they could be easily detected on enhanced CT. These lesions were HCC just under the diaphragm of the lateral segment with liver cirrhosis. Benign lesions, by contrast, were significantly more poorly visualized on DWI than on CT. Similar to the results of several articles published previously<sup>[13]</sup>, cysts showed low or no signal intensities in six of eight cases on DWI (in these cases, no signal intensity means lower signal intensities than background signal intensities.). DWI visualized most hemangiomas; however, CT was better than DWI for the visualization of hemangiomas.

### RCR measurements

The average RCR on DWI before and after SPIO administration is shown in Figure 2. RCR seemed to be significantly improved after SPIO administration in malignant lesions that were metastatic liver cancers (before:  $2.39 \pm 1.28$ , after:  $4.23 \pm 1.34$ , *n* = 26) and HCC (before:  $1.85 \pm 0.58$ , after:  $2.59 \pm 1.28$ , *n* = 18). On the other hand, the RCR was not significantly improved in benign lesions after SPIO administration. The RCR of cysts was  $0.86 \pm 0.30$  (*n* = 7) before SPIO administration, because the SI of cysts was lower than background SI on DWI. Therefore, the RCR of cysts increased after SPIO



**Figure 2** Relative contrast ratio (RCR) of focal hepatic lesions visualized by diffusion-weighted imaging (DWI) before and after the administration of superparamagnetic iron oxide (SPIO). Meta: Metastatic liver cancer; HCC: Hepatocellular carcinoma; hemangi: Hepatic hemangioma. <sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01.

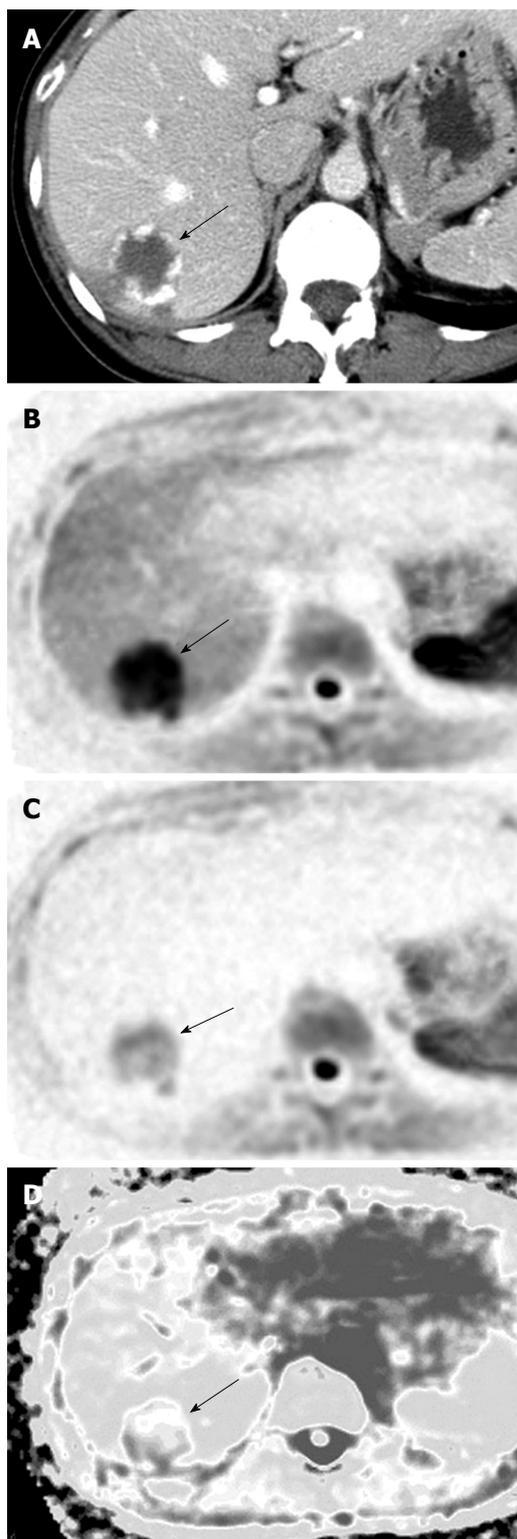
administration ( $1.48 \pm 0.65$ ) as a result of a decrease in the SI of the liver. The RCR of hemangiomas was  $1.58 \pm 0.38$  ( $n = 10$ ) before and  $2.15 \pm 1.10$  after SPIO administration. This was not statistically significant. The RCRs of hemangiomas were reduced in three lesions after SPIO administration. One example is shown in Figure 3.

Figure 4 is a case in which multiple small cysts and metastatic tumors of colon cancer were colocalized in the liver. Both cysts and cancers expressed high signal intensities in T2-weighted MR imaging. In contrast, only the metastatic nodules were expressed on DWI after SPIO administration.

**ADC measurements**

Generally speaking, the lesions showing high signal intensity on DWI demonstrate low ADCs. The average ADCs in our study are shown in Figure 5. The ADCs (mean  $\pm$  SD  $\times 10^3$  mm<sup>2</sup>/s) of malignant lesions, both HCC ( $1.31 \pm 0.28$ ,  $n = 26$ ) and metastatic liver cancer ( $1.11 \pm 0.22$ ,  $n = 32$ ), were significantly lower than the ADCs of the surrounding hepatic tissues. The ADC of cholangiocarcinomas was  $1.33 \pm 0.23$  ( $n = 4$ ). Although this was lower than the ADC of the surrounding hepatic tissue, the difference was not significant.

In benign lesions, the ADCs of both cysts ( $2.61 \pm 0.45$ ,  $n = 8$ ) and hemangiomas ( $1.84 \pm 0.37$ ,  $n = 10$ ) were significantly higher than the ADC of the surrounding hepatic tissue. A representative hemangioma is shown in Figure 3, which expressed high signal intensity and high RCR on DWI. The ADC of the hemangioma was high even though their signal intensity was higher than the surrounding hepatic tissue on DWI. A representative hepatic cyst case is shown in Figure 6. The cyst expressed nearly no signal intensity on DWI after SPIO administration. However, the cyst revealed a higher ADC value than the surrounding hepatic tissue on the ADC map.

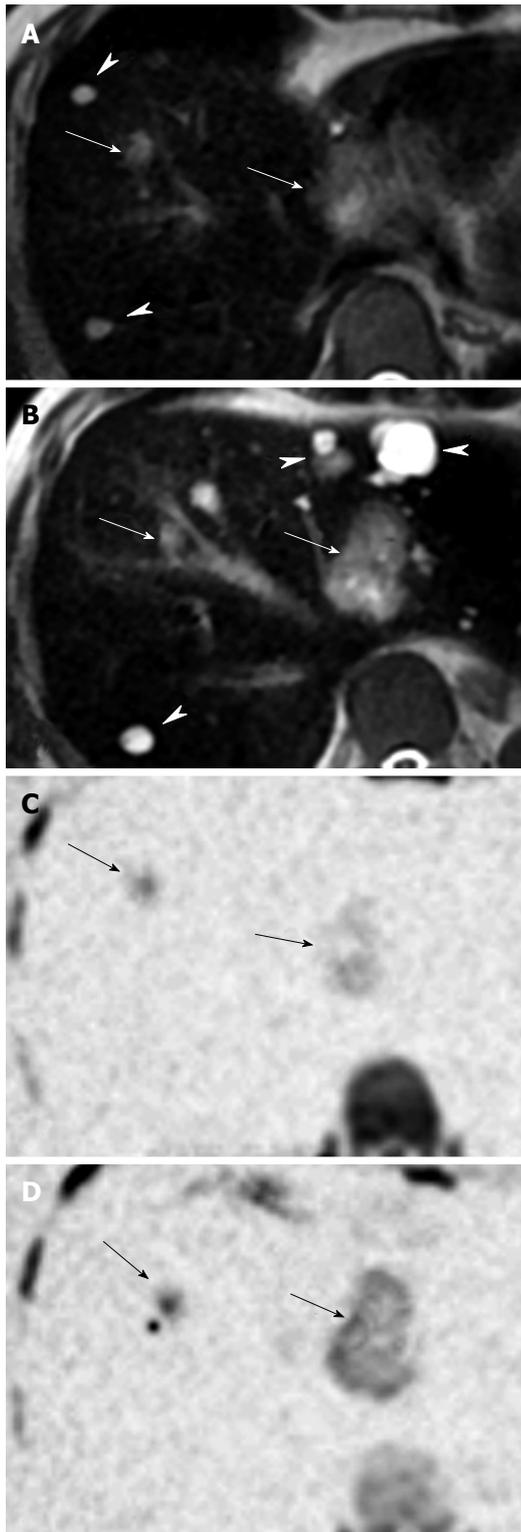


**Figure 3** A case of hepatic hemangioma. A: Dynamic computed tomography in the portal phase showing a low-density mass with a marginal stain at the S7 lobe (arrow). This is a typical staining pattern for hemangiomas; B: The hemangioma (arrow) expressed high signal intensity on diffusion-weighted imaging; C: The intensity of this signal was reduced after administration of superparamagnetic iron oxide (arrow); D: The hemangioma showed a high apparent diffusion coefficient (ADC) value on the ADC map (arrow).

and concisely evaluate malignant lesions. DWI has recently emerged as a tool for detecting cancers in the abdominal organ field<sup>[5,13,14]</sup>. Positron emission imaging is currently used as a powerful screening tool for malignancy<sup>[15]</sup>. Some

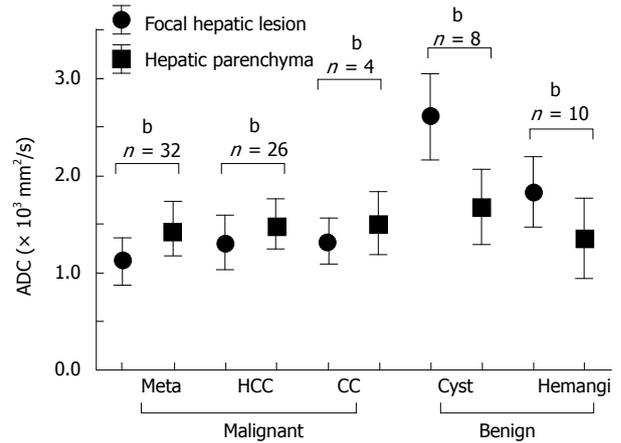
**DISCUSSION**

Surgeons need new imaging modalities that can precisely

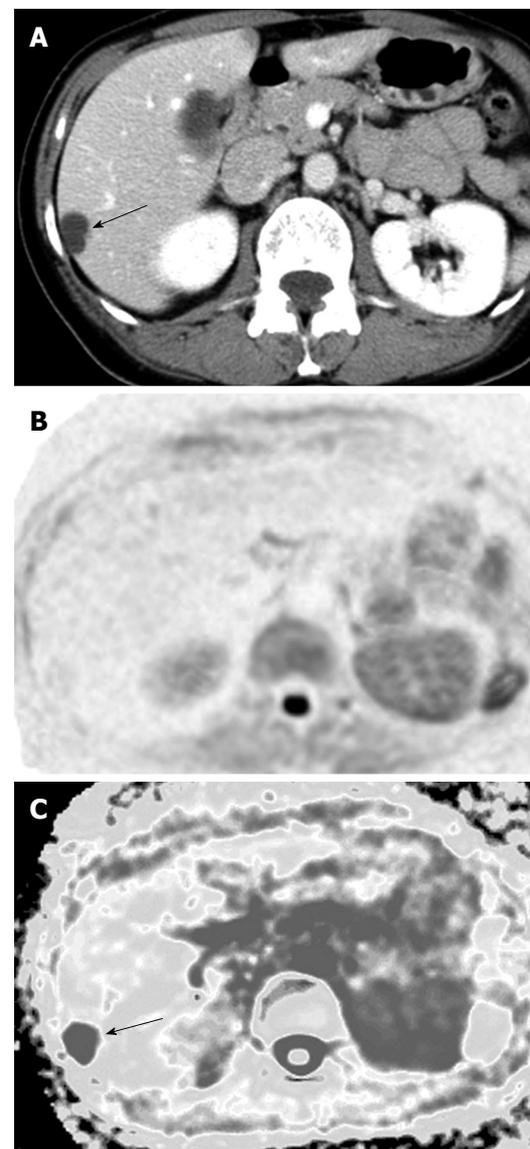


**Figure 4** A case of multiple hepatic metastases of colon cancer with multiple hepatic cysts. A, B: T2-weighted magnetic resonance imaging showing high signal intensities on both metastatic lesions (arrows) and cysts (arrow heads); C, D: Diffusion-weighted imaging after administration of superparamagnetic iron oxide showing high signal intensities on metastatic lesions only (arrows).

articles have reported that SPIO-enhanced T2-weighted MR imaging and CT during arteriportography have the best ability to diagnose metastatic liver cancer<sup>[8,9,16]</sup>. However, even with use of these modalities, differentiating



**Figure 5** Comparison of apparent diffusion coefficient (ADC) between focal hepatic lesions and surrounding hepatic parenchyma. Meta: Metastatic liver cancer; HCC: Hepatocellular carcinoma; CC: Cholangiocarcinoma; Hemangi: Hepatic hemangioma. <sup>b</sup>P < 0.01.



**Figure 6** A case of hepatic cysts. A: Dynamic computed tomography in the portal phase showing the hepatic cyst as a low-density lesion (arrow); B: The lesion expressed no signal intensity on diffusion-weighted imaging; C: The lesion showed a high apparent diffusion coefficient (ADC) on the ADC map (arrow).

between malignant tumors and intrahepatic vascular structures (e.g. arteriportal shunts and thin vessels) or benign lesions (e.g. small cysts and hemangiomas) is sometimes difficult. One of the purposes of the current study was to evaluate the potential of DWI for differentiating malignant from benign lesions.

Nasu *et al*<sup>[17]</sup> reported the sensitivity of DWI for detecting small metastases to be higher than that of SPIO-enhanced T2-weighted MR imaging because metastatic tumors tended to appear larger on DWI than on T2-weighted images, and intrahepatic vascular signals were suppressed on DWI. Parikh *et al*<sup>[18]</sup> also reported that DWI was better than standard breath-hold T2-weighted imaging for the detection of focal hepatic lesions. In particular, they found much higher contrast between HCC and cirrhotic liver on DWI than on T2-weighted images. The contrasts between HCC and cirrhotic liver on DWI in the present study, however, were not higher than that between metastatic liver cancer and surrounding hepatic tissue. This finding might have been because the signal intensity of HCC was not so high as metastatic tumors and the signal intensity of the cirrhotic liver was irregularly increased in our study (data not shown), probably because of marked liver fibrosis, as previously noted<sup>[19-21]</sup>. High signal intensities were found in most malignant focal hepatic lesions and DWI was useful for identifying them, as it was in previous studies<sup>[17,18]</sup>.

However, the findings of benign lesions differed from malignant lesions. Cysts and hemangiomas are most frequently found in the benign lesions of the liver. In the present study, the cysts had low or no signal intensities and hemangiomas had high or low signal intensities, similar to previous studies<sup>[22]</sup>. Such lesions are usually diagnosed using major imaging modalities, such as ultrasonography and enhanced CT. However, because these lesions are small and multiple, differential diagnosis against malignant focal hepatic lesions is often difficult, even using these modalities. The characteristics of cysts on DWI, however, in which the cysts express low or no signal intensities, can enable differential diagnosis against malignant focal hepatic lesions, as shown in Figure 4.

Administration of SPIO in the present study reduced the signal intensity of liver parenchyma on DWI and increased the RCR of malignant focal hepatic lesions, but did not often increase the RCR of hepatic hemangiomas. Therefore, SPIO was quite useful for differential diagnosis, especially between small metastatic liver cancers and hepatic hemangiomas.

We used a high *b* value (1000 s/mm<sup>2</sup>) for DWI because a high *b* value allows delineation of malignant tumors with excellent conspicuity owing to the generally suppressed background noise. Furthermore, the differences in the RCR between malignant and benign lesions were increased with a high *b* value. However, this characteristic of DWI occasionally hinders defining the anatomical location of the abnormal signal on DWI. Therefore, the location of the signal must be correlated with ordinary T2 images by making the slice thickness, interslice gap, and fields of view uniform among these

images. Recent developments in fusion software can readily resolve this problem by anatomically overlapping DWI onto ordinary T2 images. Such fused images might allow improved detection of malignant tumors<sup>[22]</sup>.

ADC is a quantitative parameter calculated from DWI. ADC combines the effects of capillary perfusion and water diffusion in the extracellular and extravascular space<sup>[1]</sup>. ADC is helpful for characterizing focal and diffuse diseases in the body. Several studies have reported that ADC can contribute to the differential diagnosis of benign and malignant focal lesions in the liver<sup>[7,18,23]</sup>.

Our results also revealed the contribution of ADC to the characterization of focal hepatic lesions. The ADC of malignant lesions was significantly lower than that of the surrounding hepatic tissue, whereas the ADC of benign lesions was higher than that of the surrounding hepatic tissue. Benign lesions, hemangiomas, and cysts in particular had high ADCs, even if they had high signal intensities on conventional DWI. The signal intensity of DWI includes both restricted diffusion and the effect of tissues with high T2 relaxation times, which is called the "T2 shine-through" effect. This might be why hemangiomas and cysts sometimes have high signal intensities on DWI. In any case, those results will be useful for differential diagnosis of malignant and benign focal hepatic lesions. For large lesions, however, the ADC was often markedly inhomogeneous on the locations and slices in the lesion. ADCs also seem to differ according to the machine used, the set-up conditions of the machine, and each human body<sup>[24]</sup>. Thus, ADC must be carefully used in the evaluation of lesion characteristics.

A few malignant lesions could not be visualized by DWI in our study. These lesions were mainly located on the left lobe, just under the diaphragm. DWI is known to be extremely sensitive to motion. During clinical image interpretation of MRI, a signal drop is often encountered in the lateral segment of the liver, which is assumed to be due to cardiac pulsation. Not only the signal intensity of DWI, but also ADC, is thought to be incorrect for the lateral segment just under the diaphragm<sup>[12,23,25]</sup>. Further studies need to be conducted to better understand the characteristics of DWI.

This study had some limitations. First, the subjects were a heterogeneous group, and the number of cases was relatively small. Second, histological proofs were not completely obtained, especially for the benign lesions. Therefore, the sensitivities and specificities of DWI cannot be reported here. Third, placement of the region of interest cursor might not be objective enough for measuring ADC and RCR. Additionally, inhomogeneity of hepatic lesions and motion may have affected the accuracy of the calculations, because the DWI was acquired with free breathing<sup>[25]</sup>.

Despite these limitations, we believe that DWI is a very useful imaging modality for identifying malignant focal lesions in the liver. Recently, a new MRI contrast medium, gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid (Gd-EOB-DTPA), has been used for diagnosis of HCC. We plan to combine both Gd-EOB-

DTPA and DWI for diagnosing focal hepatic lesions.

In conclusion, DWI is a simple and sensitive method for screening focal hepatic lesions. SPIO administration can effectively improve the RCR of metastatic liver cancers. ADC measurement is occasionally helpful for differential diagnosis of malignant and benign small focal hepatic lesions.

## COMMENTS

### Background

Screening, accurate detection and characterization of focal hepatic lesions are important for planning treatment of malignant hepatic lesions. Recently, some studies have reported that the apparent diffusion coefficient (ADC), which is one of calculated parameters of diffusion-weighted imaging (DWI), might be useful for differential diagnosis of benign and malignant lesions in the liver. Superparamagnetic iron oxide (SPIO) has been reported to further improve the performance of DWI of the liver because SPIO reduces the signal in normal liver parenchyma. This study aimed to evaluate the usefulness of DWI in screening and differential diagnosis of benign and malignant focal hepatic lesions.

### Research frontiers

In some previous studies, the ADC of benign lesions, such as hepatic cysts and hemangiomas, was reported to be higher than that of malignant lesions, such as hepatocellular carcinoma and metastasis. However, ADC seems differ according to the machine used, the set-up conditions of the machine, and each human body. In this study, the authors compared ADC of focal hepatic lesions with its surrounding hepatic parenchyma for this differential diagnosis. Only a few studies have statistically evaluated the contrast changes in focal hepatic lesions after SPIO administration.

### Innovations and breakthroughs

This is the first report indicating that these simple ADC and RCR patterns are useful for the differential diagnosis of benign and malignant focal hepatic lesions.

### Applications

DWI can be used for differential diagnosis of focal hepatic lesions using calculation of ADC or RCR after SPIO administration. This is a simple and concise method that is easily available, not only to the radiologist, but also the physician and surgeon. DWI also should become a good diagnostic tool for other intra-abdominal organs.

### Terminology

Diffusion is the thermally induced motion of water molecules, which is also referred to as Brownian motion. DWI is a new magnetic resonance imaging (MRI) technique that provides images of the diffusion in biological tissues. The ADC is a quantitative parameter calculated from DWI and it combines the effects of capillary perfusion and water diffusion in the extracellular extravascular space. SPIO is a liver-specific particulate MRI contrast agent that is taken up by the reticuloendothelial system of the liver and improves the focal hepatic lesion-to-liver contrast-to-noise ration and hepatic tumor detection.

### Peer review

This is a retrospective study. The authors presented the results of their study and they reviewed the literature related with this subject. As a conclusion; the authors recommended that DWI by MR was a simple, sensitive and useful method for screening focal hepatic lesions and their differential diagnosis.

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## NT4(Si)-p53(N15)-antennapedia induces cell death in a human hepatocellular carcinoma cell line

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### Abstract

**AIM:** To construct the recombinant lentivirus expression plasmid, pLenti6/V5-NT4 p53(N15)-antennapedia (Ant), and study its effect on HepG2 cells.

**METHODS:** Plasmid pLenti6/V5-NT4 p53(N15)-Ant was constructed incorporating the following functional regions, including signal peptide sequence and pro-region of neurotrophin 4, N-terminal residues 12-26 of p53 and 17 amino acid drosophila carrier protein, Ant. Hepatocellular carcinoma (HepG2) cells were used for transfection. 3-[4,5-dimethyl-thiazol-2yl]-2,5 diphenyl tetrazolium bromide (MTT) assay, lactate dehydrogenase (LDH) release assay, transmission electron microscopy (TEM) and flow cytometric analysis (FCM) were employed to investigate the effects of LV-NT4(Si)-p53(N15)-Ant *in vitro* on HepG2 cells. *In vivo* experiment was also performed to investigate the inhibitory effect of LV-NT4(Si)-p53(N15)-Ant on tumor growth in nude mice.

**RESULTS:** LV-NT4(Si)-p53(N15)-Ant significantly suppressed the growth of HepG2 cells. MTT assay showed that the growth of HepG2 cells was much more significantly inhibited by LV-NT4(Si)-p53(N15)-Ant than by LV-EGFP. The inhibition rate for HepG2 cell growth in the two groups was 46.9% and 94.5%, respectively, 48 h after infection with LV-NT4(Si)-p53(N15)-Ant, and was 33.9% and 95.8%, respectively, 72 h after infection with LV-NT4(Si)-p53(N15)-Ant ( $P < 0.01$ ). Light microscopy and TEM showed morphological changes in HepG2 cells infected with LV-NT4(Si)-p53(N15)-Ant, but no significant changes in HepG2 cells infected with LV-EGFP. Changes were observed in ultra-structure of HepG2 cells infected with LV-NT4(Si)-p53(N15)-Ant, with degraded membranes, resulting in necrosis. LDH release from HepG2 cells was analyzed at 24, 48, 72 and 96 h after infection with LV-NT4(Si)-p53(N15)-Ant and LV-EGFP, which showed that LDH release was significantly higher in LV-NT4(Si)-p53(N15)-Ant treatment group (682 IU/L) than in control group (45 IU/L,  $P < 0.01$ ). The longer the time was after infection, the bigger the difference was in LDH release. FCM analysis showed that LV-NT4(Si)-p53(N15)-Ant could induce two different kinds of cell death: necrosis and apoptosis, with apoptosis being the minor type and necrosis being the main type, suggesting that LV-NT4(Si)-p53(N15)-Ant exerts its anticancer effect on HepG2 cells by inducing necrosis. The *in vivo* study showed that LV-NT4(Si)-p53(N15)-Ant significantly inhibited tumor growth with an inhibition rate of 66.14% in terms of tumor size and weight.

**CONCLUSION:** LV-NT4(Si)-p53(N15)-Ant is a novel recombinant lentivirus expression plasmid and can be used in gene therapy for cancer.

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**Key words:** Gene therapy; Lentivirus vector; Anticancer; Necrosis; LV-NT4(Si)-p53(N15)-Ant; Hepatocellular carcinoma cell line

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common causes of death worldwide, especially in Asian countries<sup>[1]</sup>. To date, none of the conventional treatment modalities can completely eradicate HCC cells. In recent years, gene therapy has been evaluated as a novel treatment modality for HCC<sup>[2-5]</sup>, while conventional treatment modalities, including surgery, chemotherapy and liver transplantation, are still used<sup>[6-8]</sup>.

*p53* is an important tumor suppressor gene which regulates many important cellular activities, including apoptosis. Mutations and deletions of the *p53* gene are common in HCC in a number of geographic regions. Since the mutation incidence ranges 5%-50%, *p53* has become an ideal target for gene therapy. Kanovsky *et al*<sup>[9]</sup> and Do *et al*<sup>[10]</sup> reported that a *p53* peptide, synthesized from residues 12-26 and fused with the *Drosophila* carrier protein antennapedia (Ant), can induce rapid tumor cell necrosis in all breast and pancreatic cancer cell lines irrespective of its status and exhibits a low cytotoxicity to normal cells, which is uncommonly observed in traditional cancer therapy. Human HCC cell line, HepG2, contains the wild-type *p53* gene and can thus be used in research of the relation between HCC and *p53* peptide gene therapy.

It is important to study the transfer of fusion gene and the secretion of expressed protein for the assessment of enhanced cancer-killing effects of a protein. In this study, NT4 signal peptide and its pro-region were used as the regions responsible for protein and peptide secretion from cells. Lentivirus gene expression plasmids were constructed for NT4(Si)-ADNF-9 and NT4(Si)-NAP fusion proteins containing the NT4 signal peptide and pro-region to enhance their expression. The restriction enzyme site *Nae*I at the NT4 signal peptidase fissure site and two restriction enzyme sites, *Bam*H I and *Xho* I, in NT4(Si)-*p53*(N15)-Ant could ensure the correct construct. A novel recombinant lentivirus expression plasmid, pLenti6/V5-NT4 *p53*(N15)-Ant, was constructed, containing a signal peptide sequence and pro-region of neurotrophin 4 (NT4) fused to *p53*(N15)-Ant peptide. Its effect on HepG2 cells, both *in vitro* and *in vivo*, was investigated.

## MATERIALS AND METHODS

### Cells and cell culture

Human hepatoma HepG2 cells containing wild-type *p53* were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS). 293T human kidney cells were

grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in a humidified atmosphere containing 50 mL/L CO<sub>2</sub>. All cell lines were supplied by Xi'an Huaguang Bioengineering Company (Xi'an, China).

### Plasmid construction and virus titration

*p53*(N15)-Ant was synthesized by Beijing Sun Biotechnology Co, according to Kanovsky *et al*<sup>[9]</sup> and Genbank. Two restriction enzyme sites (*Nae* I and *Xho* I) were introduced upstream and downstream of this sequence, respectively. *p53*(N15)-Ant was subcloned into pGEM-T-easy for sequencing, then into pBV220/NT4. Signal peptide sequence and pro-region of NT4 were cloned from human genomic DNA and subcloned into the *Bam*H I / *Nae* I sites of pBV220. After digestion with restriction enzymes, the resulting NT4 *p53*(N15)-Ant gene with *Bam*H I and *Xho* I sites from the pBV220/NT4-*p53*(N15)-Ant plasmid was inserted into the multiple cloning site of the expression plasmid, pLenti6/V5-D-TOPO (Invitrogen) containing a cytomegalovirus (CMV) promoter, upstream of the inserted gene. The resulting plasmid was named pLenti6/V5-NT4 *p53*(N15)-Ant.

To generate a control plasmid containing green fluorescent protein, EGFP was amplified from EGFP-C2 by PCR. The sequences of sense and anti-sense primers are 5'CGGGATCCATGGTGAGCAAGGGCGAGG-3' and 5'CGCTCGAGTCAAGTCCGGCCGGACTTGTAC-3', respectively. The resultant PCR fragments were digested with *Bam*H I and *Xho* I (underlined), subcloned into pLenti6/V5-D-TOPO, and verified by DNA sequencing.

The four-plasmid-based lentiviral expression system was purchased from Invitrogen. Briefly, four kinds of plasmid including 4.2 µg of pLP1, 2 µg of pLP2, 2.8 µg of pLP/VSVG and 3 µg of lentivector pLenti6/V5-NT4 *p53*(N15)-Ant or pLenti6/EGFP, were cotransfected into 293T cells using the calcium phosphate coprecipitation method. The conditioned medium was harvested 72 h after transfection and filtered through a 0.45 µm filter. Concentrated viral stocks were prepared by ultra-centrifugation of 3 mL conditioned medium at 50 000 *g* for 1.5 h at 4°C in a SW41 rotor (Beckman). The pellet was resuspended in 30 µL complete medium and stored at -80°C. The resulting recombinant lentivirus was named LV-NT4 *p53*(N15)-Ant and LV-EGFP, respectively.

Viral titer was measured by assessing the viral p24 antigen concentration using ELISA (Beckman Coulter, Fullerton, California, USA), showing that one microgram per milliliter of p24 corresponds to approximately  $2 \times 10^6$  transducing units per milliliter of EGFP virus, as assessed by titration in 293 T cells.

### Lentivirus-mediated gene transfer and expression in HepG2 cells

HepG2 cells were seeded into 6-well plates at a density of  $1 \times 10^6$  cells/well, allowed to adhere for 24 h, and then infected with EGFP lentivirus at multiplicities of infection (MOI) of 4 transducing units (TU)/cell. EGFP-positive cells were observed and counted under a fluorescence microscope 48 h after infection.

Expression of the *NT4 p53(N15)-Ant* gene was detected by reverse transcription-polymerase chain reaction (RT-PCR). HepG2 cells were seeded in 100 mL culture flasks at a density of  $1 \times 10^6$  cells/flask and infected with NT4-p53(N15)-Ant lentivirus at a MOI of 4 TU/cell. Forty-eight hours after infection, HepG2 cells were scraped from the flask surface with a brush (Paro-Isola, Thalwil, Switzerland). Total RNA was extracted using Trizol reagent (Gibco). A reverse transcription (RT) step was carried out for 60 min at 37°C using 1 µg total RNA treated with DNase and M-MLV reverse transcriptase (Invitrogen, USA) in the presence of random primers. PCR was performed in 50 µL reaction volume containing 10 µL (about 2.5 ng) cDNA, 1 µL of 10 mmol/L dNTP, 1 U of Taq DNA polymerase, 5 µL of  $10 \times$  Taq buffer, and 1 µL 50 pmol of forward primer (5'-CG GATCCATGCTCCCTCTCCCTCATGC-3') and reverse primer (5'-CCTCGAGTCATCCGCGCTGTACCTTTACC-3') of the *NT4 p53(N15)-Ant* gene. Samples were subjected to RT-PCR at the following conditions: pre-denaturation for 5 min at 94°C, followed by 30 cycles of denaturation for 60 s at 94°C, annealing for 60 s at 60°C, extension for 90 s at 72°C, and a final extension for 5 min at 72°C. The RT-PCR products were separated by electrophoresis on 2% agarose gels (Qiagen, USA).

#### **Immunohistochemical detection of p53(N15)-Ant expression in lung cancer H1299 cells**

H1299 cells (null for p53) were seeded onto cover slips and infected with LV-NT4(Si)-p53(N15)-Ant at a MOI of 4 TU/well. Polybrene was added to a final concentration of 8 µg/mL. Twelve hours after infection, 2 mL of a new DMEM medium was added. Forty-eight hours after infection, H1299 cells were washed and fixed with cold acetone. Protein expression of p53(N15)-Ant in H1299 cells was determined by immunocytochemical staining with anti-p53 antibody (DO-1) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using the Vectastain Elite ABC kit following its manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA).

#### **MTT assay**

To determine the effect of lentivirus-NT4(Si)-p53(N15)-Ant on HCC, HepG2 cells were seeded in 96-well plates at a concentration of  $5 \times 10^3$  cells, grown for 24 h, infected with lentivirus-NT4(Si)-p53(N15)-Ant or lentivirus-EGFP at a MOI of 4 TU/cell with polybrene (8 µg/mL) for 12 h. The culture medium was then replaced with a fresh medium. MTT assay was performed at different time points (24, 48 and 72 h) after infection with lentivirus according to its manufacturer's instructions (Xi'an, China).

#### **Examination of HepG2 cell morphology under light microscope**

After infection with NT4(Si)-p53(N15)-Ant, morphology of HepG2 cells was observed under an inverted light microscope and recorded.

#### **Examination of ultra-structure of HepG2 cells under electron microscope**

Forty-eight hours after infection with NT4(Si)-p53(N15)-Ant, HepG2 cells were trypsinized, fixed with 2.5% glutara-

ldehyde for 2 h at 4°C, washed with 0.1 mol/L dimethyl arsenic trioxide buffer, fixed with 1% osmium acid for 2 h, dehydrated, and then embedded in 618 domestic epoxy resin, followed by polymerization for 24 h. The embedded block was cut into ultra-thin sections. The sections were double stained with uranyl acetate and lead citrate, and observed under a transmission electron microscope.

#### **LDH release assay**

To determine LDH leakage into the extra-cellular fluid, supernatant was collected at different time points (24, 48, 72, and 96 h) after infection with NT4(Si)-p53(N15)-Ant. LDH in the supernatant was detected with a RA-100 automatic biochemical analyzer.

#### **Annexin V and PI double staining**

When HepG2 cells were grown to  $2 \times 10^5$ /mL, they were seeded into 9-well plates at a volume of 3 mL/well, and left to adhere for 24 h. The cells were then divided into control group and lentivirus treatment group. The cells in control group were grown in a serum-free medium. Forty-eight hours after infection with lentivirus, the cells were washed twice with PBS at 4°C, re-suspended in 250 µL of a combination buffer solution, and the cell concentration was adjusted to  $1 \times 10^5$ /mL. Five microliter of Annexin V/FITC and 5 µL of 20 µg/mL propidium iodide were added to 100 µL of cell suspension. After incubation in the dark for 15 min, the cells were analyzed by flow cytometry (FCM).

#### **Inhibition of HepG2 cells in nude mice by LV-NT4(Si)-p53(N15)-Ant**

Twenty 6-8 wk old male and female BALB/c (nu/nu) mice, weighing  $20.3 \pm 2$  g, were purchased from Laboratory Animal Center, Fourth Military Medical University (Xi'an, China).

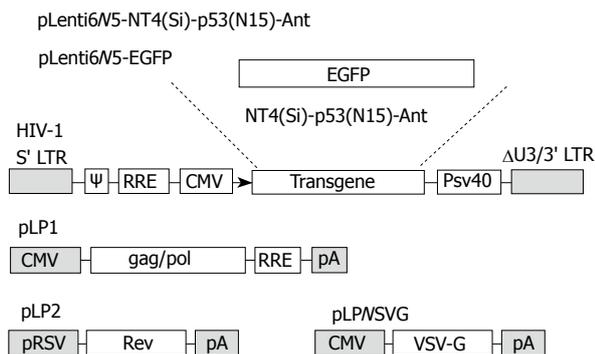
HepG2 cells ( $2 \times 10^5$ ) were injected subcutaneously on the right side of each mouse's back to induce tumor formation. The animals were divided into three groups when the tumor volume reached 30 mm<sup>3</sup> with 0.1 mL LV-NT4(Si)-p53(N15)-Ant ( $2 \times 10^6$  TU/mL) injected into their tumor. Mice treated with PBS and LV-EGFP served as controls with 0.1 mL LV-EGFP ( $2 \times 10^6$  TU/mL) injected into their tumor (injection at different directions). Infection efficiency of lentivirus was observed under a confocal laser scanning microscope one week after infection.

The animals were sacrificed 3 wk after injection of lentivirus. Tumors were removed and weighed with their size measured using a vernier caliper and their volume calculated according to the following formula:  $(3.14 \times L \times W \times H)/6$ . Tumors were fixed in 4% formaldehyde and embedded in paraffin. Tumor tissue was cut into 5-µm thick sections which were stained with HE.

Tumor growth inhibition rate =  $(A-B)/A \times K$ , where A is the average tumor weight of control group, B is the average tumor weight of treatment group, K represents 100%.

#### **Statistical analysis**

The data were expressed as mean  $\pm$  SD. LDH release and



**Figure 1** Packaging system of LV-NT4(Si)-p53(N15)-Ant and LV-EGFP. The packaging system: lentiviral vector, pLenti6/V5-D-TOPO, contains HIV-1 5'LTR, packaging signal ( $\psi$ ), RRE sequence, CMV promoter and 3'LTR from which some regulatory sequences are deleted, resulting in "self-inactivation" of lentivirus after transduction of the target cells. pLP1, pLP2 and pLP/VSVG are three helper plasmids, which provide structural and replication proteins to produce lentivirus.

MTT were analyzed by *t*-test and  $\chi^2$  test, respectively. The data for animal experiment were processed by analysis of variance. Data analysis was performed using the SPSS 15.0.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Construction of lentivirus vector expressing NT4-p53(N15)-Ant or EGFP

The lentivirus expression vector, pLenti6 V5-D-TOPO we constructed, contains the elements required for virion packaging, such as 5' and 3' long terminal repeats and  $\psi$  packaging signal. The packaging system of LV-NT4(Si)-p53(N15)-Ant and LV-EGFP is shown in Figure 1.

The DNA sequence of the *p53(N15)-Ant* gene was identical to those of *p53(N15)* and 17 amino acid Ant<sup>[9]</sup>. The length of NT4-p53(N15)-Ant was 349 bp, including a 247 bp fragment of the signal peptide sequence and pro-region of neurotrophin 4 with the two restriction sites (*Bam*H I and *Nae* I). The DNA fragment of *p53(N15)-Ant* was 108 bp with *Nae* I and *Xho* I. Digestion of pLenti6/V5-NT4-p53(N15)-Ant with *Bam*H I and *Nae* I followed by electrophoresis verified that the NT4-p53(N15)-Ant secretory expression cassette was correctly inserted into the multiple cloning site (MCS) of pLenti6/V5-D-TOPO, and pLenti6/V5-EGFP was confirmed in the same way as pLenti6/V5-NT4-p53(N15)-Ant.

### Gene transfer and lentivirus expression in HepG2 cells

Fluorescent microscopic analysis showed that EGFP was expressed in over 60% of HepG2 cells 48 h after infection with pLenti6/V5-EGFP at a MOI of 4 TU/cell (Figure 2A and B).

### Expression of NT4-p53(N15)-Ant in lentivirus-delivered H1299 cells

Since H1299 cells lack of the *p53* gene (null-*p53*), H1299 lung cancer cells were used as target cells for the expression of *p53(N15)-Ant*. *p53* protein could not be detected with immunohistochemical staining before or after infection with LV-EGFP (Figure 2C). However, 48 h

after infection with LV-NT4(Si)-*p53(N15)-Ant*, *p53* protein was mainly expressed in cytoplasm of H1299 lung cancer cells (Figure 2D). Anti-DO-1 could identify and bind to amino acids 11-25 of *p53*, suggesting that LV-NT4(Si)-*p53(N15)-Ant* can effectively infect the target cells and express the *p53* fusion peptide.

### Killing effect of lentivirus-NT4 p53(N15)-Ant on HepG2 cells in vitro

HepG2 cells, infected with pLenti6/V5-NT4-p53(N15)-Ant in our study, were significantly killed, whereas pLenti6/V5-EGFP had no effect on cell viability, suggesting that growth of HepG2 cells can be inhibited by the transferred exogenous gene rather by viral toxicity (Figure 3).

### Morphological changes in HepG2 cells observed under light microscope

Significant morphological changes were observed in HepG2 cells at different time points after infection with LV-NT4(Si)-*p53(N15)-Ant* but not after infection with LV-EGFP and LV-NT4(Si)-*p53(N15)-Ant* (Figure 4). However, the cells became swollen and their boundary was blurred 48 h after infection with LV-NT4(Si)-*p53(N15)-Ant*, while their viability was significantly declined with noticeable cell shrinkage, fragments and detachment 72 h after infection with LV-NT4(Si)-*p53(N15)-Ant*, but without significant morphological changes 72 h after infection with LV-EGFP infection.

### Changes in ultra-structure of HepG2 cells observed under electron microscope

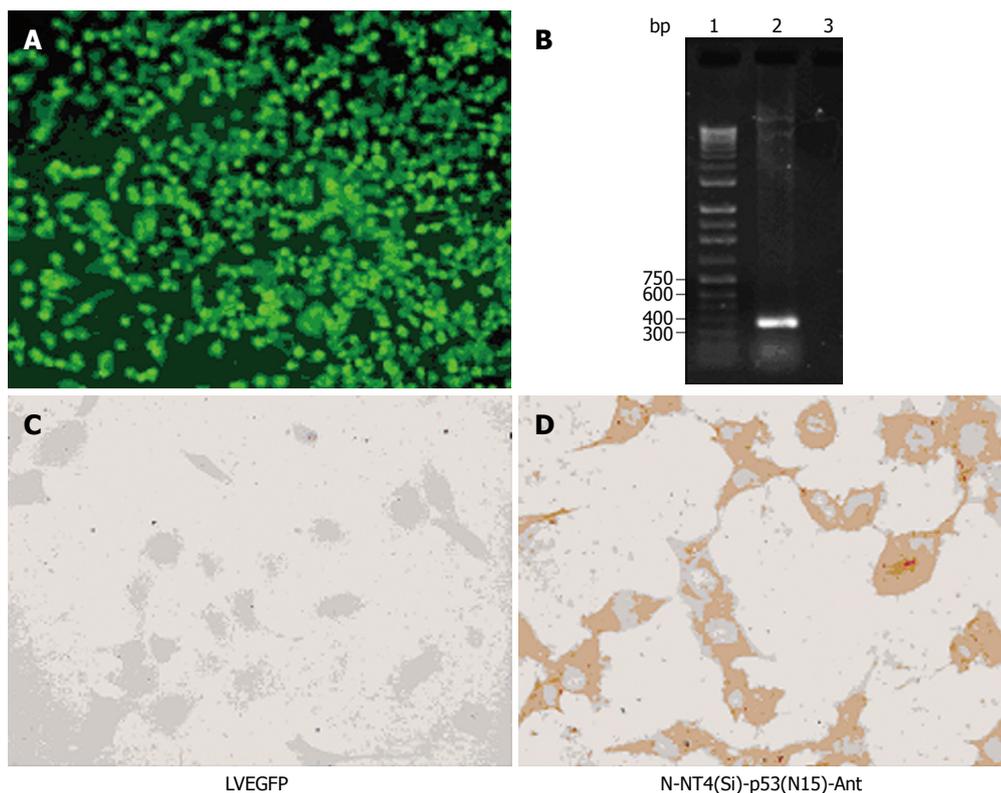
Forty-eight hours after infection with LV-EGFP, the membranes and nuclear membranes of HepG2 cells were intact with some crimples in the nuclear membranes, some microvilli on the surface of HepG2 cells, normal structure of mitochondria and ER (Figure 5A), and 2 completely divided HepG2 cells (Figure 5B), suggesting that LV-EGFP has no significant effect on cell growth and division. Forty-eight hours after infection with LV-NT4(Si)-*p53(N15)-Ant*, significant changes in ultra-structure of HepG2 cells, as well as in cell membranes and mitochondria, were observed under electron microscope. Cell membranes were incomplete with several fractures and the cells appeared to leak contents. Mitochondria were swollen with vacuolization. Vacuoles were also seen under the nuclear membrane with bare nuclei (Figure 5C and D). These findings suggest that NT4(Si)-*p53(N15)-Ant* may exert its anticancer effect mainly by inducing necrosis of cell membranes.

### Determination of LDH release

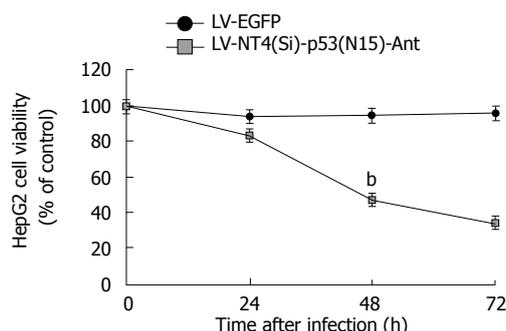
LDH was significantly increased in LV-NT4(Si)-*p53(N15)-Ant* treatment group, but not in LV-EGFP treatment group, with no significant difference between the two groups. The longer the time was after the infection, the greater the difference was between the groups, suggesting that cell membranes are damaged.

### Annexin V-PI double staining

Flow cytometry (FCM) was employed to detect the



**Figure 2** Fluorescent microscopic analysis and immunohistochemical staining. Fluorescence microscopy analysis showing EGFP expression in HepG2 cells (A), agarose gel electrophoresis of RT-PCR products from HepG2 cells (B) [lane 1: 1.0 kb DNA ladder; lane 2: RT-PCR products from cells infected with LV-NT4(Si)-p53(N15)-Ant; lane 3: negative controls infected with LV-EGFP], immunohistochemical staining showing no p53 protein (C), and p53 protein expression in cytoplasm of HepG2 cells (D) 48 h after infection with LV-NT4(Si)-p53(N15)-Ant.



**Figure 3** Cytotoxicity of LV-NT4(Si)-p53(N15)-Ant to HepG2 cells at different time points (24, 48 and 72 h) after infection with lentivirus. <sup>b</sup>*P* < 0.01 vs LV-EGFP.

fluorescence intensity after HepG2 cells were double-stained with Annexin V and PI. A plot composed of 4 quadrants was detected by flow cytometry, which showed that the number of necrotic cells was significantly greater in LV-NT4(Si)-p53(N15)-Ant treatment group than in LV-EGFP treatment group 48 h after infection with lentivirus. The apoptosis level was also higher in cells expressing LV-p53(N15) than in controls. However, the number of necrotic cells was still much greater than that of apoptotic cells (Figure 6), suggesting that the NT4(Si)-p53(N15)-Ant gene kills tumor cells mainly by inducing necrosis of cell membrane.

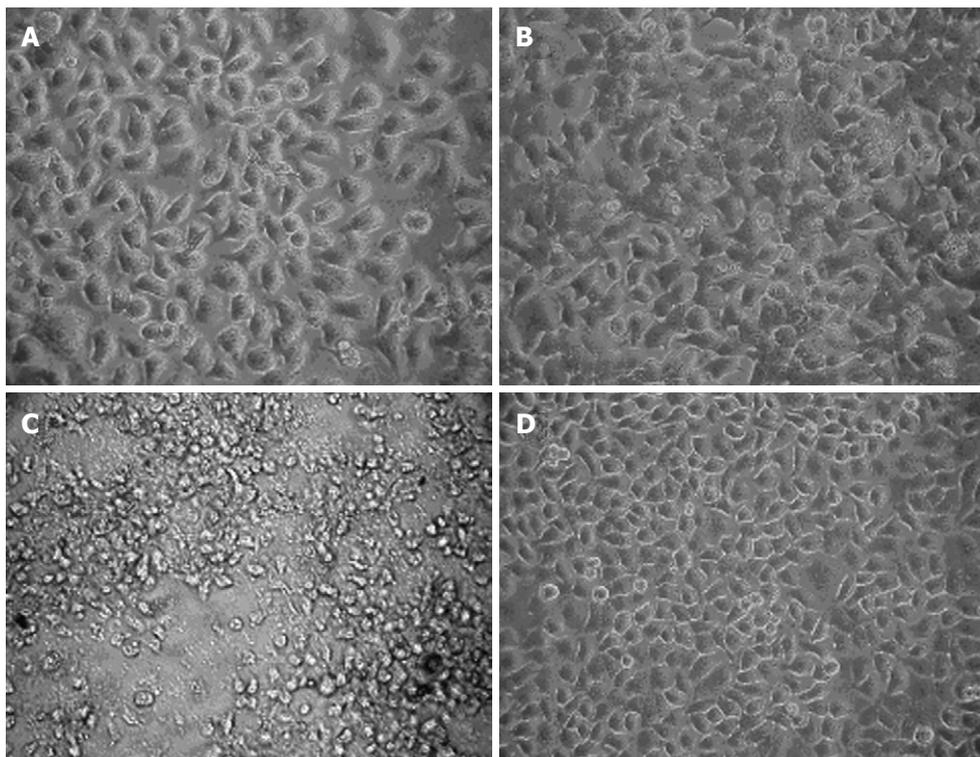
**Inhibition of HepG2 cell growth in nude mice by LV-NT4(Si)-p53(N15)-Ant**

Nude mice developed tumor mass within 10 d after inoculation with HepG2 cells. Three weeks after infection with lentivirus, the size and weight of tumor mass were measured (Figure 7A and B). The inhibition

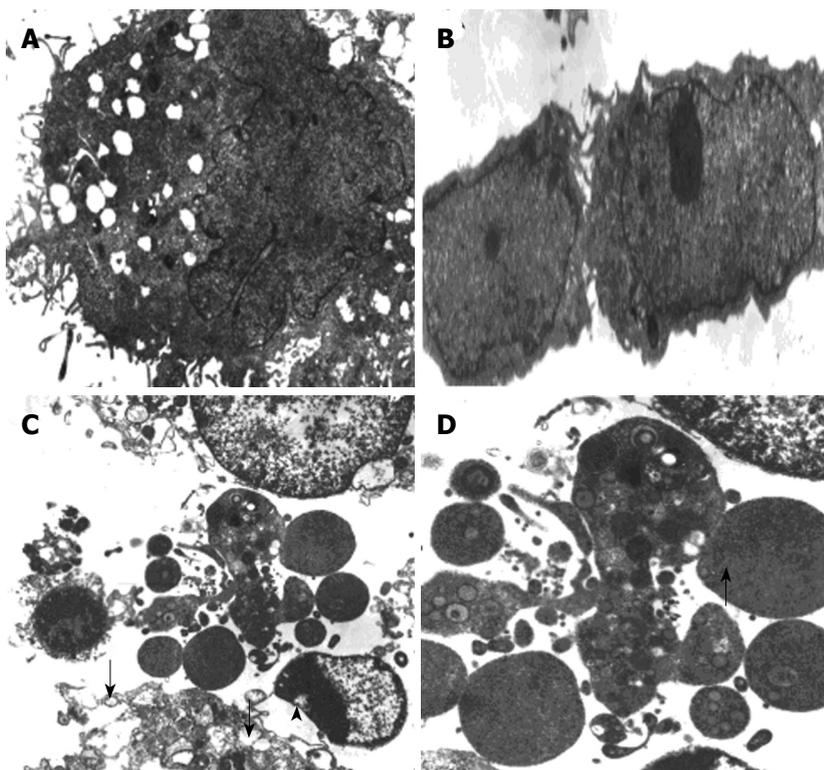
rate of tumor growth for LV-NT4(Si)-p53(N15)-Ant was 66.14%.

**DISCUSSION**

p53 protein plays an essential role in cell activities<sup>[11,12]</sup>. Under various stress conditions, such as genotoxic damage, hypoxia, ribonucleotide depletion or oncogene activation, p53 molecule accumulates and is activated. Activated p53 acts as a transcription factor and activates or represses a variety of genes involved in cell-cycle regulation, induction of apoptosis or senescence. Therefore, the *p53* gene represents an ideal target for cancer therapy. In non-stressed cells, p53 protein is present at a very low cellular concentration, because it interacts with the hdm2 protein, the human analogue of murine mdm2, which acts as a ligase of ubiquitin<sup>[13,14]</sup> and promotes p53 degradation<sup>[9,10]</sup>. Recent advances in regulation of p53 by hdm2 offer the possibility of generating new anticancer agents that activate wild-type p53 in tumors. Since hdm2 down-regulates p53, inhibition of this interaction would lead to an accumulation of p53 in tumor cells, eventually inducing their death<sup>[10,15]</sup>. p53(N15)-Ant has been considered a novel cancer therapeutic peptide because it induces cancer cell death and does not seem to be cytotoxic to normal cells and may, therefore, prove useful as a general anticancer agent. In this study, we focused on how to improve the anticancer effect of peptide therapy by employing two strategies. The first was to enable the expressed therapeutic peptide to be secreted, and the second was to use the lentivirus gene transfer system. It is well-known that the signal peptide is located at the terminal of amino acid sequence of a secreted protein and plays an important role in protein targeting



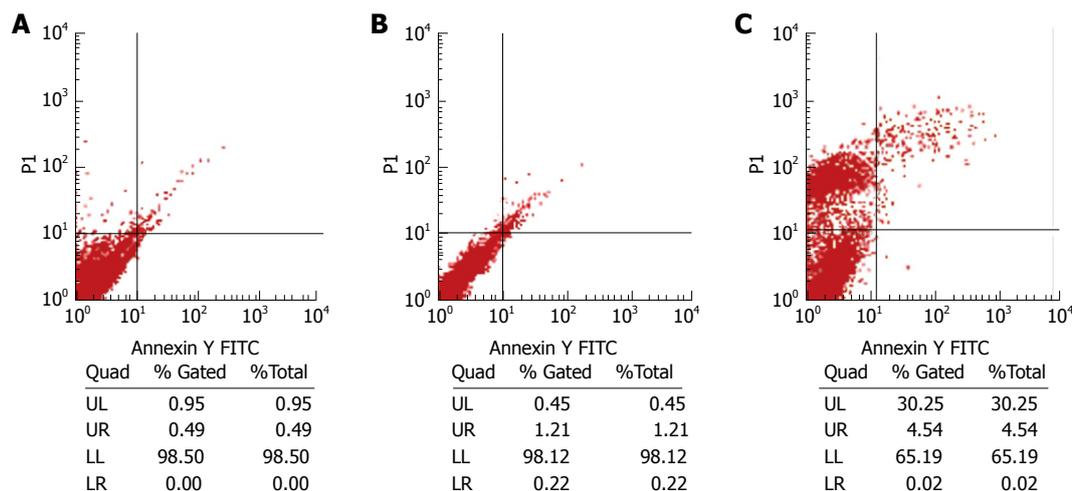
**Figure 4** Morphological changes in HepG2 cells 24 h (A), 48 h (B), 72 h (C) after infection with LV-NT4(Si)-p53(N15)-Ant and 72 h (D) after infection with LV-EGFP ( $\times 400$ ).



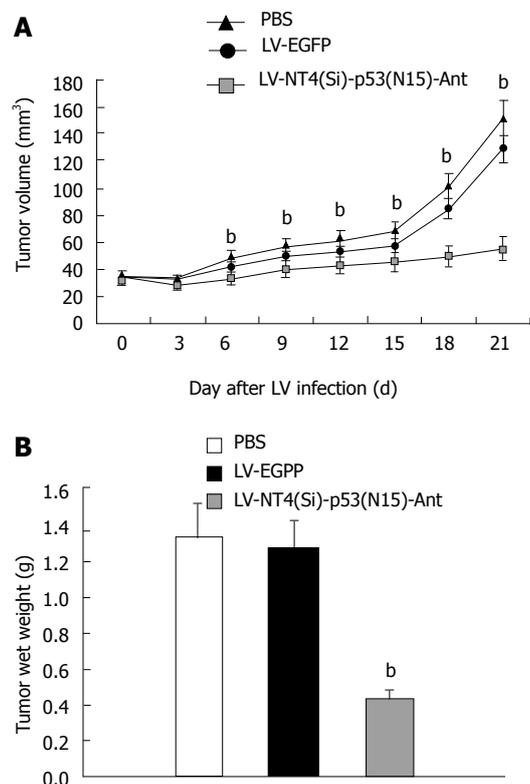
**Figure 5** Ultra-structure of HepG2 cells 48 h after infection with LV-EGFP (A, B) and LV-NT4(Si)-p53(N15)-Ant (C, D) ( $\times 5000$ ). A: Forty-eight hours after LV-EGFP infection, the cell membranes and nuclear membranes of HepG2 cells were intact; there were some crimples in the nuclear membranes and some microvilli on the surface of cells. The structure of mitochondria and ER was normal; B: Forty-eight hours after LV-EGFP infection, there were two HepG2 cells which had just completed division and this phenomenon suggested that LV-EGFP did not have a significant effect on cell growth and division; C: Forty-eight hours after LV-NT4(Si)-p53(N15)-Ant infection, it can be seen that cell membranes were incomplete and several fractures (arrows) were seen. Cell contents also appeared to be leaking. Mitochondria were swollen and showed vacuolization. Vacuoles were also seen under the nuclear membrane (arrowhead); D: Forty-eight hours after LV-NT4(Si)-p53(N15)-Ant infection, the remaining bare nuclei (arrow), after cytoplasm collapse can be seen.

and translocation in both prokaryotic and eukaryotic cells. NT4 is a member of the neurotrophin family, involved in controlling survival and differentiation of vertebrate neurons<sup>[16]</sup>. Besides the common features of neurotrophins, NT4 has many unusual features. Unlike other neurotrophins, the expression of NT4 is ubiquitous and less influenced by environmental signals<sup>[17]</sup>. In the present study, NT4 structure analysis showed that the human NT4 initiation codon was followed by a signal

peptide sequence, a pro-region and an apparent dibasic cleavage site, which was followed by the sequence of the mature NT4. The signal peptide sequence and pro-region of human NT4 are notably shorter (by = 243 bp) than those of other neurotrophins<sup>[18]</sup>. In our previous study, the pre-protein NT4 could be cleaved into the signal peptide and the mature protein by *Escherichia coli* (*E. coli*) endopeptidase. There is a native NaeI enzyme site in front of the site in the NT4 signal peptide,



**Figure 6** Flow cytometry analysis of HepG2 cells without treatment (A), 48 h after infection with LV-EGFP (B) and LV-NT4(Si)-p53(N15)-Ant (C) after stained with Annexin V and propidium iodide. The left lower quadrant represents normal cells (An-PI-), the right lower quadrant represents early apoptotic cells (An+ PI-), the right upper quadrant represents apoptotic cells and necrotic cells (An + PI +), the upper left quadrant represents early necrotic cells (An-PI +).



**Figure 7** Tumor growth curve (A) and tumor wet weight (B) after treatment with LV-NT4(Si)-p53(N15)-Ant. A: Tumor growth curve after treatment with LV-NT4(Si)-p53(N15)-Ant. Tumors grew rapidly in the PBS and LV-EGFP groups, while the tumor growth was significantly inhibited in the LV-NT4(Si)-p53(N15)-Ant group 3 wk after infection. There was no statistical significance between the PBS group (151.0 ± 50.1 mm<sup>3</sup>) and the LV-EGFP group (128.5 ± 45.3 mm<sup>3</sup>) in terms of tumor volume (*P* > 0.05). But the tumor volume in the LV-NT4(Si)-p53(N15)-Ant group (55.1 ± 18.7 mm<sup>3</sup>) was significantly less than that of the PBS and LV-EGFP groups (<sup>b</sup>*P* < 0.01); B: Tumor wet weight after treatment with LV-NT4(Si)-p53(N15)-Ant. There were no significant differences in the wet weight of tumors between the two control groups: PBS group 1.33 ± 0.93 g vs LV-EGFP group 1.27 ± 1.04 g (*P* > 0.05). But the wet weight of the LV-NT4(Si)-p53(N15)-Ant treatment group (0.43 ± 0.77 g) was significantly less than that of the two control groups (<sup>b</sup>*P* < 0.01).

convenient for the expression of other exogenous peptides in a secretory manner. It has been shown that it is not only feasible, but also improves the therapeutic effect<sup>[19]</sup>. In this study, we successfully constructed a LV containing the NT4(Si)-p53(N15) fusion gene with *in vitro* recombinant DNA technology.

To transfer the NT4(Si)-p53(N15)-Ant gene into the target cells and to achieve a consistent expression, selection of the gene transfer vector is a critical step. Lentiviruses, such as HIV and SIV, are a new generation of vectors and have many advantages over the traditional virus vectors, such as retroviral or adenovirus vector. These LVs can infect both dividing and non-dividing cells and efficiently integrate into the host DNA, thus providing stable transgene expression over several months<sup>[20,21]</sup>. At the same time, LV is very safe because all lentivirus accessory genes have been removed, virus production components have been split into 3 or 4 separate parts, and self-inactivating deletions have been introduced into the vector<sup>[21,22]</sup>. Since the aim of cancer gene therapy is to remove all cancer cells including those in a precancerous stage, only extensive gene transfer and long-term gene expression in cancer cells are appropriate. Thus, LV has a greater promise to become an ideal vector for gene delivery.

In this study, MTT assay showed that LV-NT4(Si)-p53(N15)-Ant could significantly induce HepG2 cell death 48 h after infection, LDH was significantly increased in the LV-NT4(Si)-p53(N15)-Ant treatment group but not in the LV-EGFP treatment group, suggesting that the cell membranes are damaged. Moreover, cytotoxicity occurred in a time-dependent manner. The ultra-structure of HepG2 cells, especially cell membranes and mitochondria, was significantly changed after infection with LV-NT4(Si)-p53(N15)-Ant, which was different from that during apoptosis and necrosis. In addition, FCM revealed that two cell death modes (apoptosis and necrosis) occurred 48 h after infection with LV-NT4(Si)-p53(N15)-Ant with necrosis being common and apoptosis being rare, revealing that LV-NT4(Si)-p53(N15)-Ant exerts its effect on HCC mainly by inducing necrosis of cell membranes.

thus ensuring the correct cleavage of therapeutic peptide, and making this signal peptide sequence highly

This is probably related to the process of LV infection, integration of the therapeutic gene into the host genome and gene expression.

The *in vivo* study also showed that LV-NT4(Si)-p53(N15)-Ant significantly inhibited tumor growth. Since p53(N15) contains overlapping sequences from the p53 mdm-2 binding domain, p53(N15)-Ant peptide may block the interaction of mdm-2 with other proteins. Recently, a study investigating the secondary structure of the p53 fusion peptide has revealed that p53(N15)-Ant peptide plays an important role in membrane interaction<sup>[15]</sup>, showing that p53(N15)-Ant peptide forms distinctive S-shape helix-loop-helix structures, which can rapidly disrupt cancer cell membranes by forming a toroidal-like pore, resulting in necrosis. The two mechanisms exist simultaneously.

In conclusion, we have established a way to express lentivirus-introduced p53(N15)-Ant 32-peptide. The growth of HepG2 cells can be significantly inhibited by LV-NT4(Si)-p53(N15)-Ant. LV-NT4(Si)-p53(N15)-Ant gene therapy may be used as a novel anticancer strategy. Further study on the biological characteristics of the p53 peptide is needed by transfecting LV-NT4(Si)-p53(N15)-Ant into other cancer cell lines.

## COMMENTS

### Background

Peptide has emerged as a new anticancer agent in recent years. Numerous reports suggest that many low molecular weight peptides possess an anticancer effect but have almost no cytotoxic effect on normal cells. p53(N15)-Ant has been considered a novel cancer therapeutic peptide because it induces cancer cell death and does not seem to be cytotoxic to normal cells.

### Research frontiers

It has been reported that p53 peptide, synthesized from residues 12-26 and fused with *Drosophila* carrier protein antennapedia (Ant), induces rapid tumor cell necrosis in all breast and pancreatic cancer cell lines tested, irrespective of the p53 status, whereas it shows a low cytotoxicity to normal cells. In this study, the authors constructed a novel recombinant lentivirus expression plasmid LV-NT4(Si)-p53(N15)-Ant and demonstrated its anticancer effect.

### Innovations and breakthroughs

The signal peptide plays an important role in protein targeting and translocation in both prokaryotic and eukaryotic cells. In this experiment, the authors used the NT4 signal peptide to enable the therapeutic peptide to be secreted. Lentivirus vectors were also employed. LV-NT4(Si)-p53(N15)-Ant was constructed and successfully cultured at a high titer and verified to induce necrosis of cancer cells.

### Applications

This work has established a way to express lentivirus-introduced p53(N15)-Ant 32-peptide. LV-NT4(Si)-p53(N15)-Ant dependent gene therapy may be a promising novel anticancer strategy. p53(N15)-Ant may represent a promising agent of gene therapy for hepatocellular carcinoma.

### Terminology

NT4 signal peptide and its pro-region are protein sequences that can direct the secretion of proteins and peptides from cells. Such a mechanism is thought to be helpful in fusion peptide gene therapy as it can enhance the therapeutic effect.

### Peer review

In this study, Song *et al* showed that the constructed lentivirus vector expressed in cancer cells could inhibit the growth of HepG2 cells. This vector can induce necrosis and apoptosis, and inhibit tumor growth *in vivo*. The study in general is significant and interesting.

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## Separate basolateral and apical phosphatidylcholine secretion routes in intestinally differentiated tumor cells

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Gotthardt D, Braun A, Tietje A, Weiss KH, Ehehalt R, Stremmel WR. Separate basolateral and apical phosphatidylcholine secretion routes in intestinally differentiated tumor cells. *World J Gastroenterol* 2009; 15(46): 5821-5826 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5821.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5821>

### Abstract

**AIM:** To investigate whether the secretion of phosphatidylcholine (PC) in intestinal mucus occurs by apical secretion or *via* basolateral excretion and to determine its subsequent passage across the tight junctions to the apical mucus.

**METHODS:** We addressed this question using the polarized intestinally differentiated tumor cell line CaCo-2 grown on filters to confluence in Transwell culture chambers. The released PC and sphingomyelin (Sph) from apical and basolateral media were analyzed by mass spectrometry.

**RESULTS:** The secreted PC species were identical in both compartments indicating the same intracellular origin of PC. However, PC secretion into the basolateral compartment was more effective, and the PC:Sph ratio in the basolateral compartment was significantly higher than that in the apical compartment ( $8.18 \pm 1.84$  vs  $4.31 \pm 1.22$ ,  $P = 0.01$ ). Both pathways were temperature sensitive and were unaltered in the presence of cyclosporine.

**CONCLUSION:** The data demonstrate the PC secretion capacity of CaCo-2 cells and indicate two separated apical and basolateral release mechanisms.

### INTRODUCTION

The presence of phosphatidylcholine (PC) in intestinal mucus serves to protect the underlying mucosa from attack by the commensal bacterial flora which is of particular importance in the colon<sup>[1]</sup>. Recently it was shown that the mucus PC content is markedly reduced (up to 70%) in patients with ulcerative colitis (UC), irrespective of whether acute inflammation of the colon is present - when compared to controls or patients with Crohn's disease<sup>[2,3]</sup>. This suggests its significance in the pathogenesis of UC. Accordingly, therapeutic replacement of PC in UC was shown to be very effective<sup>[4-6]</sup>.

Furthermore, it was shown that apical PC secretion occurs mainly in the ileum, and is stimulated by bile acids<sup>[7]</sup>. It is assumed that lumenally secreted PC -after absorption of bile acids in the terminal ileum- is attracted to the mucosal surface and moves along the colonic wall towards the rectum driven by colonic motility<sup>[1]</sup>. The exposure of PC to phospholipases of commensal colonic bacteria thins the PC layer towards the rectum, the "last lawn" with least PC content<sup>[1]</sup>. This could explain the continuous spread of inflammation starting from the rectum in UC where an intrinsic low PC content in the mucus is already present. Moreover, the fact that PC is mainly secreted in ileum<sup>[7]</sup> and that it is significantly impaired in UC<sup>[2,3]</sup> could explain the occurrence of pouchitis after colectomy due to the lack of protective

PC in mucus. In contrast, pouchitis is not observed after colectomy for familial adenomatous polyposis (FAP).

PC within mucus is arranged as lamellar structures facing the luminal and mucosal surface of the mucus as well as liposomal structures within the layer. Of all phospholipids within the mucus, 60%-90% are reported to represent PC species<sup>[8,9]</sup>. This indicates a specific PC secretion mechanism. The conventional view of such a transport process would suggest apical secretion *via* vesicular excretion or by ABC-transporters similar to MDR 2/3 mediated translocation of PC at the apical pole of hepatocytes<sup>[10,11]</sup>. However, Alpers *et al*<sup>[12]</sup> claimed a predominant basolateral secretion mechanism with secondary back diffusion across the tight junctions and subsequent release into the mucus space.

To evaluate this secretion mechanism, we used the intestinally differentiated tumor cell line CaCo-2 which revealed a polarized growth in tissue culture. We addressed the following questions: (1) Is PC secretion detectable in CaCo-2 cells? (2) Does it occur apically, basolaterally or on both sides? (3) Are these secretion pathways identical? (4) What are the kinetic characteristics?

## MATERIALS AND METHODS

### Cell culture

CaCo-2 cells were cultured routinely at 37°C, 5% CO<sub>2</sub> in DMEM (GIBCO Invitrogen, Karlsruhe, Germany), containing 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 1% sodium pyruvate (Sigma-Aldrich, Munich, Germany) and 1% nonessential amino acids (Sigma-Aldrich, Munich, Germany). Cells were seeded in 6-well Corning® Transwell® polyester membrane inserts (pore size 3.0 μm, membrane diameter 24 mm) (Corning COSTAR, USA), at a density of 10<sup>5</sup> cells/cm<sup>2</sup>. Cells were cultured for up to 21 d after reaching confluency to allow complete apical/basolateral polarization. Transepithelial electric resistance was checked regularly to verify appropriate polarization (World Precision Instruments, Berlin, Germany).

### Sampling for lipid measurement

For measurement of PC secretion, polarized CaCo-2 cells on filters were washed with FCS-free medium three times to remove extracellular lipids. They were then incubated in FCS-free medium containing 1% bovine serum albumin (BSA) (Sigma-Aldrich, Munich, Germany) for 16 h unless otherwise stated. Apical and basolateral media were collected and detached cells removed by centrifugation at 300 g for 5 min. All samples were stored at -80°C until further processing.

For experiments at different temperatures, CO<sub>2</sub>-independent medium (GIBCO Invitrogen, Karlsruhe, Germany) containing 1% w/v BSA was used. Where indicated, BSA was replaced by taurocholate (Sigma-Aldrich, Munich, Germany) at a concentration of 2.5 mmol/L. In addition, the following drugs were used at the following concentrations: (1) verapamil 500 μmol/L, (2) glibenclamide 50 μmol/L, (3) hydrocortisone

100 ng/mL, 500 ng/mL, and 1000 ng/mL, (4) cyclosporine 80 μmol/L (all Sigma-Aldrich, Munich, Germany). In experiments using hydrocortisone, cells were pre-incubated in regular medium containing the respective concentrations of hydrocortisone for 24 h to allow for changes in transcription. Experiments were performed in quadruplicate, except for the time-course experiments, which were performed in triplicate.

### Differential centrifugation

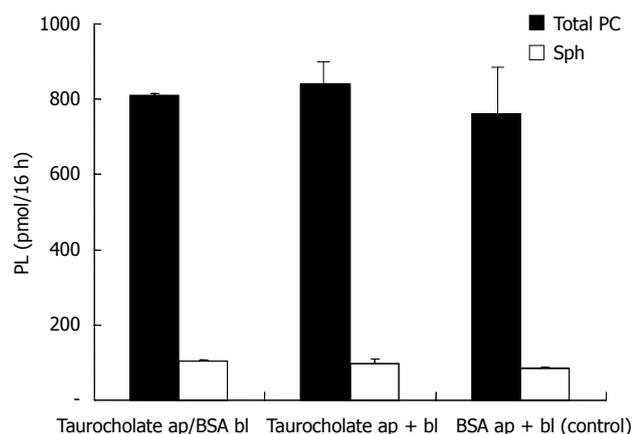
To retrieve particles of different sizes, the following standard setup was used. Cells were incubated as described above. The supernatant (SN) of the 300 g/5 min centrifugation was centrifuged at 1200 g for 20 min (P2). To obtain the next pellet the resulting SN was centrifuged at 10000 g for 30 min (P3). This SN was then centrifuged at 100000 g for 1 h (P4). Resulting pellets (P2-4) and the final supernatant were then subjected to lipid analysis.

### Lipid analysis

Lipids from the respective medium samples were extracted according to Folch<sup>[13]</sup>. Before lipid extraction, non-physiologic 1,2-didodecanoyl-sn-glycero-3-PC, 1,2-tetradecanoyl-sn-glycero-3-PC, 1,2-dieicosanoyl-sn-glycero-3-PC, 1,2-dido-cosanoyl-sn-glycero-3-PC as well as 1-pentadecanoyl-2-hydroxy-sn-glycero-3-PC, 1-arachidonyl-2-hydroxy-sn-glycero-3-PC and sphingomyelin (Sph) were added for internal standardization. The apical and basolateral media were dried with a speed vac. In brief 75 μL aqua dest and 50 μL of the lipid standard were added to each sample. 500 μL of methanol were added and the sample was vortexed for 5 min. 1000 μL chloroform was added and vortexed for 5 min. The supernatant of the 5 min/17000 g centrifugation was transferred to a new reaction tube. 300 μL aqua dest was added and vortexed again for 5 min. Phase separation was achieved by centrifugation at 500 g for 5 min. The lower organic phases were transferred to a separate glass tube and dried before resuspension for mass spectrometry.

### Nano-ESI tandem mass spectrometric analyses

Mass spectrometric analyses were performed with a triple quadrupole instrument [Finnegan MAT (San Jose, CA, USA) model TSQ 7000] equipped with a nano-electrospray source operating at a typical flow rate of 20 to 50 nL/min. The electrospray capillary was positioned at a distance of 0.5 to 1 mm away from the orifice of the heated transfer capillary which was maintained at 140°C. The instrument was used in the tandem MS mode. Argon was used as the collision gas at a nominal pressure of 2 mTorr. Crude lipid extracts, which had been dried completely, were resolved in 50 μL methanol/chloroform 2:1 (v/v), vortexed thoroughly, and infused into the capillary. The mass spectrometric resolution was set to about nominal mass resolution for the scan range of m/z 400 to 900. All specimens were analyzed in precursor ion scan mode for m/z 184. At least 150 consecutive scans, if possible more than 200 scans of 4-s duration, were averaged for each quantitative measurement.



**Figure 1 Release of phospholipids (PL) into the apical (ap) and basolateral (bl) media in the presence of BSA or taurocholate as solubilizing agents.** The figure illustrates no differences in the amount of PC and Sph secreted regardless of whether taurocholate was applied only apically (left), both apically and basolaterally (middle) compared to control (right) ( $P = ns$ ).

For quantification of the physiological PC and sphingomyelin species, regression curves were determined from the non physiological standards. Due to a general loss in sensitivity with increasing molecular weight, a set of four PC species was added as internal standards to bracket the profile of naturally occurring PCs. For the quantitative determination of Sph, we used a single internal standard. For correction of the measured values to the molar abundances, we used a calibration curve with the slope corresponding to that of PC.

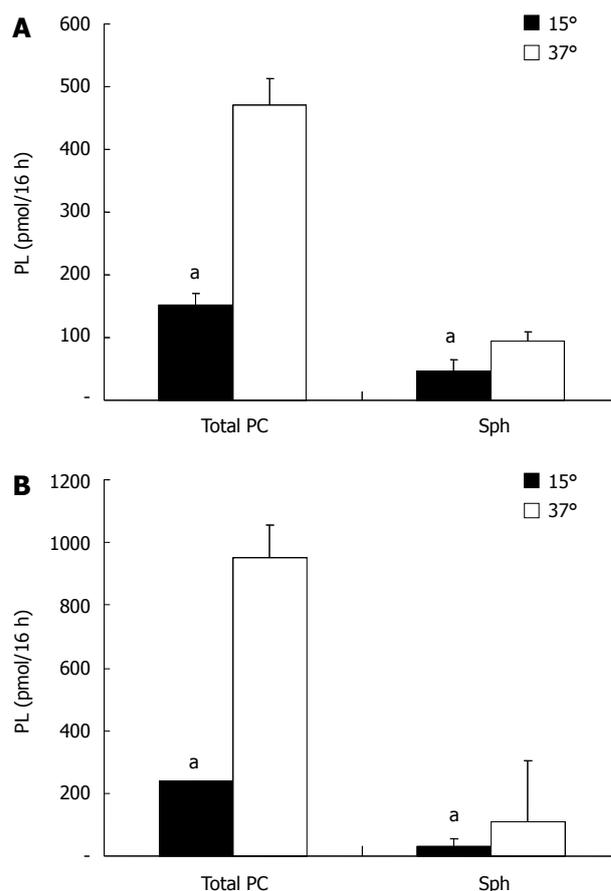
The most abundant physiological PC and Sph species were determined after comparison of all spectra. The molecular weights were related to the number of carbon atoms and double bonds with the help of a lipid data bank.

**Statistical analysis**

SPSS 16.0 was used for statistical analysis of the data. A Mann-Whitney-U-test was performed to analyze the differences between different conditions. Differences were considered significant, if  $P$  was  $< 0.05$ . In the time-course experiments a linear regression assuming a zero-crossing and applying the method of least squares was performed.

**RESULTS**

For polarization, CaCo-2 cells were grown on filters in Transwell culture dishes. Secretion of PC was examined in the apical and basolateral media containing, as lipid acceptors, taurocholate (2.5 mmol/L) or albumin (1% BSA). CaCo-2 cells showed PC secretion into both compartments. With regard to the acceptors of PC in both compartments (taurocholate or albumin), there was no difference recorded in the amount of PC or Sph released into the media (Figure 1). This indicated that under the experimental conditions employed, both acceptor molecules were present in excess and their capacity for PC and Sph solubilization was not

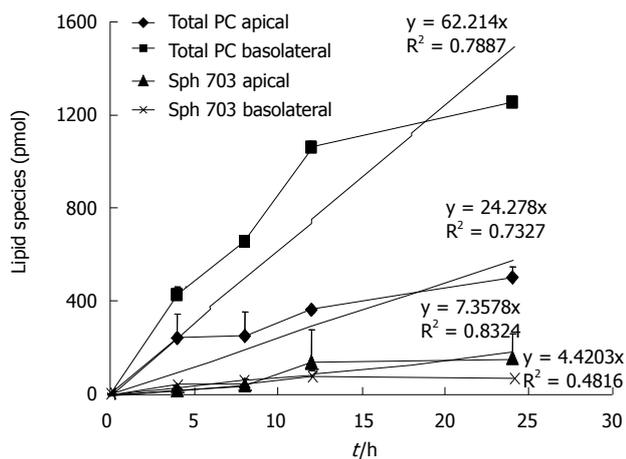


**Figure 2 Temperature dependency of phospholipid (PL) secretion.** Caco-2 cells were incubated either at 37°C or 15°C and secreted PC and Sph were measured. Both apical (A) and basolateral secretion (B) were drastically reduced at 15°C; apical PC by 68%, basolateral PC by 75%, apical Sph by 53% and basolateral Sph by 73% ( $^*P < 0.05$ ).

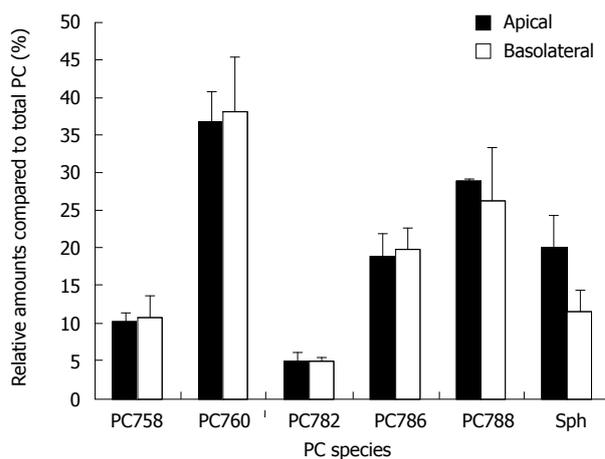
compromised. The secretion of PC and Sph in both compartments was temperature sensitive with a significant reduction in the secretion rate at 15°C. The degree of temperature-induced acceleration/deceleration of the transport rates was comparable for PC and Sph at each side. However, the total secretion rates towards the basolateral side were significantly more inhibited at 15°C than those at the apical side. This observed drop in secretion is typical for vesicular transport, because much lower transport rates in membrane carrier systems are expected at 15°C (Figure 2).

At 37°C the rate of secretion was significantly higher at the basolateral side compared to the apical side - also an indication of two different secretion capacities (Figure 3).

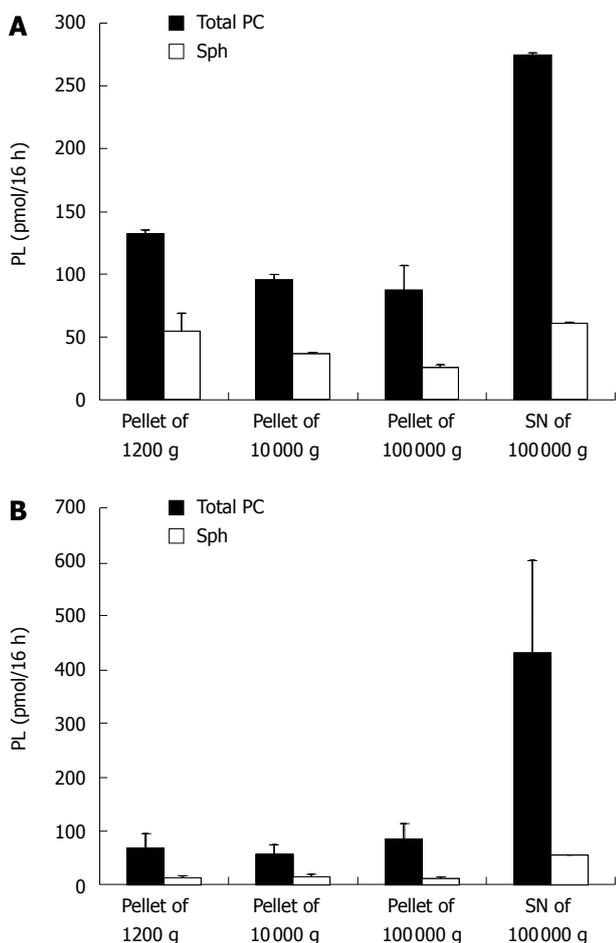
When the PC species and their secretion distribution on both sides were compared, they were virtually superimposable (Figure 4). This supports the concept that they originate from the same intracellular source. However, the relative fraction of Sph release compared to total PC release was significantly higher in the apical compartment. This corresponded to a higher level of Sph in the different pellets and the 100000 g supernatant of the apical compartment vs the basolateral compartment (Figure 5). Sphingomyelins are typical phospholipids of vesicular membranes in the secretory pathway<sup>[14]</sup>. A



**Figure 3 Time-course of phospholipid secretion.** An almost linear secretion up to 12 h with some decline in secretion towards 24 h was registered. The average secretion rate of PC was 24.3 pmol/h on the apical side and 62.2 pmol/h on the basolateral side. Sph was secreted apically at 4.4 pmol/h and basolaterally at 7.4 pmol/h. The regression line for each setup was drawn.



**Figure 4 Typical distribution pattern of phospholipid species secreted.** The PC species composition is similar in the apical and basolateral compartments. The relative amount of Sph 703 secreted is higher in the apical compartment ( $P < 0.05$  when comparing the ratio of Sph 703 to total PC in the apical and basolateral compartments).



**Figure 5 Distribution of secreted PL in pellets and supernatants obtained from the apical (A) and basolateral (B) compartments.** The majority of the PL is retrieved in the 100 000 g supernatant resembling a rather soluble fraction. In the medium of the basolateral compartment a reduced amount of PC and Sph could be found in the pellets, but more PC was found in the 100 000 g supernatant.

higher proportion of Sph compared to the “cargo”-PC is suggestive of smaller sized secretory vesicles (“containers”).

This is in line with the observation of a lower sensitivity towards temperature changes and a lower total apical PC secretion rate.

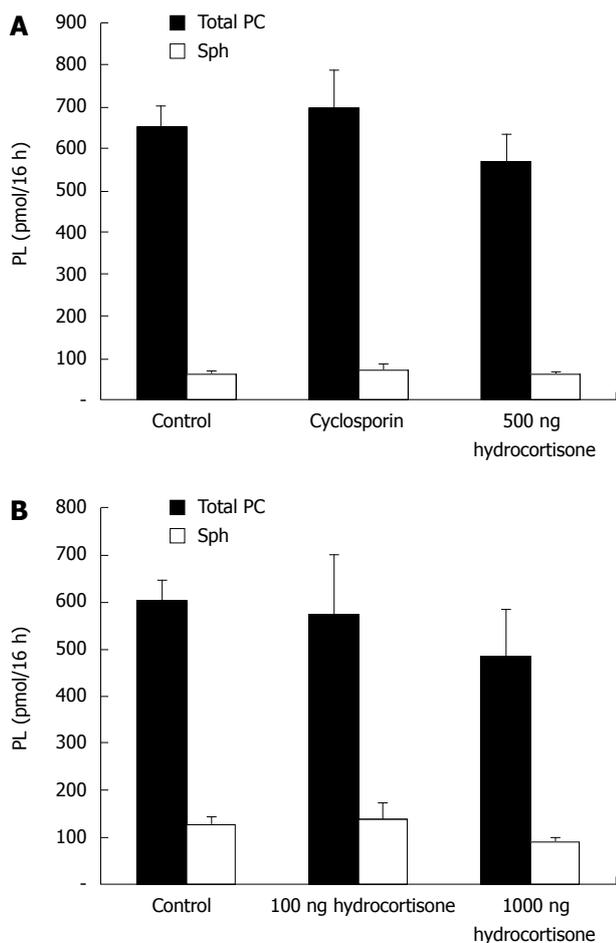
From a structural point of view, it would be easier for small sized vesicles to pass through the apical microvillous plasma membrane compared to large vesicles, which easily pass through the rather smooth basolateral side of the plasma membrane.

To further characterize the PC secretory pathway, we evaluated the effect of cyclosporine - a known inhibitor of ABC-transporters (Figure 6). No differences in transport rates were observed, underlying the hypothesis that PC secretion in intestinal mucosal cells more likely represents a vesicular excretion route. Similar results were obtained when other ABC-transport inhibitors (verapamil, glibenclamide and PSC833) were used (data not shown).

Since glucocorticoids are known to increase PC synthesis and excretion in the lung (surfactant), we evaluated their effects in CaCo-2 cells. Under the experimental conditions employed, no differences in secretion were detected (Figure 6). However, our experimental approach may have been too simple and unrefined to draw any further conclusions. It is possible that CaCo-2 cells do not express a glucocorticoid receptor.

## DISCUSSION

The small intestine is an organ which is highly active in transport-mainly from the luminal to the basolateral side. It manages the complete absorption of all food constituents. The fact that the intestinal mucosa also has a secretory pathway towards the luminal site is a rather unrecognized feature. Thus, the high secretion rate at the basolateral side could be explained by the essential role of intestinal mucosal cells in the absorption process, in particular of lipids (high lipid throughput). After uptake of fatty acids, monoglycerides, lysophospholipids, and fat soluble vitamins into the mucosal cell, they are



**Figure 6** Effect of cyclosporine and hydrocortisone on PL secretion. Cyclosporine and hydrocortisone did not affect the amount or distribution of PL secretion at the apical (A) and basolateral side (B) ( $P = ns$ ).

resynthesized to complex lipids (triglycerides, phospholipids), bound to apolipoproteins, and secreted at the basolateral side of mucosal cells *via* a vesicular pathway<sup>[14,15]</sup>. In contrast, at the microvillous side of the plasma membrane, it is sufficient to utilize a low capacity PC secretory pathway because mucus resembles a low PC-turnover compartment. Our experiments support the notion, that there are two separate PC secretion pathways in intestinal epithelial cells: a high capacity basolateral and a low capacity apical vesicular excretion route. This now needs confirmation by cellular imaging techniques and the use of native intestinal mucosal cells. The fact that 60%-90% of the phospholipids in mucus represent PC<sup>[8,9]</sup>, indicates a specific PC secretion mechanism. The highly enriched PC arrangement within lamellar membranes establishes the protective hydrophobic surface layer of the mucus<sup>[8,9]</sup>. In the case of ulcerative colitis, where a decrease in the mucus PC levels was detected, this may be due to impairment of PC synthesis, PC loading of secretory vesicles, apical membrane fusion and PC release into the mucus or adherence to mucus proteins. After verification of the data in native mucosal cell models, the experimental system can be used to examine whether the PC secretion mechanism can be modified for therapeutic purposes.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Intestinal mucus and the mucosal barrier are very important aspects of intestinal disease.

### Research frontiers

Impaired mucosal barrier function, its pathophysiology and its impact on inflammatory bowel disease have become a very important focus of research.

### Innovations and breakthroughs

The pathway by which phosphatidylcholine is secreted into the intestine to form mucus is not yet known. In this paper we provide evidence, that there are two separate secretion routes.

### Applications

The used methodology and the acquired results might be the basis for future work, to confirm the data *in vivo* and to elucidate the role of phosphatidylcholine in pathophysiology.

### Terminology

Phospholipids, mainly phosphatidylcholine are the constituents of intestinal mucus. Due to their hydrophobicity they are part of the intestinal barrier.

### Peer review

The reviewers appreciated the innovative approach and techniques of this work. These data need to be confirmed in other experimental setups.

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## Value of three-dimensional reconstructions in pancreatic carcinoma using multidetector CT: Initial results

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### Abstract

**AIM:** To evaluate the use of three-dimensional imaging of pancreatic carcinoma using multidetector computed tomography (CT) in a prospective study.

**METHODS:** Ten patients with suspected pancreatic tumors were examined prospectively using multidetector CT (Somatom Sensation 16, Siemens, Erlangen, Germany). The images were evaluated for the presence of a pancreatic carcinoma and invasion of the peripancreatic vessels and surrounding organs. Using the isotropic CT data sets, a three-dimensional image was created with automatic vascular analysis and semi-automatic segmentation of the organs and pancreatic tumor by a radiologist. The CT examinations and the three-dimensional images were presented to the surgeon directly before and during the patient's operation using the Medical Imaging Interaction Toolkit-based software "ReLiver". Immediately after surgery, the value of the two images was judged by the surgeon. The operation and the histological results served as the gold standard.

**RESULTS:** Nine patients had a pancreatic carcinoma

(all pT3), and one patient had a serous cystadenoma. One tumor infiltrated the superior mesenteric vein. The infiltration was correctly evaluated. All carcinomas were resectable. In comparison to the CT image with axial and coronal reconstructions, the three-dimensional image was judged by the surgeons as better for operation planning and consistently described as useful.

**CONCLUSION:** A 3D-image of the pancreas represents an invaluable aid to the surgeon. However, the 3D-software must be further developed in order to be integrated into daily clinical routine.

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**Key words:** Pancreatic carcinoma; 3D-reconstruction; Multidetector computed tomography; Pancreatic carcinoma invasion; Segmentation

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### INTRODUCTION

Pancreatic carcinomas are the fourth most frequent cause of death from cancer worldwide. The prognosis remains poor with a relative 5-year survival rate of about 5%. The only procedure resulting in significantly longer survival is R0 resection with adjuvant chemotherapy<sup>[1]</sup>.

Computed tomography (CT) is considered the method of choice for the detection and preoperative staging of pancreatic carcinomas<sup>[1-6]</sup>. Currently, 16- and 64-row spiral CTs are increasingly becoming available, and these offer improved local resolution over older models. The high-resolution data sets gained from these also offer the option of three-dimensional image post-processing<sup>[7,8]</sup>.

In planning an operation, the location of the pancreatic tumor relative to the surroundings, surgically relevant vessels and adjacent organs is of utmost importance to the surgeon. The option of being able to assess the tumor volume in relation to the pancreatic tissue can also represent further valuable information.

In liver surgery, three-dimensional imaging is increasingly being used for the interactive planning of surgery in complex partial liver resections and living donors. Special procedures are used which - in addition to visualization - permit volumetric assessment of the primary data. Systems which can be used to gain additional information from the layer data include HepaVision2 (MeVis GmbH, Bremen, Germany)<sup>[9]</sup>, LiverLive (Navidez Ltd., Ljubljana, Slovenia), and Medical Imaging Interaction Toolkit (MITK) ReLiver (Deutsches Krebsforschungszentrum, Heidelberg, Germany).

The prerequisite for such procedures is the pre-processing step of segmentation, during which interesting regions of the image are marked on the section images. In a previous study, we have already shown that three-dimensional imaging of pancreatic tumors with semi-automatic segmentation is possible analogously to hepatic imaging<sup>[10]</sup>.

The aim of this study is to test in a prospective, clinically controlled study, whether the three-dimensional images of the pancreas and the surrounding structures gained using the CT data sets are useful to the surgeon in planning operations and for intraoperative orientation in patients with pancreatic carcinoma.

## MATERIALS AND METHODS

Between March 2006 and August 2006, ten patients were included in the study with suspected operable pancreatic carcinoma. The criterion for inclusion in the study was urgent clinical suspicion of a pancreatic carcinoma. The study had the approval of the local ethics committee.

All patients underwent 16-row multislice CT (Somatom Sensation 16, Siemens, Erlangen, Germany), using the hydro technique with administration of Ultravist 370<sup>®</sup> (Schering, Berlin, Germany).

The operative findings and the histological results obtained during the operation served as the gold standard.

The evaluation was carried out by two experienced radiologists using a standardized evaluation form. This covered the location, size, and peripancreatic extent of the tumor. The resectability of the carcinoma was also assessed.

### 16-row spiral CT

Patients were examined using 16-row spiral CT (Somatom Sensation 16, Siemens, Erlangen, Germany) using the hydro technique. This involves distension of the stomach and duodenum using 1.5 L of still water, intestinal paralysis through intravenous administration of 40 mg N-butylscopolaminium bromide (Buscopan<sup>®</sup>) and positioning the patient on the right side at an angle of 30°<sup>[4,11]</sup>.

Firstly, a native spiral CT of the abdomen was carried out. Then an examination was carried out in the arterial

Table 1 Examination parameters

	Native CT	Arterial phase	Portal venous phase
Anode voltage (kV)	120	120	120
Anode current (mAs)	140	170	185
Detector collimation	1.5 (slice: 6 mm)	0.75 (slice: 3 mm)	0.75 (slice: 3 mm)
Table feed (mm)	24	12	12
Reconstructed slice thickness (mm)	6	2/1	1.3/3; 2.1/0.5
Delay (s)		8	35

phase (delay 8 s) and in the portal venous phase (delay 35 s) using the Combined Application Reduced Exposure bolus technique with 120 mL contrast medium (Ultravist<sup>®</sup> 370, Schering AG, Berlin, 5 mL/s) (Table 1).

### Three-dimensional imaging

The data sets obtained from CT were transmitted *via* an internal data connection (Chili, Heidelberg, Germany) to the Department of Medical and Biological Informatics at the German Cancer Research Center. There, the vascular structures were transferred to a semantic model in which the vascular branches could be individually colored, shown, or removed from view. All segmentations were carried out by a radiologist using the software program MITK ReLiver. During the process, the liver, kidneys, duodenum, stomach, pancreas, and pancreatic tumor were segmented using interactive segmenting tools in the section images. It was found to be particularly time-saving to use a combination of an interactive regional growth procedure and a form-based interpolation procedure<sup>[12]</sup>. On this basis, it was possible to reduce the interaction time to a necessary minimum. Subsequently, the data from the vascular trees, the individually segmented organs and the tumor were fused into a three-dimensional scene.

These freely rotatable images were presented to the surgeon before and during the operation above the OP field, analogous to the three-dimensional image of the liver<sup>[11]</sup>. The surgeon also saw the conventional CT images before the operation, which were also available to him in the operating theater on a monitor. After the operation, on a questionnaire, the surgeon assessed the value of the three-dimensional image compared with that of the conventional CT images (Table 2).

The questionnaire included comments about representation of the tumor, tumor location, tumor invasion of the vessels and surrounding organs, how well the imaging material matched the operative site, the manageability of the images, a score by which the surgeon could grade how secure he felt looking at the CT or three-dimensional images, and possible changes in the operative strategy.

The individual points on the questionnaire were graded with marks ranging from 1 for excellent to 5 for very bad.

### Statistical analysis

Using the Wilcoxon test, we examined whether the

**Table 2** Questionnaire put to the surgeon assessing the value of the three-dimensional image *vs* conventional CT images

Depiction of the tumor	
How well can the tumor localization be assessed?	1 = excellent; 2 = good; 3 = mediocre; 4 = bad; 5 = very bad
How well can vascular invasion be assessed?	
How well can organ invasion be assessed?	
How comfortable are you with the image?	
How well does the imaging material match the operative situation?	Completely; partially; not at all
How manageable was the 3D view in comparison with CT?	Less complicated; equally complicated; more complicated
Was the operating strategy changed due to the images?	Yes; no

**Table 3** Three-dimensional reconstruction *vs* CT

Patients	Tumor image		Tumor localization		Vascular invasion		Organ invasion		Feel-good score	
	CT	3D	CT	3D	CT	3D	CT	3D	CT	3D
Φ	2.2	1.4	1.8	1.6	2.2	2.2	2.0	1.6	2.4	1.2
<i>P</i> -value	0.157		0.564		1.0		0.157		0.063	

1 = excellent, 2 = good, 3 = mediocre, 4 = bad, 5 = very bad.

differences between the CT and three-dimensional images were statistically relevant. The *P* values were calculated using SPSS for Windows XP.

## RESULTS

In nine patients, a pylorus-preserving Whipple procedure was carried out. In one patient, explorative laparotomy was performed with placement of a biliodigestive anastomosis and a gastrojejunostomy, since the patient's advanced age (80 years) made a portal vein resection seem too stressful.

Nine patients had adenocarcinomas of the pancreas and one had a serous cystadenoma. All carcinomas had grown beyond the margins of the organ and invaded the peripancreatic lipid tissue and the duodenum, and were thus staged histologically as T3. In one patient, invasion of the superior mesenteric vein was found intraoperatively.

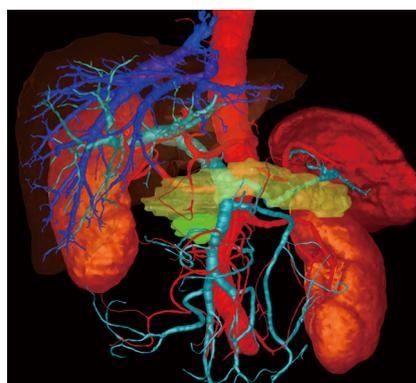
### Three dimensional imaging

The three-dimensional images of the pancreatic tumors, as evaluated on the questionnaire, were found to be graded more favorably with regard to assessment of tumor imaging, location, and invasion of the surrounding organs, and to the score indicating how secure the surgeon felt in assessing the images than the conventional CT examination. However, the differences were not statistically significant (*P* = 0.157, 0.564, 0.157, 0.063, respectively). In evaluating vascular invasion, the results were equally good for both procedures (*P* = 1.0) (Table 3).

The three-dimensional imaging correctly and completely matched the operative site in all cases.

The manageability of the three-dimensional image was deemed to be less complicated in four out of five cases and in one case to be equally good.

In one case the surgeon found that the three-dimensional image demonstrated much more clearly than the conventional CT image that there was no invasion of the portal vein (Figure 1). In a second case, extensive inva-



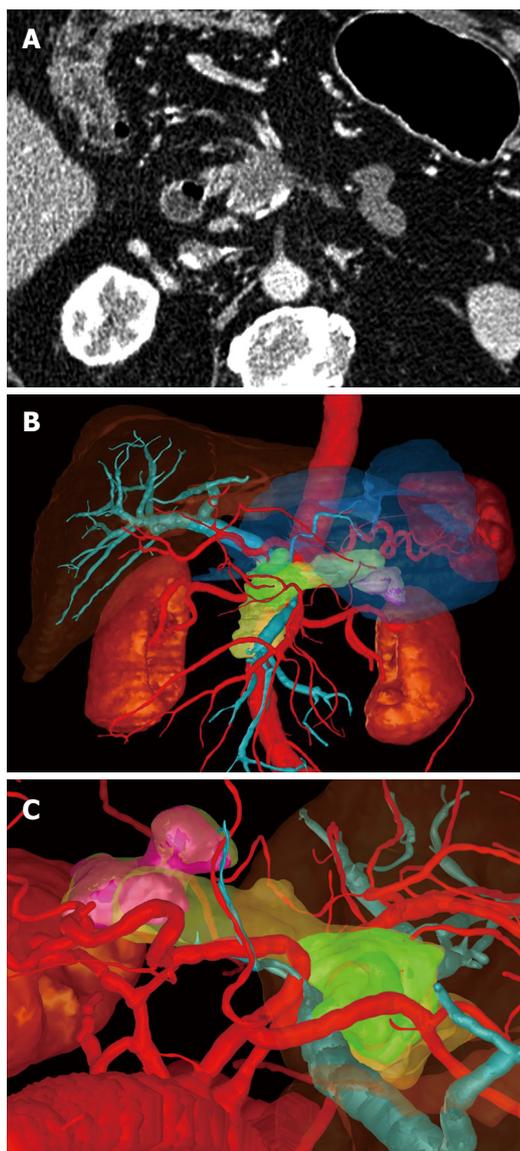
**Figure 1** Lateral view of the three-dimensional model of a pancreatic carcinoma (pale green) in the processus uncinatus. There is contact between the tumor and the superior mesenteric vein but no invasion.

sion of the portal vein by the tumor could be established in both types of image, but the extent of the invasion was demonstrated to the surgeon better in the three-dimensional image, enabling the tumor to be classified as a resectable stage T3 and explorative laparotomy was performed. Intraoperatively, this long-distance invasion was confirmed but no tumor resection was carried out due to the patient's advanced age; vascular replacement of the portal vein was deemed too stressful (Figure 2).

## DISCUSSION

Despite all the progress made in diagnosis and surgery, the prognosis for pancreatic carcinomas today is still poor. Surgical resection is the treatment of choice as a curative approach to pancreatic carcinomas. Thus, the main aim of diagnosis consists of correctly evaluating the surgically relevant vessels with regard to possible invasion in order to be able to safely distinguish between resectable and non-resectable pancreatic carcinomas.

Spiral CT is considered to be state of the art in the diagnosis of pancreatic carcinoma and in the evaluation of resectability in most centers<sup>[6]</sup>. The sensitivity of spiral



**Figure 2** Patient with stage T3 pancreatic carcinoma with portal vein invasion; the preoperative assessment was correct. A: Axial slice through tumor; B: Three-dimensional reconstruction, frontal view (tumor = pale green); C: Three-dimensional reconstruction, cranial view (tumor = pale green).

CT, as cited in the literature, for evaluating resectability in pancreatic carcinoma is between 81% and 96.3%<sup>[4,11,13-15]</sup>.

Due to the particular anatomical relationship between the pancreas and the surrounding vessels, three-dimensional reconstructions are helpful in presenting additional information about this relationship<sup>[16,17]</sup>.

For the surgeon, it is valuable to be able to see the tumor, both by itself and in the context of its surrounding structures, in three dimensions from all sides whereby particular interest is obviously accorded to the presence and extent of contact to the relevant vessels or surrounding organs. In addition, the surgeon can more easily assess the tumor volume in relation to healthy pancreatic parenchyma. With the aid of a freely rotatable, three-dimensional image, he can picture the extent of venous invasion more clearly (even before the operation) than is possible with two-dimensional, axial, coronal and sagittal images.

Thus, the surgeon is provided with as detailed a picture as possible of the field of operation even before the operation takes place. As far as we know, there are no studies so far available assessing the clinical value of three-dimensional image processing of pancreatic tumors. To date, the Harvard Medical School has produced reconstructions of the pancreas and the neighboring vascular systems, but has not clinically evaluated them<sup>[18]</sup>.

Three-dimensional reconstruction already has a firm place in orthopedics and neurosurgery, where only unmoving and fixed anatomical structures are represented without any great range of variation.

The use of this method in visceral surgery is more difficult, since the organs can move and in some cases are shifted and reshaped during respiration and since the rate of anatomical variability is high<sup>[10]</sup>.

In the field of hepatic imaging and in the context of living liver donors and before complex partial liver resections, three-dimensional imaging of the liver, hepatic vessels, and bile ducts has managed to become established in some centers. In this case, in addition to visualization, the volumetry of various liver sections is of interest. Moreover, the three-dimensional reconstruction can be used preoperatively to consider various resection options and to evaluate their technical feasibility with regard to vascular and bile duct anatomy and to the expected liver volume after surgery.

The prerequisite for three-dimensional imaging of the primary data is semiautomatic segmentation of the structures of interest such as pancreas, tumor, stomach, liver, spleen, kidneys and any cysts that might be present. In pancreatic tumors, however, the segmentation process is particularly time-consuming and problematic, since the margins between organ tissue and inflammatory processes or of the tumor and surrounding structures are hard to recognize due to growth beyond the organ borders. In addition, the preparation of data includes all important organs and vascular structures in the abdominal cavity in order to provide the surgeon with a maximum amount of context information so that possible problems in exposure of the pancreas can be included in the surgical planning.

This means that the reconstruction procedure for visualizing pancreatic carcinomas today still requires a great amount of effort both technically and in terms of time required. In addition, an objective and precise localization of the tumor is not possible because segmentation is always conducted according to the subjective interpretation of the radiologist.

An automatic image processing procedure cannot be used for segmenting the tumor since the differences in thickness between organ tissue and tumor are sometimes very slight and cannot be recognized by a computer program. However, there are very promising approaches to segmenting the organs which make a largely automatic procedure seem tangible. Statistical form models learn the mean organ form with its variants from training data. Using even the current models, it can be shown that the work of segmentation can be significantly reduced for the liver<sup>[19]</sup>.

For vessels, no automatic segmentation program

exists that can independently mark vascular cross-sections by using the good contrast provided by the various contrast medium phases of the CT examination and the high contrast differences associated with it. Three-dimensional imaging of the vessels, moreover, only depicts the inside of the visualized vessel since the contrast medium that is used for processing the reconstruction only fills the vascular lumen.

Tumor invasion of the relevant vessel can thus only be indirectly visualized, just as with two-dimensional CT images, e.g. on the basis of a sudden difference in vessel caliber or complete vascular occlusion.

In the present study, three-dimensional post-processing of the data sets was highly acclaimed by the surgeons. Analysis of the questionnaire showed that the three-dimensional image was graded better in most points than conventional CT images. Only evaluation of the vessels was graded equally for both procedures.

The so-called “feelgood score” was better for the three-dimensional image than the two-dimensional image in all the cases.

This explains why in two cases the surgeon indicated that he found extensive portal vein invasion and exclusion of portal vein invasion to be much clearer for him on the three-dimensional image than on the conventional CT images.

The manageability of the three-dimensional image in comparison to the conventional CT images was deemed less complicated in four out of five cases. In all the cases the findings presented in the three-dimensional image material matched the intraoperative situation completely.

In complex liver surgery, three-dimensional visualization has already been shown to meet with high acclaim by surgeons. Its advantage consists in providing the surgeon with information about vascular anatomy that is indispensable for planning living liver transplantation or in providing additional information about the percentage of residual hepatic tissue following resection.

In their clinical study of the value of three-dimensional data sets before complex liver resections and before living liver donations, Fischer and Lamade found that although three-dimensional presentation did not lead to an improved segment allocation of hepatic tumors in comparison to axial sections, the three-dimensional presentation meant that the position of the tumor within the liver as marked on a liver model was significantly improved. It was shown that the three-dimensional presentation did indeed lead to improved OP representation. Overall, three-dimensional presentation led to approaching a 31% higher precision in tumor localization and improvement in resection recommendations.<sup>[20,21]</sup>

This study also illustrated the disadvantage of this method. The three-dimensional representation of the findings can only be as good as the primary data sets from CT in conjunction with the experience of the radiologist in evaluating CT data. If contact between the tumor and the encircling wall of a vessel can be visualized over a long distance on CT, this will also be visible in the three-dimensional image and can be interpreted in most cases, and in both procedures, as invasion of the relevant vessel.

The three-dimensional reconstruction cannot improve on the examination; it can only present the situation in a more plastic form.

A three-dimensional image will thus generally not improve the assessment of the resectability of pancreatic carcinomas. Overall, this new procedure, however, seems to provide a good aid in preoperative planning for the surgeon in surgical therapy of pancreatic carcinoma.

It remains to be seen whether the method, which still involves a great deal of effort, will stand the test of practical daily routine or if the time and technical input necessary for post-processing the image material is too high to be integrated practically into clinical routine or if these factors can be significantly reduced.

One restriction on the present study is the very small case number. The calculated *P* values showed no significant differences. Thus further studies should be conducted with a larger number of patients.

In summary, it can be said that three-dimensional imaging of pancreatic carcinomas with the surrounding vessels and organs is currently constrained by the great deal of effort involved, but it does primarily provide the surgeon with valuable additional information.

## COMMENTS

### Background

In planning a resection of a pancreatic carcinoma, the location of the pancreatic tumor relative to the surroundings, surgically relevant vessels and adjacent organs is of utmost importance to the surgeon.

### Research frontiers

The aim is to provide better visualization of pancreatic carcinoma and peripancreatic vessels for the surgeon in the preoperative period.

### Innovations and breakthroughs

In complex liver surgery, three-dimensional visualization has already been shown to meet with high acclaim by surgeons, but this study is the first which used this technique in pancreatic carcinoma patients. Its advantage consists in providing the surgeon with information about peripancreatic vascular anatomy that is helpful for planning the resection of a pancreatic carcinoma.

### Applications

A three-dimensional image of the pancreas represents an additional, valuable aid to the surgeon. However, this method is still time-consuming. The software must be further developed to allow further automation of the segmentation to enable it to be integrated into daily clinical routine.

### Terminology

Segmentation: To circumscribe a freehand region-of-interest on each single CT-slice, where, for example, the tumour or the pancreas is seen.

### Peer review

This is a pilot study of 10 consecutive patients with suspected pancreatic carcinoma subjected to preoperative 3-D spiral CT reconstruction. This is the first such clinical study utilizing this technique.

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## Iodized oil uptake assessment with cone-beam CT in chemoembolization of small hepatocellular carcinomas

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iodized oil uptake in 22 of the lesions (sensitivity 85%). The degree of iodized oil uptake was overestimated (9%, 2/22) or underestimated (14%, 3/22) on spot image in five nodules compared with that of cone-beam CT.

**CONCLUSION:** Cone-beam CT is a useful and convenient tool for assessing the iodized oil uptake of small hepatic tumors (< 3 cm) directly after TACE.

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**Key words:** Chemoembolization; Computed tomography; Hepatocellular carcinoma; Liver

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### Abstract

**AIM:** To evaluate the utility of assessing iodized oil uptake with cone-beam computed tomography (CT) in transarterial chemoembolization (TACE) for small hepatocellular carcinoma (HCC).

**METHODS:** Cone-beam CT provided by a biplane flat-panel detector angiography suite was performed on eighteen patients (sixteen men and two women; 41-76 years; mean age, 58.9 years) directly after TACE for small HCC (26 nodules under 30 mm; mean diameter, 11.9 mm; range, 5-28 mm). The pre-procedural locations of the tumors were evaluated using triphasic multi-detector row helical computed tomography (MDCT). The tumor locations on MDCT and the iodized oil uptake by the tumors were analyzed on cone-beam CT and on spot image directly after the procedures.

**RESULTS:** All lesions on preprocedural MDCT were detected using iodized oil uptake in the lesions on cone-beam CT (sensitivity 100%, 26/26). Spot image depicted

### INTRODUCTION

Transarterial chemoembolization (TACE) is a regional therapeutic modality that has become an accepted treatment for unresectable hepatocellular carcinoma (HCC). Triphasic multi-detector computed tomography (MDCT) is commonly used for the detection and preprocedural localization of hypervascular HCCs greater than 1 cm in diameter<sup>[1-3]</sup>.

Diagnostic angiography using digital subtraction angiography (DSA) is performed before TACE in order to detect hypervascular HCCs. Occasionally, the small neoplastic foci are uncertain, and difficult to detect. Some studies have reported that the detection rate of small HCCs, less than 3 cm, in DSA was about 70%<sup>[4,5]</sup>, and other studies showed higher sensitivity in nodule detection in helical biphasic CT than in DSA<sup>[6]</sup>. This discrepancy can make it difficult to determine whether or not the same nodules exist on MDCT in cases of small tumor nodules.

Iodized oil retention pattern after TACE is a post-treatment prognostic marker and significant factors affect

local recurrence<sup>[7-9]</sup>, however, a method for evaluating this immediately after TACE, is to take a spot image. Follow-up unenhanced CT is usually performed within 1 mo after TACE, but it is sometimes difficult to determine whether there is washout of iodized oil uptake or initial failure of iodized oil uptake when there is partial iodized oil uptake in a lesion on the first follow-up CT. The exact method for comparing iodized oil retention in hypervascular nodules on preprocedural MDCT is to check the postprocedural CT directly after TACE and for this the patient must be transported from the angiography suite to the nearest CT scanner.

Cone-beam CT is a new technology provided by the combined angiography/CT suite that uses flat-panel detector (FD) technology. It provides images similar to those of CT and is able to obtain 3D reconstructions such as multiplanar reformat (MPR), maximum intensity projection (MIP), and 3D volume rendering (VR) with these data sets.

The purpose of this study was to evaluate the clinical value of cone-beam CT in the assessment of iodized oil uptake in TACE for small HCCs.

## MATERIALS AND METHODS

### Patients

From March 2006 to June 2006, eighteen patients (sixteen males and two females; 41-76 years; mean age, 58 years) with small HCCs (26 nodules; mean diameter 11.9 mm; range, 5-28 mm), underwent cone-beam CT and TACE consecutively. All patients had underlying liver cirrhosis. The diagnosis of HCC was made clinically in these patients by using typical imaging findings on MDCT and an elevated level of serum alpha fetoprotein. The size and location of the tumors were evaluated with triphasic MDCT before TACE (Figures 1 and 2). Eleven patients had previously undergone TACE for multiple nodules. The first TACE was performed in seven patients who were not candidates for surgery. According to our institutional guidelines, institutional review board approval was not required for this report.

### TACE

Angiography was conducted using the AXIOM Artis FD Biplane Angio suite with cone-beam CT (DynaCT, Siemens Medical Solutions, Erlangen, Germany). For angiography, a 5-Fr catheter was inserted using the Seldinger technique from the right common femoral artery into the celiac trunk. After confirming the location of the tumor and its feeding artery, a 3-Fr 100-cm-long microcatheter (Progreat; Terumo, Tokyo, Japan) with a 0.016-inch, 150-cm long guidewire covered by hydrophilic polymer (Radiofocus; Terumo, Tokyo, Japan), was advanced through a 5-Fr catheter into the peripheral portion of the feeding artery as close to the lesion as possible (Figures 1 and 2). If tumor staining or feeders were uncertain, cone-beam CT hepatic arteriography (cone-beam CTHA) was performed (contrast media injection rate was 2 mL/s; X-ray delay used in 1.5 s). After these baseline studies, TACE using an emulsion of epirubicin hydrochloride (Pharmorubicin; Ildong, Seoul, Korea) dissolved in me-

Table 1 Degree of iodized oil uptake by the tumors

Degree	Spot image	Cone-beam CT
Excellent	11	16
Good	8	7
Poor	3	3
Total	22	26

Discordance between the two modalities-5 nodules [over-: 9% (2/22), underestimated: 14% (3/22) in spot image]. CT: Computed tomography.

glumine ioxitalamate (Telebrix; Guerbet, Aulnay-sous-Bois, France) mixed with iodized oil (Lipiodol Ultra Fluid; Guerbet, Aulnay-sous-Bois, France) was performed. The end point of TACE was when an "oily portogram" was achieved. Finally, spot image and cone-beam CT were performed to determine the deposition of iodized oil.

### Cone-beam CT

Cone-beam CT acquisition was obtained using the following parameters: 10-s rotation; 0.4° increment; 1024 × 793 matrix in projections at zoom 0 after resampling; 217° total angle; and 11°/s, 27 frames/s, system dose 0.36 μGy/pulse, total 273 projections. The image reconstruction was performed on a commercially available dedicated workstation (X-Leonardo with DynaCT; Siemens Medical Solutions). The volume dataset was displayed on the monitor in the MPR.

### Image analysis

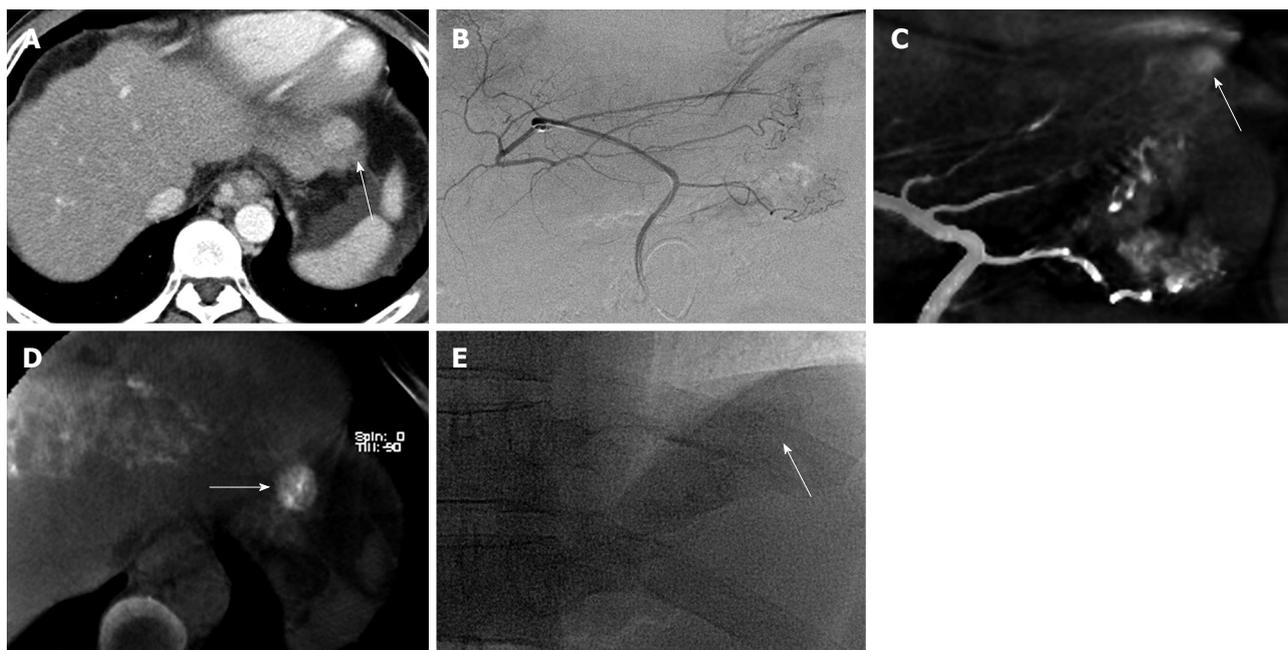
The tumor locations, as shown on MDCT, iodized oil uptake by the tumor in the lesion on cone-beam CT and spot image, were determined directly after the procedures were analyzed. The enhancing tumor locations as shown on MDCT and iodized oil uptake by the tumors were compared. The degree of iodized oil uptake by the tumor (excellent, 100% deposition of iodized oil in the lesion; good, 51%-99%; poor, ≤ 50%) on cone-beam CT and spot image were also compared. The spot images were evaluated at the workstation, while cone-beam CT images were evaluated by images created by MPR, and MIP (maximum intensity projection). All images were graded by two radiologists, and decisions were reached by consensus.

## RESULTS

Cone-beam CT was successfully completed in all study patients. It only took about 5 min to obtain MPR images. Cone-beam CT visualized the iodized oil uptake in all the nodules (26/26, sensitivity 100%), but spot image only visualized iodized oil uptake in 22 of the lesions (sensitivity 85%) (Figures 1 and 2).

### Degree of iodized oil uptake in tumors

The degree of iodized oil uptake was somewhat different in the two modalities (Table 1). Cone-beam CT depicted iodized oil uptake in the nodules more clearly than spot image. Two nodules were not seen in spot image but were clearly evaluated with cone-beam CT. There was discordance between the two modalities in 5 nodules and



**Figure 1** A 47-year-old man with hepatocellular carcinoma in S2 for 2nd TACE. A: Preprocedural late arterial phase MDCT scan reveals hypervascular HCC near the diaphragm (arrow); B: Left gastric angiogram shows aberrant left hepatic artery with no tumor staining in suspected area; C: Tumor staining (arrow) and feeder artery are found after acquiring MIP image from cone-beam CT hepatic arteriography (Cone-beam CTHA); D: Cone-beam CT directly after TACE shows good grade iodized oil uptake in S2 (arrow); E: Spot image shows subtle lipiodol uptake near the left hemidiaphragm (arrow), but nodular iodized oil uptake is not observed.

**Table 2** Degree of iodized oil uptake by the tumors according to size

Size	Degree of iodized oil uptake in spot image			Degree of iodized oil uptake in cone-beam CT		
	Excellent	Good	Poor	Excellent	Good	Poor
≤ 1	5	3	1	7	4	
≤ 2	6	4	2	8	2	3
≤ 3		1		1	1	
Total	11	8	3	16	7	3

Discordance in degree of iodized oil uptake occurred in nodules less than 2 cm (3 nodules less than 1 cm).

iodized oil uptake was over-(9%, 2/22) or underestimated (14%, 3/22) in spot image compared with cone-beam CT.

The iodized oil uptake in 4 nodules was not seen in spot image, two nodules were less than 1 cm, one was between 1 cm and 2 cm, and one was over 2 cm. The discordance between the two modalities occurred in nodules less than 2 cm (Table 2).

**Nodules with atypical enhancement patterns in MDCT**

Three nodules showed atypical enhancement patterns in MDCT, and additional dynamic magnetic resonance imaging (MRI) was performed for one nodule (Figure 2). Their feeders were uncertain on selective right or left hepatic arteriography, but were detected with cone-beam CTHA. Enhancement patterns and iodized oil uptake in the nodules are shown in Table 3.

**DISCUSSION**

Several treatment options for small HCCs have been

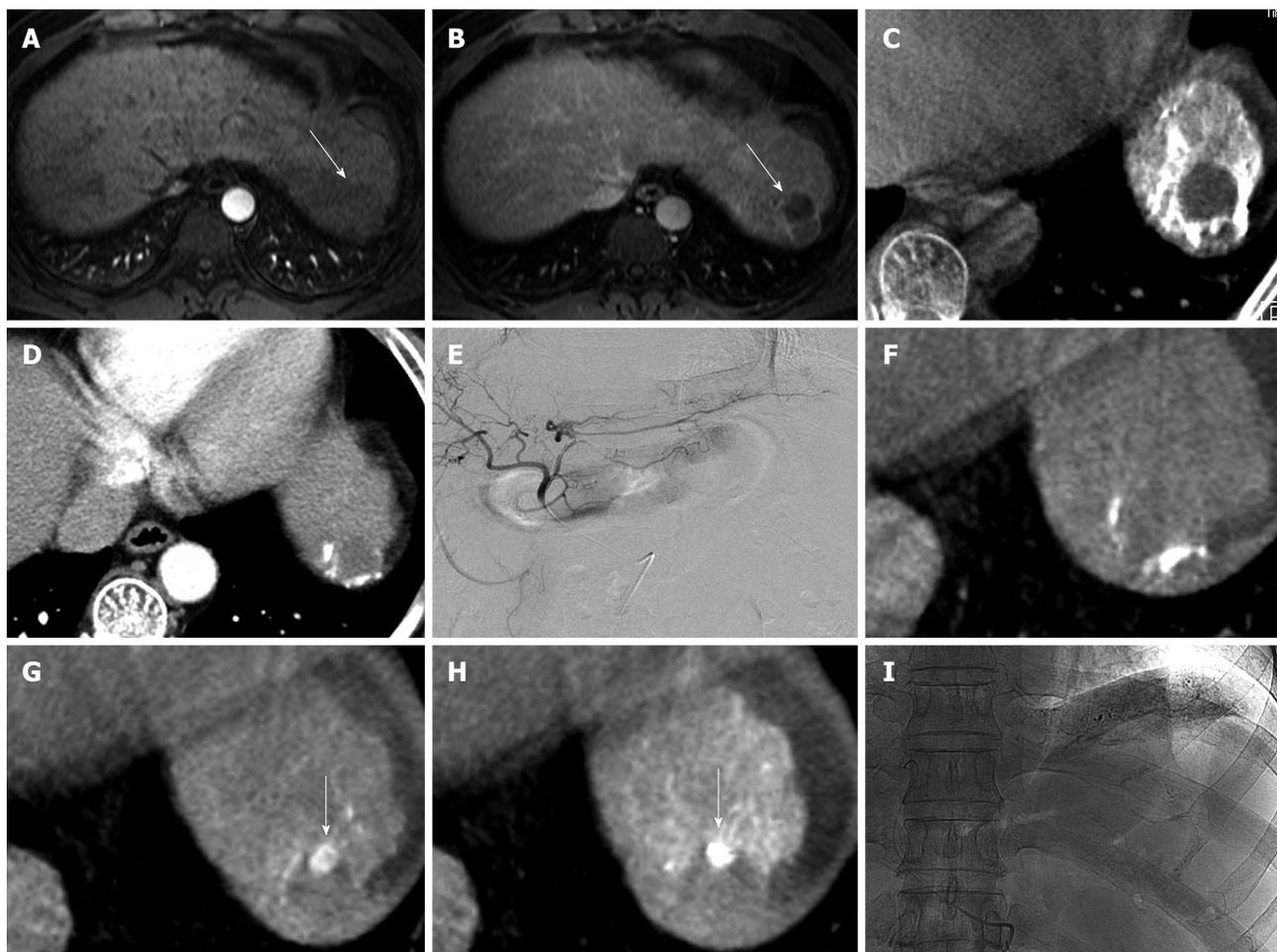
**Table 3** Enhancement patterns and iodized oil uptake in three nodules without typical enhancement patterns of HCCs on MDCT and MRI

Nodule	Modality	Phase		Degree of iodized oil uptake	
		EA	Delayed	Spot image	Cone-beam CT
1	CT	Subtle E	Washout	Poor	Poor
2 <sup>1</sup>	CT	No E	No E	Poor	Excellent
3	MRI	Subtle E	Washout	Invisible	Good

<sup>1</sup>Same patient in Figure 2. EA: Early arterial phase; Delayed: Delayed phase; Subtle E: Subtle enhancement; No E: No enhancement. HCC: Hepatocellular carcinoma; MDCT: Multi-detector row helical computed tomography; MRI: Magnetic resonance imaging.

introduced for patients who are not surgical candidates. These options include TACE, percutaneous ethanol injection (PEI), radiofrequency ablation (RFA), microwave coagulation therapy (MCT), laser thermal ablation (LTA), and combination therapy<sup>[10,11]</sup>. Among them, TACE is widely performed, because it is minimally invasive, repeatable, and more effective in combination with other treatments<sup>[10-13]</sup>.

TACE was found to be as effective as hepatic resection for early stage tumors when iodized oil was compactly retained within the tumor<sup>[8]</sup>. Iodized oil uptake pattern can be a prognostic index<sup>[7]</sup>. Various studies have suggested that iodized oil uptake in a tumor can correlate well with hepatic necrosis, and compact iodized oil uptake on unenhanced CT may represent necrosis<sup>[16,17]</sup>. Takayasu *et al*<sup>[6]</sup> reported that the highest degree of necrosis usually occurs immediately after TACE and the regrowth of viable cancer cells will occur later if complete necrosis of the tumor was not achieved. At this



**Figure 2** A 52-year-old man with HCC in S2 for 2nd TACE. A, B: Early arterial phase (A) and delayed phase (B) of MRI reveals nodule (arrow) in segment 2 with no enhancement (arrow); C: Cone-beam CT after TACE shows poor grade of iodized oil uptake by the tumor; D: Early arterial phase of MDCT shows washout of iodized oil uptake near nodule, but no enhancing portion within the nodule 3 mo later; E: Left hepatic angiogram during TACE reveals no tumor and feeding artery 1 mo later; F, G: Cone-beam CT with (F) and without (G) hepatic arteriography shows small enhancing nodule (arrow) in the nodule; H, I: Cone-beam CT after infusion of emulsion (H) shows excellent grade iodized oil uptake by the nodule (arrow). This case shows typical "nodule-in-nodule" appearance of HCC, but spot image (I) did not distinguish nodular uptake.

point, compact iodized oil uptake by the tumor directly after the procedure prevents regrowth of tumors, but to confirm this, only spot image is usually acquired. If the tumor is large enough to appear on spot images, it is easy to determine the degree of iodized oil uptake, but if the tumor is small, especially less than 2 cm, the degree of iodized oil uptake is incorrectly determined, which can cause tumor regrowth. It is also impossible to refuse chemotherapeutic agents directly if there is partial iodized oil uptake in a lesion on the first follow-up CT.

Cone-beam CT was first used in neuroendovascular procedures, and the image quality is sufficient to make a diagnosis when a complication is suspected<sup>[18]</sup>. In the era of assessing the degree of iodized oil uptake, cone-beam CT is also a convenient tool during and after TACE and clearly correlates with MDCT. If cone-beam CT images shows non-compact iodized oil uptake, immediate re-intervention can be achieved. The disadvantages of this CT-like image are low temporal resolution and a small field of view, however, it is sufficient to locate lesions previously diagnosed on MDCT<sup>[19]</sup>.

Takayasu *et al*<sup>[9]</sup> reported "targeted transarterial oily chemoembolization" which is a similar method to ours,

but these authors used a unified helical CT and angiography system. Our system is only a DSA machine without a helical CT system and has more simple structures.

The radiation dose from cone-beam CT was not measured, however, Hirota *et al*<sup>[20]</sup> measured the radiation dose of cone-beam CT with a flat-panel-detector digital angiography system (similar system with ours) and single helical CT using a cylindrical phantom model of CT dose index with a dosimeter. They reported that the radiation dose by cone-beam CT was less than that of single helical CT. In addition, the radiation dose from cone-beam CT can be calculated *via* a pre-set radiation dose (0.36  $\mu$ Gy/pulse) and total fluoro time (7.5 pulse/s, 10 s). Compared with nonenhanced MDCT for TACE follow-up, the calculated radiation dose of cone-beam CT is low.

Cone-beam CTHA placing the microcatheter in the nearest arteries was performed in only three nodules in this study. Another study<sup>[20]</sup> also reported this method, and this technique is very useful to confirm a perfusion area in the artery. Although a small number of cases were included in this study, this method is a very useful and time-saving technique in TACE and other interventional procedures, especially for small lesions.

In conclusion, cone-beam CT is a useful and convenient tool for assessing the iodized oil uptake by small hepatic tumors (< 3 cm) directly after TACE. In addition, in cases with suspected small HCC nodules without typical enhancement patterns on CT, cone-beam CTHA will be very useful, however, further study is required.

## COMMENTS

### Background

Iodized oil retention pattern after transarterial chemoembolization (TACE) is a posttreatment prognostic marker, and significant factors affect local recurrence, however, a method for evaluating this immediately after TACE is to take a spot image. Follow-up unenhanced computed tomography (CT) is usually performed within 1 mo after TACE, but it is sometimes difficult to determine whether there is washout of iodized oil uptake or initial failure of iodized oil uptake when there is partial iodized oil uptake in a lesion on the first follow-up CT.

### Research frontiers

Comparing iodized oil retention in small hypervascular nodules on preprocedural multi-detector row helical computed tomography (MDCT) directly after TACE is possible without transporting patients from the angiography suite to the nearest CT scanner.

### Innovations and breakthroughs

In the present study, the authors investigated the efficacy of cone-beam CT with regard to iodized oil uptake after TACE for small hepatocellular carcinoma (HCC)s.

### Applications

The study showed that cone-beam CT is a useful and convenient tool for assessing the iodized oil uptake by small hepatic tumors directly after TACE.

### Terminology

Cone-beam CT is a new technology provided by the combined angiography/CT suite that uses flat-panel detector technology. It provides images similar to those of CT and is able to make 3D reconstructions such as multiplanar reformat, maximum intensity projection, and 3D volume rendering with these data sets.

### Peer review

This is an interesting study which shows the advantages of assessing iodized oil uptake after TACE for small HCC with cone-beam CT. It may provide useful information for us.

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BRIEF ARTICLE

## Image-guided conservative management of right colonic diverticulitis

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**CONCLUSION:** Our results indicate that right colonic diverticulitis is essentially benign and image-guided conservative treatment is primarily required.

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**Key words:** Ascending colon; Cecum; Medical therapy; Colonic diverticulitis

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Park SJ, Choi SI, Lee SH, Lee KY. Image-guided conservative management of right colonic diverticulitis. *World J Gastroenterol* 2009; 15(46): 5838-5842 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5838.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5838>

### Abstract

**AIM:** To study the clinical outcomes of medical therapy in patients with right colonic diverticulitis.

**METHODS:** The records of 189 patients with right colonic diverticulitis which was finally diagnosed by computed tomography, ultrasonography, or operative findings were retrospectively reviewed.

**RESULTS:** Of the 189 patients hospitalized for right colonic diverticulitis, the stages of diverticulitis by a modified Hinchey classification were 26 patients (13.8%) in stage 0, 139 patients (73.5%) in stage I a, 23 patients (12.2%) in stage I b, and 1 patient (0.5%) in stage III. Medical therapy was undertaken in 185 of 189 patients (97.9%). One hundred and eighty three of 185 patients were successfully treated with bowel rest and antibiotics. Two patients in stage I b required a resection or surgical drainage because of an inadequate response to conservative treatment. Recurrent diverticulitis developed in 15 of 183 patients (8.2%) who responded to medical therapy. All 15 patients who suffered a second attack had uncomplicated diverticulitis, and were successfully treated with medical therapy.

### INTRODUCTION

Interestingly, there is a unique predilection for diverticular disease of the colon in Western and Asian populations, and is predominant in the left colon in Caucasians<sup>[1]</sup>, while much more common in the right colon in Asians<sup>[2]</sup>. Many studies have focused on left colonic diverticulitis and subsequently, therapeutic guidelines have been established, while that of right colonic diverticulitis still remains controversial<sup>[2-8]</sup>. In the past, the majority of patients with right colonic diverticulitis were faced with an operation for presumed appendicitis<sup>[3,4,9]</sup>. Thus, there is a lack of objective information for patients with right colonic diverticulitis compared with left colonic diverticulitis. Much of this information is based on case series data, which have relatively small sample sizes and the preoperative diagnosis was not made using imaging studies. Recent studies suggest that colonic diverticulitis can be correctly diagnosed by computed tomography (CT) scan<sup>[6,7,10]</sup>, or ultrasonography (US)<sup>[11-14]</sup>, and with the use of these imaging studies, right colonic diverticulitis is more common than has been previously assumed<sup>[7]</sup>. The aim of this study was to evaluate the clinical course and results of medical therapy in patients with right colonic diverticulitis, of which the final diagnosis was based on radiographic evidence from CT or US, or operative findings.

## MATERIALS AND METHODS

### Patients

Using computerized patient databases, we searched for all patients who were hospitalized with the diagnosis of colonic diverticulitis from January 1998 to August 2007 at Kyung Hee University Hospital, Seoul, Korea. Excluded were patients who were clinically suspected of having colonic diverticulitis without operative findings or radiologic evidence from CT or US, patients whose colonic diverticulosis alone was present without any evidence of inflammation, and those whose follow-up records were unobtainable. A total of 189 patients were retrospectively reviewed and data were collected with regard to age and sex, clinical presentation, location of disease, diagnostic studies (CT, US, barium enema, and colonoscopy), laboratory findings, type of complication, treatment modality, preoperative diagnosis, operative findings, type of operation, and outcome. The final diagnosis was based on radiographic evidence from CT or US, or operative findings. CT was performed in 138 patients (73%) and US was performed in 114 patients (60.3%). Both CT and US were performed in 80 patients (42.3%) and 17 patients underwent surgery without CT or US. Recurrence of diverticulitis was defined as the presence of the same symptoms and signs leading to re-hospitalization. Recurrence was tracked either by interviewing the patient or by telephone contact.

A modification of the Hinchey classification system was used to define the patients<sup>15,16</sup>. Patients were categorized into the six stages according to CT, US, or operative findings. Complicated diverticulitis is defined as diverticulitis associated with abscess, fistula, obstruction, or free perforation<sup>17</sup>. Therefore, uncomplicated diverticulitis included stage 0 and I a, whereas complicated diverticulitis included stage I b, II, III, and IV.

The data were analyzed using the chi-square test or Fisher's exact test. All *P* values of less than 0.05 were considered to be statistically significant.

## RESULTS

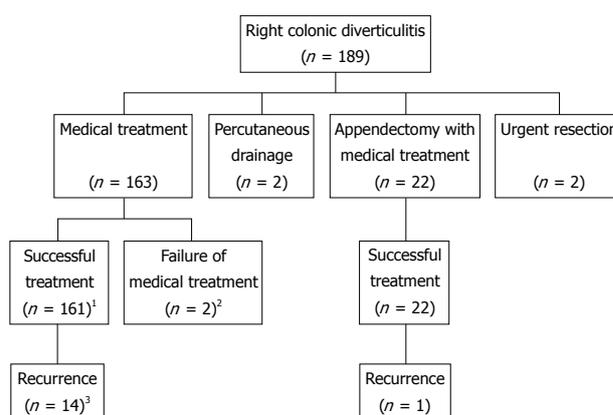
### Characteristics and presentation of patients

Of the 189 patients hospitalized for right colonic diverticulitis, 111 were men and 78 were women. The median age of the patients was 37 years (range, 14–88 years). The mean age of women (40.4 years) was not significantly different from that of men (36.7 years) (*P* = 0.088). One hundred and eight patients (57.1%) were under the age of 40 years. By a modified Hinchey classification, stages of diverticulitis present on admission were as follows: 26 patients (13.8%) in stage 0, 139 patients (73.5%) in stage I a, 23 patients (12.2%) in stage I b, and 0 (0%), 1 (0.5%), 0 (0%) patient in stage II, stage III and stage IV, respectively (Table 1). The majority of patients commonly presented with phlegmon. The majority of patients (87.3%) had mild diverticulitis (stage 0 or I a) on admission and only 24 patients (12.7%) had complicated diverticulitis. The average white blood cell count was  $11417 \pm 275$ . Fever was seen in 20.6% of patients.

**Table 1** Presentation of patients by a modified Hinchey classification<sup>15</sup>

Modified Hinchey classification	Total (n = 189)
0 Direct visualization of the diverticulum with Sx or Sign <sup>1</sup>	26
I a Confined pericolic inflammation (phlegmon)	139
I b Confined pericolic abscess	23
II Distant intraabdominal or retroperitoneal abscess	0
III Generalized purulent peritonitis	1
IV Fecal peritonitis	0

<sup>1</sup>Right abdominal pain, leukocytosis, or fever with no radiologic evidence of appendicitis. Sx: Symptom.



**Figure 1** Treatment and outcome of 189 patients with right colonic diverticulitis. <sup>1</sup>After successful medical treatment, seven patients had elective surgery at the surgeon's request; <sup>2</sup>One patient required a resection and another underwent surgical drainage; <sup>3</sup>After successful medical treatment, five patients had elective surgery at the surgeon's request.

### Treatment

Patients were initially managed with medical therapy alone, percutaneous drainage, or an urgent operation. Elective surgery was determined after a cooling-off period. Medical therapy was undertaken in 185 of 189 patients (97.9%), including 22 patients who incidentally underwent an appendectomy for presumed appendicitis (Figure 1). Two patients in stage I b underwent percutaneous drainage and two patients (one in stage I b and one in stage III) underwent urgent surgery (1 ileocecal resection, 1 right hemicolectomy). One hundred and eighty three of 185 patients were successfully treated with bowel rest and antibiotics. However, two patients in stage I b required a resection or surgical drainage because of an inadequate response to conservative treatment. Seven patients who were successfully treated with bowel rest and antibiotics had an elective operation at the surgeon's request.

### Recurrence

Recurrent diverticulitis developed in 15 of 183 patients (8.2%) who responded to medical therapy. The median interval to the onset of recurrence was 11 mo (range, 0.5–96 mo). The median disease free period was 44 mo (range, 0.5–129 mo). All 15 patients who suffered a

second attack had uncomplicated diverticulitis, and were successfully treated with medical therapy. One patient, an 80-year-old woman, experienced five episodes of right colonic diverticulitis, which were uncomplicated and successfully treated with medical therapy. Five of these 15 patients had an elective operation at the surgeon's request after successful conservative treatment. There were no deaths in these patients with right colonic diverticulitis.

### **Pathology and follow-up study**

A total of 15 patients underwent colonic resection. Nine of these 15 patients were reported to have multiple false diverticula. The pathologic reports of the other six patients contained no mention of the type of diverticulum.

During the original hospitalization, CT or US was supplemented by colonoscopy or double-contrast barium enema in 31 patients. In addition, 1 mo after recovery from an initial episode of diverticulitis, 60 patients agreed to re-evaluation by double-contrast barium enema (54 patients) or colonoscopy (six patients). In a total of 87 patients, including four patients duplicated, multiple diverticula were reported in 62 patients (71.3%) and a solitary diverticulum was reported in 13 patients (14.9%). The number of diverticulum could not be identified in 12 patients due to poor bowel preparation.

## **DISCUSSION**

The Hinchey classification and its several modifications have been used to define the stages of acute diverticulitis although the systems were mostly applied to left colonic diverticulitis<sup>[15,18]</sup>. In the present study, the diagnosis of right colonic diverticulitis was made in 167 of 172 (97%) patients using CT or US. The original Hinchey classification is not detailed enough to reflect right colonic diverticulitis with which the majority of patients have mild forms. We therefore used a modified system including subcategories in the early stage<sup>[15,16]</sup>. The majority of patients (87.3%) had uncomplicated diverticulitis (stage 0 or I a) on admission and the other patients even those with complicated diverticulitis had a relatively early stage (stage I b) such as pericolic abscess with the exception of one patient (stage III).

To date, no therapeutic guidelines for patients with right colonic diverticulitis have been established<sup>[5,6,9,19-21]</sup>. In contrast, practice parameters for the treatment of left colonic diverticulitis do exist<sup>[17]</sup>. Conservative treatment is recommended for uncomplicated left colonic diverticulitis because it results in resolution of the problem in 70% to 100% of patients<sup>[22-29]</sup>. In our study, all of the 165 patients with uncomplicated right colonic diverticulitis (stage 0 or I a) were successfully treated with bowel rest and antibiotics, including 22 patients who underwent an appendectomy for presumed appendicitis. We believe that patients with uncomplicated right colonic diverticulitis can be successfully treated with medical treatment in the same way as those with uncomplicated left colonic diverticulitis.

However, some authors advocate surgical resection for right colonic diverticulitis encountered during surgery

for presumed appendicitis<sup>[3-5]</sup>. Lo *et al*<sup>[3]</sup> reported their experience of 22 patients over an 11-year period. They performed preoperative US and CT in only one patient. At operation, an inflammatory phlegmon or indurated mass was found in 18 patients, however, colectomy with primary ileocolic anastomosis was performed in 21 patients including these 18 patients. Lane *et al*<sup>[4]</sup> reported a series of 49 patients over a 22-year period. The authors stated that because the pathophysiology of cecal diverticula may be different in the Asian population and the recurrence of symptoms may be more common in the Western population, conservative management may not be applicable to the Western population. A correct radiologic diagnosis was preoperatively made in only three patients. Immediate right hemicolectomy was performed in 39 patients at the time of laparotomy, but operative findings or the severity of diverticulitis was not provided. Fang *et al*<sup>[5]</sup> analyzed 85 patients during a 5-year period. Thirty four patients had right hemicolectomy, 9 patients had diverticulectomy, and 42 patients received conservative treatment (antibiotics or appendectomy plus antibiotics). In 34 patients receiving right hemicolectomy, the indications for surgery included repeated attack of symptoms in six and phlegmon, abscess, or perforation of the diverticulum in ten. In 42 patients receiving conservative treatment, ten patients developed recurrent diverticulitis and only three of these ten patients had complicated diverticulitis. However, the authors mentioned that the disease process of cecal diverticulitis might not be benign and recurrence of diverticulitis should be an indication for aggressive resection. In the above studies, we guess that all patients who underwent colonic resection would not essentially require surgery, and conservative treatment alone might have been sufficient in some patients. We believe that the correct pretreatment diagnosis for right colonic diverticulitis does not only avoid unnecessary surgery but also allows clinicians to determine optimal management according to the severity of the diverticulitis.

It is uncertain whether the pathophysiology of right colonic diverticulitis is really different between Asian and Western populations. Oudenhoven *et al*<sup>[7]</sup> reviewed 44 patients with right colonic diverticulitis in a Western population (including one Asian patient). Forty one patients were successfully treated conservatively and three patients underwent diverticulectomy. They concluded that the natural history of right colonic diverticulitis is benign and surgical intervention can be avoided in the vast majority of patients. In a large post-mortem survey of diverticular disease, Hughes<sup>[1]</sup> reported that the incidence of solitary cecal diverticula lies between 2.5% and 5%. Histologically all the diverticula were thin-walled, false diverticula and no case of cecal diverticulum had muscle in its wall. The author mentioned that congenital cecal diverticulum may largely be a pathological myth. Graham and Ballantyne<sup>[9]</sup> reviewed the American experiences. Among 128 histologic cases compiled from the medical literature, they found that 52 (41%) were true diverticula while 76 (59%) were, in fact, false diverticula. In addition, among 288 cases gathered from the literature, 233 (81%) were solitary, while 55 (19%) were multiple. In the

present study, nine of 15 patients who underwent colonic resection were reported to have multiple false diverticula. Of 87 patients receiving double-contrast barium enema or colonoscopy, multiple diverticula were reported in 62 patients (71.3%) and solitary diverticulum was reported in 13 patients (14.9%). The incidence of true or false diverticula in the right colon is not well known, particularly between Western and Asian populations. This is because the pathologic differentiation between true and false diverticula may be difficult once inflammation occurs<sup>[4]</sup>, and to our knowledge, the type of diverticula in right colonic diverticulitis has not been pathologically described in the Asian literature.

Some authors who advocate aggressive resection for right colonic diverticulitis assert that leaving the diseased foci *in situ* with the possibility of developing some serious complications later seems to be impractical and recurrence of diverticulitis should be an indication for aggressive resection. If recurrence is high and complications frequent, surgical resection is essentially required. However, after successful conservative treatment, a recurrence rate of 3.6% to 23.8% has been reported in the literature<sup>[5,7,8,20,21,30]</sup>. Ngoi *et al.*<sup>[31]</sup> reported a recurrence rate of 1.5%, but 38% of their patients underwent diverticulectomy. It is interesting to note that in these studies, there was little complicated diverticulitis in recurrent cases: Fang *et al.*<sup>[5]</sup> reported three complicated cases in 10 recurrences in 42 patients receiving conservative treatments, and Harada *et al.*<sup>[30]</sup> reported one complicated case in four recurrences in 29 patients. The other authors reported that all of the recurrent cases were uncomplicated diverticulitis, which responded well to medical therapy. Komuta *et al.*<sup>[8]</sup> mentioned that recurrent uncomplicated right colonic diverticulitis responded well to medical therapy regardless of the number of recurrences. In our study, 15 of 183 patients (8.2%) who responded to medical therapy developed recurrent diverticulitis. All 15 patients had uncomplicated diverticulitis, and were successfully treated with medical therapy. Therefore, we believe that if the recurrence rate after conservative treatment is not high and if complications are not frequent even after recurrence occurs, recurrence of right colonic diverticulitis should initially be an indication for medical treatment and not for surgery.

In conclusion, our results indicate that right colonic diverticulitis is essentially benign and image-guided conservative treatment is primarily required. Although our study is limited by the retrospective design and relatively few recurrent patients, our results suggest that recurrence after conservative treatment of right colonic diverticulitis is low, and rarely associated with complicated diverticulitis. Thus, recurrence of right colonic diverticulitis should initially be an indication for medical treatment, while surgical resection should be selectively considered for patients with complicated diverticulitis.

is much more common in Asian populations. There is a lack of information available on the clinical course and results of medical therapy for patients with right colonic diverticulitis compared with left colonic diverticulitis.

### Research frontiers

The final diagnosis of all patients with right colonic diverticulitis was based on radiographic evidence from computed tomography or ultrasonography, or operative findings. The majority of patients with right colonic diverticulitis had a mild form on admission and initially required medical therapy. Recurrent diverticulitis developed in 8.2%, but all recurrent patients had uncomplicated diverticulitis, and were successfully treated with medical therapy.

### Innovations and breakthroughs

Right colonic diverticulitis is essentially benign and image-guided conservative treatment is primarily required. The correct pretreatment diagnosis for right colonic diverticulitis does not only avoid unnecessary surgery but also allows clinicians to determine optimal management according to the severity of the diverticulitis. Even recurrence of right colonic diverticulitis should initially be an indication for medical treatment, while surgical resection should be selectively considered for patients with complicated diverticulitis.

### Terminology

Complicated diverticulitis is defined as diverticulitis associated with abscess, fistula, obstruction, or free perforation.

### Peer review

This is an interesting study about right colonic diverticulitis management that has been quite rarely discussed.

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## COMMENTS

### Background

Right colonic diverticulitis is a rare condition in Western populations, while it

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## Changes in intestinal mucosal immune barrier in rats with endotoxemia

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### Abstract

**AIM:** To investigate the dysfunction of the immunological barrier of the intestinal mucosa during endotoxemia and to elucidate the potential mechanism of this dysfunction.

**METHODS:** Male Wistar rats were randomly distributed into two groups: control group and lipopolysaccharide (LPS) group. Endotoxemia was induced by a single caudal venous injection of LPS. Animals were sacrificed in batches 2, 6, 12 and 24 h after LPS infusion. The number of microfold (M)-cells, dendritic cells (DCs), CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, regulatory T (Tr) cells and IgA<sup>+</sup> B cells in the intestinal mucosa were counted after immunohistochemical staining. Apoptotic lymphocytes were counted after TUNEL staining. The levels of interleukin (IL)-4, interferon (IFN)- $\gamma$  and forkhead box P3 (Foxp3) in mucosal homogenates were measured by ELISA. The secretory IgA (sIgA) content in the total protein of one milligram of small intestinal mucus was detected using a radioimmunological assay.

**RESULTS:** This research demonstrated that LPS-

induced endotoxemia results in small intestinal mucosa injury. The number of M-cells, DCs, CD8<sup>+</sup> T cells, and IgA<sup>+</sup> B cells were decreased while Tr cell and apoptotic lymphocyte numbers were increased significantly. The number of CD4<sup>+</sup> T cells increased in the early stages and then slightly decreased by 24 h. The level of IL-4 significantly increased in the early stages and then reversed by the end of the study period. The level of IFN- $\gamma$  increased slightly in the early stages and then decreased markedly by the 24 h time point. Level of Foxp3 increased whereas sIgA level decreased.

**CONCLUSION:** Mucosal immune dysfunction forms part of the intestinal barrier injury during endotoxemia. The increased number and function of Tr cells as well as lymphocyte apoptosis result in mucosal immunodeficiency.

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**Key words:** Endotoxemia; Rats; Intestinal mucosa; Immunity

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Liu C, Li A, Weng YB, Duan ML, Wang BE, Zhang SW. Changes in intestinal mucosal immune barrier in rats with endotoxemia. *World J Gastroenterol* 2009; 15(46): 5843-5850 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5843.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5843>

### INTRODUCTION

Endotoxemia can induce sepsis which is among the leading causes of death in noncardiac intensive care units (ICUs) in the US, with approximately 750 000 cases and up to 200 000 deaths per year<sup>[1]</sup>. An epidemiological investigation in 3665 ICUs in China showed that the overall hospital mortality of severe sepsis is 48.7%; the mean hospital cost is \$11 390 per patient at a mean cost of \$502 per patient per day<sup>[2]</sup>. Despite hospital mortality of 20% for simple sepsis and 40% or higher for severe sepsis or septic shock, there has nonetheless been improvement over the past decade<sup>[3]</sup>.

Lipopolysaccharide (LPS) is a component of the outer cell wall of gram-negative bacteria, which gives rise to

various manifestations of gram-negative endotoxemia and septic shock<sup>[4]</sup>. Endotoxemia-induced sepsis has been associated with deleterious functional and structural changes in many organs, such as the gastrointestinal tract<sup>[5]</sup>, lungs, and other organs. During sepsis, the most frequent complications within the gastrointestinal tract are mucosal barrier dysfunction and ileus<sup>[6]</sup>. One of the most important functions of the gastrointestinal tract is the ability to act as a mucosal barrier to infections. Mucosal barrier dysfunction plays an important role in the pathophysiology of sepsis by promoting bacterial stasis, bacterial overgrowth, and bacterial translocation, which can lead to the development of secondary infections and multiple organ failure<sup>[7]</sup>. Intestinal mucosal barriers consist of a mechanical barrier, a chemical barrier, an immunological barrier, and a biological barrier<sup>[8]</sup>. Damage to any one of these components causes mucosal barrier dysfunction. The immunological barrier is considered as the first line of defense of the intestinal mucosa from bacterial invasion<sup>[9]</sup> and plays an important role in the overall defense. It consists of Peyer's patches, which are the induction sites of the immune response, and diffused lymphoid tissue which are the effector sites. Intestinal mucosa immune responses rely largely on humoral immunity. The primary functions of the immunological barrier include<sup>[10-12]</sup> inhibiting bacterial adhesion to the mucosa so that they can be eliminated, neutralizing viruses and toxins, enclosing some antigens of acquired extraneous material to prevent systemic reactions, activating the complement 3 (C3) pathway, participating in the anti-infection effect, and protecting probiotics. Therefore, damage to the intestinal immune barrier will result in bacterial translocation and gut-derived endotoxemia.

Previous studies have discussed the changes to immunity during sepsis, but what happens to immunity, specifically the gut immunity, during endotoxemia before sepsis is not clear. Thus in the present study, changes to the number and function of intestinal mucosal immune cells in rats with endotoxemia were observed to investigate whether dysfunction of immunological barrier occurred during endotoxemia and to elucidate the potential mechanism of this dysfunction.

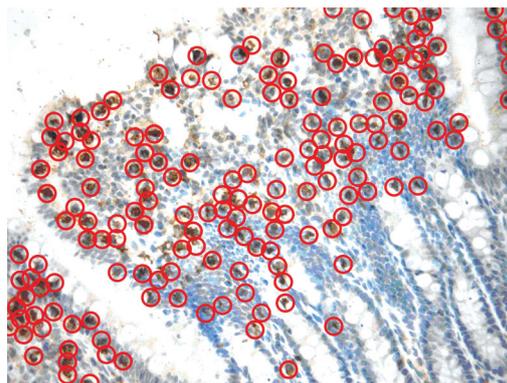
## MATERIALS AND METHODS

### Animals

Male Wistar rats weighing  $200.5 \pm 12.3$  g were purchased from Vital River Laboratories (Beijing, China). The rats were fed a standard laboratory chow diet (Vital River Laboratories) for 72 h before the experiment and maintained at  $24 \pm 1^\circ\text{C}$ , at a relative humidity of  $50\% \pm 1\%$  with a 12/12-h light/dark cycle. The animals were fasted for 12 h before the experiment, allowing free access to water. All animals were handled according to the institutional criteria for the care and use of laboratory animals in research.

### Methods

**Establishment of animal model:** A total of 80 animals were included and randomly distributed into the control



**Figure 1** A sample image of immunohistochemical staining and TUNEL staining. Some sample cells are circled to illustrate the positive cells that were counted (400 ×).

group (40 rats) and LPS (*Escherichia coli*, O55: B5, Sigma, St Louis, MO, USA) group (40 rats). In accordance with the study of Cheng *et al*<sup>[13]</sup>, endotoxemia was induced by a single caudal venous injection of LPS at 10 mg/kg, while control animals received caudal venous injections of saline. Ten animals were sacrificed in each group at each time point (2, 6, 12 and 24 h) after LPS infusion.

**Histological testing:** Small intestinal tissue was obtained from the middle part of the ileum. Samples were fixed in 4% paraformaldehyde and then observed by hematoxylin and eosin (HE) staining to explore the histopathological changes in the intestinal mucosa.

**Cytological testing:** A 5-cm-long tissue section with Peyer's patches cut off was obtained from the ileum near the cecum and observed by immunohistochemical staining after fixation in 4% paraformaldehyde to investigate immune cell changes in the mucosa. The fixed tissue was transferred to phosphate buffer solution (PBS) overnight at  $4^\circ\text{C}$  and then transferred to 30% sucrose for 2 h at  $4^\circ\text{C}$ . Then the tissue was mounted in optimum cutting temperature (OCT) embedding medium and placed on dry ice until frozen before cryosectioning of 4  $\mu\text{m}$  thick continuous slides. The slides were washed  $3 \times 5'$  (3 times, 5 min each) with PBT (Phosphate Buffer Saline with 0.02% Tween 20). Next, 500  $\mu\text{L}$  of diluted primary antibody in blocking buffer (containing 40 mL PBT, 400  $\mu\text{L}$  heat-inactivated goat serum, 400  $\mu\text{L}$  heat-inactivated donkey serum, 400  $\mu\text{L}$  10% Triton X-100) was added to each slide before they were covered and stored at  $4^\circ\text{C}$  overnight. The slides were then washed  $6 \times 5'$  with PBT before the addition of 500  $\mu\text{L}$  of diluted secondary antibody in blocking buffer to each slide. The slides were then covered and incubated at room temperature for 90 min before being washed  $6 \times 5'$  with PBT. The slides were then mounted with Vectashield. After staining, six clear-viewed slides were selected from each group and two viewing fields for each slide at high magnification (400 ×) were randomly selected for counting immune cells. Brownish-yellow stained lymphocytes were an indication of positive cells (Figure 1). Rabbit anti-rat cytokeratin 8<sup>[14]</sup>, integrin  $\alpha\text{E}2$ <sup>[15]</sup>, cluster of differentiation (CD) 4, CD8,

neuropilin-1 (NRP-1)<sup>[16]</sup> and immunoglobulin A (IgA) polyclonal antibodies were purchased from Biosynthesis Biotechnology (Beijing, China) to indicate microfold cells (M-cells), dendritic cells (DC), CD4<sup>+</sup> thymus dependent lymphocytes (T cells), CD8<sup>+</sup> T cells, CD4<sup>+</sup>CD25<sup>+</sup> T cells (regulatory T cell, Tr) and IgA<sup>+</sup> bursa dependent lymphocytes (B cells), respectively.

The terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling (TUNEL) assay was used to observe apoptotic lymphocytes in the small intestinal mucosa. The TUNEL kit was obtained from Roche Diagnostics (Indianapolis, IN, USA) to label apoptotic lymphocytes. The slides were deparaffinized in two changes of xylene for 5 min each, hydrated with two changes of 100% ethanol for 3 min each and 95% ethanol for 1 min, and then rinsed in distilled water. Slides were then incubated in TdT reaction buffer for 10 min before incubation in TdT reaction mixture for 1-2 h at 37-40°C in a humidified chamber. The reaction was stopped by rinsing the slides in stop wash buffer for 10 min. The slides were then rinsed 3 × 2' in PBT before incubation with FITC-Avidin D in PBS for 30 min at room temperature. Slides were then rinsed 3 × 2' in PBT, counterstained with PI or DAPI for 20 min and rinsed in PBS for 5 min. Slides were then mounted with Vectashield. Brownish-yellow stained lymphocytes were an indication of positive cells. The intraepithelial apoptotic lymphocytes were identified and counted.

**Detection of IL-4, IFN- $\gamma$  and Foxp3:** The small intestinal mucosa was stripped off by circumferentially pushing the muscularis with a moist cotton applicator, as described previously<sup>[17]</sup> and then weighed to prepare a 10% homogenate by adding an appropriate amount of normal saline. The homogenate was then centrifuged at 3000 × *g* for 10 min at 0°C. The supernatant was harvested and diluted with normal saline to make a 1% homogenate. The levels of interferon- $\gamma$  (IFN- $\gamma$ ), interleukin-4 (IL-4) and forkhead box P3 (Foxp3) in the homogenate were measured by enzyme-linked immunosorbent assays (ELISA) to evaluate the function of Helper T-cell (TH) 1, TH2 and Tr cells. The IFN- $\gamma$  and IL-4 ELISA kits were obtained from R&D Systems (Minneapolis, MN, USA). The Foxp3 ELISA kit was obtained from Adlitteram Diagnostic Laboratories (San Diego, CA, USA). The ELISA assays were performed according to the manufacturer's instructions.

**Detection of sIgA:** Secretory IgA (sIgA) was detected using a radioimmunological assay (RIA). A 10-cm-long tissue section was obtained from the small intestine, dissected and carefully washed with normal saline. Small intestinal mucus was collected into an Eppendorf tube, and 1 mL of 0.01 mol/L PBS was added into the Eppendorf tube. The solution was then centrifuged at 3000 × *g* for 10 min at 0°C. The supernatant was then harvested and the level of sIgA was measured by a double antibody sandwich RIA purchased from Beijing Nuclear Research Center (Beijing, China). The total protein level

of the intestinal mucus was assayed by the Bradford brilliant blue method simultaneously. The sIgA content in 1 mg of total protein from small intestinal mucus was detected.

### Statistical analysis

All statistical analyses were performed using the SPSS 15.0 software package. All data were expressed as the mean ± SD. Group comparisons were carried out using a one-factor analysis of variance. *P* < 0.05 was considered statistically significant.

## RESULTS

### Mortality of rats in different groups

During the course of the study there were no rats that died in the control group. A total of 5 rats died in the LPS group; 3 at 12 h and 2 at 24 h after LPS injection.

### Histological changes

As shown in Figure 2, the intestinal mucosa of saline-treated rats was complete and the villi were presented in an orderly fashion. Samples displayed no abnormal epithelial cell morphology and there was no evidence of congestion, edema or infiltration of inflammatory cells (Figure 2A). In contrast, the intestinal mucosal villi of rats with endotoxemia were loosened and atrophic where the epithelial cells were necrotic. The mucosa was edematous and infiltrated with inflammatory cells (Figure 2B-E). These abnormal changes to the intestinal mucosa were most obvious 12 h after LPS injection (Figure 2D).

### Numbers of immune cells and apoptotic lymphocytes in the intestinal mucosa

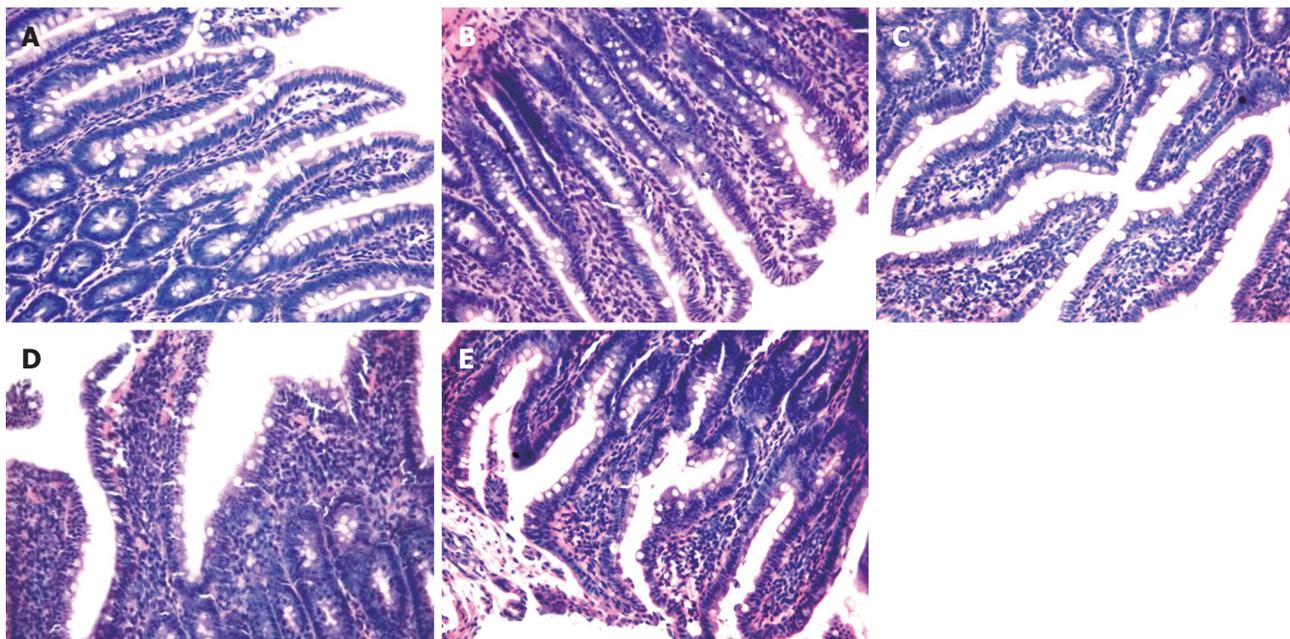
The effects on the immune system induced by LPS were assessed in the rat intestinal mucosa (Figure 3). We found that M-cell and CD8<sup>+</sup> T cell numbers in the small intestinal mucosa were significantly decreased 6 and 12 h after LPS challenge compared to controls. Furthermore, the number of DCs was significantly decreased after 2, 6 and 12 h. In contrast, the number of CD4<sup>+</sup> T cells was significantly increased after 6 and 12 h, before decreasing slightly by 24 h, although this decrease was not statistically significant. The number of Tr cells was significantly increased at 2, 6 and 12 h. The number of IgA<sup>+</sup> B cells and apoptotic lymphocytes were significantly increased at all time points.

### Levels of IL-4, IFN- $\gamma$ and Foxp3 in the small intestinal mucosa

As shown in Figure 4, the level of IL-4 was significantly increased in the small intestinal mucosa after 2 h before significantly decreasing after 6 and 12 h in LPS-treated animals compared with controls. The level of IFN- $\gamma$  was increased slightly after 2, 6 and 12 h before decreasing markedly by 24 h. The level of Foxp3 was significantly increased after 12 and 24 h.

### Level of intestinal mucus sIgA

As shown in Figure 5, LPS induced a significant decrease



**Figure 2** Representative images of the histological changes in the small intestinal mucosa of rats after LPS injection. A: The intestinal mucosa of normal rats was complete and the villi were in an orderly fashion with no abnormal morphology present in the epithelial cells, as well as no manifestation of congestion, edema or infiltration of inflammatory cells; B-E: Representative of the changes 2, 6, 12 and 24 h after LPS treatment. The intestinal mucosal villi of rats with endotoxemia were loosened and atrophic while the epithelial cells were necrotic and the mucosa was edematous and infiltrated with inflammatory cells. The LPS-induced changes to the intestinal mucosa were most obvious in the rats after 12 h.

in sIgA levels in rat intestinal mucus after 2, 12 and 24 h compared with controls.

## DISCUSSION

This study has demonstrated that LPS-induced endotoxemia results in small intestinal mucosa injury. The number of M-cells, DCs, CD8<sup>+</sup> T cells and IgA<sup>+</sup> B cells were decreased and the number of Tr cells and apoptotic lymphocytes were increased significantly. The number of CD4<sup>+</sup> T cells was increased in the early stages before slightly decreasing by the end of the study period. Similarly, the level of IL-4 was significantly increased at early time points and then reversed by the 24 h time point, while IFN- $\gamma$  levels were also increased slightly at early time points before decreasing markedly by the end of the study. The level of Foxp3 was increased whereas the level of sIgA decreased.

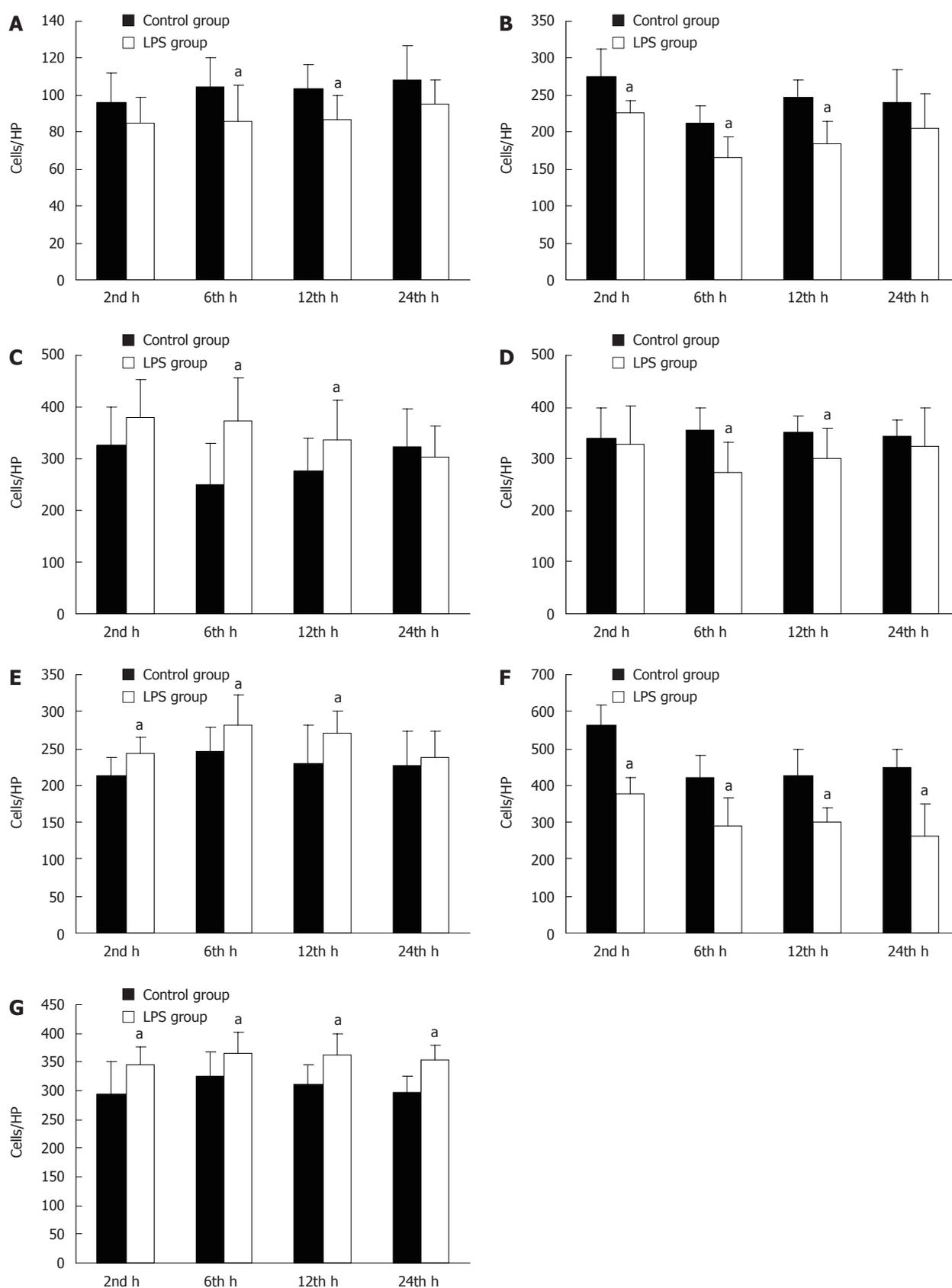
Many researchers have demonstrated that the digestive tract is the largest immune organ in the body<sup>[18]</sup>. The immune response of the digestive tract mucosa primarily relies on humoral immunity. The induction site of mucosal immune responses is at the Peyer's patches and the effector site is at the mucosa<sup>[19]</sup>. The mucosal immune response involves a number of processes: the M-cells of the Peyer's patches collect granular antigens and pass them to DCs and macrophages which can then activate the T cells. The activated T cells will further activate B cells and the latter will produce antigen-specific sIgA after homing to the effector site.

During endotoxemia, oxidative stress causes direct damage to cells and tissues and is involved in inflammatory cytokine production. The response of the immune

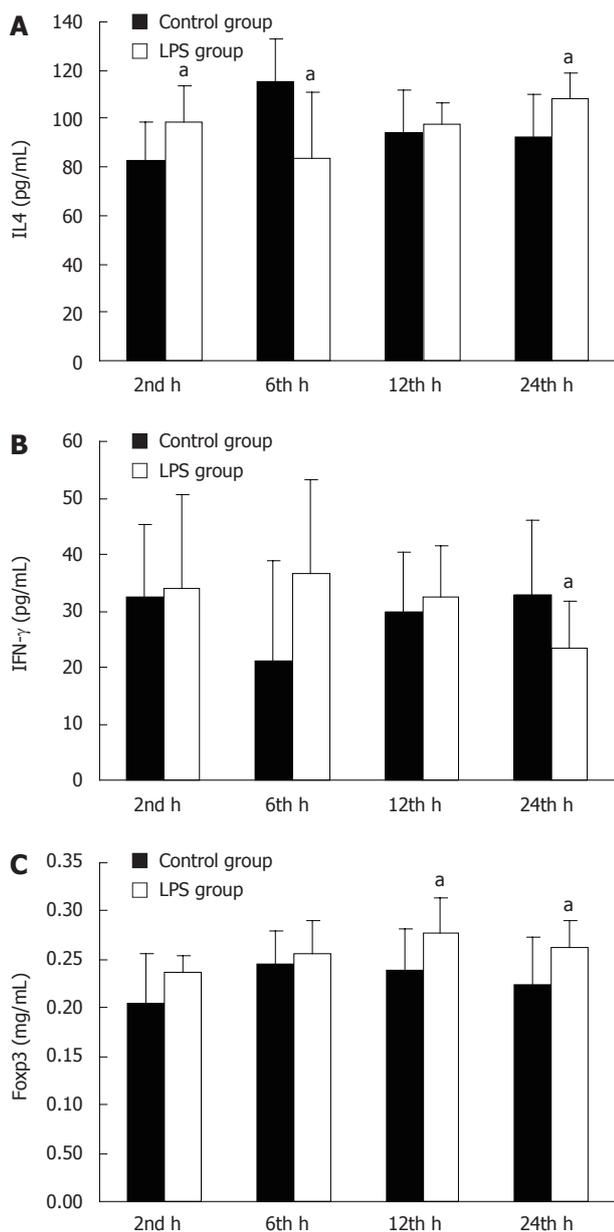
system to LPS is an inflammatory reaction in the early phase and anti-inflammatory reaction in the later period<sup>[20]</sup>.

LPS can activate phagocytes, stimulating them to release large amounts of cytokines and inflammatory factors which can further induce microcirculatory disturbances and intestinal epithelium injury, resulting in intestinal barrier damage<sup>[21]</sup>. In this study, it was observed that the small intestinal mucosa was injured in rats with endotoxemia, suggesting that the intestinal barrier was damaged and that the most severe damage occurred 12 h after LPS injection. This study also demonstrated that the immune function of the small intestinal mucosa changed most significantly after 12 h, indicating that the immune barrier dysfunction was a part of intestinal mucosal barrier injury.

To investigate the changes to local immune induction during endotoxemia we observed the numbers of M-cells and DCs in the intestinal mucosa. M-cells are a special kind of intestinal epithelium mucosa cell that can play a role by delivering antigen to antigen presenting cells (APCs). It is believed that the M-cell is the first step in intestinal immunity<sup>[22]</sup>. It has been observed that the number of M-cells decreased and the intestinal barrier was damaged during chronic intestinal inflammation and bacterial invasion<sup>[23]</sup>. In this study, we found the same change in M-cells during acute LPS stimulation, suggesting that endotoxemia could impair the first step of intestinal immunity. DCs are a kind of APC and a previous study by Hotchkiss *et al*<sup>[24]</sup> demonstrated DC depletion in patients with sepsis. In this study, a decrease in DC number was observed in the intestinal mucosa, suggesting the same change in rats with endotoxemia. The decreases in M-cell and DC numbers in the small intestinal mucosa in



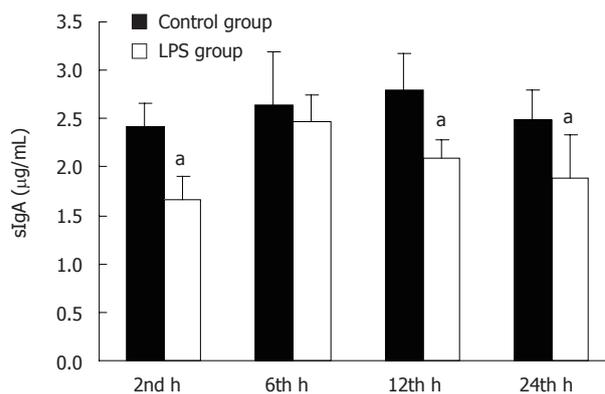
**Figure 3** The number of immune cells and apoptotic lymphocytes in the intestinal mucosa. A: The number of M-cells was significantly decreased after 6 and 12 h in the LPS group; B: The number of DCs was significantly decreased after 2, 6 and 12 h in the LPS group; C: The number of CD4<sup>+</sup> T cells was significantly increased after 6 and 12 h before slightly decreasing by 24 h in the LPS group; D: The number of CD8<sup>+</sup> T cells was significantly decreased after 6 and 12 h in the LPS group; E: The number of Tr cells was significantly increased after 2, 6 and 12 h in the LPS group; F: The number of IgA<sup>+</sup> B cells was significantly increased at all time points in the LPS group; G: The number of apoptotic lymphocytes was significantly increased at all time points in the LPS group. <sup>a</sup>*P* < 0.05.



**Figure 4** Levels of IL-4, IFN- $\gamma$  and Foxp3 in small intestinal mucosa. A: The level of IL-4 was significantly increased after 2 h and significantly decreased by 6 and 12 h in the LPS group; B: The level of IFN- $\gamma$  was increased slightly after 2, 6 and 12 h and decreased markedly by 24 h in the LPS group; C: The level of Foxp3 was significantly increased after 12 and 24 h in the LPS group. <sup>a</sup> $P < 0.05$ .

rats with endotoxemia implied that the induction of local immune responses was impaired.

T lymphocytes, especially the Tr cells, play a major regulatory role in mucosal immunity. T cells can be classified into CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells according to the different protein markers on their cell surface. TH cells and Tr cells belong to the CD4<sup>+</sup> T cell family. TH cells can be further divided into TH1 and TH2, where TH1 secretes inflammatory factors such as IFN- $\gamma$  to mediate the protective immune response while TH2 secretes anti-inflammatory factors such as IL-4 to mediate the non-specific immune response<sup>[25]</sup>. A previous study demonstrated a TH1/TH2 drift<sup>[26]</sup>, suggesting that the immune response progressed from being active to a sup-



**Figure 5** Small intestinal mucosa sIgA levels. The level of intestinal mucus sIgA was significantly decreased after 2, 12 and 24 h in the LPS group. <sup>a</sup> $P < 0.05$ .

pressed state during sepsis, while a further study demonstrated that it was Tr cells that mediated this drift<sup>[27]</sup>. In addition, other studies have demonstrated that Tr cells could selectively kill and directly suppress B cells<sup>[28,29]</sup>. It was reported that circulating Tr numbers were markedly elevated and induced immunoparalysis in sepsis<sup>[30]</sup>. The development and function of Tr cells can be investigated by the expression level of Foxp3<sup>[31]</sup>. CD8<sup>+</sup> T cells include cytotoxic T (Tc) cells and suppressor T (Ts) cells, which are the effector cells of cell immunity and suppressors of TH cells, respectively. In accordance with Bruder *et al*<sup>[16]</sup> study, we inferred Tr cell numbers from a single marker of NPR-1 positive cells in our study. We only observed the intraepithelial NPR-1 positive cells to exclude interference since NPR-1 can be expressed by other cells such as DCs. Our study showed that CD4<sup>+</sup> T cells increased in the early stages of endotoxemia before sepsis and then slightly decreased in the later phase, while CD8<sup>+</sup> T cells decreased and Tr cells increased in the small intestinal mucosa in rats with endotoxemia. It should also be noted that in this study the level of IL-4 increased at the beginning and then decreased by the end of the study whereas the level of IFN- $\gamma$  demonstrated the opposite change and the level of Foxp3 increased. Though a lot of activated immunological cells can secrete IL-4 and IFN- $\gamma$ , the effects of IL-4 and IFN- $\gamma$  remained unchanged. Similarly, Foxp3 is not exclusively expressed by Tr cells but also by activated effector T cells. The combination of an increase in NPR-1 cells and intestinal Foxp3 levels could suggest that Tr cells are increased. These results suggest a trend of immune changes whereby cellular immunity progressed from an active to a suppressed state during endotoxemia before sepsis. It can be concluded from this study that the rise in the number and function of Tr cells is one of the major reasons for immunosuppression in the small intestinal mucosa of rats with endotoxemia before sepsis.

IgA<sup>+</sup> B cells are identified by positive IgA staining, because only B cells produce IgA or have surface IgA. A previous study showing decreases in the level of sIgA, the number of IgA<sup>+</sup> B cells and the number of Gram-negative bacteria enclosed by sIgA in the intestinal tract during stress suggested that humoral immune function

was inhibited dramatically<sup>[32]</sup>. Our study demonstrated that the level of sIgA and the number of IgA<sup>+</sup> B cells diminished during endotoxemia, suggesting that LPS-induced endotoxemia inhibited humoral immune function of the digestive tract in accordance with the previous report. One explanation for this could be due to the rise in the number and function of Tr cells, as demonstrated in our study, which could lead to the suppression of B cell function.

Mongini *et al.*<sup>[33]</sup> reported that relocation of endotoxin after injury can increase the number of apoptotic lymphocytes. Our research demonstrated a similar change, with an increase in the number of apoptotic lymphocytes in the intestinal mucosa of rats with endotoxemia. This could contribute to the immunosuppression of the small intestine in rats with endotoxemia.

In conclusion, our study showed that mucosal immune barrier dysfunction was a part of intestinal mucosal barrier injury. Cellular immunity was active in the early phase of endotoxemia and suppressed in later periods, humoral immunity was abnormal and lymphocyte apoptosis was increased. Our study suggests that the increased number and function of Tr cells and the increase in lymphocyte apoptosis are the reasons for intestinal mucosal immunodeficiency. Based on these findings, an earlier protective or immunoregulative treatment aimed at gastrointestinal immune function may be of benefit to severely infected patients. We suggest future studies could be designed to test this hypothesis.

## COMMENTS

### Background

Immune dysfunction is one of the most frequent complications within the gastrointestinal mucosal barrier during sepsis. Mucosal barrier dysfunction plays an important role in the pathophysiology of sepsis by promoting bacterial stasis, bacterial overgrowth, and bacterial translocation, which can lead to the development of secondary infections and multiple organ failure. The changes of gastrointestinal immune function may appear at the beginning or even before sepsis.

### Research frontiers

Intestinal mucosal barriers consist of a mechanical barrier, a chemical barrier, an immunological barrier, and a biological barrier. The immunological barrier is considered as the first line of defense of the intestinal mucosa from bacterial invasion and plays an important role in the overall defense. This study observed the changes in the gastrointestinal immunological barrier during endotoxemia.

### Innovations and breakthroughs

Previous studies have discussed the changes to immunity during sepsis, but what happens to immunity, specifically the gut immunity, during endotoxemia before sepsis is not clear. In this study, changes to the number and function of intestinal mucosal immune cells in rats with endotoxemia were observed to investigate whether dysfunction of the immunological barrier occurred during endotoxemia and to elucidate the potential mechanism of this dysfunction.

### Applications

By observing the changes of gastrointestinal immune function, the study shows that the gastrointestinal immune dysfunction occurs during endotoxemia before sepsis. Thus, a protective or an immunoregulative treatment of gastrointestinal immune function should be used earlier in severely infected patients.

### Terminology

Lipopolysaccharides (LPS) are large molecules consisting of a lipid and a polysaccharide joined by a covalent bond; they are found in the outer membrane of Gram-negative bacteria, act as endotoxins and elicit strong immune responses in animals. Endotoxemia is the immune responses to LPS in animals. Cytokines: non-antibody proteins secreted by inflammatory leukocytes

and some non-leukocytic cells, which act as intercellular mediators. Sepsis is defined as infection plus systemic manifestations of infection.

### Peer review

This is a descriptive study of the effect of intravenous LPS on intestinal immune cells and cytokine levels in the rat. Straightforward and generally well written paper.

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## Effect of implanting fibrin sealant with ropivacaine on pain after laparoscopic cholecystectomy

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### Abstract

**AIM:** To investigate the safety and efficacy of implanting fibrin sealant with sustained-release ropivacaine in the gallbladder bed for pain after laparoscopic cholecystectomy (LC).

**METHODS:** Sixty patients (American Society of Anesthesiologists physical status was I or II and underwent LC) were randomly divided into three equal groups: group A (implantation of fibrin sealant in the gallbladder bed), group B (implantation of fibrin sealant carrying ropivacaine in the gallbladder bed), and group C (normal saline in the gallbladder bed). Postoperative pain was evaluated, and pain relief was assessed by visual analog scale (VAS) scoring.

**RESULTS:** The findings showed that 81.7% of patients had visceral pain, 50% experienced parietal, and 26.7% reported shoulder pain after LC. Visceral pain was significantly less in group B patients than in the other groups ( $P < 0.05$ ), and only one patient in this group experienced shoulder pain. The mean VAS score in group B patients was lower than that in the other groups.

**CONCLUSION:** Visceral pain is prominent after LC and can be effectively controlled by implanting fibrin sealant combined with ropivacaine in the gallbladder bed.

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**Key words:** Analgesia; Fibrin sealant; Laparoscopic cholecystectomy; Pain; Ropivacaine

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### INTRODUCTION

Since the widespread adoption of laparoscopic cholecystectomy (LC) in the late 1980s, LC has become the gold standard for chronic cholecystitis<sup>[1]</sup>. Postoperative pain after LC is generally less than that after open cholecystectomy, however, the postoperative pain experienced by patients still causes preventable distress. Treating postoperative pain is an important and primary objective, because it affects patients' comfort, postoperative morbidity, and, inevitably, social costs due to prolonged hospitalization and work inactivity. Pain after LC can be divided into three components, namely, visceral, parietal, and shoulder pain, with different intensities and time courses<sup>[2]</sup>. LC is mainly associated with visceral pain<sup>[3]</sup> which may refer to the shoulder in 35% to 60% of cases<sup>[4,5]</sup>. Various treatments have been proposed to make this surgery as pain-free as possible<sup>[6-8]</sup>. The main objective of this study was to assess the effectiveness of implanting fibrin sealant combined with ropivacaine in the gallbladder bed for pain control after LC.

### MATERIALS AND METHODS

The study was designed as a single-center, randomized trial. Of the 78 patients who underwent LC from October 2008 to August 2009, 60 patients (42 women, 18 men) were enrolled in this study, which was performed after approval was received from the Ethics Committee of Beijing Tongren Hospital, Capital Medical University. All patients whose American Society of Anesthesiologists (ASA) physical status were I or II underwent diagnostic abdominal ultrasound, liver function tests, and coagulation profile along with hematologic and biochemical investigations. Patients with previous major upper abdominal surgeries, choledocholithiasis, acute

cholecystitis, or conversion to open cholecystectomy were excluded from the study. Patients with a body mass index higher than 35, and those with diminished liver and kidney functions were not evaluated in this study. The visual analog pain evaluation scale (VAS) was introduced to the patients before surgery and the details of the study were explained to the patients. All patients stated that they understood the VAS. Patients who were unable to comprehend the scale were not included in the study. Only patients who were suitable and compatible with the study design were included. The patients were randomly divided into 3 equal groups with the help of computer-generated randomization numbers.

All patients underwent LC under a standard general anesthetic technique for premedication and during the intraoperative period. The anesthetist performed intraoperative, noninvasive monitoring. Ventilation was adjusted to maintain an end-tidal CO<sub>2</sub> pressure below 38 mmHg. Second-generation cephalosporin (cefoxitin) 1 g was injected intravenously before the induction of anesthesia. LC was carried out using the standard three-port technique, and CO<sub>2</sub> pneumoperitoneum pressure was maintained at 14 mmHg throughout the procedure. The procedures were performed by the same experienced surgeon. After complete hemostasis and the gallbladder bed washed with normal saline, the different treatments were performed according to the different groups. Group A: Fibrin sealant (5 mL) (Guangzhou Bioseal Biotech, Guangzhou, China) was implanted in the gallbladder bed. Group B: Fibrin sealant (5 mL) combined with ropivacaine (1 mg/kg body weight) was implanted in the gallbladder bed. Group C: The gallbladder bed was doused with normal saline only.

Following LC, carbon dioxide was evacuated through the ports by applying gentle pressure all over the abdomen. Gallbladders were taken out of the peritoneal cavity *via* the umbilical incision. Rescue analgesia (intramuscular dolantin 50 mg) or rescue antiemetic (intramuscular ondansetron 8 mg) was administered if the VAS was higher than 10 or the patient complained of vomiting. The amount of dolantin used was noted.

The degree of postoperative pain was assessed every 4 h in the first 12 h after surgery and then every 12 to 48 h, using a VAS (0 = no pain, 10 = worst possible pain), by nursing staff who were unaware of the perioperative intervention. The character of the pain was also assessed simultaneously. Visceral pain was defined as deep-seated pain located in the right hypochondrium or referred to the shoulder. Parietal pain was defined as incisional pain located at the trocar sites.

### Statistical analysis

Data were expressed as the mean  $\pm$  SE. All data were prepared and compiled using SPSS 16.0 software. Statistical differences were determined by ANOVA using the Dunnett procedure in non-repeated measures obtained by the mean postoperative VAS scores for the various groups. Categorical variables were recorded as numbers (percentages) and compared by using the  $\chi^2$  test with

Table 1 Patient characteristic data (mean  $\pm$  SE)

Variables	Group A	Group B	Group C
Age (yr)	41.6 $\pm$ 16.1	42.2 $\pm$ 14.7	40.8 $\pm$ 17.6
Weight (kg)	62.6 $\pm$ 18.3	65.3 $\pm$ 16.8	64.7 $\pm$ 19.1
Sex (M/F)	14/6	14/6	14/6
Duration of anesthesia (min)	106.7 $\pm$ 21.4	98.2 $\pm$ 24.3	103.7 $\pm$ 25.7
Duration of surgery (min)	66.4 $\pm$ 20.1	62.8 $\pm$ 19.5	68.5 $\pm$ 18.3

Table 2 Multiple comparisons of visual analog scale (VAS) scores for various groups *vs* group C (the control) (mean  $\pm$  SE)

Time (h)	Group	n	VAS	P value
4	G1	20	7.2 $\pm$ 2.8	0.547
	G2	20	4.3 $\pm$ 1.2	0.007
	G3	20	8.3 $\pm$ 3.5	
8	G1	20	6.5 $\pm$ 3.7	0.435
	G2	20	3.4 $\pm$ 1.6	0.008
	G3	20	7.0 $\pm$ 3.1	
12	G1	20	5.1 $\pm$ 1.9	0.327
	G2	20	2.5 $\pm$ 0.8	0.001
	G3	20	5.9 $\pm$ 2.0	
24	G1	20	3.5 $\pm$ 1.3	0.264
	G2	20	1.8 $\pm$ 0.9	0.001
	G3	20	4.0 $\pm$ 1.8	
36	G1	20	2.7 $\pm$ 1.2	0.362
	G2	20	1.2 $\pm$ 0.6	0.001
	G3	20	2.9 $\pm$ 1.0	
48	G1	20	2.0 $\pm$ 0.8	0.538
	G2	20	0.8 $\pm$ 0.3	0.001
	G3	20	2.2 $\pm$ 0.8	

Yates correction. The threshold for statistical significance was considered  $P < 0.05$ .

## RESULTS

The 60 study patients (42 women and 18 men) varied in age from 25 to 63 years (median, 41.2 years). The three groups did not differ in mean age, body weight, or ASA status. None of the patients had a history of jaundice or gallstone pancreatitis. Eleven patients (18.3%) had previously undergone lower abdominal surgery. There was no significant difference in the duration of surgery among the three groups ( $P = 0.587$ ): 66.4  $\pm$  20.1 min for group A, 62.8  $\pm$  19.5 min for group B, 68.5  $\pm$  18.3 min for group C (Table 1).

The VAS score decreased after surgery in all patients. Analysis of variance followed by multiple comparisons using the Dunnett procedure, with group C used as a control, suggested that the mean VAS score for group B was significantly less than that for group A and C. The mean VAS scores for group A were lower than Group C, but the difference was not significant (Table 2).

The overall incidence of visceral, parietal, and shoulder pain in our study were 81.7%, 50.0%, and 26.7%, respectively. However, the incidence of visceral pain in group B was less than that in the other groups ( $P < 0.05$ ) (Table 3). The number of patients in group B experiencing visceral pain after surgery was also significantly lower

**Table 3** Character of pain after laparoscopic cholecystectomy *n* (%)

Group	<i>n</i>	Visceral	Parietal	Shoulder
A	20	18 (90.0)	10 (50.0)	7 (35.0)
B	20	12 (60.0)	9 (45.0)	1 (5.0)
C	20	19 (95.0)	11 (55.0)	8 (40.0)
Total	60	49 (81.7)	30 (50.0)	16 (26.7)

than that in the other groups ( $P < 0.05$ ). The overall incidence of parietal pain was 50.0% in group A, 45.0% in group B and 55.0% in group C ( $P > 0.05$ ). There was no difference in the incidence of parietal pain between the three groups. Only one patient in group B reported shoulder pain, as compared with 16 (26.7%) of the 40 patients in groups A and C ( $P < 0.01$ ).

Rescue analgesia (intramuscular pethidine hydrochloride 50 mg once) was administered if the VAS was higher than 10. The amount of pethidine hydrochloride used per capita in group B ( $2.5 \pm 11.2$  mg) was significantly lower than that in group A ( $30.0 \pm 25.1$  mg) and group C ( $25.0 \pm 25.6$  mg).

## DISCUSSION

Even though postoperative pain after LC is markedly less than that after open cholecystectomy, pain is still the patient's first complaint after LC<sup>[9]</sup>. Although postoperative pain is reduced compared to laparotomic surgery<sup>[10]</sup>, effective analgesic treatment still remains crucial for early patient discharge<sup>[11]</sup>. Usually, postoperative pain following LC peaks immediately after surgery, and decreases within 24 h, and then increases to a second or even a third peak later<sup>[12]</sup>.

The incidence of pain after laparoscopy may be attributed to the carbon dioxide gas (CO<sub>2</sub>) used to induce pneumoperitoneum<sup>[13,14]</sup>. CO<sub>2</sub> remains in the peritoneal cavity for several days after surgery and causes stretching of the phrenic nerve endings<sup>[15]</sup>, local hypothermia, and diaphragmatic irritation *via* carbonic acid<sup>[16]</sup>. The benefit of using intraperitoneal local anesthetics for shoulder and abdominal pain control has been proven<sup>[7,17-19]</sup>, however, several other studies did not confirm these findings<sup>[20-23]</sup>.

Pain after LC includes three components, visceral, parietal, and shoulder pain<sup>[3]</sup>. In the early postoperative period, many studies report that visceral pain is predominant, especially during the first hours after surgery<sup>[3]</sup>. At the same time, parietal pain is less intense because of the small incisions and limited damage to the abdominal wall. Shoulder pain may occur later with visceral pain. The most common location of postoperative pain is in the right upper quadrant, followed by the trocar site and the shoulder<sup>[24]</sup>.

In this study, all operations were progressed according to the line of least tissue damage. We took the gallbladder out of the peritoneal cavity *via* the umbilical incision which was less sensitive than the other incisions. Thus, we observed that parietal pain was mild in this study and did not contribute substantially to the VAS score.

Fibrin sealant has been an extremely effective and

widely used adjunct to surgical procedures for the control of diffuse slow bleeding over large surfaces. In addition, fibrin sealant has been used as a carrier for other compounds. Thus, it has been used to release medicines slowly at a fixed site which are therefore effective for a long time.

We observed a significant reduction in pain after gallbladder bed implantation of fibrin sealant combined with ropivacaine. This effect was indirectly reflected in the progressive reduction in both the VAS score and visceral pain in this group of patients. This suggests that the progressive reduction in the VAS score in this group of patients was primarily attributable to the effective control of visceral pain.

The VAS score for the patients with fibrin sealant alone implanted in the gallbladder bed was less than that of the control group, although the differences were not statistically significant. This suggests that the gallbladder bed with implanted fibrin sealant alone may lead to a slight relief in postoperative pain.

Verma *et al*<sup>[19]</sup> reported that visceral pain is prominent after LC and can be effectively controlled by 0.5% bupivacaine-soaked Surgicel in the gallbladder bed alone. They used bupivacaine 0.5% (2 mg/kg) instilled over the oxidized regenerated cellulose strips (Surgicel) in the gallbladder bed, and found that the postoperative pain was significantly less in these patients than in the control groups (bupivacaine infiltrated at the trocar sites and normal saline in the gallbladder bed and at the trocar sites).

These findings are in accordance with the anatomical characteristics of the phrenic nerve which supplies the gallbladder, porta hepatis, and liver, while sharing the root of nerves to the shoulder<sup>[25]</sup>. We used fibrin sealant carrying ropivacaine adhered to the gallbladder bed. Using this method, ropivacaine was released slowly and the stickiness of the fibrin sealant ensured that the drug remained in contact with the wound for a longer period of time. In our study, implanting fibrin sealant combined with ropivacaine in the gallbladder bed was effective in controlling shoulder pain, and only one of the patients in this treatment group experienced shoulder pain.

In conclusion, we conclude that implanting fibrin sealant combined with ropivacaine in the gallbladder bed is effective in controlling both visceral and shoulder pain after LC.

## COMMENTS

### Background

Even though postoperative pain after laparoscopic cholecystectomy (LC) is markedly less than that after open cholecystectomy, pain is still the patient's first complaint after LC.

### Research frontiers

According to the anatomical characteristics of the nerve which supplies the gallbladder, the use of fibrin sealant carrying ropivacaine adhered to the gallbladder bed can relieve postoperative pain.

### Innovations and breakthroughs

This study determined that implantation of fibrin sealant with sustained-release ropivacaine in the gallbladder bed could relieve the pain after LC.

### Applications

The implantation of 5 mL fibrin sealant combined with ropivacaine (1 mg/kg

body weight) in the gallbladder bed provided significant postoperative pain relief compared with the implantation of fibrin sealant alone.

### Terminology

Fibrin sealant can be used as a carrier for ropivacaine. With fibrin sealant, ropivacaine could be released slowly, and the stickiness of the fibrin sealant ensured that the drug remained in contact with the wound for a longer period of time.

### Peer review

The authors have assessed the use of a fibrin sealant with local anaesthetic in the gallbladder bed on post-operative pain after LC. Fibrin sealant with local anaesthetic was associated with lower post-operative pain scores.

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## Multidrug resistance protein 3 R652G may reduce susceptibility to idiopathic infant cholestasis

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### Abstract

**AIM:** To evaluate the role of genetic factors in the pathogenesis of idiopathic infant cholestasis.

**METHODS:** We performed a case-control study, including 78 infants with idiopathic infant cholestasis and 113 healthy infants as controls. Genomic DNA was extracted from peripheral venous blood leukocytes using phenol chloroform methodology. Polymerase chain reaction was used to amplify the multidrug resistance protein 3 (MDR3) R652G fragment, and products were sequenced using the ABI 3100 Sequencer.

**RESULTS:** The R652G single nucleotide polymorphism (SNP) was significantly more frequent in healthy infants (allele frequency 8.0%) than in patients (allele frequency 2.60%) ( $P < 0.05$ ), odds ratio, 0.29; 95% confidence interval, 0.12-0.84. The conjugated bilirubin in patients with the AG genotype was significantly lower than in those with the AA genotype ( $44.70 \pm 6.15 \mu\text{mol/L}$  vs  $95.52 \pm 5.93 \mu\text{mol/L}$ ,  $P < 0.05$ ).

**CONCLUSION:** MDR3 R652G is negatively correlated with idiopathic infant cholestasis. Children with the R652G SNP in Guangxi of China may have reduced susceptibility to infant intrahepatic cholestasis.

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**Key words:** Multidrug resistance protein 3; Single nucleotide polymorphisms; R652G; Infant; Cholestasis

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Chen XQ, Wang LL, Shan QW, Tang Q, Lian SJ. Multidrug resistance protein 3 R652G may reduce susceptibility to idiopathic infant cholestasis. *World J Gastroenterol* 2009; 15(46): 5855-5858 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5855.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5855>

### INTRODUCTION

Idiopathic infant cholestasis is of unknown etiology and can occur in either a sporadic or a familial form. Most affected infants have a good prognosis. Approximately 5%-10% have persistent inflammation or fibrosis, and a few develop more severe liver disease, such as cirrhosis. Although the cause of idiopathic infant cholestasis is unknown, genetic factors have been suggested by the existence of some other entities with cholestasis, such as familial intrahepatic cholestasis-1 mutation in progressive familial intrahepatic cholestasis type 1 (PFIC-1), bile salt export pump deficiency in PFIC-2<sup>[1-3]</sup>, multidrug resistance protein 3 (MDR3) deficiency in PFIC-3<sup>[4-6]</sup>.

Studies have investigated the involvement of MDR3 variants in the development of disease, and we were interested to note that the MDR3 R652G single nucleotide polymorphism (SNP) has a high allele frequency in generally healthy people<sup>[7]</sup>. Our aim was to study the MDR3 R652G SNP distribution frequency in idiopathic infant cholestasis and healthy infants and to determine whether there were any differences between them and, if so, their relevance.

### MATERIALS AND METHODS

#### Patients

Seventy eight infants were diagnosed with idiopathic infant cholestasis in the case group and there were 113 healthy infants in the control group. The patients were included in the study on the basis of the following crite-

ria: (1) a history of chronic cholestasis onset in the neonatal/infant period with unknown origin; (2) presence of hepatomegaly or hepatosplenomegaly; (3) persistent marked serum alanine aminotransferase (ALT) activity; (4) elevated total bile acid concentration and total bilirubin (TB), mainly marked conjugated bilirubin (CB); (5) absence of viral infections (such as hepatitis A virus, hepatitis B virus, hepatitis C virus, cytomegalovirus).

For patients and healthy infants, the following biochemical parameters were recorded: serum ALT and aspartate aminotransferase (AST);  $\gamma$ -glutamyltransferase ( $\gamma$ -GT) and serum TB and CB.

We received informed consent from all the subjects' guardians to take part in the study, and the protocol was approved by the local ethical committee of the Hospital.

### Polymerase chain reaction (PCR) amplified the MDR3 R652G and sequence analysis

Genomic DNA was extracted from peripheral venous blood leukocytes using standard phenol chloroform procedures. The DNA concentration was quantified by spectrophotometry. Primers for MDR3 R652G were forward (5'-CATCCATTTGGAGACACACACAC-3') and reverse (5'-GTAGCAGTCATCTGTGCCTGAA A-3')<sup>[7]</sup>. The primary PCR product fragments were 348 bp. PCRs for generating the R652G fragments were generally performed in a reaction volume of 50  $\mu$ L with 100 ng of genomic DNA, 1.5 U of *Taq* polymerase (Fermentas), 10  $\times$  PCR buffer (Fermentas), 1.5 mmol/L of MgCl<sub>2</sub> (Fermentas), 200  $\mu$ mol/L deoxynucleoside-5-triphosphate (Takara) and 20  $\mu$ mol of each primer. PCR conditions included an initial denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s and extension at 72°C for 1 min. The PCR reaction was terminated after an extension step at 72°C for 10 min. The PCR products were analyzed by 2% agarose gel with 0.5  $\mu$ g/mL ethidium bromide and quantitated approximately with a DNA marker (Takara, DaLiang). The PCR products sequence analysis was run in the ABI 3100 automated DNA sequencer. Sequences were compared with the sequence of the MDR3 in GenBank (NG\_007118.1, GI: 169234676).

### Statistical analysis

Data are given as mean  $\pm$  SD. Comparison of the frequency of the R652G SNP was made between patients and controls, and the analysis of association with the phenotype was performed by  $\chi^2$  test or Fisher's exact test when appropriate. A *P*-value < 0.05 was considered statistically significant. The data was analyzed using SPSS 13.0. The odds ratio (OR) was calculated with the corresponding 95% confidence intervals (95% CI). Allele frequency was tested for the Hardy-Weinberg equilibrium.

## RESULTS

The study comprised 78 infants with idiopathic infant cholestasis and 113 controls. Details of the study groups are shown in Table 1. The mean total bilirubin was

Table 1 Clinical characteristics of the 78 idiopathic infant cholestasis patients (mean  $\pm$  SD)

	Patients	Controls
Age (mo)	1.8 $\pm$ 0.31	1.2 $\pm$ 0.25
TB ( $\mu$ mol/L)	194.43 $\pm$ 13.33 <sup>a</sup>	12.94 $\pm$ 5.15
ALT (U/L)	123.80 $\pm$ 11.63 <sup>a</sup>	29.09 $\pm$ 2.62
AST (U/L)	273.86 $\pm$ 27.90 <sup>a</sup>	34.43 $\pm$ 2.43
$\gamma$ -GT (U/L)	308.60 $\pm$ 46.30 <sup>a</sup>	30.22 $\pm$ 9.90

<sup>a</sup>*P* < 0.05 vs control. TB: Total bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase;  $\gamma$ -GT:  $\gamma$ -glutamyltransferase.

Table 2 MDR3 R652G genotypes and allele frequencies in patients and controls *n* (%)

Groups	<i>n</i>	Genotypes			Allele frequency (%)	<i>P</i> -value
		A/A	A/G	G/G		
Patients	78	74 (94.9)	4 (5.1)	0	2.6	< 0.05 <sup>1</sup>
Controls	113	95 (84.1)	18 (15.9)	0	8.0	

<sup>1</sup>Fisher's exact test between patients and controls for genotype frequency. *P* = 0.022; OR, 0.29; 95% CI, 0.12-0.84.

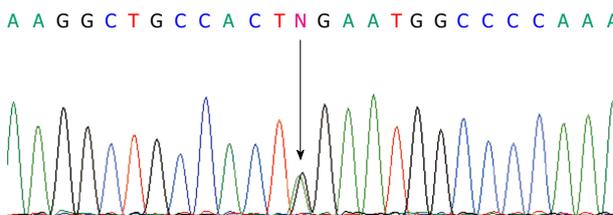


Figure 1 Sequence of the multidrug resistance protein-3 (MDR3) single nucleotide polymorphism R652G (A>G).

194.43  $\pm$  13.33  $\mu$ mol/L (normal  $\leq$  20  $\mu$ mol/L). The mean CB was 105.75  $\pm$  6.44  $\mu$ mol/L. The mean serum transaminase levels were 273.86  $\pm$  27.90 and 123.80  $\pm$  11.63 U/L for AST and ALT, respectively (normal  $\leq$  40 U/L), and mean serum  $\gamma$ -GT was 308.60  $\pm$  46.30 U/L (normal  $\leq$  50 U/L). The levels were all normal in the control group. All the patients had hepatomegaly, and 62 of the 78 cases had splenomegaly.

PCR products sequence analysis showed a heterozygous substitution A>G (Figure 1) in codon 652 which creates an amino acid substitution in codon R652G in exon 16. Distribution of the genetic polymorphisms in patients and controls is shown in Table 2. Test results showed that the R652G genotype distribution in the 2 groups was in line with the Hardy-Weinberg equilibrium, indicating that the selected sample was representative of the population. The patient AG genotype frequency was significantly lower than in the control group (*P* < 0.05). The AG genotype was negatively correlated with idiopathic infant cholestasis (OR, 0.29, 95% CI: 0.12-0.84) showing that the AG genotype has a protective effect in the normal population (Table 2).

### Biochemical markers comparison in patients with different genotypes

The patient group was divided into 2 sub-groups ac-

**Table 3** Comparison of clinical characteristics in patient sub-groups according to genotype (mean  $\pm$  SD)

	Patients A/A	Patients A/G
TB ( $\mu\text{mol/L}$ )	198.94 $\pm$ 14.85	91.65 $\pm$ 15.70
CB ( $\mu\text{mol/L}$ )	95.52 $\pm$ 5.93	44.70 $\pm$ 6.15*
ALT (U/L)	131.54 $\pm$ 11.91	92.50 $\pm$ 10.01
AST (U/L)	281.29 $\pm$ 28.26	129.25 $\pm$ 12.43
$\gamma$ -GT (U/L)	317.68 $\pm$ 47.64	77.00 $\pm$ 18.78

CB: Conjugated bilirubin. \* $P < 0.05$ .

according to genotype and serum biochemical parameters were compared. The CB of the AG genotype ( $44.70 \pm 6.15 \mu\text{mol/L}$ ) was significantly lower compared with that of the AA genotype ( $95.52 \pm 5.93 \mu\text{mol/L}$ ) ( $P < 0.05$ ). Other serum markers in the sub-groups with the 2 genotypes were not significantly different ( $P > 0.05$ ) (Table 3).

## DISCUSSION

Our study is the first report of the MDR3 R652G SNP in children in China. Our data showed that the R652G SNP had a significantly higher proportional distribution in normal infants than in idiopathic infant cholestasis patients. Moreover, in the cholestasis group, the CB of AG genotype patients had a lower mean value than in the AA genotype patients. It is known that CB is a very important index that can reflect the extent of intrahepatic cholestasis. For this reason, we can infer that the R652G variant has a specific protective effect in the normal population.

In previous studies, in the analysis of MDR3 gene sequence variants in different countries, the R652G SNP was the only protein-altering variant with high allele frequency in all groups. It was 7.3% in Caucasians, 1.4% in Japanese and 2.3% in Koreans<sup>[7]</sup>. In another study, the variant was 7.3% in healthy Caucasians, 8.6% in primary biliary cirrhosis patients and 12.8% primary sclerosing cholangitis patients<sup>[8]</sup>. R652G is a MDR3 gene mutation that results in non-synonymous amino acid substitutions but is not associated with disease. In Switzerland, a study showed that the MDR3 R652G variant was 7% in healthy Caucasians, 9% in drug-induced cholestatic patients and 4% in hepatocellular/mixed liver injury<sup>[9]</sup>. Pauli-Magnus *et al*<sup>[10]</sup> reported the R652G variant in 10% of intrahepatic cholestasis pregnancy cases and 16.3% in healthy controls.

The previous studies indicated that the R652G variant was prevalent in the general population. Furthermore, studies of MDR3 R652G in a variety of diseases showed no evidence that the R652G variant was related to disease. In our study, the R652G variant was 15.9% in healthy children, with a higher frequency than in infant cholestasis patients. Therefore, we believe that the AG genotype has a protective effect in infant cholestasis, and the AG genotype is likely to reduce the risk of children suffering from cholestasis. In this study, the proportion of the AG gene type in the normal population was higher than in

previous reports from other regions, possibly because of the existence of racial and geographic differences.

There is also another phenomenon whereby R652G may have a protective effect and reduce the risk of suffering from disease. Jacquemin *et al*<sup>[11]</sup> reported one patient with intrahepatic cholestasis of pregnancy carrying a R652G substitution, and whose biliary phospholipids were lower than other patients. Another study showed the A/G allele frequency was 6% in intrahepatic cholestasis of pregnancy patients and 17% in healthy controls in Sweden. The AA genotype subjects were more likely to suffer from cholestasis<sup>[12]</sup>. Our result also confirmed this conclusion.

A recent study of gallstones in sibling pairs and controls showed that R652G variant frequency was 18% in patients and 25% in controls. In the control sub-groups, subjects with the GG genotype had significantly lower cholesterol compared with those with the AA genotype. It is known that cholesterol is the raw material for synthesis of bile acids<sup>[13]</sup>. This gives an indication of the direction of future research.

It is worth noting that this is an interesting new result. Most of the MDR3 gene variants have reported an association with disease causation, such as PFIC and intrahepatic cholestasis of pregnancy<sup>[14,15]</sup>. In contrast, the R652G variant seems to have a protective effect. It has been assumed that the missense mutation R652G is a neutral polymorphism. Sequence comparisons of MDR3 genes identified that the glycine residue is conserved in other species such as rat and hamster. More research will be required to confirm that in the future.

In conclusion, the MDR3 R652G SNP is negatively correlated with cholestasis (OR, 0.29, 95% CI, 0.12-0.84). Children in Guangxi of China with the R652G variant may have reduced susceptibility to infant intrahepatic cholestasis.

## COMMENTS

### Background

Idiopathic infant intrahepatic cholestasis is a common clinical disease that can occur in either a sporadic or a familial form. The incidence of idiopathic infant intrahepatic cholestasis is very high in China. If not treated early, approximately 5%-10% of patients can have persistent inflammation or fibrosis, and a few develop more severe liver disease, such as cirrhosis.

### Research frontiers

All studies in recent years on the multidrug resistance protein 3 (MDR3) variants have involved the development of disease. However, an interesting phenomenon is that there was no positive evidence for the MDR3 R652G variant being involved in the pathogenesis of intrahepatic cholestasis. This study investigated the MDR3 R652G distributed allele frequency in idiopathic infant cholestasis and healthy infants to determine whether there were any difference and, if so, their relevance.

### Innovations and breakthroughs

Most studies have concentrated on MDR3 mutations in the pathogenesis of progressive familial intrahepatic cholestasis type 3, intrahepatic cholestasis of pregnancy and cholelithiasis. There have been few studies investigating MDR3 and idiopathic infant intrahepatic cholestasis. In order to evaluate the role of the MDR3 R652G variant in the pathogenesis of idiopathic infant cholestasis, the authors analyzed the MDR3 R652G polymorphism in a case-control study in Guangxi Chinese infants. The authors found that the R652G variant was significantly more frequent in healthy infants than in patients. The results showed that the R652G variant has a protective effect in healthy infants and reduces the possibility of suffering from idiopathic infant cholestasis. The result

was not the same as a previous study. The reason may be a result of ethnic population differences and the variability in geographical location.

### Applications

The study results suggest that the MDR3 R652G variant has a protective effect in healthy infants. This will give further information for comparing geographical regions, and it is very important to establish particular characteristics of MDR3 R652G that can be useful in the differential diagnosis of idiopathic infant cholestasis, and furthermore, may establish the influence of such a single nucleotide polymorphism (SNP) in prognosis.

### Terminology

MDR3 R652G: MDR3 R652G is a SNP of the MDR3 in the 652 coding site. The MDR3 is encoded by the ABCB4 gene, and the 652 coding site is located in ABCB4 exon 16. The R652G is a gene mutation where the adenine (A) mutates into guanine (G) which causes AGA>GGA resulting in arginine substitution by glycine (R652G).

### Peer review

The paper gives a significant contribution to basic science knowledge, as well as clinical practice. Publishing this manuscript will allow further comparison with data from other regions and help to clarify the pathogenesis of idiopathic infant cholestasis.

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## Two synchronous somatostatinomas of the duodenum and pancreatic head in one patient

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### Abstract

Somatostatinomas are extremely rare neuroendocrine tumors of the gastrointestinal tract, first described in the pancreas in 1977 and in the duodenum in 1979. They may be functional and cause somatostatinoma or inhibitory syndrome, but more frequently are non-functioning pancreatic endocrine tumors that produce somatostatin alone. They are usually single, malignant, large lesions, frequently associated with metastases, and generally with poor prognosis. We present the unique case of a 57-year-old woman with two synchronous non-functioning somatostatinomas, one solid duodenal lesion and one cystic lesion within the head of the pancreas, that were successfully resected with a pylorus-preserving Whipple's procedure. No secondaries were found in the liver, or in any of the removed regional lymph nodes. The patient had an uneventful recovery, and remains well and symptom-free at 18 mo postoperatively. This is an extremely rare case of a patient with two synchronous somatostatinomas of the duodenum and the pancreas. The condition is discussed with reference to the literature.

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**Key words:** Somatostatinoma; Duodenal neoplasms; Pancreatic neoplasms

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### INTRODUCTION

Somatostatinomas are extremely rare and account for < 1% of functional APUD (amine precursor uptake and decarboxylation) cell neuroendocrine tumors of the gastrointestinal tract<sup>[1,2]</sup>, with an annual incidence of 1 case per 40 million people<sup>[3]</sup>. Somatostatinomas usually occur within the pancreas but can also manifest in the duodenum, and less frequently in the jejunum and cystic duct<sup>[4-6]</sup>. Since the first description of somatostatinoma of the pancreas in 1977<sup>[7]</sup>, and duodenum in 1979<sup>[8]</sup>, 100-200 cases<sup>[1,2,9]</sup> have been described in the world literature, each occurring as a single lesion. A PubMed search resulted in a single case report of two somatostatinomas in the same patient, associated with type I neurofibromatosis<sup>[10]</sup>.

We present an extremely rare case with two synchronous non-functioning somatostatinomas, one solid duodenal lesion and one cystic lesion within the head of the pancreas, without other disorders such as von Recklinghausen's disease.

### CASE REPORT

A 57-year-old woman presented with moderate epigastric pain in January 2007. Clinically, she was found to have only mild tenderness in the epigastrium, and all laboratory data were within normal limits. Ultrasound (US) and computed tomography (CT) revealed a 3 cm × 3 cm tumor in the duodenum, which was causing narrowing of its lumen to 1 cm, as well as a mass with a gas-filled cyst within the head of the pancreas (Figure 1). A cyst was also found within the head of the pancreas. A barium meal confirmed the duodenal luminal narrowing, and the tumor position above the ampulla of Vater, and



**Figure 1** Computed tomography (CT) showing well-vascularized mass within the duodenum and in the head of the pancreas. There is also a cystic lesion containing gas within the pancreatic mass.

upper gastrointestinal endoscopy provided a histological specimen that indicated a neuroendocrine tumor.

At laparotomy, two separate masses were found, one within the medial wall of the descending duodenum, and the second within the head of the pancreas. There were no visible or palpable secondaries within the liver or the peritoneal cavity. As both tumors appeared resectable, we proceeded with a pylorus-preserving Whipple's procedure, as well as regional lymph node dissection. The postoperative recovery was uneventful and the patient remains well at 18 mo.

Macroscopically, the first lesion was a well-demarcated solid nodular suprapapillary tumor of the duodenum, 42 mm in its greatest diameter, 25 mm from the pylorus, and penetrating 15 mm through the duodenal wall to reach the superior margin of the head of the pancreas. There was no obvious intrapancreatic or choledochal infiltration of the tumor. A transverse section showed the tumor to be composed of soft grey/yellow granular and light brown nodular tissues (Figure 2A-D).

The second was a distinctive cystic and hemorrhagic lesion, with a tiny fistula with the duodenum, within the enlarged head of the pancreas. The lesion measured 36 mm in its greatest diameter, contained necrotic and hemorrhagic coagula, and was surrounded by a thin fibrotic pseudocapsule zone. The inner surface of the cyst contained a layer of spongy tan/light brown tissue. There were no other notable micromorphological findings in the remainder of the resected pancreatic specimen.

Histopathology of the duodenal tumor revealed a dominant micronodular and insular growth of epithelioid cells, with rare trabecular or solid microorganization, and without necrotic areas (Figure 3A). The tumor cells were packed tightly with a high degree of uniformity and ill-defined ovoid/polygonal shapes. Their round nuclei were situated centrally, with low nuclear anaplasia and two mitotic figures per 10 high-power fields. However, despite expansive growth and a clearly demarcated border, peritumoral venous infiltration and microinfiltration of the periduodenal fatty tissue was found frequently.

The pseudocystic lesion of the pancreas showed an

obvious neoplastic proliferation with similar histology seen within the thin neoplastic peripheral rim. This narrow peripheral zone was surrounded by a pseudocapsule, and the vast majority of cells contained within the cyst showed hemorrhagic and necrotic changes (Figure 3B). No extrapancreatic spread or involvement of the regional lymph nodes was found, although focal venous infiltration was observed.

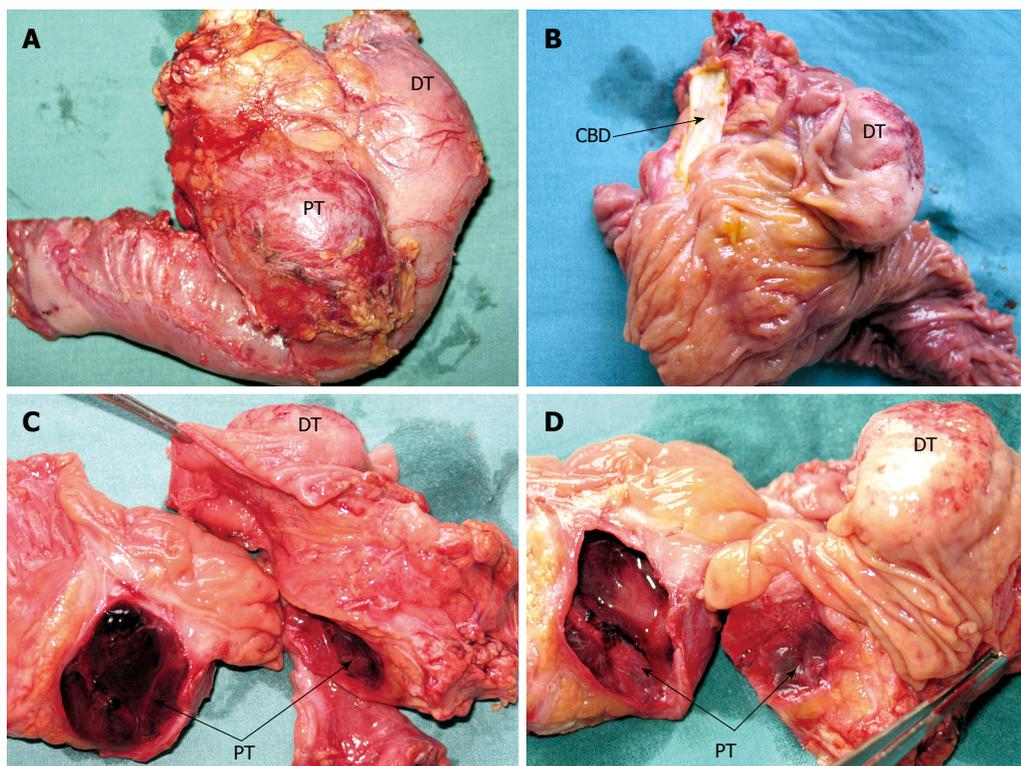
Immunohistochemistry confirmed that both tumors were derived from a similar cell origin. There was a significant cytoplasmic immunoreactivity of pan-neuroendocrine markers: chromogranin A occurred more intensely and frequently than synaptophysin (Figure 4A). There was strong somatostatin immunoreactivity in over half of the duodenal tumor cells (Figure 4B) and 70% of the pancreatic tumor cells (Figure 5A), where a small percentage of cells that coexpressed glucagon and pancreatic polypeptide immunoreactivity were also found. Proliferative activity measured using Ki-67 protein labeling indices was approximately 3.5% in both tumors (Figure 5B). The final histopathological diagnosis of somatostatinoma of the duodenum and the pancreas was made in tumor stages pTNP: 2T (T3+T2) N0 Mx LOVI. It was concluded that despite the absence of regional and distant metastases, neither tumor could be categorized as benign, given their histopathological features, size, mitotic counts and proliferative activities.

One and a half years after surgery, the patient is symptom-free, with normal US and scintigraphy of the liver.

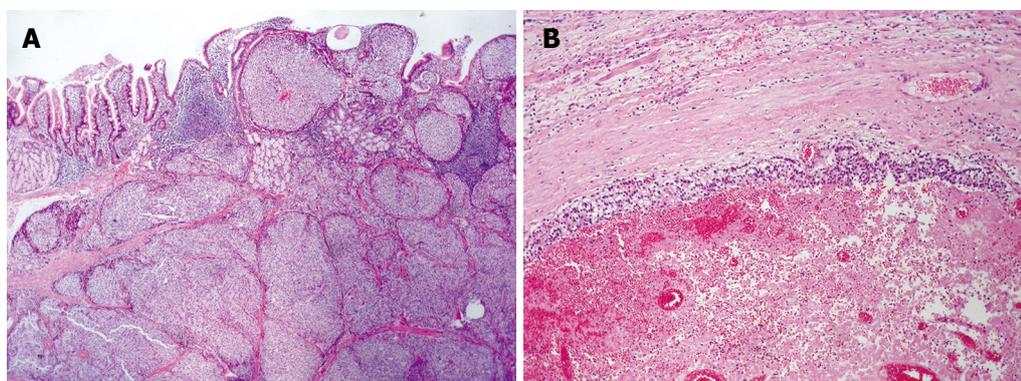
## DISCUSSION

Somatostatinomas are extremely rare gastrointestinal tumors with an annual incidence of 1 case per 40 million adults<sup>[5]</sup>. Two-thirds of somatostatinomas occur within the pancreas, with the duodenum being the next most common site<sup>[9]</sup>; although some authors have found equal frequencies<sup>[11]</sup>. They can occur at any age (range: 21-91 years)<sup>[1,9]</sup>, and pancreatic somatostatinomas show a slight female predominance (67%), with extrapancreatic sites being more often present in men<sup>[1]</sup>. Forty-five percent of cases exist as part of multiple endocrine neoplasia type I syndrome or von Hippel-Lindau disease<sup>[1]</sup> and it is vital to confirm or refute these associated conditions. Duodenal somatostatinomas may be associated with von Recklinghausen's neurofibromatosis<sup>[1,12,13]</sup>.

Differences exist between the pancreatic and extra-pancreatic types. Duodenal somatostatinomas usually are located in the second part of the duodenum, and most frequently in the ampullary or periampullary regions. Approximately 30% have lymph node or liver secondaries at the time of diagnosis, although these are less frequent when tumors are < 2 cm<sup>[9]</sup>. About two-thirds of these tumors contain psammoma bodies within the lumina of their glandular structures, and their biological behavior is similar to that of carcinoid tumors. As a result of their location, patients often present earlier with bile duct obstruction and jaundice, hence the reason for their earlier discovery, earlier surgery, smaller



**Figure 2** Resected duodenum and pancreatic head specimen containing both tumors. A: Posterior aspect; B: Resected duodenum highlighting the duodenal tumor and common bile duct; C, D: Transected head of the pancreas showing the cystic pancreatic and duodenal tumors. DT: Duodenal tumor; PT: Pancreatic tumor; CBD: Common bile duct.



**Figure 3** Histopathology. A: Mucosal and submucosal tumor infiltration of the duodenum, revealing an insular growth of rather uniform epithelioid cells (HE,  $\times 25$ ); B: The pancreatic tumor presenting as a hemorrhagic pseudocyst, with a thin peripheral layer of neoplastic proliferation (HE,  $\times 56$ ).

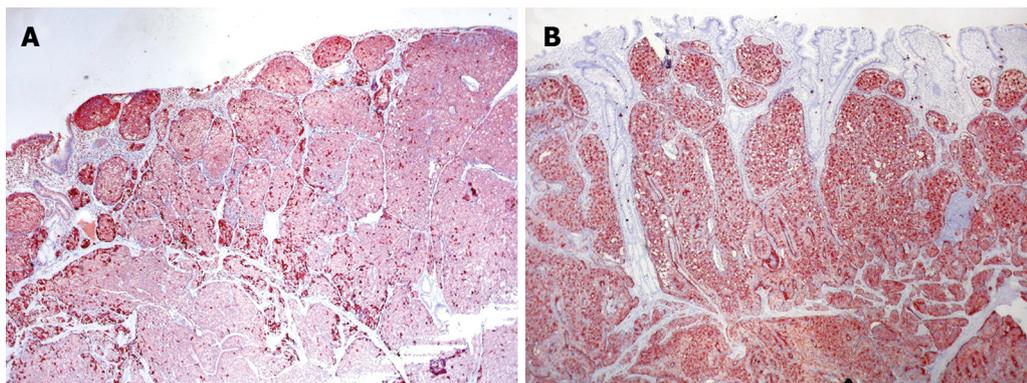
sizes and lower rates of metastasis<sup>[1,2,9]</sup>.

Almost 60% of pancreatic somatostatinomas are located in the head of the pancreas, and about 30% in the tail<sup>[1]</sup>. They usually are larger (mean size: 5-6 cm), and are considered more malignant with a 70% incidence of metastases<sup>[9]</sup>. Often the mass effect of the tumor itself leads to its diagnosis<sup>[11]</sup>.

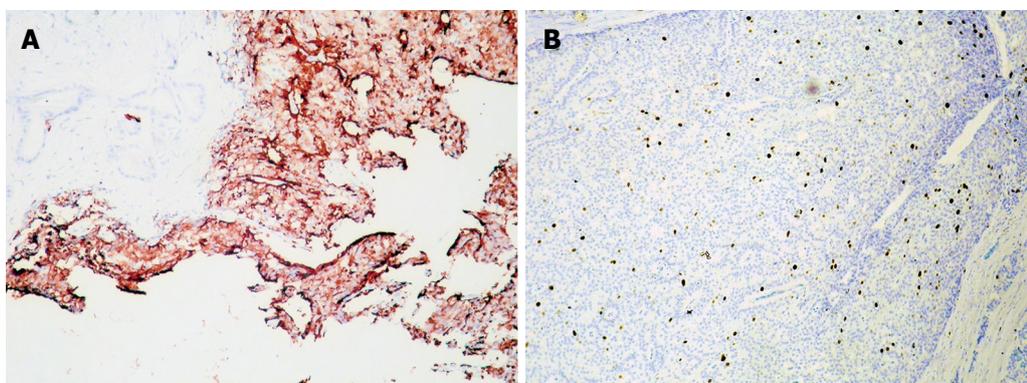
Although somatostatin is the main secretory product, at least 10% of somatostatinomas produce other hormones, including glucagon, gastrin, vasoactive intestinal peptide (VIP), insulin, calcitonin, and adrenocorticotrophic hormones<sup>[14-16]</sup>, and the clinical picture may be dominated by the effects of these secreted hormones<sup>[4]</sup>. Duodenal somatostatinomas more frequently secrete gastrin, VIP

and calcitonin<sup>[9]</sup>, while pancreatic somatostatinomas are also more likely to be multihormonal<sup>[11]</sup>.

Regardless of their plasma somatostatin levels, most patients present with indolent, nonspecific symptoms including vague abdominal pain, weight loss and a change in bowel habits<sup>[1,2]</sup>. The classical somatostatinoma or inhibitory syndrome, which includes cholelithiasis, mild diabetes mellitus and steatorrhea with diarrhea, is present in only a small number of patients<sup>[1,4]</sup>. The syndrome is the result of the inhibitory effects of somatostatin on endocrine and exocrine secretion, as well as its suppression of stomach and gallbladder motility<sup>[9]</sup>. It occurs far less with duodenal than other extrapancreatic somatostatinomas<sup>[1]</sup>, and this may be due to their earlier



**Figure 4** Duodenal tumor cells showing a non-homogeneous immunoreactivity against anti-chromogranin A antibody, but clear diffuse cytoplasmic immunohistochemical reactivity with anti-somatostatin antibody. A: Chromogranin A immunoreactivity,  $\times 56$ ; B: Somatostatin immunoreactivity, labeled streptavidin biotin (LSAB) method, aminoethylcarbazole (AEC) visualization,  $\times 56$ .



**Figure 5** Pancreatic tumor cells showing strong immunoreactivity with anti-somatostatin antibody. A: Somatostatin immunoreactivity,  $\times 100$ ; B: Ki-67 protein immunoreactivity, LSAB+ method, AEC visualization,  $\times 100$ .

diagnosis, release of inactive peptides, or lower secretory activity of the smaller duodenal tumors<sup>[2]</sup>.

The preoperative diagnosis of somatostatinoma is extremely difficult to establish unless there is a high clinical suspicion based on the patient's symptomatology<sup>[1]</sup>, although elevated fasting serum levels of somatostatin that cause the somatostatinoma syndrome can be diagnostic, particularly if associated with tumors larger than 4 cm<sup>[1]</sup>. CT and magnetic resonance imaging often fail to identify small tumors of the duodenum<sup>[13]</sup>, and these are often discovered during upper gastrointestinal endoscopy. The diagnosis is then made based upon histological assessment of biopsy specimens during upper gastrointestinal endoscopy<sup>[1,13]</sup> or endoscopic US<sup>[17]</sup>, with the exact histopathological diagnosis ultimately based on the resected specimen<sup>[1,2]</sup>. Once the preoperative diagnosis is established, somatostatin receptor scintigraphy and positron emission tomography can localize accurately the disease<sup>[18]</sup>, and are particularly useful in identifying occult metastatic spread and recurrent disease<sup>[19]</sup>. Scintigraphy positive for metastatic spread can obviate unnecessary surgery<sup>[2]</sup>; similarly a negative scan can indicate that curative resection is a possibility<sup>[19]</sup>.

Surgical resection is the only form of curative treatment<sup>[1]</sup> smaller lesions (< 2 cm) by enucleation larger lesions with extensive Whipple's type resections<sup>[20]</sup> (due to

their common localization in the head of the pancreas and duodenum), or other similar anastomoses. Unfortunately, in only 60%-70% of surgical cases is the tumor completely resected<sup>[1,3]</sup>. Debulking surgery should also be considered in the presence of metastatic secondaries because this may lead to improved survival and quality of life, especially in those with somatostatinoma syndrome<sup>[1,21]</sup>. Indeed, palliation for bile duct obstruction, pain or hormonal excess is beneficial in most unresectable cases<sup>[1]</sup>.

For patients with liver secondaries that are not amenable to resection or ablation, hepatic artery embolization or chemoembolization may be an effective therapy for symptomatic palliation<sup>[22]</sup>. Adjuvant chemotherapy is not advocated after complete resection<sup>[1]</sup>, however, in locally unresectable cases or in metastatic somatostatinoma, chemotherapeutic agents have been used with moderate clinical responses<sup>[1,23]</sup>.

Somatostatinomas are slow-growing lesions and long-term survival is possible<sup>[1]</sup>, even with no intervention. Unfavorable prognostic predictors include tumor size > 3 cm, poor cytological differentiation, regional and/or portal vein metastases, a non-functioning tumor, and incomplete surgical resection<sup>[1]</sup>.

Ultimately, patients with localized disease and a successful resection are the most likely to achieve a cure<sup>[2]</sup>, however, even in the presence of metastases, the 5-year

survival for patients undergoing surgical resection with adjuvant chemotherapy approaches 40%<sup>[1]</sup>.

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CASE REPORT

## Is iron overload in alcohol-related cirrhosis mediated by hepcidin?

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### Abstract

In this case report we describe the relationship between ferritin levels and hepcidin in a patient with alcohol-related spur cell anemia who underwent liver transplantation. We demonstrate a reciprocal relationship between serum or urinary hepcidin and serum ferritin, which indicates that inadequate hepcidin production by the diseased liver is associated with elevated serum ferritin. The ferritin level falls with increasing hepcidin production after transplantation. Neither inflammatory indices (IL6) nor erythropoietin appear to be related to hepcidin expression in this case. We suggest that inappropriately low hepcidin production by the cirrhotic liver may contribute substantially to elevated tissue iron stores in cirrhosis and speculate that hepcidin replacement in these patients may be of therapeutic benefit in the future.

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**Key words:** Alcohol; Iron; Anaemia; Hepcidin; Cirrhosis

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### INTRODUCTION

Hepatic iron overload in alcohol related cirrhosis and the detrimental effect of excessive iron on hepatic tissues in this condition is well described<sup>[1]</sup>. Previous studies investigating whole-body retention of iron showed a two-fold increase in intestinal iron absorption in chronic alcoholism, and the underlying mechanism has not been identified<sup>[2]</sup>.

Hepcidin, a 25 amino acid peptide, is the recently described body iron storage regulator. Under physiological conditions, hepcidin expression is stimulated by stored tissue iron, inflammatory stimuli and repressed by anemia and tissue hypoxia<sup>[3]</sup>. Recently, interactions between various signals affecting hepcidin production by hepatocytes have been elucidated<sup>[4]</sup>. Hepcidin binds to the iron exporter protein ferroportin on the basolateral aspect of macrophages and enterocytes and causes internalization and degradation of this molecule<sup>[5]</sup>, rendering cells iron-loaded. The two major systemic effects of elevated hepcidin production are therefore reduction of intestinal iron uptake and reticulo-endothelial sequestration of iron.

As the erythron is the main user of iron, its requirement for red cell production is paramount. In the presence of anemia due to ineffective erythropoiesis, animal experiments have shown that an unidentified signal from bone marrow seems able to override the stimulus of elevated body iron stores, repressing hepcidin and allowing duodenal iron absorption to continue even in the presence of elevated iron stores<sup>[6]</sup>.

Spur cell anemia (SCA) is an interesting clinical condition in which both erythropoiesis and tissue iron stores are altered by excessive alcohol consumption such that sufferers develop severe tissue iron overload and ongoing hemolysis<sup>[7]</sup>. The hemolysis is due to structural abnormalities of red cell membranes, resulting in spiculated erythrocytes (acanthocytes) which undergo early splenic destruction<sup>[8]</sup>. Treatment of SCA has been disappointing and it is commonly viewed as an indicator of end-stage liver disease<sup>[9]</sup>.

We postulate that reduced hepcidin production in the cirrhotic liver might be a significant factor contributing to the body iron excess in this patient with end stage alcohol-induced cirrhosis and SCA who underwent orthotopic liver transplantation (OLT).

## CASE REPORT

The patient was a 55-year old female with a 10-year history of excessive alcohol consumption who, following hospitalization due to decompensation and alcoholic hepatitis, became abstinent in May 2005. She remained stable until March 2006 when she developed worsening jaundice. Hematological findings indicated that hemolysis was a significant contributory factor in the patient's jaundice. Subsequent investigations confirmed the diagnosis of SCA in the context of cirrhosis. She was referred for transplantation assessment in November 2006. She had an unconjugated hyperbilirubinaemia (230 U/L) and raised reticulocyte count ( $125 \times 10^9/L$ ). Hepatic synthetic function was relatively preserved with an INR of 1.5 and serum albumin concentration of 38 g/L. A blood film showed many acanthocytes with polychromasia and spherocytes. A transjugular liver biopsy showed micronodular cirrhosis and grade 1 siderosis. Her serum ferritin level at this time was greatly elevated at 600 ng/mL.

The patient decompensated further and was listed for transplantation. The blood film immediately before transplantation showed a predominance of acanthocytes (70%-80% of total red blood cells (RBC)), and there was a gradual reduction in acanthocytes over the 48 h following transplantation so that examination of a blood film at 48 h after surgery indicated that acanthocytes made up 50%-60% of total circulating RBCs.

The patient gave consent for blood and urine samples collection for the study (LREC 08/MRE09/2). Samples were taken immediately before and at 12, 36, 48 h and 2 mo after transplantation. Blood was analyzed for ferritin, hemoglobin, erythropoietin, IL-6 and CRP to assess the extent of hemolysis and inflammation. Hepcidin was measured by SELDI-TOF using  $Cu^{2+}$  loaded IMAC ProteinChip Arrays and stable isotope labeled hepcidin as an internal standard as previously described<sup>[10]</sup> and available *via* <http://www.hepcidin.bham.ac.uk/>. Urine samples were normalized with respect to protein concentration (20  $\mu$ g/mL, Bradford assay).

A sample of the explant liver was analyzed for hepcidin mRNA by q-RTPCR and this was compared with samples taken from 8 normal livers.

The hepcidin level in relation to hematological parameters is shown in Table 1. Hepcidin levels before transplantation were very low compared with the elevated ferritin. Following transplantation, hepcidin production rose with falling ferritin. At 2 mo after transplantation, the hepcidin production had fallen in the context of the normal serum ferritin. The urinary hepcidin/ferritin ratio rose progressively following transplantation.

Figure 1 shows that the rise and subsequent fall in urinary hepcidin following transplantation does not seem to be related to erythropoietin production which remains quite stable (except for one dip at 12 h coincident with a

Table 1 Urinary hepcidin levels in relation to haemoglobin and ferritin

Time	Urinary hepcidin (ng/mg)	Ferritin (ng/mL)	Hepcidin/ferritin ratio	Hb (g/dL)
0 h	5	542	0.009	7.0
12 h	8	349	0.023	10.7
36 h	100	404	0.248	9.8
48 h	112	413	0.272	9.3
2 mo	12	81	0.148	14.3

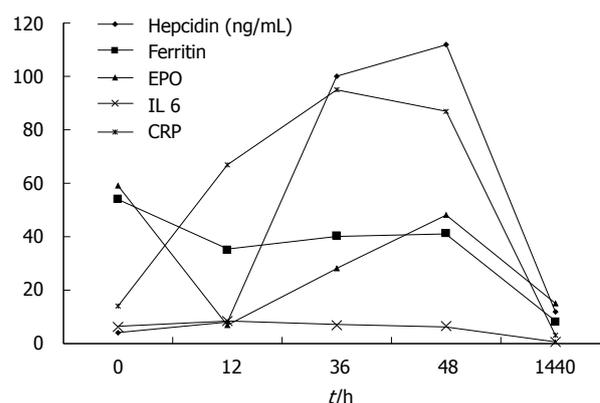


Figure 1 Variation in iron parameters with time.

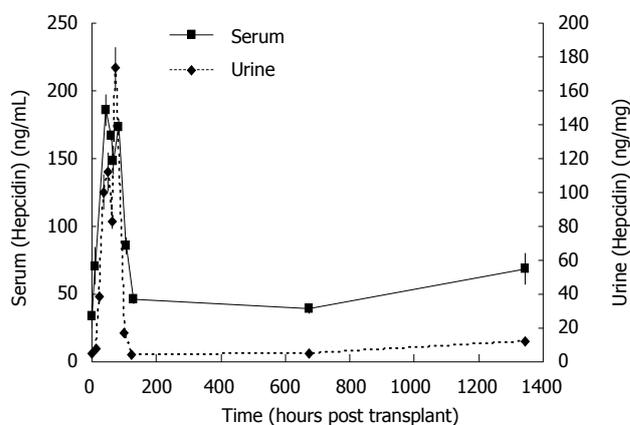


Figure 2 Correlation between urinary and serum ferritin.

two unit peri-operative blood transfusion). Similarly, IL6 levels did not vary greatly. CRP rose as expected in relation to the operation. This rise was not immediately accompanied with the dramatic rise in hepcidin production which was delayed for 12 h. Figure 2 illustrates a close correlation between urinary and serum hepcidin levels in this patient (Pearson correlation coefficient  $r = 0.958$ ).

We compared hepcidin mRNA expression in the explant liver with that in 8 normal livers from transplant donor tissues. Hepcidin mRNA expression in the explant liver from our patient was significantly lower than that in normal liver (data not shown).

## DISCUSSION

This case illustrates "inappropriate" low hepcidin production in an iron-over loaded cirrhotic patient with

alcohol-related SCA. Following successful transplantation, hepcidin production by the new liver increased accompanied with falling serum ferritin. "Appropriate" hepcidin production by the new liver would act to sequester iron in reticulo-endothelial stores and to repress duodenal iron uptake. At 2 mo after OLT, both hepcidin and ferritin fell to low levels.

One potential mechanism for pre-operative hepcidin repression would be high circulating erythropoietin level in the hemolyzing patient. Apart from a small reduction in EPO in the peri-operative period which co-incided with a blood transfusion, EPO remained relatively high in the post-operative period consistent with observed persistence of spur cells and the ongoing anemia. This hepcidin expression in the new liver increased progressively. It is likely that hepcidin production in the new liver during the early post-transplantation period remains somewhat muted in relation to the ongoing EPO stimulus.

IL6 stimulates hepcidin production acutely through the STAT pathway. In our patient IL6 was low before transplantation and did not rise in the peri-operative period. CRP did rise in the immediate post-transplantation period as expected. This may have had a bearing on the observed increase in hepcidin as inflammation is a major trigger to hepcidin production.

In our case, although the cirrhosis was undoubtedly alcohol-related, the patient had been abstinent for 2 years. This raises the possibility that, even in the absence of an ongoing stimulus, a persistent hepatic oxidative state, perhaps induced by iron overload, could trigger ongoing repression of hepcidin production. This illustrates potentially differential effects of acute stimulation of hepcidin perhaps *via* the IL6/STAT pathway and chronic repression mediated by oxidative stress<sup>[11]</sup>.

In summary, the cirrhotic liver in this patient with alcohol-induced spur cell anemia produced very little hepcidin in relation to circulating high ferritin levels and this deficiency was corrected by transplantation.

The mechanisms underlying reduced hepcidin production in this situation, in particular the role of oxidative stress as mediated through CHOP/CEBP, deserve further

studies in the setting of human alcohol-related cirrhosis. The influence of alcoholic cirrhosis on duodenal expression of iron transporter proteins and the influence of local effects related to oxidative stress and external factors such as erythropoiesis on human hepcidin production clearly deserve further prospective examinations.

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## Neoadjuvant peptide receptor radionuclide therapy for an inoperable neuroendocrine pancreatic tumor

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### Abstract

Pancreatic endocrine tumors are rare but are among the most common neuroendocrine neoplasms of the abdomen. At diagnosis many of them are already advanced and difficult to treat. We report on an initially inoperable malignant pancreatic endocrine tumor in a 33-year-old woman, who received neoadjuvant peptide receptor radionuclide therapy (PRRT) as first-line treatment. This resulted in a significant downstaging of the tumor and allowed its subsequent complete surgical removal. Follow-up for eighteen months revealed a complete remission. This is the first report on neoadjuvant PRRT in a neuroendocrine neoplasm with subsequent successful complete resection.

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**Key words:** Endocrine pancreatic carcinoma; Peptide receptor radionuclide therapy; Neoadjuvant treatment; Pancreatic surgery; Molecular imaging; Receptor pancreatic endocrine tumor; Computed tomography

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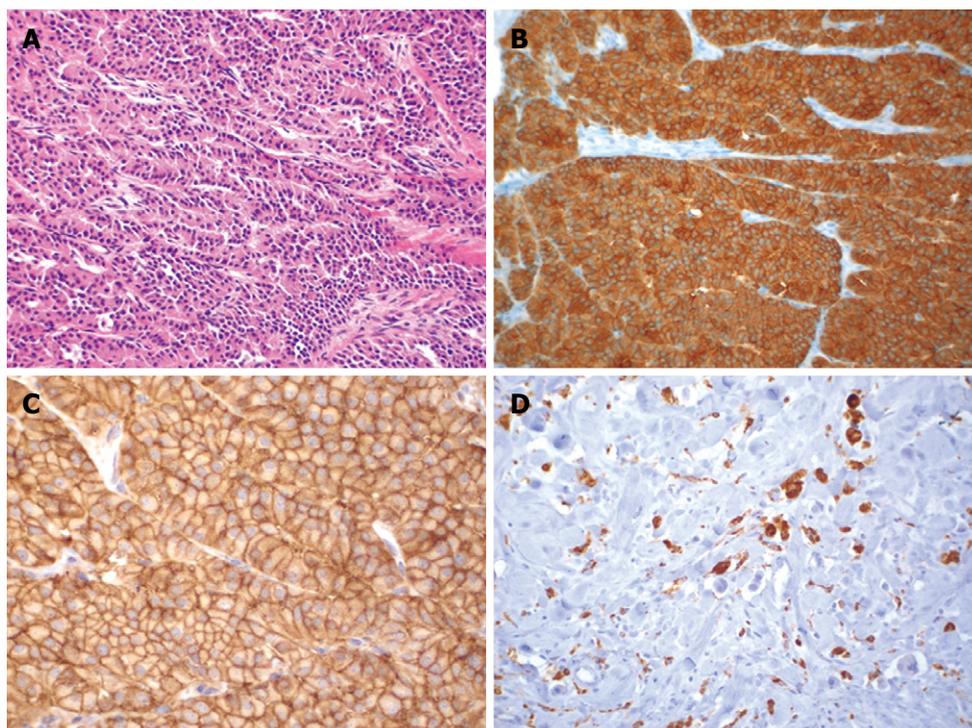
Kaemmerer D, Prasad V, Daffner W, Hörsch D, Klöppel G, Hommann M, Baum RP. Neoadjuvant peptide receptor radionuclide therapy for an inoperable neuroendocrine pancreatic tumor. *World J Gastroenterol* 2009; 15(46): 5867-5870 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5867.asp>  
DOI: <http://dx.doi.org/10.3748/wjg.15.5867>

### INTRODUCTION

Although pancreatic endocrine tumors (pNETs) are rare, they are among the most common neuroendocrine neoplasms of the abdomen<sup>[1]</sup>. pNETs account for less than 5% of all primary pancreatic malignancies. They are separated into functional (insulinomas, gastrinomas, glucagonomas, somatostatinomas) and non-functional pNETs. Non-functioning pNETs are pancreatic tumors with endocrine differentiation that lack a clinical syndrome of hormone hypersecretion. Up to 60% of non-functioning endocrine pancreatic tumors are already metastasized at diagnosis. In the advanced stage the pNETs still remains an interdisciplinary challenge. Pancreatic neuroendocrine tumors treatment by chemotherapy and radiation therapy has not demonstrated significant antitumoral effects. Because well differentiated neuroendocrine neoplasms usually express somatostatin receptors, they can be targeted with peptide receptor radionuclide therapy (PRRT) with palliative intention<sup>[2,3]</sup>. Several studies showed promising results in patients with advanced neuroendocrine tumors, with a partial response or disease stabilisation in palliative treatment<sup>[3,4]</sup>. But surgery still remains the gold standard in the management of pNETs<sup>[5]</sup>. Aggressive surgical resection can be performed safely and may improve both symptomatic disease and overall survival<sup>[6]</sup>. Prognostic indices such as tumor differentiation and the ability to achieve R0 resection have been linked to survival outcome. Here we describe the treatment of an initially inoperable pNET with peptide receptor radionuclides and show that this neoadjuvant treatment resulted in downstaging of the tumor and curative surgery.

### CASE REPORT

A 33-year-old woman was admitted to an external hospital



**Figure 1** Histological images.

A: Lymph node metastasis of a high differentiated neuroendocrine carcinoma showing a trabecular pattern (HE,  $\times 120$ ); B The cells are positive for synaptophysin ( $\times 120$ ); C: High membranous expression of somatostatin-receptor SSTR-2 ( $\times 120$ ); D: Macrophages (CD68-positive) as a sign for tumor necrosis after PRRT ( $\times 120$ ).

because of recurrent abdominal pain, flush attacks and diarrhea. CT revealed enlarged paraaortic lymph nodes, but no primary tumor. On exploratory laparotomy a tumor in the head of the pancreas was found and was thought to be a pancreatic ductal adenocarcinoma. This tumor was deemed inoperable because of the involvement of the mesenteric vessels and the paraaortic lymph nodes. Therefore only a lymph node biopsy was obtained. Histological investigation of the biopsy specimen revealed a lymph node metastasis (peripancreatic) of a highly differentiated neuroendocrine carcinoma that stained positive for chromogranin A and synaptophysin (Figure 1A and B). There was no expression of insulin, glucagon, somatostatin, or pancreatic polypeptide. The somatostatin receptor SSTR2 showed distinct membranous expression (Figure 1C). The tumor cells were only supported by small stromal tissue bands. A [ $^{68}\text{Ga}$ ]DOTA-1-Nal $^3$ -octreotide (NOC) PET/CT was performed to evaluate the somatostatin receptor status. Chromogranin A and serotonin serum levels were normal at all times. Finally, the patient was diagnosed as having a highly differentiated neuroendocrine pancreatic carcinoma, stage IV. Biotherapy with a somatostatin analogue failed. The patient still complained about flushes and diarrhea.

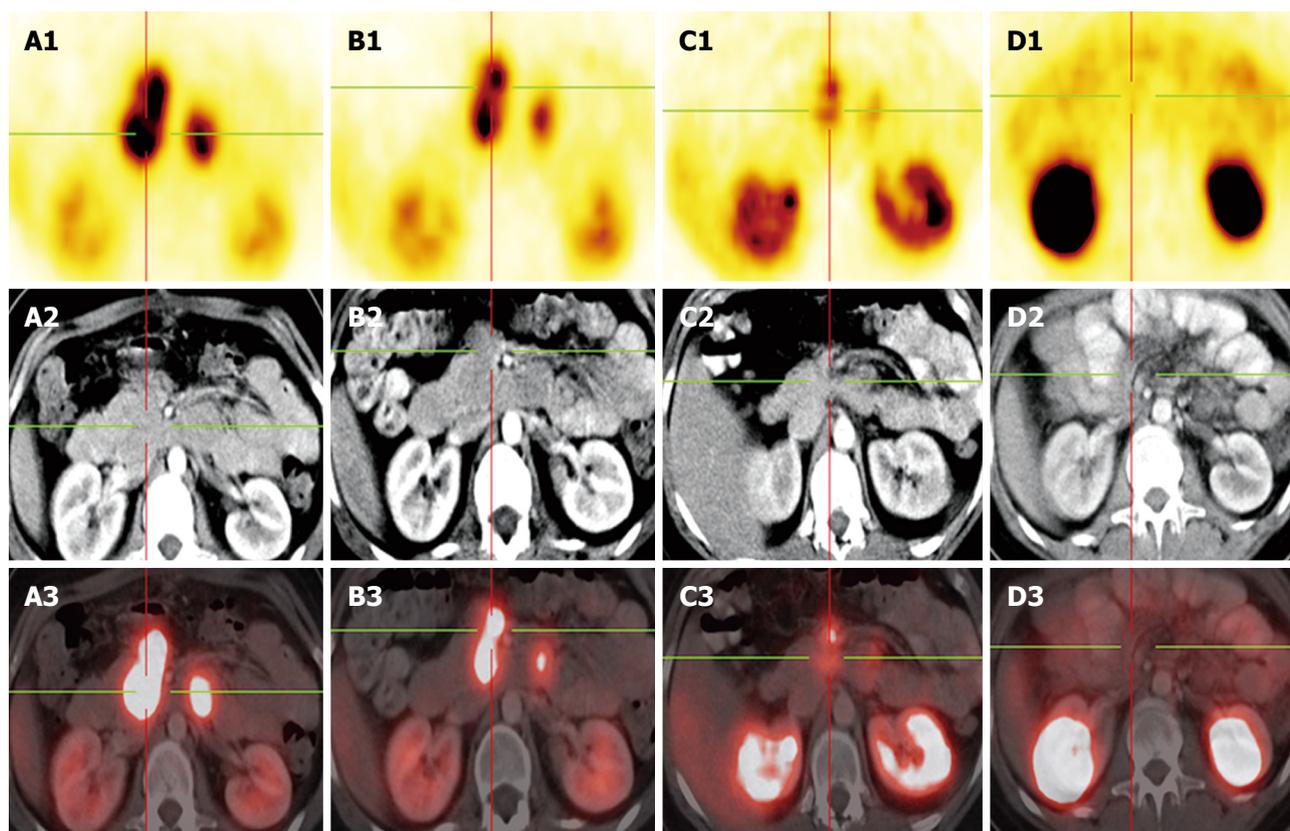
As the patient resolutely refused any chemotherapy, but was willing to undergo radioreceptor therapy, two cycles of PRRT were administered intravenously. The first cycle, administering 6000 MBq (162.1 mCi) [ $^{90}\text{Y}$ ]DOTA-TATE (DOTA-[Tyr $^3$ ] octreotate) was given in February 2007 and the second one, injecting 4500 MBq (121.6 mCi), in June 2007. To prevent nephrotoxicity, an amino acid infusion based on the protocol of Jamar *et al*<sup>[7]</sup> was used. Kidney function was measured by  $^{99\text{m}}\text{Tc}$ -DTPA using the single sample plasma clearance method for GFR calculation.  $^{99\text{m}}\text{Tc}$ -MAG3 was used for dynamic renal scintigraphy and for determination of

the tubular extraction rate (TER). The blood profile and routine laboratory parameters (electrolytes, liver function tests, creatinine, BUN *etc.*) were checked prior to therapy and then every month.

Mild grade 1 anemia and erythrocytopenia were noted as the only side effects. There was no other hematotoxicity or nephrotoxicity.

After 2 cycles the abdominal lymph node metastases had regressed significantly and PET/CT using [ $^{68}\text{Ga}$ ]DOTA-NOC and  $^{18}\text{F}$ -FDG ([ $^{18}\text{F}$ ]fluor-2-deoxy-glucose) performed in October 2007 indicated that the patient was operable. After every PRRT cycle a partial remission (EORTC criteria 1999) was detected (Figure 2B and C).

In November 2007, a pylorus-preserving pancreatoduodenectomy (Traverso-Longmire) was performed with en bloc resection of parts of the jejunum and its mesentery and lymph nodes (Figure 3). The operation specimen revealed a tumor in the head of the pancreas that was more than 2 cm in diameter and had metastasized to one mesenteric lymph node. Histologically, the endocrine tumor formed a trabecular pattern. The cell structures were embedded in well developed hyaline connective tissue, some parts of which were myxomatous. In the myxomatous area there were aggregates of CD68 positive macrophages that were occasionally positive for iron (Figure 1D). The tumor tissue infiltrated the surrounding pancreatic and interstitial tissue and also showed perineural infiltration. Immunohistochemically, about 5%-10% of the tumor cells expressed glucagon or somatostatin and were negative for insulin, serotonin and pancreatic polypeptide. The somatostatin receptor SSTR2 showed a distinct membranous staining pattern in all tumor cells. The lymph node was filled with tumor tissue. The final stage was ypT2 pN1 pM1(LYM) G3 R0 L0 V0<sup>[8]</sup>. The patient left the hospital 19 d after the operation. Six, twelve and



**Figure 2** Gallium-68 DOTA-NOC PET-CT in the follow up. A: Octreotide scan prior to PRRT-1, Gallium-68 DOTA NOC PET-CT and prior operation, showing multiple paraaortal lymph nodes next to the pancreas head; B: 3 mo after PRRT-1; C: 5 mo after PRRT-2 showing consistently decreasing mesenterial lymph nodes metastases with a decreasing SUV; D: Octreotide scan as follow up 18 mo after operation-complete remission.



**Figure 3** The pancreatic tumor with lymph node metastases (peripancreatic and one distal mesenteric lymph node metastasis) after pylorus-preserving duodenopancreatectomy and mesenteric lymphadenectomy *en bloc*.

eighteen months later a [<sup>68</sup>Ga]DOTA NOC PET/CT revealed a complete remission (Figure 2D).

## DISCUSSION

Advanced malignant pNETs are difficult to treat<sup>[9,10]</sup> and there are only a few studies on the resection of pNETs with extensive local spread. It seems, however, that patients with advanced malignant pNETs benefit from resection, because postoperative actuarial 5-year survival rates of up to 80% have been reported after extended tumor resection, even with synchronous metastasis<sup>[11,12]</sup>.

Nonetheless, the presence of liver or distant metastases is the prognostic factor determining survival<sup>[13]</sup>. In our patient, the diagnosis of a malignant well differentiated pNET was made on a biopsy specimen obtained during an explorative laparotomy. Tumor tissue showed a high expression of the somatostatin receptor SSTR2. Since the patient refused any chemotherapy, treatment with PRRT was performed. In the course of therapy an excellent downstaging of the initially inoperable tumor was reached. The oncology concept was changed from palliative to curative intent considering operability. PRRT is usually used as a palliative treatment<sup>[14]</sup>. Currently, there are no reports that deal with the use of radionuclide therapy as a neoadjuvant strategy in oncological surgery. Our case indicates that this therapy may be useful for downstaging and downsizing well differentiated neuroendocrine tumors, thereby achieving a higher operability rate. We recommend first performing [<sup>68</sup>Ga]DOTA-NOC PET/CT to evaluate the somatostatin receptor status<sup>[15,16]</sup>. If there is high SSTR expression PRRT will be effective<sup>[17]</sup>. In addition, staging of the patient after every cycle is required in order to evaluate the response and the operability. The tumor should be downsized to an extent in which a R0-resection should be feasible. In case of a relapse, which is not unlikely<sup>[11]</sup>, another course of PRRT is again a therapeutic option.

In conclusion, based on the encouraging results obtained in this case we believe that neoadjuvant PRRT is a novel approach in the treatment of surgically unrese-

ctable pNETs in patients with positive somatostatin receptor status.

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## Cronkhite-Canada syndrome associated with myelodysplastic syndrome

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### Abstract

We report a case of Cronkhite-Canada syndrome (CCS) associated with myelodysplastic syndrome (MDS). A 54-year-old woman, diagnosed as MDS the prior year after evaluation of anemia, visited our hospital with the chief complaint of epigastric discomfort. She also had dysgeusia, alopecia, atrophic nail change, and pigmentation of the palm, all of which began several months ago. Blood tests revealed severe hypoalbuminemia. Colonoscopy (CS) showed numerous, dense, red polyps throughout the colon and rectum. Biopsy specimens showed stromal edema, infiltration of lymphocytes, and cystic dilatation of the crypt. Her clinical manifestations and histology were consistent with CCS. We prescribed corticosteroids, which dramatically improved her physical findings, laboratory data, and endoscopic findings. This is the first report of CCS in a patient with MDS.

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**Key words:** Cronkhite-Canada syndrome; Myelodysplastic syndrome; Polyposis; Steroid therapy

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### INTRODUCTION

Cronkhite-Canada syndrome (CCS), first described by Cronkhite and Canada in 1955<sup>[1]</sup>, is a rare, acquired, nonfamilial syndrome with diffuse gastrointestinal (GI) polyposis, atrophic nail change, alopecia, cutaneous hyperpigmentation, diarrhea, abdominal pain, and other GI complications such as protein-losing enteropathy and malnutrition. Goto<sup>[2]</sup> and Takeuchi *et al*<sup>[3]</sup> reviewed 278 cases of CCS patients up to 1993 and found that 212 (76.3%) of them were Japanese. Except for cases of anemia caused by malnutrition, CCS with hematologic disorder has not been reported. This report is the first to describe a case of CCS in a patient with myelodysplastic syndrome (MDS).

### CASE REPORT

A 54-year-old woman visited our hospital with the chief complaint of epigastric discomfort for a month. She was diagnosed as MDS the prior year after evaluation of anemia following a routine check-up. She also suffered from dysgeusia, alopecia, and pigmentation of the palms several months ago. She and her family had no history of GI disease. Physical examination revealed a partial loss of capillus and supercilia, with blackish brown pigmentation in both palms (Figure 1).

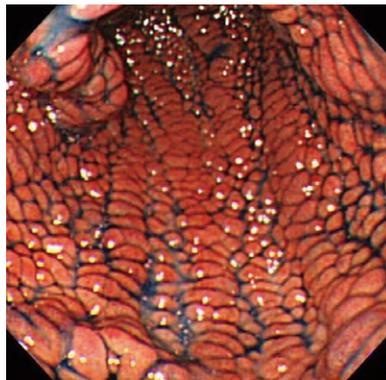
Partial loss of body hair including capillus and supercilia (Figure 1A) with blackish brown pigmentation was found in both palms (Figure 1B). Atrophic nail change was observed later (Figure 1C). Laboratory test showed that her white blood cell (WBC) count was 6400/ $\mu\text{L}$  (3000-6000), red blood cell (RBC) count was  $349 \times 10^4/\mu\text{L}$  ( $380-500 \times 10^4$ ), platelet count was  $9.7 \times 10^4/\mu\text{L}$  ( $12-38 \times 10^4$ ), C-reactive protein (CRP) was negative and erythrocyte sedimentation rate (ESR) was 40 mm/1 h, total protein was 5.7 g/dL (6.5-8.0), and serum albumin was 3.2 g/dL



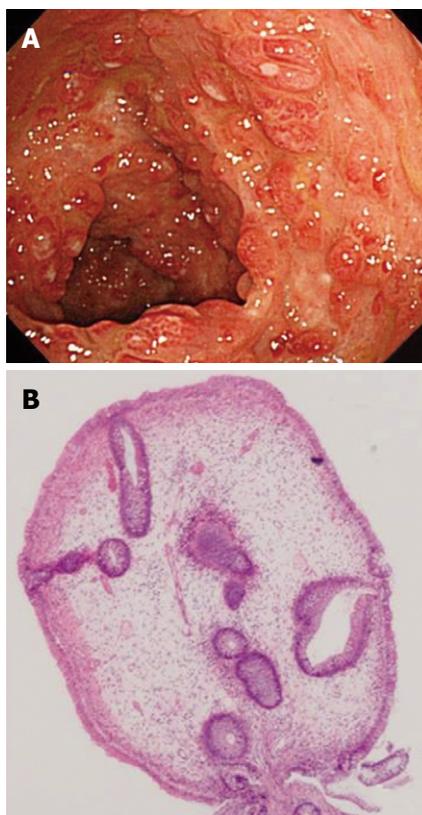
**Figure 1** Physical findings in our case at her first visit. A: Partial loss of the capillus and supercilia; B: Blackish brown pigmentation in both palms (black arrow); C: Atrophic nail change.

(4.0-5.0). Esophagogastroduodenoscopy (EGD), performed for further evaluation of the GI tract, revealed red and edematous granular polyps with giant folds, the so-called red-carpet-like polyposis of the stomach (Figure 2). A biopsy specimen displayed proliferation of connective tissue, edema, and infiltration of lymphocytes in the lamina propria. Since these findings could not confirm the diagnosis, we prescribed famotidine (20 mg per day) for nonspecific gastritis. Watery diarrhea gradually worsened, occurring up to 7 times per day at 2 wk after her first visit. Then, alopecia also worsened and atrophic nail change was observed.

Laboratory test displayed not only elevated CRP and ESR, but also hypoalbuminemia (Alb 3.2 g/dL). We suspected protein-losing enteropathy and performed colonoscopy (CS) for differential diagnosis, which showed numerous, dense, red polyps throughout the colon and rectum (Figure 3A). Biopsy specimens from the colon displayed cystic dilation of crypts and edematous stroma with inflammatory cell infiltration (Figure 3B). These physical and endoscopic findings were consistent with CCS, but CS findings did not exclude ulcerative colitis. We added salazosulfapyridine (3 g per day) and probiot-



**Figure 2** Esophagogastroduodenoscopy. Red and edematous granular polyps with giant folds, the so-called red-carpet-like polyposis of the stomach before treatment.



**Figure 3** Colonoscopy (CS) findings. A: Numerous, dense, red polyps throughout the colon and rectum; B: Biopsy specimen from colon displaying cystic dilation of crypts and edematous stroma with inflammatory cell infiltration before treatment (HE,  $\times 100$ ).

ics for diagnostic therapy. Diarrhea and alopecia were gradually relieved, but hypoalbuminemia increased to 1.8 g/dL. Three months after salazosulfapyridine treatment, we started corticosteroid therapy with intravenous prednisolone (40 mg per day) and then exchanged salazosulfapyridine to mesalazine (1500 mg per day). We tapered the dosage of prednisolone at two-week intervals in consideration of the clinical and laboratory changes in our patient. Diarrhea gradually became solid and the serum albumin level increased steadily to 2.5 g/dL one month later. At three months after treatment, we tapered prednisolone to 2.5 mg/d. Her clinical manifestations were



Figure 4 Clinical manifestations of the patient after treatment.

dramatically relieved (Figure 4). The CS findings were relieved and no neoplastic change was observed (Figure 5). The clinical course of this patient is depicted in Figure 6.

## DISCUSSION

CCS, first described by Cronkhite and Canada in 1955<sup>[1]</sup>, is a rare, acquired, nonfamilial syndrome with diffuse GI polyposis, atrophic nail change, alopecia, and cutaneous hyperpigmentation. Its etiology is apparently associated with the ectoderm abnormality<sup>[2,3]</sup>.

Therapeutic options comprise nutrition support, steroid therapy<sup>[4-6]</sup>, antiplasmin therapy<sup>[4,7]</sup>, mesalazine<sup>[8]</sup> and surgery<sup>[9]</sup>. Numerous cases for which corticosteroid treatment was effective have been reported, but corticosteroid therapy is regarded as a first-line therapy. In this case, although an insufficient effect of salazosulfapyridine and antiplasmin agents was observed, steroid therapy was also found to have dramatic effects.

The etiology of CCS remains unknown. Infection, mental stress, concomitant neoplasm, and immune abnormalities are regarded as triggering factors for CCS<sup>[3,10]</sup>. In addition, MDS has a high incidence (10%-20%) ac-

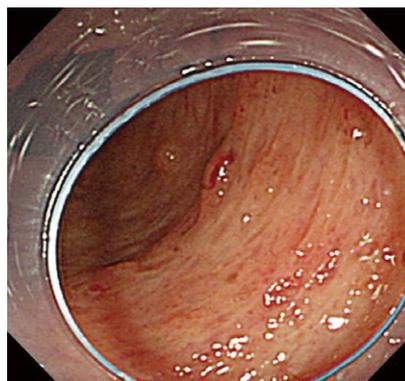


Figure 5 CS image after treatment.

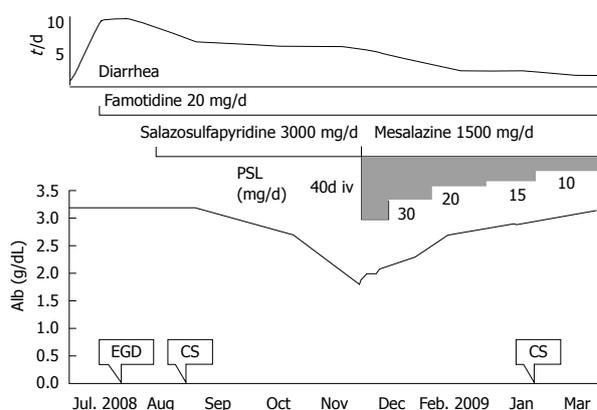


Figure 6 Clinical course of the patient.

companying autoimmune diseases, such as systemic lupus erythematosus and aortitis<sup>[11-13]</sup>. It is particularly interesting that GI autoimmune diseases, such as inflammatory bowel disease (IBD) and Behcet's disease, are also reported in patients with MDS. The etiology is considered to be an imbalance of various cytokines attributable to the abnormal level of multipotent stem cells. Reportedly, up-regulation of cytokines, such as TGF- $\beta$ , TGF- $\beta$  receptor, IL-6, and IL-7 receptors mapped to chromosome 8, related to inflammation and cell proliferation, plays an important role in the pathogenesis of patients having Behcet's disease with MDS and trisomy 8<sup>[14-16]</sup>.

This case was consistent with CCS, considering its typical clinical manifestations and histology. We infer the possibility that cytokine abnormalities induced by earlier MDS may have caused CCS in this case. However, the karyotype was not trisomy 8, 46, XX, or i(7) (q10). Moreover, inflammatory cytokine levels (IFN $\gamma$ , IL-6, IL-10, TNF- $\alpha$ ) were within normal limits. These results suggest that the etiology of this case is not associated with cytokine imbalance, as reported for Behcet's disease with MDS of trisomy 8.

In conclusion, we report a case of CCS in a patient with MDS. Although the association of CCS and MDS in this case remains uncertain, clarification of the CCS etiology is possible through accumulation of similar cases and results of further studies of the pathogenesis of MDS associated with autoimmune disease.

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## Spontaneous liver rupture in hypereosinophilic syndrome: A rare but fatal complication

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### INTRODUCTION

Spontaneous intrahepatic hemorrhage and liver rupture usually occur in patients with underlying hepatocellular carcinoma or adenoma<sup>[1,2]</sup>. This has also been described in patients with HELLP syndrome, Ehlers Danlos disease and graft-*vs*-host disease<sup>[3-6]</sup>. In this report, we described a rare case of spontaneous liver rupture in a patient with hypereosinophilic syndrome (HES), of which the diagnosis was delayed, resulting in a fatal outcome.

### CASE REPORT

A 48-year-old man with good past health was admitted because of fever associated with flu-like symptoms and left loin pain for a few days. Initial physical examination showed mild suprapubic and left loin tenderness, and urine dipsticks revealed microscopic haematuria. The chest radiograph was normal and initial blood tests showed eosinophilia and mildly deranged liver function (Table 1). The patient had no clinical signs of allergic reaction. Ultrasound examination of the abdomen revealed no abnormality in the hepatobiliary and urinary system. Urine microscopy showed microscopic haematuria. Ova or parasites were not detected in stool samples. The cultures from blood, sputum and urine were all negative.

Five days after admission, while awaiting further investigations, the patient suddenly developed hypovolemic shock. He rapidly deteriorated to pulseless electrical activity. Cardiopulmonary resuscitation was initiated immediately. He was pale and his abdomen was distended. His pulse returned after resuscitation with 2 L gelofusine. His hemoglobin level dropped from 14.5 to 5 g/dL. In addition, ultrasound examination of the abdomen confirmed the presence of free intraperitoneal fluid. The patient was given six units of unmatched blood during resuscitation. Owing to the unstable hemodynamic state and the diagnosis of exsanguinating intra-abdominal pathology, emergency laparotomy was arranged.

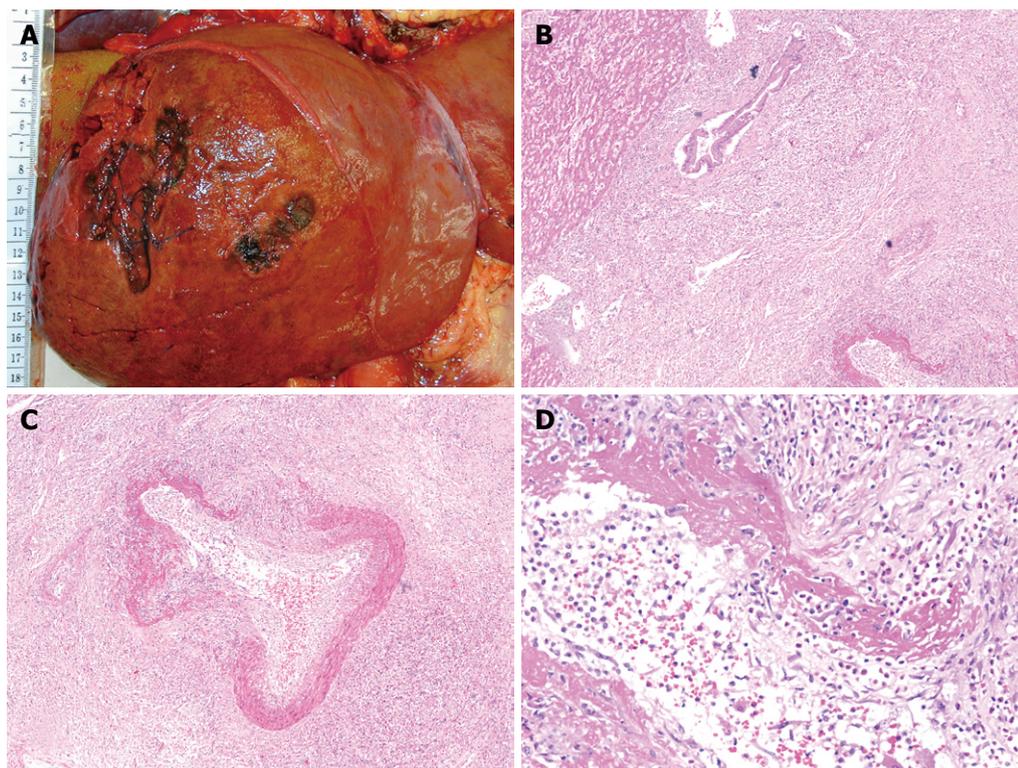
### Abstract

We report a rare case of spontaneous liver rupture in a patient with hypereosinophilic syndrome (HES), of which the diagnosis was delayed, resulting in a fatal outcome. The diagnostic criteria and treatment of HES with hepatic involvement were reviewed. The possible cause of spontaneous liver rupture in HES and its management were also discussed. To our knowledge, this is the first case report of spontaneous liver rupture in HES. We emphasized the need of a high index of suspicion in diagnosing HES, so that early treatment could be initiated.

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**Key words:** Hypereosinophilic syndrome; Eosinophilia; Hemoperitoneum; Complication; Spontaneous liver rupture

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**Figure 1** Post-mortem examination of liver. A: The ruptured site over the right lobe of liver; B: Expanded portal tracts with fibrosis and inflammation (microscopy, HE stain, 100 × magnification); C: Hepatic artery with fibrinoid necrosis (microscopy, HE stain, 200 × magnification); D: Eosinophilic infiltration of the hepatic artery (microscopy, HE stain, 400 × magnification).

**Table 1** Blood tests on admission

Blood test	Value	Unit
WBC	22.6 <sup>1</sup>	10 <sup>9</sup> /L
Eosinophil	6.6 <sup>1</sup>	10 <sup>9</sup> /L
Haemoglobin	13.9	g/dL
Platelet	179	10 <sup>9</sup> /L
PT	12.9	s
INR	1.33	
APTT	38.7	s
Na	133	mmol/L
K	3.7	mmol/L
Urea	3.6	mmol/L
Creatinine	68	umol/L
Protein	74	g/L
Albumin	34	g/L
Bilirubin	25 <sup>1</sup>	umol/L
ALP	207 <sup>1</sup>	IU/L
ALT	55	IU/L
GGT	504 <sup>1</sup>	U/L
CRP	190.1 <sup>1</sup>	mg/L

<sup>1</sup>Elevated values.

On laparotomy, 4 L of blood in the peritoneal cavity and a 10 cm ruptured subcapsular haematoma at anterior sector of right lobe with capsular tear were found. Active bleeding was found at a 9 cm laceration of 5 cm deep at segment VI/VII of the liver (Figure 1A). The liver was not cirrhotic with no palpable space occupying lesion. No abnormality and retroperitoneal haematoma were detected in other intra-abdominal organs. Hemostasis was attempted by suturing the liver laceration and packing. Bleeding from

the raw surface was coagulated with a TissueLink device (TissueLink Medical Inc, Dover, U.S.). However, the patient developed coagulopathy with diffuse oozing after massive transfusion with blood products (10 units of platelet concentrates, 16 units of fresh frozen plasma, and 16 units of pack cells). He required high dose trabecular support during operation. Perihepatic packing was performed and the abdomen was closed. He finally succumbed 1 h after the operation at the intensive care unit.

Post-mortem examination of the patient confirmed the diagnosis of HES with diffuse eosinophilic infiltration to the heart, liver, pancreas, mesentery, kidneys and urinary bladder. Microscopic examination of the liver showed marked eosinophilic expansion in the portal tracts and dilated sinusoids. The portal tract hepatic arteries showed fibrinoid necrosis with eosinophilic infiltration (Figure 1A-D). The lacerated areas showed extensive tissue necrosis and eosinophilic infiltration.

## DISCUSSION

Eosinophilia, defined as an increased eosinophil count in peripheral blood and accumulation in various tissues<sup>[7]</sup>, can be caused by atopic disease, hypersensitivity reaction, parasitic infection, vasculitis and hematological disorders. It is also found in uncommon conditions, such as eosinophilic gastrointestinal disease, Churg-Strauss syndrome and HES<sup>[8]</sup>. The diagnostic criteria for HES, first described by Chusid *et al*<sup>[9]</sup> in 1975, include persistent peripheral blood eosinophilia for more than 6 mo with an absolute

eosinophil count greater than 1500 cells/ $\mu\text{L}$ , the presence of organ involvement by eosinophilic infiltration, and exclusion of secondary causes of eosinophilia<sup>[8]</sup>.

The common organ systems involved in HES are hematologic (100%), cardiovascular (58%), cutaneous (56%), neurologic (54%) and pulmonary (49%) systems<sup>[8]</sup>. Liver and gastrointestinal tract are involved in only 20%-30% of patients. Patients with hepatic involvement may develop chronic active hepatitis-like picture and some may suffer from Budd-Chiari syndrome secondary to strictures in inferior vena cava or hepatic veins as a result of eosinophilic infiltration<sup>[10]</sup>. Spontaneous rupture of bladder and esophagus due to eosinophilic infiltration has been reported in the literature<sup>[11,12]</sup>. To our knowledge, this is the first case report of spontaneous liver rupture in HES.

Patients with HES usually present with vague symptoms<sup>[8]</sup>, making its diagnosis difficult and delayed. In our case, the patient presented with fever and flu-like symptoms, which were not specific of any disease. Although his liver function was mildly deranged, the normal initial sonographic appearance of the hepatobiliary system gave further misleading reassurance to the clinicians in identifying the hepatic involvement. Without a high index of suspicion, it was difficult to diagnose HES early and to start treatment before the catastrophic event in our case, namely liver rupture and subsequent mortality.

In order to avoid end organ damage by HES, it is important to establish the diagnosis and start treatment accordingly. Secondary causes of eosinophilia, such as atopic disease, hypersensitivity reaction or parasitic infestation, should be excluded. For patients with deranged liver function, non-invasive investigations including ultrasound of the liver and biliary system and hepatitis serology should be performed to exclude common disorders of the hepatobiliary system. In patients suspicious of HES with hepatic involvement, liver biopsy can be performed to demonstrate eosinophilic infiltration of the liver<sup>[13,14]</sup>. After the diagnosis of HES is confirmed, specific tests on Fip1-like-1 and platelet-derived growth factor receptor  $\alpha$  (FIP1L1-PDGFRA) fusion gene mutation can guide further treatment using targeted therapy<sup>[8]</sup>.

Successful treatment using corticosteroids has been reported in patients with hepatic HES<sup>[13,14]</sup>. Studies also showed that patients with HES have a good response to targeted therapy according to the result of FIP1L1-PDGFRA<sup>[15,16]</sup>. HES patients showing positive FIP1L1-PDGFRA have a good response to imatinib mesylate, resulting in a normal eosinophil count<sup>[15]</sup>. For patients with negative FIP1L1-PDGFRA, mepolizumab (an anti-interleukin 5 antibody) can effectively stabilize the eosinophil count and reduce the daily steroid dose to less than approximately 7.5 mg prednisolone<sup>[16]</sup>.

The present case of liver rupture was likely caused by eosinophilic infiltration and fibrinoid necrosis of the vascular wall, leading to rupture of hepatic arteries as demonstrated in Figure 1B-D. Although reports are available on cardiopulmonary resuscitation (CPR)-related major liver injury<sup>[16]</sup>, this was unlikely in our case because

the event of deterioration occurred abruptly before the initiation of CPR. The right posterolateral located liver laceration in the absence of ribs fractures further made traumatic cause of the liver rupture unlikely. CPR-related liver trauma usually occurs in the left lobe where it is anatomically close to the point of chest compression<sup>[17]</sup>.

Management and prognosis of spontaneous liver rupture heavily depend on its severity and the hemodynamic stability of patients. For stable patients, non-operative management with transfusion or transarterial selective embolization of the feeding artery has been described with promising results<sup>[1,4]</sup>. For patients with hemodynamic instability or failure in non-operative treatments, surgery for haemostasis is recommended as in our case. Hemostasis can be achieved by temporary tamponade of the liver using packs and portal triad occlusion (Pringle manoeuvre)<sup>[18]</sup>. After initial operative resuscitation and identification of the site of bleeding, different surgical techniques, including direct suture ligation, hepatic resection, selective hepatic artery ligation and perihepatic packing, can be employed for hemostasis depending on the case scenario<sup>[17]</sup>. Despite all these methods, if patients develop coagulopathy, acidosis and multi-organ failure, the chance of survival is low.

Although the clinical course of HES is highly variable and dependent on the degree of organ involvement, early diagnosis and initiation of treatment are of paramount importance. Delay in diagnosis may lead to catastrophic complications. A high index of suspicion is crucial in the management of patients with eosinophilia.

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## Surgery for rare aneurysm associated with colorectal cancer

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### INTRODUCTION

The surgical treatment of coexistent aortic aneurysm and colorectal cancer (CRC) needs special consideration. The controversy is mainly whether to treat both diseases or treat either of them alone. If we choose to treat both, should we treat them simultaneously or as staged procedures? For a two-stage process, there is a significant risk of aortic aneurysm rupture, and a single-stage procedure has the disadvantages of technical difficulty and graft infection<sup>[1-3]</sup>. Lin *et al*<sup>[4]</sup> have proposed that the treatment priority should be given to life-threatening lesions. Such cases include the presence of an abdominal aorta aneurysm with a dangerously large diameter and synchronous obstructing or perforating CRC. Veraldi *et al*<sup>[5]</sup> have indicated that a one-stage procedure reduces the length of stay, avoids further surgical and anesthetic trauma, and does not increase graft infection.

We report a case of rectal cancer and concomitant aneurysm from the descending aorta to the common iliac artery, which is similar to a DeBakey type I aortic dissecting aneurysm, with a maximum diameter of 4.06 cm in the abdominal aortic segment and 6.51 cm in thoracic aortic segment. The patient was treated with the Dixon operation under conditions of controlled hypotension, without aneurysm rupture. At 2 years follow-up, the patient remained free of CRC and his aneurysm was stable.

### CASE REPORT

A 69-year-old man underwent anal examination after complaining of a change in bowel habits and rectal bleeding 2 years ago. Anal examination revealed a cauliflower-like lesion located 7 cm from the edge of the anus, which involved more than half of the rectal circumference. Abdominal computed tomography (CT) revealed the presence of a rectal tumor, which almost obstructed the bowel lumen (Figure 1). Medical history included smoking, diabetes, hypertension and thoracoabdominal aortic aneurysm for 6 years. After admission, the patient underwent chest radiography, which confirmed concomitant aneurysm and tracheal

### Abstract

The occurrence of concomitant aortic aneurysm and colorectal cancer is a rare medical entity, and controversy surrounds its optimal treatment. We report a case of rectal cancer and concomitant aneurysm from the ascending aorta to the common iliac artery. As with DeBakey type I aortic dissecting aneurysm, our patient was treated by rectal cancer resection, with preservation of the anus (Dixon operation) under controlled hypotension. Blood pressure was maintained at 80-90/50-60 mmHg and the pulse at 70-90 beats/min. The pathological examination of the surgical specimen showed a poorly differentiated T3N0 tumor. The patient had an uneventful recovery without aneurysm rupture, and was discharged from hospital on postoperative day 15 after 3 d adjuvant chemotherapy with oxaliplatin combined with calcium folinate and fluorouracil. The patient was given six courses of adjuvant chemotherapy in 6 mo, without recurrence or metastasis, and the aneurysm was still stable after 2 years follow-up.

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**Key words:** Colorectal cancer; DeBakey I aneurysm; Aortic aneurysm

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Figure 1 Thickening of the rectal wall, with narrowing of the lumen.



Figure 2 Chest radiography confirmed concomitant aneurysm and tracheal compression.

compression (Figure 2). Advanced CT angiography revealed large ectasia of the ascending aorta, aortic arch and descending aorta, with a maximum diameter of 6.51 cm and mural thrombosis. In addition, the aneurysm extended to the abdominal aorta, internal-organ arteries and the right common iliac artery, with a length of 60 cm and a diameter of 4.06 cm in the abdominal aortic aneurysm (Figure 3). Elevation of carcinoembryonic antigen and blood urea nitrogen to 11.79 ng/mL and 13.60 mmol/L, respectively, allowed diagnosis of digestive tract tumor and renal inadequacy. A complete preoperative metastatic work-up was negative.

Complicating aneurysm increases the difficulty of managing advanced malignant disease. We treated this patient with the Dixon operation under conditions of controlled hypotension, with minimal blood pressure fluctuation, and renal function protection and blood sugar regulation. Intraoperative blood pressure was maintained at 80-90/50-60 mmHg and the pulse at 70-90 beats/min. The surgery was completed in 1.5 h. Postoperative blood pressure was < 100/60 mmHg and was maintained between 100/60 mmHg and 110/70 mmHg by glyceryl trinitrate administered by mini pump. The pathological examination of the surgical specimen showed a poorly differentiated T3N0 tumor.

The patient had an uneventful recovery, and he was discharged from hospital on postoperative day 15 after 3 d adjuvant chemotherapy with oxaliplatin combined with calcium folinate and fluorouracil. The patient was given six courses of adjuvant chemotherapy without

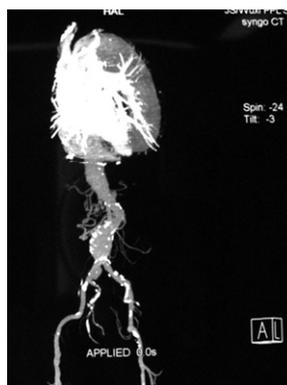


Figure 3 CT angiography of the aorta showed large ectasia from the ascending aorta to the right common iliac artery.

tumor recurrence or metastasis and the aneurysm was still stable after 2 years follow-up.

## DISCUSSION

The concomitant occurrence of aortic aneurysm and CRC appears to be increasing, from 0.5% to 2%<sup>[2]</sup>, as a result of the increase in the two individual diseases, which is caused by increased life expectancy and improved imaging capability in revealing additional asymptomatic disease<sup>[5]</sup>. Published studies are either small case series or limited retrospective reports<sup>[3,6]</sup>, therefore, the ideal treatment strategy represents a therapeutic dilemma<sup>[2]</sup>. Initial aortic aneurysm repair followed by CRC resection exposes patients to the risk of tumor progression before resection, although no study has shown the oncological implications of such a delay. Resection of CRC followed by staged aneurysmorrhaphy carries a significant risk of aortic aneurysm rupture in the perioperative period, particularly when the aortic aneurysm is > 5 cm in diameter<sup>[2]</sup>. Synchronous treatment of both lesions has been described but has the disadvantages of technical difficulties and graft infection, especially when the patient is in poor general condition.

Veraldi *et al*<sup>[5]</sup> have found, from Medline 1987-2005, a total of 229 cases of associated aortic aneurysm and CRC. Four emergency operations have been reported, one for rupture of the aortic aneurysm, two for unstable aneurysm, and one for diastatic rupture of the cecum by the stenosing neoplasm in the sigmoid colon. Including these four, there were 25 patients in all with only one disease treated. In 24 of these, intervention was carried out for CRC because of the advanced stage of the disease or poor general condition of the patient. In the remaining case, because of postoperative death, only the aneurysm was treated. From January 1988 to May 2006, 14 patients with both diseases were observed and treated in the University of Verona<sup>[5]</sup>. In one of these patients, only CRC was treated because of poor cardiac conditions, and the patient died after 2 mo from myocardial infarction.

Patients in poor general condition, who are treated for both diseases simultaneously or in stages face the risk of surgical fatality. Our patient had a rare true aneurysm similar to a dissecting aneurysm<sup>[7]</sup>. Usually, dissecting aneurysm is caused by an intimal tear or interstitial hemorrhage and requires emergency surgery, and has

a low survival rate. He had been protected by blood pressure control since his aneurysm was diagnosed 8 years ago. CT revealed that the aneurysm was in a steady state, and the diameter and distribution of the aneurysm showed no obvious changes in these 8 years. The aneurysm in our patient began from the ascending aorta and extended to the common iliac artery, which is a very rare occurrence, and the prognosis was beyond our expectation. This kind of aneurysm usually requires Bentall's procedure and the elephant trunk technique for artificial blood vessel replacement. Bentall's procedure is a cardiac surgery technique that involves composite graft replacement of the aortic valve, aortic root and ascending aorta, with reimplantation of the coronary arteries into the graft. For the elephant trunk technique, excess tubular graft material is inserted during ascending aortic and arch repair, to facilitate the subsequent treatment of distal aortic aneurysms. These operations are extremely hazardous.

Davies *et al*<sup>[8]</sup> have pointed out that treatment decisions should involve a balance between the risk of complications caused by the dilated aorta and those from the operation itself. The most devastating complication of an aneurysm is dissection, which leads to arterial occlusion and rupture and is almost invariably fatal. However, the risk of operation is also unmanageable. The risk of spinal cord injury, particularly in operations on the descending aorta<sup>[9]</sup>, is also significant. As a result of surgical risk and expense, our patient elected for conservative treatment for his aneurysm 8 years ago. When we were faced with the rare aortic aneurysm and CRC, we chose only to resect the CRC as a result of the great risks of the operation because of the patient's age, mural thrombosis, renal inadequacy, diabetes and hypertension. The postoperative recovery was satisfactory, with stable aneurysm and no cancer recurrence during follow-up.

Most surgeons agree that treatment priority should be focused on symptomatic or more life-threatening lesions in CRC and aneurysm. The largest retrospective study to date was reported by Lin *et al*<sup>[4]</sup> in 2007. In that study, 108 patients with synchronous aortic aneurysm and CRC were identified, and 92 were treated for both lesions. Thirty-five patients had CRC removed first, following two patients with aneurysm rupture while 2/35 people with aneurysm rupture had CRC removed first. Twenty-three patients with endovascular aortic repair were associated with shorter recovery and lower postoperative mortality rate. This modality offered potential treatment benefits in patients with suitable anatomy who have concomitant CRC. However, the treatment should be offered with caution because of the risk of sigmoid ischemia caused by inferior mesenteric artery occlusion. Sometimes the

single operation decision may be a better one. In the study of Lin *et al*<sup>[4]</sup>, 16 patients were treated for only one disease, because of advanced CRC, hepatic metastasis or ruptured aneurysm, and these patients had a good prognosis. The most important thing is that treatment decisions should involve striking a balance between the risk of the disease itself and the complications of the operation. However, likely aneurysm rupture presents a great challenge, and urgent aneurysm repair is required to preserve the patient's life.

In summary, in patients with aortic aneurysm and CRC, priority should be given to the more life-threatening lesion. If patients have heart disease or are in poor general condition, attempting to treat both CRC and aneurysm carries a great risk, and it may be possible only to treat one of the conditions.

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LETTERS TO THE EDITOR

## Lethal neuroendocrine carcinoma in ulcerative colitis

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### Abstract

A 48-year old male with longstanding and extensive pancolitis developed a high grade and rapidly lethal malignant lesion in the ascending colon characterized by a neuroendocrine carcinoma. Prior biopsies obtained from multiple sites in the colon during endoscopic surveillance were reported to show only inflammatory changes without dysplasia. Although operator-dependent, repeated endoscopic studies may have limitations during surveillance programs because the biological behavior of some colonic neoplastic lesions may have a rapid and very aggressive clinical course.

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**Key words:** Colorectal cancer; Neuroendocrine carcinoma; Ulcerative colitis; Surveillance colonoscopy; Dysplasia

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### TO THE EDITOR

An article recently published in *World Journal Gastroenterology* concerning two patients, aged 35 and 77 years

respectively, with left-sided ulcerative colitis, was of special importance<sup>[1]</sup>. In spite of colonoscopic and histologic follow-up in the previous year, both developed large neuroendocrine carcinomas in the rectum, and one patient was reported to have died due to multiple liver metastases.

We had a similar experience in a 48-year old male with longstanding extensive pan-ulcerative colitis for 16 years, first diagnosed in 1992. During the clinical course, his symptoms were initially treated and controlled with 5-aminosalicylates and corticosteroids. Endoscopic studies eventually showed virtually complete mucosal healing and biopsies showed only minimal inflammatory changes. No other immunosuppressive drugs or biological agents were used. During the last decade, he continued to use 5-aminosalicylates alone and remained completely asymptomatic. He underwent repeated surveillance colonoscopies with multiple site biopsies throughout the colon, which showed minimal inflammatory changes. Dysplasia was not reported in any colonic biopsy specimens. Approximately ten months after his last endoscopic procedure, he developed right upper quadrant abdominal pain. Blood studies, including liver chemistry tests, were normal, but an ultrasound and a computerized tomographic (CT) scan suggested possible liver metastases. In addition, the CT suggested a focal thickening area in the ascending colon. Colonoscopy confirmed an ulcerated sessile lesion. Histologic examination of the endoscopic biopsies showed an ulcerating tumor with predominant trebecular architecture and vascular stroma. The tumor cells had hyperchromatic nuclei with small nucleoli and scant pale-stained cytoplasm. Mitoses were numerous and there was abundant apoptosis, consistent with a high grade malignancy. Immunohistochemical stain for chromogranin and synaptophysin showed moderately intense staining of a neuroendocrine carcinoma. Tumor cells were positive for CK7 and negative for CK-20. Subsequent studies also showed pulmonary metastases and palliative chemotherapy was provided with FOLFOX B (12 cycles), but the disease remained progressive so FOLFIRI (10 cycles) was given. He died fourteen months after diagnosis.

Neuroendocrine carcinomas of the colon and rectum, accounting for less than 1% of colon and rectal cancers reported over more than a decade from Memorial Sloan-Kettering in New York, United States<sup>[2]</sup>, are very distinct from well-differentiated carcinoid tumors (or neuroendocrine tumors, using the World Health Organization schema discussed elsewhere<sup>[3]</sup>) seen with inflammatory bowel disease but often detected incidentally during surgi-

cal treatment<sup>[4,5]</sup>. About 70% of those classified as neuroendocrine carcinomas present with metastatic disease and appear to have a dismal prognosis with a reported overall mean survival of about ten months<sup>[2]</sup>. These carcinomas have been subdivided into small and large cell types based on their histological and immunohistochemical features, similar to those of pulmonary neuroendocrine cancers with most positively stained for neuroendocrine markers, such as chromogranin, synaptophysin and/or neuron-specific enolase<sup>[2]</sup>. Interestingly, in a report from Taiwan, there were 2 patients with small cell carcinomas that were believed to represent gastrointestinal metastases from a primary pulmonary site, possibly emphasizing the difficulty in defining their origin in some cases<sup>[6]</sup>.

Scattered reports are available on poorly differentiated neuroendocrine carcinoma with inflammatory bowel disease have been noted with an equally dismal outcome<sup>[7-9]</sup>. In a recent report by Grassia *et al*<sup>[1]</sup>, however, surveillance studies were completed during the preceding year, and yet, large lesions in the most distal colon were eventually detected later. Although the present case of pancolitis developed a carcinoma in the ascending colon, the surveillance efforts for longstanding extensive colitis failed, in spite of multiple site endoscopic biopsies for dysplasia over many years. While colonoscopic evaluation, especially in surveillance programs, remains operator-dependent, these cases emphasize that repeated and systematic endoscopic and histological evaluations have limits because the underlying biological behavior of some colonic neoplastic lesions

may result in a rapidly developing and aggressive clinical course.

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- 9 **Sigel JE**, Goldblum JR. Neuroendocrine neoplasms arising in inflammatory bowel disease: a report of 14 cases. *Mod Pathol* 1998; **11**: 537-542

S- Editor Wang JL L- Editor Wang XL E- Editor Lin YP

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## Meetings

### Events Calendar 2009

January 12-15, 2009  
Hyatt Regency San Francisco, San Francisco, CA  
Mouse Models of Cancer

January 21-24, 2009  
Westin San Diego Hotel, San Diego, CA  
Advances in Prostate Cancer Research

February 3-6, 2009  
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)  
Second AACR Conference  
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009  
Hyatt Regency Boston, Boston, MA  
Translation of the Cancer Genome

February 8-11, 2009  
Westin New Orleans Canal Place, New Orleans, LA  
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009  
Hong Kong Convention and Exhibition Centre, Hong Kong, China  
19th Conference of the APASL  
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009  
Orlando, Florida  
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009  
Vienna, Austria  
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease  
[www.easl.ch/vienna2009](http://www.easl.ch/vienna2009)

March 13-14, 2009  
Phoenix, Arizona  
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009  
Marriott Wardman Park Hotel  
Washington, DC  
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009  
Glasgow, Scotland  
British Society of Gastroenterology (BSG) Annual Meeting  
Email: [bsg@mailbox.ulcc.ac.uk](mailto:bsg@mailbox.ulcc.ac.uk)

April 8-9, 2009  
Silver Spring, Maryland  
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009  
Colorado Convention Center, Denver, CO  
AACR 100th Annual Meeting 2009

April 22-26, 2009  
Copenhagen, Denmark  
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)  
<http://www.easl.ch/>

May 17-20, 2009  
Denver, Colorado, USA  
Digestive Disease Week 2009

May 29-June 2, 2009  
Orange County Convention Center  
Orlando, Florida  
45th ASCO Annual Meeting  
[www.asco.org/annualmeeting](http://www.asco.org/annualmeeting)

May 30, 2009  
Chicago, Illinois  
Endpoints Workshop: NASH

May 30-June 4, 2009  
McCormick Place, Chicago, IL  
DDW 2009  
<http://www.ddw.org>

June 17-19, 2009  
North Bethesda, MD  
Accelerating Anticancer Agent Development

June 20-26, 2009  
Flims, Switzerland  
Methods in Clinical Cancer Research (Europe)

June 24-27 2009  
Barcelona, Spain  
ESMO Conference: 11th World Congress on Gastrointestinal Cancer  
[www.worldgicancer.com](http://www.worldgicancer.com)

June 25-28, 2009  
Beijing International Convention Center (BICC), Beijing, China  
World Conference on Interventional Oncology  
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009  
Snowmass, CO, United States  
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009  
Aspen, CO, United States  
Molecular Biology in Clinical Oncology

August 1-7, 2009  
Vail Marriott Mountain Resort, Vail, CO, United States  
Methods in Clinical Cancer Research

August 14-16, 2009  
Bell Harbor Conference Center, Seattle, Washington, United States  
Practical Solutions for Successful Management  
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009  
Beijing International Convention Center (BICC), Beijing, China  
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists (IASGO)  
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009  
Taipei, China  
Asian Pacific Digestive Week  
<http://www.apdwcongress.org/2009/index.shtml>

October 7-11, 2009  
Boston Park Plaza Hotel and Towers, Boston, MA, United States  
Frontiers in Basic Cancer Research

October 13-16, 2009  
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States  
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009  
Versailles, France  
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009  
Boston, MA, United States  
The Liver Meeting

November 15-19, 2009  
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States  
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009  
London, UK  
Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

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#### Format

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- 1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

*Chinese journal article (list all authors and include the PMID where applicable)*

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

*In press*

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of

balancing selection in *Arabidopsis*. *Proc Natl Acad Sci USA* 2006; In press

#### Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

#### Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

#### No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

#### Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

#### Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

#### No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

### Books

#### Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

#### Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

#### Author(s) and editor(s)

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wicczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

#### Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

#### Conference paper

- 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

#### Electronic journal (list all authors)

- 15 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>

#### Patent (list all authors)

- 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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Write as mean  $\pm$  SD or mean  $\pm$  SE.

### Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as  $\chi^2$  (in Greek), related coefficient as *r* (in italics), degree of freedom as  $\nu$  (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

### Units

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose)  $6.4 \pm 2.1$  mmol/L; blood CEA mass concentration, *p* (CEA) =  $8.6 \pm 2.5$   $\mu$ g/L; CO<sub>2</sub> volume fraction, 50 mL/L CO<sub>2</sub>, not 5% CO<sub>2</sub>; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23243641.

The format for how to accurately write common units and quantumms can be found at: <http://www.wjgnet.com/wjg/help/14.doc>.

### Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

### Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

Restriction enzymes: *EcoRI*, *HindI*, *BamHI*, *Kpn I*, *Kpn I*, etc.

Biology: *H. pylori*, *E. coli*, etc.

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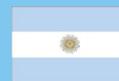
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## Role of transcatheter arterial embolization for massive bleeding from gastroduodenal ulcers

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### Abstract

Intractable bleeding from gastric and duodenal ulcers is associated with significant morbidity and mortality. Aggressive treatment with early endoscopic hemostasis is essential for a favourable outcome. In as many as 12%-17% of patients, endoscopy is either not available or unsuccessful. Endovascular therapy with selective catheterization of the culprit vessel and injection of embolic material has emerged as an alternative to emergent operative intervention in high-risk patients. There has not been a systematic literature review to assess the role for embolotherapy in the treatment of acute upper gastrointestinal bleeding from gastroduodenal ulcers after failed endoscopic hemostasis. Here, we present an overview of indications, techniques, and clinical outcomes after endovascular embolization of acute peptic-ulcer bleeding. Topics of particular relevance to technical and clinical success are also discussed. Our review shows that transcatheter arterial embolization is a safe alternative to surgery for massive gastroduodenal bleeding that is refractory to endoscopic treatment, can be performed with high technical and clinical success rates, and should be considered the salvage treatment of choice in patients at high surgical risk.

### INTRODUCTION

Acute bleeding is the most common complication of peptic ulcer disease and about half the cases of upper gastrointestinal bleeding (UGI) are caused by gastric and duodenal ulcers<sup>[1,2]</sup>. First-line endoscopy achieves bleeding control in as many as 98% of patients<sup>[3,4]</sup>. Despite these measures, the mortality rate in patients with bleeding peptic ulcers remains as high as 5% to 10%<sup>[5,6]</sup> due to a combination of advanced age, multiple co-morbidities, and high transfusion requirements<sup>[7]</sup>. Current treatment algorithms for massive UGI bleeding recommend aggressive correction of coagulation disorders followed by endoscopy<sup>[8,9]</sup>. Endoscopic therapy with epinephrine injection and heat probe coagulation is the most reliable method. Re-bleeding is usually managed with a second endoscopic attempt. Severe bleeding despite conservative medical treatment or endoscopic intervention occurs in 5% of patients<sup>[10]</sup> and requires surgery or transcatheter arterial embolization. Surgery is associated with mortality rates as high as 20% to 40%<sup>[11]</sup>. Although endovascular management is not included in the treatment algorithms for UGI bleeding described in surgical textbooks, selective catheter-directed embolization has been proposed as a less hazardous alternative to surgery, especially for high-risk patients<sup>[12,13]</sup>, and is now considered in many institutions as the first-line intervention for massive gastroduodenal bleeding after failed endoscopic treatment<sup>[12-15]</sup>. The

obvious advantage of transcatheter embolization is avoidance of a laparotomy in a critically ill patient. With the advent of metallic coils, gelfoam, and surgical glue, outcomes after embolization have compared favourably with those of surgery. The purpose of this review is to review the data on the indications, safety, effectiveness, and outcomes of embolotherapy in the treatment of acute UGI from gastroduodenal ulcers.

## KEYWORD SEARCH

We searched PubMed for studies of embolization for peptic ulcer bleeding published in English from 1992 to 2009. Further studies were sought by manually searching the reference lists of articles retrieved *via* PubMed. We then selected the articles that had well-defined indications for the intervention and offered a detailed description of the outcomes, including technical and clinical success rates, re-bleeding, re-intervention, need for surgery for bleeding control, and morbidity and mortality rates. To avoid selection bias associated with a small series, we excluded studies with fewer than 10 patients and anecdotal case-reports. Studies of patients with bleeding from causes other than peptic ulcers were also excluded. The results were tabulated as absolute numbers and percentages. Mean values of the outcome variables of interest were computed. Information on indications, technique, complications, and a variety of other topics of interest is presented as a narrative, in order to provide a better understanding of the current status and controversial aspects of the endovascular treatment of UGI bleeding from peptic ulcers.

## INDICATIONS

Transcatheter arterial embolization as an alternative to surgery for the control of UGI bleeding was introduced by Rösch *et al*<sup>[16]</sup> in 1972. Since then, arterial catheterization has become a useful diagnostic and therapeutic tool in selected populations<sup>[17]</sup>. The typical candidate presents with massive bleeding (transfusion requirement of at least four units of blood per 24 h) or hemodynamic instability (hypotension with systolic pressure less than 100 mmHg and heart rate of 100/min or clinical shock secondary to blood loss) that has not responded to conservative medical treatment combining volume replacement, proton pump inhibitors, and at least one endoscopic procedure aimed at controlling the bleeding<sup>[18]</sup>. At this point, surgery is offered to low-risk patients and percutaneous embolotherapy to high-risk patients. Finally, endovascular treatment can be used if the bleeding recurs after surgery<sup>[19]</sup>.

## TECHNIQUE

A transfemoral approach was used in most of the case-series retrieved by our literature search. A 5-French sheath is placed in the common femoral artery. Brachial access may be necessary when there is an acute angulation at the origin of the celiac axis. A variety of selective catheters can be used to cannulate the celiac artery

and to access the common hepatic artery. Once access is obtained, arteriography is performed to delineate the arterial anatomy and to identify contrast extravasation. If no extravasation is seen, then superselective catheterization of the gastroduodenal artery (GDA) (Figure 1), left gastric artery (Figure 2), or splenic artery (Figure 3) is performed, depending on the endoscopic evidence concerning the probable bleeding site. During this step, a microcatheter is useful but not indispensable. Arteriography after superselective cannulation might reveal extravasation that was missed during contrast injection into the main hepatic artery. When a dual supply to the bleeding area is suspected, both arterial sources must be embolized. This typically occurs with ulcers that erode the GDA: embolization in this case needs to start distally to prevent persistent “backdoor” bleeding from the right gastroepiploic and superior pancreaticoduodenal arteries, and should then move to the proximal side of the erosion. If no evidence of bleeding is found on the pre-embolization arteriogram, then blind embolization is performed, typically guided by the endoscopic findings regarding the bleeding site (Figure 4). Another useful manoeuvre in this scenario is the placement, during the pre-embolization endoscopy, of clips around the bleeding site. The clips remain in position for several hours and allow for an educated guess about the location of the bleeding arterial branch<sup>[20]</sup>. If, despite the injection of a contrast agent, no extravasation is seen, then the branches terminating at each clip are selectively catheterized using microcatheter techniques and embolized. Arteriography with multiple projections is necessary at this step to assess the relationship between each clip and the adjacent branches. Infusion of a fibrinolytic agent such as t-PA, intra-arterial anticoagulants, or vasodilators to temporarily increase the bleeding rate during angiography has been reported to facilitate the angiographic identification and localisation of the bleeding vessel<sup>[21]</sup>.

## COMPLICATIONS

Groin hematomas and contrast-related complications occur with the same frequency as during other endovascular procedures. Acute renal failure may develop as a result of multiple factors including contrast injection and intravascular volume depletion. Duodenal ischemia can result from embolization of terminal muscular branches or from embolization of the main GDA with polyvinyl alcohol (PVA) particles. Typical symptoms include persistent epigastric pain, nausea and, occasionally, vomiting. Endoscopy shows small multiple duodenal erosions consistent with healing ischemic lesions. Predisposing factors include previous abdominal surgery and/or radiation therapy. Conservative treatment with proton pump inhibitors and maintenance of NPO status is usually sufficient. Inadvertent embolization of the main hepatic artery can result in a broad spectrum of manifestations, ranging from temporary liver enzyme elevation to life-threatening hepatic failure, for which risk factors include cirrhosis and associated portal vein compromise<sup>[12]</sup>. Inadvertent placement of coils in the main branches of the



**Figure 1** Arteriogram images of bleeding from a bulbar duodenal ulcer in a 76-year-old man. A, B: Arteriogram showing contrast medium extravasated from a slender branch of the gastroduodenal artery (GDA) into the duodenum (arrows); C, D: After microcatheterization, selective glue embolization (radiopaque because of associated lipiodol (arrows) preserving the GDA ensured control of the bleeding, with no early or late recurrences.

celiac axis has been reported. Given the rich collateral circulation, however, coils in the left gastric or splenic artery rarely produce organ-threatening ischemia<sup>[22]</sup>. Duodenal stenosis can become a serious problem after GDA embolization. It is more common after superselective embolization of terminal muscular branches with surgical glue and can occur several years after successful embolization. Balloon dilatation can be attempted initially, although surgery is required in patients with persistent symptoms of duodenal obstruction<sup>[23]</sup>.

## OUTCOMES IN CASE-SERIES

We identified 13 studies (422 patients; mean age, 69 years) on the endovascular management of intractable gastrointestinal bleeding from gastroduodenal ulcers. Endoscopy was performed unsuccessfully in 98% of these patients (Table 1). The vast majority of the patients with major comorbidities and were considered at high surgical risk. Endovascular embolization was technically successful in 392 (93%) patients. A variety of embolic materials including coils, PVA particles, blood clot, gelfoam, and cyanoacrylate glue were used. The “sandwich” technique with placement of embolic material on either side of the bleeding vessel was used in most case-series to minimize the risk of recurrent bleeding due to collaterals. Active extravasation was present at the time of embolization in 53% of patients. The other patients underwent blind embolization guided by the endoscopy findings or by clips placed around the bleeding site. In the subgroup with technically successful embolization, the rate of bleeding cessation (clinical success rate) was 81% (Table 2).

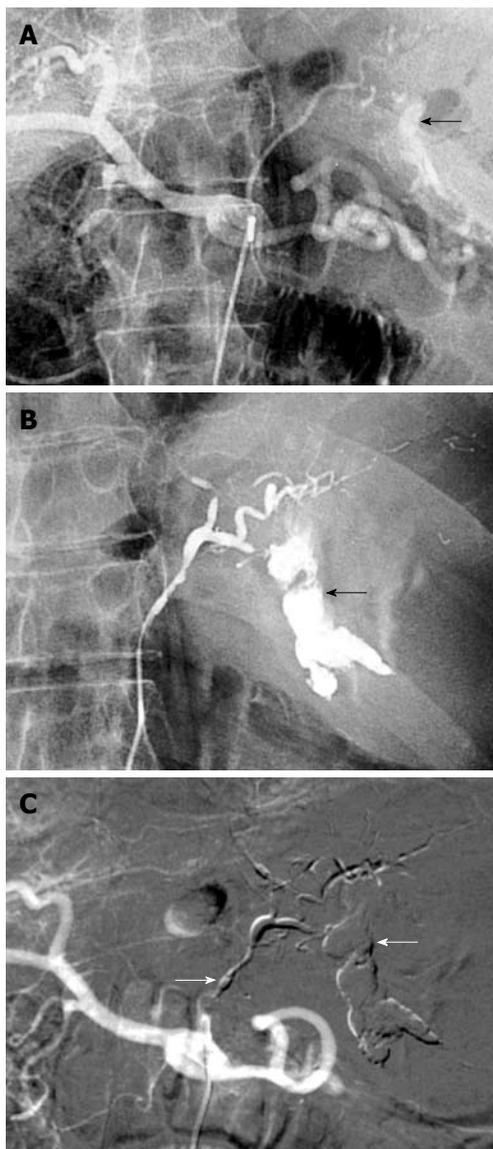
Overall, 25% (106/422) of patients had persistent bleeding. However, almost half of them responded to

**Table 1** Synopsis of the studies under review

Ref., yr	Patients (n)	Mean age (yr)	Previous endoscopy (%)	Active extravasation (%)	Technical success (%)
Lang <i>et al</i> <sup>[23]</sup> , 1992	57	52	NA	100	91
Toyoda <i>et al</i> <sup>[33]</sup> , 1995	11	65	100	54	100
Toyoda <i>et al</i> <sup>[37]</sup> , 1996	30	62	100	NA	100
Walsh <i>et al</i> <sup>[43]</sup> , 1999	50	64	100	50	92
De Wispelaere <i>et al</i> <sup>[40]</sup> , 2002	28	69	100	39	89
Ljungdahl <i>et al</i> <sup>[39]</sup> , 2002	18	78	72	50	72
Ripoll <i>et al</i> <sup>[24]</sup> , 2004	31	75	100	NA	100
Holme <i>et al</i> <sup>[25]</sup> , 2006	40	70	100	30	100
Eriksson <i>et al</i> <sup>[20]</sup> , 2006	10	75	100	10	100
Loffroy <i>et al</i> <sup>[13]</sup> , 2008	35	71	100	66	94
Larssen <i>et al</i> <sup>[15]</sup> , 2008	36	80	100	42	92
van Vugt <i>et al</i> <sup>[35]</sup> , 2009	16	71	100	75	88
Loffroy <i>et al</i> <sup>[26]</sup> , 2009	60	69	100	63	95
All studies	422	69	98	53	93

NA: Not available.

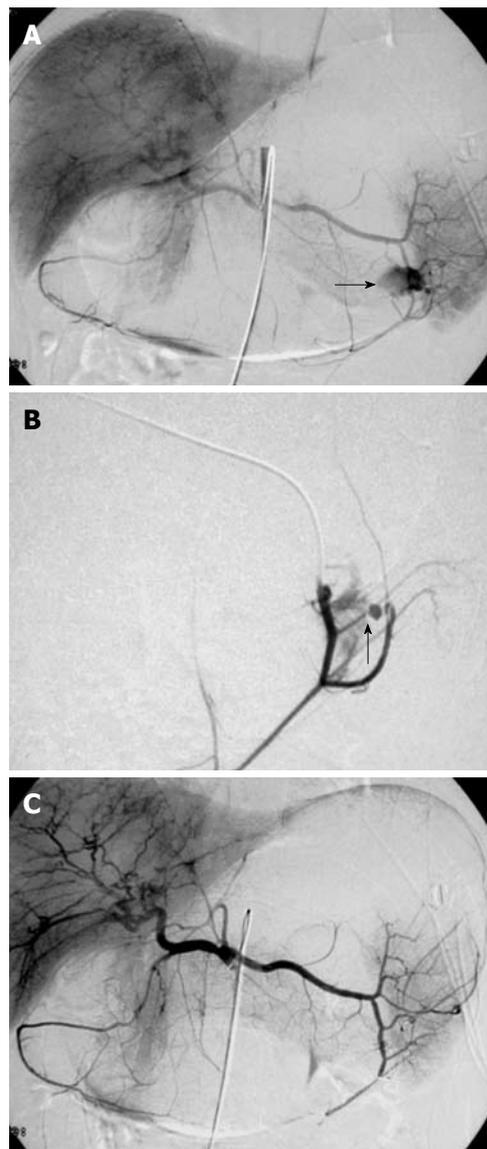
repeat embolization. Finally, 18% of patients overall underwent surgery for bleeding control (Table 2). Major and minor embolization-related complications developed in 4% of patients and included access-site complications,



**Figure 2** Bleeding Dieulafoy lesion in an 87-year-old man. A, B: Selective angiography shows contrast medium extravasation from the left gastric artery at the celiac trunk, indicating active bleeding (arrows); C: After arterial microcatheterization, bleeding was controlled after embolization of the left gastric artery using a Glubran/Lipiodol mixture (1:3) (arrows).

dissection of the target vessel, and hepatic or splenic infarction. The most significant long-term complication was duodenal stenosis, particularly after glue embolization of terminal muscular branches of the GDA. Overall 30-d mortality was 25% (Table 2). The data available in the study reports did not allow us to assess the causes of death or their relationship with the result of the embolization or need for further intervention. Although the mortality rates seem as high as those in several case-series of emergent surgery for UGI bleeding, they should be interpreted with the knowledge that most of the patients treated with embolotherapy had been turned down for surgery due to major co-morbidities and advanced age.

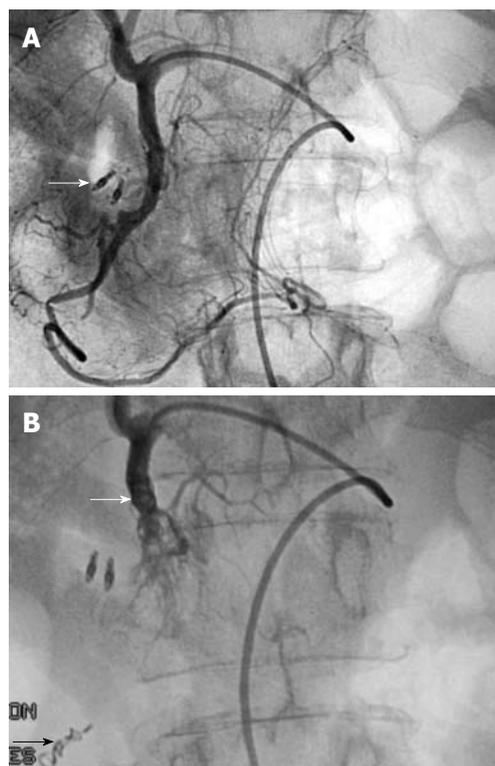
Given the variability in the way the results are reported and incomplete data on risk factors in the patient populations, we cannot draw conclusions regarding the impact of embolotherapy on mortality. Nevertheless,



**Figure 3** Digital subtraction images from a 37-year-old man with massive hematemesis. A, B: Selective angiography shows a bleeding ulcer in the fundus of the stomach. Extravasation of contrast medium from a branch of the left gastroepiploic artery is seen (arrows); C: The control angiogram after glue embolization throughout the splenic artery shows complete and selective occlusion of the bleeding branch, with no active bleeding. The patient was discharged from the hospital 4 d later.

several important points can be made. First, mortality and complication rates varied widely across the series, highlighting the influence of individual expertise and center volume on the outcomes. Second, the visualization of active extravasation followed by selective embolization was not consistently associated with a higher short-term clinical success rate. Possible explanations are the intermittent nature of gastrointestinal bleeding and the presence of bleeders missed by highly selective embolotherapy. Lastly, only 18% of the patients who initially underwent embolization finally needed surgery to control recurrent bleeding. Thus, embolotherapy considerably diminishes the need for laparotomy in patients with acute UGI bleeding from peptic ulcers.

A few of the most noteworthy case-series that raised



**Figure 4** Typical sandwich embolization in a 75-year-old woman with bleeding from a postbulbar duodenal ulcer at endoscopy. A: Angiography before embolization, guided by clip position (arrow): no evidence of active bleeding; B: Result after coil embolization of the distal and proximal GDA (with gelatine sponge in the arterial trunk), including the anterior and posterior superior pancreaticoduodenal arteries and the right gastropiploic artery, to prevent retrograde flow (arrows). No ischemic complications were reported.

interesting points are briefly summarized below. Ripoll *et al*<sup>[24]</sup> compared outcomes after embolization (31 patients) or surgery (39 patients) for bleeding from UGI peptic ulcers. Patients treated with embolotherapy were older (mean age, about 10 years older) and had higher rates of cardiovascular disease and anticoagulation treatment. Otherwise, co-morbidities were not different between the two groups. In the embolization group, the technical failure rate was 6.5% ( $n = 2$ ) and the re-bleeding rate was 29% ( $n = 9$ ). Four of the patients with persistent bleeding underwent surgical exploration. In the surgery group, the re-bleeding rate was 23.1% ( $n = 9$ ); five of these patients underwent a repeat surgical procedure for bleeding control. In addition, seven other patients required repeat surgery for complications from the initial operation. Overall, the embolization and surgery groups were not significantly different regarding the need for additional surgery (16.1% *vs* 30.8%, respectively) or survival (25.8% *vs* 20.5%, respectively). However, the re-intervention rate was considerably higher in the surgery group and the difference would perhaps have been statistically significant had the sample sizes been larger. Holme *et al*<sup>[25]</sup> reported on 40 consecutive patients who were referred for embolotherapy after unsuccessful endoscopic or surgical treatment. Long-term bleeding control was achieved in 26 (65%) patients. Of the 12 patients with active bleeding from a duodenal ulcer

**Table 2** Outcomes in case-series that included more than 10 patients treated with endovascular embolization for peptic ulcer bleeding over a 17-year period

Ref., yr	Clinical success (%)	Re-bleeding rate (%)	Need for surgery (%)	Complication rate (%)	30-d mortality (%)
Lang <i>et al</i> <sup>[23]</sup> , 1992	86	56	2	16	4
Toyoda <i>et al</i> <sup>[33]</sup> , 1995	91	18	18	0	27
Toyoda <i>et al</i> <sup>[37]</sup> , 1996	80	23	13	NA	23
Walsh <i>et al</i> <sup>[43]</sup> , 1999	52	52	37	4	40
De Wispelaere <i>et al</i> <sup>[40]</sup> , 2002	64	36	21	0	46
Ljungdahl <i>et al</i> <sup>[39]</sup> , 2002	67	8	8	0	6
Ripoll <i>et al</i> <sup>[24]</sup> , 2004	71	29	16	0	26
Holme <i>et al</i> <sup>[25]</sup> , 2006	65	28	35	0	25
Eriksson <i>et al</i> <sup>[20]</sup> , 2006	80	0	20	NA	NA
Loffroy <i>et al</i> <sup>[13]</sup> , 2008	94	17	14	6	21
Larssen <i>et al</i> <sup>[15]</sup> , 2008	72	9	30	8	17
van Vugt <i>et al</i> <sup>[35]</sup> , 2009	81	19	12	NA	38
Loffroy <i>et al</i> <sup>[26]</sup> , 2009	72	28	12	10	27
All studies	75	25	18	4	25

The table shows the rates of clinical success, recurrent bleeding after technically successful embolization, need for surgery to control the bleeding, complications, and peri-procedural mortality.

at the time of embolization, 10 (83%) had the bleeding controlled; one of these patients experienced re-bleeding, which was managed by surgery. In this subgroup, lasting hemostasis was achieved in eight (75%) patients. The 28 other patients had no signs of active bleeding on the diagnostic angiogram and underwent blind GDA coil embolization. Among them, 11 (39%) experienced re-bleeding. The inability to accurately identify and selectively embolize the culprit vessel in this subgroup is the most likely explanation for the high re-bleeding rate. The group with active bleeding on the angiogram had a higher likelihood of lasting hemostasis (75% *vs* 66%), underlining the limited effectiveness of blind coil embolization of the GDA. In this series, 10 patients died, including five as a result of continuous bleeding. Of note, this series included 13 patients who underwent duodenotomy for surgical bleeding control then experienced re-bleeding that was managed endovascularly. Eriksson *et al*<sup>[20]</sup> reported a series of 10 patients who were referred for embolotherapy after endoscopy failed to control bleeding from acute duodenal ulcers. To guide the endovascular treatment, a metallic clip was placed to mark the edge of the ulcer next to the bleeding site. Embolization ensured bleeding control in eight patients; the two remaining patients required surgery. In six patients, the clip played a crucial role in identifying the bleeding vessel,

as there was no evidence of contrast extravasation on the angiogram. The clip was particularly useful in three patients: two patients had bleeding from a supraduodenal artery without connection to the GDA, and the remaining patient had bleeding from an erosion in the inferior pancreaticoduodenal artery that arose from the superior mesenteric artery. One of the series with the best follow-up data was described by Lang *et al*<sup>[23]</sup>, who reported immediate and long-term results in 57 patients with bleeding duodenal ulcers. Control of bleeding was achieved in 52 of 57 patients. Superselective terminal muscular branch embolization was as effective as embolization of the main GDA. In eight of these patients, a second catheter-based intervention was needed to achieve complete bleeding cessation. Importantly, 29 of the 52 patients whose embolization procedure was successful experienced re-bleeding during follow-up (up to 7 years), underlining the need for aggressive long-term risk factor modification and treatment of the underlying peptic ulcer disease. Long-term bleeding control was more common in the subgroup of patients who underwent selective terminal muscular branch embolization, compared to the individuals treated with embolization of the main GDA trunk (53% *vs* 27%,  $P = 0.084$ ). Long-term success in this series was related to the embolic material used. For occlusion of the muscular branch arteries, 6-cyanoacrylate had the highest success rate, whereas for occlusion of the main GDA, an epsilon-aminocaproic acid-induced blood clot was superior over the other modalities (coils, gelatine sponge particles, or PVA particles). Together with re-bleeding, duodenal stenosis was the most troublesome complication and developed in nine patients between 8 mo and 7 years after the embolization procedure. This complication was more common after superselective embolization of terminal muscular branches. Surgical correction of the stenosis was necessary in eight patients to address persistent symptoms. Another patient required multiple balloon dilatations for duodenal stenosis. Balloon dilatations were performed in one additional patient for recurrent symptoms of duodenal obstruction after surgical resection. We reported our experience managing 60 patients with peptic ulcer bleeding<sup>[26]</sup>. The technical success rate was 95%. In 37% ( $n = 22$ ) of patients, the angiography showed no contrast extravasation and, therefore, empiric embolization was performed based on the endoscopic findings prior to the procedure. Approximately 28% ( $n = 16$ ) of these patients experienced re-bleeding, and only three underwent repeat embolization. Interestingly, the univariate analysis showed that early re-bleeding was associated with several of the study variables including coagulation disorders, a longer time from shock onset to angiography, a larger number of red-blood-cell units transfused before angiography, having two or more comorbid conditions, and being treated with coils as the only embolic agent. The multivariate analysis identified two factors that significantly predicted failure of embolization, namely, the presence of coagulation disorders ( $P = 0.027$ ) and the use of coils as the only embolic

agent ( $P = 0.022$ ). The mortality rate was not different in the patient group with clinically successful embolization and in the group with failed embolization (22% *vs* 37%)<sup>[26]</sup>.

## TOPICS OF INTEREST

### **Predictors of favourable outcome**

In surgical case-series, mortality rates in patients who have UGI bleeding from gastroduodenal ulcers and who do not respond to conservative therapy have ranged from 17% to 43%<sup>[27,28]</sup>. Factors influencing mortality include advanced age, trauma or sepsis, recent major operation, lung or liver disease, and massive blood transfusions<sup>[28,29]</sup>. After embolization in patients who are too sick to undergo surgery, mortality rates were similar, with a range of 10% to 45%. A number of factors have been identified as influencing post-embolization mortality. One of the most important and common factors is the absence of early re-bleeding, especially after selective embolization of a vessel with contrast extravasation on the initial angiography. Patients with angiographic extravasation and successful embolization have considerably lower mortality rates compared to patients who require surgery after failed embolization (38% *vs* 83%, respectively)<sup>[30]</sup>. Coagulopathy correlates closely with clinical failure and death after embolization. Thus, patients with impaired coagulation are three times more likely to re-bleed after initially successful embolization and 10 times more likely to die as a result of bleeding, compared to those with normal coagulation<sup>[2,12]</sup>. Rescue surgery after a failed embolization attempt has a very high mortality rate that exceeds even the 50% rate associated with emergent surgery<sup>[31,32]</sup>. In other series, underlying medical problems such as cirrhosis and malignancy, had major impacts on the mortality rate. Finally, in patients with multiorgan failure, clinically successful embolization appears to offer the only chance for survival. In the case-series reported by Schenker *et al*<sup>[22]</sup>, the mortality rate was 96% in patients with multiorgan failure who did not respond to embolization *vs* 31% in those who did.

### **Choice of embolic agent**

A focus of greater controversy is the influence of the type of embolic agent on the clinical outcome. There is general agreement that embolic therapy is superior over vasopressin infusion for the treatment of UGI bleeding from gastroduodenal ulcers<sup>[17]</sup>. The choice of the best embolic agent remains a matter of debate. Coils alone inserted into the GDA or super selectively in the pancreaticoduodenal arteries have been used successfully by several authors<sup>[33-35]</sup>. Lang *et al*<sup>[23]</sup> compared several embolic agents in a case-series of 57 patients. Safety and efficacy were best with autologous blood clot for proximal GDA embolization and with tissue adhesive for occlusion of the distal vessels from the GDA. These authors reported a 40% rate of duodenal stricture with tissue adhesives, a finding that may be related to the use of tissue adhesives to embolize the terminal muscular branches, and not to the nature of the embolization agent. The same group reported a high rate of re-bleeding

when PVA particles or gelfoam were used alone. Similarly, Encarnacion *et al*<sup>[2]</sup> obtained a low success rate (62%) in their case-series, which chiefly included patients embolized with gelatine sponge alone. Good results have also been reported with cyanoacrylate<sup>[36,37]</sup> and with the combination of gelatine sponge and coils<sup>[38]</sup>. Most of these series included small study populations; therefore, no statistical conclusions can be drawn. Finally, Aina *et al*<sup>[12]</sup> compared embolization with coils alone *vs* coils combined with PVA particles or gelfoam. By multivariate regression analysis, the use of coils alone was associated with re-bleeding in patients with coagulopathy, a finding that supports the use of PVA or gelfoam in combination with coils in this patient subgroup. We also found that using coils alone was significantly associated with early re-bleeding<sup>[26]</sup>. Otherwise, the nature of the embolic agent does not seem to affect the clinical response or re-bleeding rate.

### **Blind or empirical embolization**

Blind embolization, defined as embolization without angiographic proof of extravasation, is also controversial. In a study comparing several groups of patients, Dempsey *et al*<sup>[30]</sup> found that blind embolization was not helpful in achieving bleeding control. The proportion of patients who required surgery for bleeding control was similar in the patients without angiographic evidence of contrast extravasation who did not undergo embolization and in the patients who underwent blind embolization. However, endoscopy - a crucial procedure for selecting the target vessel for blind embolization - was non-diagnostic in 39% of patients in this case-series<sup>[30]</sup>. Massive bleeding is often intermittent<sup>[39]</sup>; therefore, most groups perform embolization based on the endoscopic findings, even when no extravasation is visible on the angiogram. In the case-series by Aina *et al*<sup>[12]</sup> and us<sup>[26]</sup>, outcomes were not different between patients who underwent blind embolization and those who underwent embolization after angiographic identification of a bleeding site. Other researchers also advocate endoscopy-directed blind embolization<sup>[2,33,40]</sup>. Based on the data in the literature and our own experience, we believe that blind embolization is appropriate. The GDA should be embolized using the "sandwich technique", in which both ends of the artery are filled with coils to avoid retrograde bleeding from the superior mesenteric circulation. If smaller muscular branches terminating at a clip are suspected culprits, then they should be embolized with any of the available materials.

### **Marking with a metallic clip**

Clip placement during endoscopy can help to localize the vessel feeding the bleeding ulcer, even when there is no contrast medium extravasation after injection with the catheter into the common hepatic artery or the main trunk of the GDA. Clip placement is also helpful when the bleeding artery arises separately from the proper hepatic artery or the GDA. Superselective angiography guided by clip position is more likely to visualize the extravasation, thus making blind coil

placement unnecessary, increasing the efficacy of the procedure, and decreasing the risk of coil misplacement and inadvertent hepatic embolization<sup>[20,26]</sup>. The only limitation of this technique is the need for around-the-clock availability of an experienced interventionalist and gastroenterologist, which is easy to achieve only at high-volume centres. This approach increases the likelihood of successful embolization and is now routinely used at our centre. Even when extravasation is not visualized, the clips can guide the blind embolization procedure, as pointed out previously.

### **Risk for gastrointestinal tract necrosis**

Arterial embolization in the UGI tract above the ligament of Treitz is generally considered very safe because of the rich collateral supply to the stomach and duodenum. However, the risk of significant ischemia after embolization is increased in patients with a history of surgery in the same area<sup>[41]</sup> or with embolic agents that can advance far into the vascular bed such as liquid agents (e.g. tissue adhesives such as cyanoacrylate) or very small particles (e.g. gelatine sponge powder)<sup>[41-44]</sup>. Although cases have been reported at the acute phase, post-embolization ischemia usually presents as duodenal stenosis at the chronic phase. Lang *et al*<sup>[23]</sup> reported duodenal stenosis in seven of 28 patients after embolization of terminal vessels, mostly when tissue adhesive was used. In this series, duodenal stenosis after GDA embolization was far less common and occurred in only two of 29 patients who underwent more proximal GDA occlusion. No major gastric or duodenal ischemic events occurred in our case-series; coils were the most often used single embolic agents, and gelatine sponge plugs were used instead of powder. A tissue adhesive was used only when angiographic extravasation was considered massive, and one part of cyanoacrylate was then diluted in two parts of lipiodol to ensure rapid polymerization. In addition, the mixture was injected selectively into the bleeding vessel while taking care not to fill the normal branches<sup>[12]</sup>.

### **Angiographic embolization vs surgery**

To date, there has been no controlled trial comparing angiographic embolization to surgery as a salvage procedure for failed endoscopic therapy. Two retrospective comparisons showed at least similar efficacy in terms of rates of re-bleeding, morbidity, and mortality. Ripoll *et al*<sup>[24]</sup> retrospectively assessed the outcomes of 70 patients with refractory peptic ulcer bleeding: 31 patients underwent angiographic embolization, and 39 patients were managed with surgery. Although the patients treated with angiographic embolization were 10 years older on average and more often had heart disease, there were no major differences in the rates of re-bleeding (29% *vs* 23%) or mortality (26% *vs* 21%). Another retrospective comparison, by Eriksson *et al*<sup>[45]</sup>, included 40 patients who underwent angiographic embolization and 51 patients who underwent surgery after failed endoscopic therapy. The angiographic embolization group was older and had a higher co-morbidity rate. Nevertheless, 30-d mortality

was lower in the angiographic embolization group (3% vs 14%). These results are promising, and we are eagerly awaiting the results of randomized, controlled trials.

## CONCLUSION

Massive bleeding from a peptic ulcer remains a challenge. Optimal management required a multidisciplinary team of skilled endoscopists, intensivists, experienced UGI surgeons, and interventional radiologists. Endoscopy is the first-line treatment. The role for early elective surgery or angiographic embolization in selected high-risk patients to prevent re-bleeding remains controversial. However, technological advances including lower-profile catheter systems will probably broaden the indications for endovascular treatment of UGI bleeding from gastroduodenal ulcers after failed endoscopy. Although prospective studies are needed to compare these management strategies, the available data suggest that transcatheter arterial embolization is not only a good alternative to surgery, but should now be considered the salvage treatment of choice after failed endoscopic treatment. However, only high volume centers, with experienced and skillful interventional radiologists, have the opportunity to use this technique as an alternative treatment.

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## Invasive front of colorectal cancer: Dynamic interface of pro-/anti-tumor factors

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### Abstract

Tumor-host interaction at the invasive front of colorectal cancer represents a critical interface encompassing a dynamic process of de-differentiation of colorectal carcinoma cells known as epithelial mesenchymal transition (EMT). EMT can be identified histologically by the presence of "tumor budding", a feature which can be highly specific for tumors showing an infiltrating tumor growth pattern. Importantly, tumor budding and tumor border configuration have generated considerable interest as additional prognostic factors and are also recognized as such by the International Union Against Cancer. Evidence seems to suggest that the presence of tumor budding or an infiltrating growth pattern is inversely correlated with the presence of immune and inflammatory responses at the invasive tumor front. In fact, several tumor-associated antigens such as CD3, CD4, CD8, CD20, Granzyme B, FOXP3 and other immunological or inflammatory cell types have been identified as potentially prognostic in patients with this disease. Evidence seems to suggest that the balance between pro-tumor (including budding and infiltrating growth pattern) and anti-tumor (immune response or certain inflammatory cell types) factors at the invasive front of colorectal cancer may be decisive in determining tumor progression and the clinical outcome of patients with colorectal cancer. On one hand, the infiltrating tumor border configuration and tumor budding promote progression and dissemination of tumor cells by penetrating the vascular and lymphatic vessels. On the other, the host attempts to fend off this attack by mounting an immune response to protect vascular and lymphatic channels from invasion by tumor buds. Whereas standard pathology reporting of breast and prostate cancer involves addi-

tional prognostic features, such as the BRE and Gleason scores, the ratio of pro- and anti-tumor factors could be a promising approach for the future development of a prognostic score for patients with colorectal cancer which could complement tumor node metastasis staging to improve the clinical management of patients with this disease.

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**Key words:** Colorectal cancer; Prognosis; Tumor invasive front; Tumor budding; Tumor growth pattern; Tumor infiltrating lymphocytes; Tumor immunity; Microsatellite instability

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### INTRODUCTION

The tumor node metastasis (TNM) staging system from the American Joint Committee on Cancer/International Union Against Cancer (UICC) remains the most reliable prognostic indicator for patients with colorectal cancer<sup>[1]</sup>. Overall 5-year survival rates are reported at 65% and correspond closely to disease progression; patients with stage I disease have more favourable prognoses with 5-year survival rates exceeding 80%-90%. In contrast, patients with stage II, III and IV disease experience progressively worse outcomes with varying 5-year survival rates of 70%-85%, 44%-80% and < 10%, respectively<sup>[2]</sup>. It is recognized, however, that patients with tumors of the same TNM stage may be variable both in terms of prognosis and response to therapy.

A range of other histomorphological, molecular and protein biomarkers have additionally been investigated for their prognostic value independently of TNM stage. These tumor-related factors such as venous and lymphatic invasion, tumor grade, perineural invasion, histological

type, loss of heterozygosity at 18q, mutation in p53, tumor expression of vascular endothelial growth factor and thymidylate synthase are recognized as essential, additional or new and promising prognostic factors by the UICC<sup>[3,4]</sup>. In particular, microsatellite instability (MSI) status has revealed itself not only as a significant prognostic factor but also as an attribute categorizing colorectal carcinogenesis into two major pathways: the chromosomal instability (or microsatellite stable; MSS) and MSI pathways, the latter including both sporadic and hereditary Lynch syndrome [Hereditary non-polyposis colorectal cancer (HNPCC)] patients both demonstrating mismatch repair deficiencies and high level MSI (MSI-H)<sup>[5]</sup>.

Tumor-host interaction at the invasive front of colorectal cancer represents a critical interface where tumor progression and tumor cell dissemination ensue. The invasive tumor front encompasses a dynamic process of de-differentiation of colorectal carcinoma cells, a process known as epithelial mesenchymal transition (EMT)<sup>[6]</sup>. EMT can be identified histologically by the presence of “tumor budding”, a feature which is specific for tumors showing an infiltrating growth pattern<sup>[7]</sup>. Importantly, tumor budding and tumor border configuration have generated considerable interest as additional prognostic factors and are also recognized as such by the UICC<sup>[3,4]</sup>. Evidence seems to suggest that the presence of tumor budding or an infiltrating growth pattern is inversely correlated with the presence of immune and inflammatory responses at the invasive tumor front<sup>[8,9]</sup>. In fact, several tumor-associated antigens such as CD3, CD4, CD8, CD20, Granzyme B, FOXP3 and other immunological or inflammatory cell types have been identified as potentially prognostic in patients with this disease<sup>[10-16]</sup>.

Together, evidence seems to suggest that the balance between pro-tumor (including budding and infiltrating growth pattern) and anti-tumor (immune response or certain inflammatory cell types) factors at the invasive front of colorectal cancer may be decisive in determining tumor progression and the clinical outcome of patients with colorectal cancer.

The aim of this review is to outline the evidence supporting a pro-/anti-tumor factor model of colorectal cancer progression, one which highlights the dynamic interface between tumor and host-related factors at the invasive front of colorectal cancer.

## THE INVASIVE FRONT OF COLORECTAL CANCER: PRO-TUMOR FACTORS

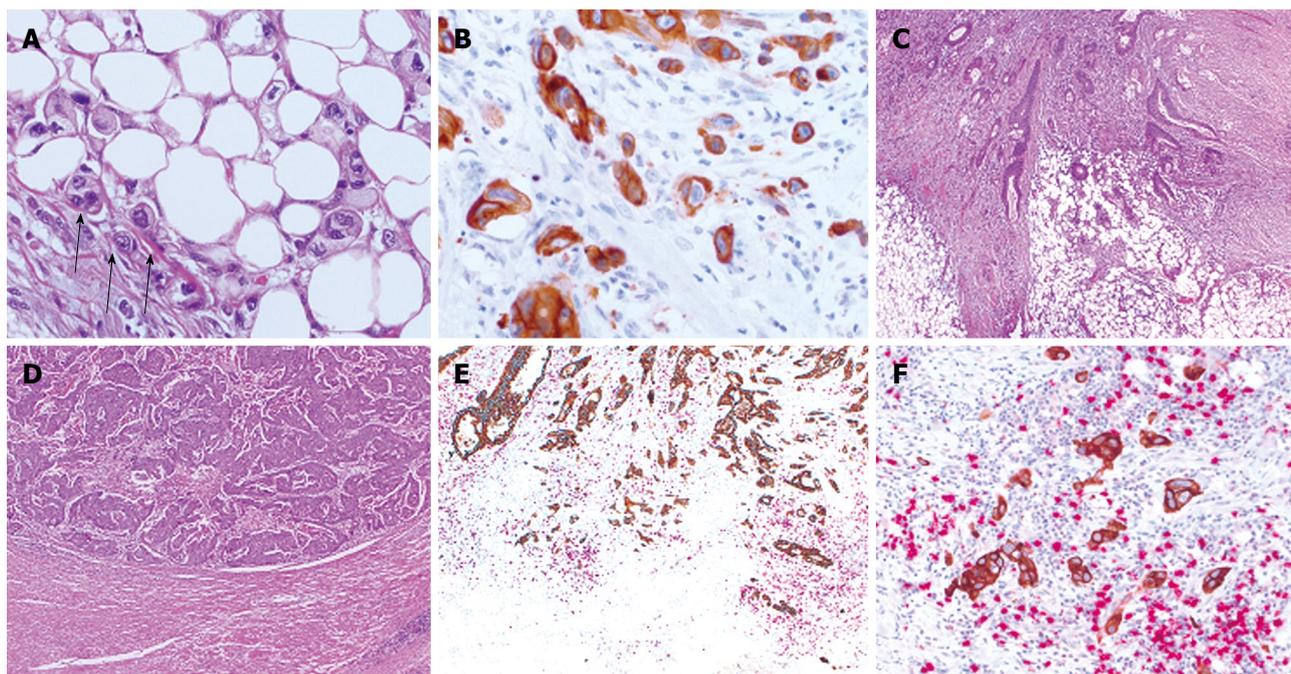
### *Tumor budding-migrating cancer stem cells (CSCs)?*

In 1985, a study of colonic adenocarcinoma in rats reported a peculiar feature at the invasive border of differentiated tumors<sup>[17,18]</sup>. Using both light and electron microscopy, Gabbert *et al*<sup>[18]</sup> observed neoplastic glands irregularly arranged into small strands and cords. In addition, they noted discontinuous small aggregates or single tumor cells which ultrastructurally did not possess junctional complexes, often had incomplete desmosomes,

missing or rudimentary basement membranes and absent or incomplete brush borders. They determined that at the invasive tumor front of colorectal cancer, differentiated tumors could acquire an undifferentiated phenotype. Their observation is credited for pioneering the concept known as de-differentiation at the invasive margin, which today is commonly referred to as “tumor budding”.

Tumor budding is a histological feature diagnosed at high magnification and is defined as single cells or clusters of up to four or five cells at the invasive tumor front<sup>[19]</sup>. Budding cells can be spotted using standard HE-stained tissue slides and their visualization is further facilitated using pan-cytokeratin stains (Figure 1A and B)<sup>[7]</sup>. The process of tumor budding is likened to EMT observed during gastrulation in embryonic development<sup>[7]</sup>. In the early phase of EMT, epithelial cells reduce intercellular contacts and cell-matrix contacts and reorganize the cytoskeleton to form cell membrane ruffles (lamellipodia) or cytoplasmic protrusions. Migration ensues and new cell-matrix contacts are formed providing cells with an anchorage for the contraction of the cell body. In colorectal cancer, tumor budding is a highly dynamic process giving temporal heterogeneity to the tumor.

Budding cells have been credited with the properties of malignant stem cells including the potential for redifferentiation both locally and at sites of distant metastasis and marking, what appears to be, the first histological event in tumor cell migration and invasion. Supporting this hypothesis further is the presence of “pseudopodia-like” cytoplasmic protrusions in tumor buds which have been identified by both electron microscopy and recently by immunohistochemistry with pan-cytokeratins<sup>[17,20]</sup>. These podia appear to be in direct contact with the adjacent interstitial tissue suggesting their formation occurs during tumor cell migration. Moreover, Shinto *et al*<sup>[21]</sup> recently suggested that cytoplasmic pseudo-fragments could be used as a marker for an activated budding phenotype that is associated with cell motility and increased invasiveness independent of the extent of budding. Not surprisingly, tumor buds have been shown to over-express proteins involved in extracellular matrix degradation and to under-express adhesion molecules. Previous studies on EMT and events occurring at the invasive tumor front implicate, in particular, the Wingless-INT (WNT) signaling pathway in the process of tumor budding evidenced by increased  $\beta$ -catenin immunohistochemical staining in tumor buds, a concomitant loss of E-cadherin and over-expression of laminin5 $\gamma$ 2 along with activation of transcriptional repressors SLUG, and ZEB1<sup>[22,23]</sup>. Over-expression of urokinase plasminogen activator receptor (uPAR), matrix metalloproteinase-7 and -9 (MMP7, MMP9), matrilysin, CD44, Ep-CAM, and extensive staining of  $\beta$ (III)-tubulin, a major constituent of microtubules, have all been reported<sup>[20,23-30]</sup> suggestive of the invasion and migration potential of tumor buds. Tumor buds seem to over-express CXCL12, a stromal cell-derived factor whose receptor CXCR4 is involved in chemotaxis and angiogenesis<sup>[31]</sup>. In addition, we recently documented the over-



**Figure 1** The invasive front of colorectal cancer. A: HE staining of colorectal cancer ( $\times 40$  magnification) showing tumor buds (arrows) at the invasive front; B: Pan-cytokeratin staining (CK22) highlighting tumor buds at the invasive front of colorectal cancer ( $\times 40$  magnification). Colorectal cancers with different tumor border configurations upon evaluation of HE staining at low magnification ( $\times 5$ ); C: Infiltrating tumor border configuration; D: Pushing tumor border configuration; E: Low ( $\times 5$ ); F: High ( $\times 20$ ) power magnification of double immunostaining for pan-cytokeratin (CK22) and anti-CD8 antibody highlighting "attackers" (tumor buds, brown) and "defenders" (CD8+ T-lymphocytes, red) at the invasive front of a colorectal cancer with infiltrating tumor border configuration.

expression of the putative colorectal CSC marker ABCG5 within tumor buds leading to a poorer outcome of patients including those with node-negative disease (Hostettler, *World J Gastroenterol*, in press). Whether a sub-population of tumor buds may in fact represent malignant stem cells is still an open question which necessitates further investigation.

### Prognostic impact of tumor budding

Since tumor budding appears to play a critical role in the initiation of metastasis, several authors have investigated the potential of this feature to predict dissemination of tumor cells to regional lymph nodes. A significant association between tumor budding and lymph node positivity has been consistently demonstrated correlating with tumor aggressiveness and more advanced TNM stage<sup>[32-43]</sup>. Tumor budding is frequently associated with poorly differentiated tumors, and with the presence of vascular and lymphatic invasion independently of disease extent<sup>[44-48]</sup>. Local tumor recurrence and distant metastasis to the lung and liver are also more commonly observed in patients with tumor budding<sup>[36,39,48-50]</sup> and additionally represent a reproducible prognostic factor in stage II patients<sup>[51]</sup>. Recently, Suzuki *et al*<sup>[52]</sup> found that tumor budding and venous invasion were significant predictors of local and distant metastases in patients with T1 stage colorectal cancers. Xu *et al*<sup>[53]</sup> demonstrated an increased rate of tumor budding in colorectal carcinomas with the aggressive micropapillary component. The presence of tumor budding has repeatedly been linked to poor clinical outcome, underlined by the adverse effect on overall survival independently of TNM stage<sup>[47,51,54]</sup>.

### Tumor growth pattern and prognosis

Tumor budding is closely linked to tumor growth pattern, a feature described by Jass *et al*<sup>[55]</sup> in 1987 which led to the proposal of an alternative prognostic classification system for rectal cancers<sup>[55,56]</sup>. The diagnosis of either a pushing (or expanding) or infiltrating tumor border configuration can be made at low magnification and is reproducible among pathologists thereby underlining its usefulness as a prognostic indicator (Figure 1C and D)<sup>[7]</sup>. The pushing tumor border is one in which margins are reasonably well-circumscribed and often associated with a well-developed inflammatory lamina. In contrast, the infiltrative tumor border is characterized by widespread dissection of normal tissue structures with loss of a clear boundary between tumor and host tissues.

Several studies have confirmed that an infiltrative tumor border configuration has a significant adverse prognostic impact in colorectal cancer and may predict local recurrence<sup>[57,58]</sup>. Our study group has also recently provided evidence for the improved stratification of stage II colorectal cancer patients based on the diagnosis of tumor border configuration. In particular, the 5-year survival rates for patients with stage II tumors decreased substantially from 80% in those with a pushing margin to 62.7% in patients with an infiltrating growth pattern, a survival rate similarly found in patients with stage III disease<sup>[59]</sup>. Considering that patients with stage III tumors are generally considered for adjuvant therapy<sup>[60]</sup>, the implications of these findings suggest that stage II patients with an infiltrating tumor margin should perhaps be considered for post-operative therapy. The addition of tumor border configuration to TNM stage improved

the prognostic classification of colorectal cancer patients by 17.9%. Since the presence of tumor budding can be strongly specific for an infiltrating, rather than a pushing/expanding growth pattern, it is not surprising that loss of cell-adhesion molecule E-cadherin and apoptosis activating factor-1, a pro-apoptotic molecule and over-expression of uPA and uPAR have all been reported as significant predictors of an infiltrating tumor border configuration in colorectal cancer<sup>[9,61]</sup>.

## THE INVASIVE FRONT OF COLORECTAL CANCER: ANTI-TUMOR FACTORS

Immunotherapy for patients with colorectal cancer represents a realistic alternative approach to treatment of this disease<sup>[62-64]</sup>. The last 20 years has seen a wide range of publications on tumor immunity and the prognostic impact of immune and inflammatory cell types in the microenvironment of colorectal tumors demonstrating promising results both *in vitro* and *in vivo*.

### Peritumoral inflammation

The presence of conspicuous peritumoral lymphocytic (PTL) inflammation, viewed as a distinctive “encapsulating” connective tissue mantle cap at the invasive front of colorectal cancer, is inversely correlated with the presence of tumor budding and positively associated with improved survival<sup>[19,65,66]</sup>. Jass<sup>[8]</sup> demonstrated that PTL infiltration in rectal cancer decreased with more advanced Dukes’ stage to 53%, 28% and 13% with Dukes’ A, B and C cases, respectively. In addition, the significantly worsened prognosis in patients lacking PTL inflammation at the tumor border was highlighted, while patients with moderate or pronounced infiltration performed significantly better independently of disease stage. The results have also been confirmed by other study groups<sup>[67]</sup>. However, the presence of PTL inflammation at the invasive front does not appear to be an independent prognostic factor in patients with this disease<sup>[59]</sup>. Nonetheless, PTL inflammation seems to be intimately linked with abundant CD8+ tumor infiltrating T-lymphocytes, further implicating tumor immunity in the defense against colorectal cancer.

### T-lymphocytes

Most studies to date confirm that a high rate of tumor infiltrating lymphocytes (TILs), in particular those located intra-epithelially characterized by CD4+ and CD8+ tumor-associated antigens are beneficial for patient outcome<sup>[68,69]</sup>. An abundant TIL count appears to be linked to earlier Dukes’ stage, decreased local recurrence rate following curative surgery and improved overall and disease-free survival time both in non-metastatic and metastatic patients undergoing hepatic resection<sup>[10,68-73]</sup>.

Galon *et al.*<sup>[13]</sup> evaluated by gene expression profiling and immunohistochemistry, the type, density and location (whether at the invasive margin or the tumor centre) of TILs in a large number of cases. They evaluated CD3, CD8, granzyme B and memory CD45RO T cells

and demonstrated a significant independent and positive effect of TILs on both recurrence and survival. Pages *et al.*<sup>[15]</sup> performed a comprehensive analysis of TILs focusing on early metastatic invasion. They found, by RT-PCR on 75 cases that mRNA levels of CD8, granzyme B and granzyme B and granzyme B were significantly greater in patients without vascular emboli, lymphatic and perineural invasion (collectively known as VELIPI) compared to those with these features and that CD45RO+ cells had independent prognostic value<sup>[15]</sup>. Diederichsen *et al.*<sup>[74]</sup> showed that a low CD4/CD8 ratio by flow cytometry was an independent prognostic factor for prolonged survival. In addition, Milasienė *et al.*<sup>[75]</sup> evaluated inter-epithelial CD3, CD4, CD8, CD20 and CD16 and found that increased levels of all these markers, particularly of the natural killer cell marker CD16 led to significantly improved overall outcome<sup>[11,75]</sup>. Moreover, regulatory T-cells expressing FOXP3+ has been shown to correlate with improved outcome independently of TNM stage<sup>[16,76]</sup>.

### Macrophages, mast cells, neutrophils and dendritic cells

In addition to T cells in colorectal cancer, a growing number of studies have demonstrated the clinical impact of dendritic cells, mast cells, macrophages and neutrophils on survival. An improved survival time and a preventative effect of mast cells on local recurrence and distant metastasis in patients with rectal tumors with high mast cell counts have been identified<sup>[77-79]</sup>. Further, the significant benefit of mast cell number on tumor progression in colorectal cancer was highlighted by Gounaris *et al.*<sup>[80]</sup> who reported that depletion of mast cells whether by pharmacological means or through generation of chimeric mice with genetic lesions in mast cell development led to remission of existing polyps. Moreover, Halazun *et al.*<sup>[81]</sup> found that an elevated neutrophil/lymphocyte ratio led to a poorer survival time and higher rate of recurrence in colorectal cancer patients undergoing surgery for liver metastasis.

Dendritic cells are the most potent antigen-presenting cells and as such are now one of the many important tools for tumor immunotherapy. Evidence is accumulating which suggests that the presence of dendritic cells may be of significant benefit to patients with colorectal cancer<sup>[82]</sup>. Using immunohistochemistry for CD83, Suzuki *et al.*<sup>[83]</sup> described the presence of mature dendritic cells at the invasive margin of cancer stroma and demonstrated by light and electron microscopy their formation into clusters with lymphocytes, the majority of which were CD45RO+ T cells. They conclude that mature CD83+ dendritic cells at the invasive margin promote T-cell activation for the generation of tumor specific immunity. Using electron microscopy, tumor-infiltrating dendritic cells were found to make contacts among themselves, with TILs and tumor cells. The presence of dendritic cells was found predominantly in early compared to later disease stages and mostly located in tumor surrounding tissue<sup>[84]</sup>. Dadabayev *et al.*<sup>[12]</sup> demonstrated that dendritic cells were significantly correlated with intra-epithelial CD4+ and CD8+ lymphocytes. Recently, HLA-DR

expressed constitutively on antigen-presenting cells such as dendritic cells and macrophages has also been found to correlate with the presence of TILs and PTLs as well as improved patient outcome<sup>[85]</sup>.

## THE INVASIVE FRONT OF COLORECTAL CANCER: MSI

Works by Banerjee *et al*<sup>[86]</sup> clearly show that MSI status (MSS; sporadic MSI-H and hereditary Lynch syndrome-associated colorectal cancers) should be taken into consideration when discussing tumor immunity in colorectal cancer. Compared to MSS tumors, both sporadic and hereditary MSI-H cancers from patients with Lynch syndrome (hereditary non-polyposis coli; HNPCC) are characterized by prolonged survival time, significantly more frequent PTL inflammation at the invasive front and by an inherent abundance of intra-epithelial TILs<sup>[87-94]</sup>. In contrast to MSS tumors which primarily arise following disruption of WNT signalling, sporadic MSI-H tumors are linked to mutations in TGF $\beta$ R II<sup>[95,96]</sup>. Baker *et al*<sup>[97]</sup> hypothesized that retention of TILs in MSI-H cancers may be a consequence of refractoriness to normal TGF- $\beta$  signalling. In a subsequent study, these authors show in more than 1000 MSS and 223 MSI tumors that an abundant CD8+ TIL infiltrate has a beneficial effect on survival time in MSS, but not MSI cancers<sup>[71]</sup>. Other authors confirm the abundance of CD8+ TILs and granzyme-positive cells as well as improved clinical outcome in patients with MSI-H compared to MSS colorectal cancers<sup>[98-100]</sup>. In addition, a positive correlation between apoptosis rates and higher TIL number has been described, a finding which could perhaps help to explain the improved prognosis seen in patients with MSI-H compared to MSS tumors<sup>[98,101]</sup>. Studies on T-reg cells such as FOXP3 and CD25+ have recently been undertaken<sup>[102]</sup>. Drescher *et al*<sup>[102]</sup>, evaluating both MSS and MSI-H cancers found that in contrast to CD8+ T-cells which may be involved in actively preventing growth and/or metastatic in MSI-H tumors, CD25+ cell counts were similar between MSS and MSI-H tumors suggesting no active immunosuppressive mechanisms in MSS cancers. Finally, the upregulation in MSI-H cancers of a large number of genes involved in immune response and increased levels of pro-inflammatory cytokines and cytotoxic mediators indicate an activated anti-tumor immune response in these tumors<sup>[86]</sup>.

## THE INVASIVE FRONT OF COLORECTAL CANCER: A BALANCE OF PRO- AND ANTI-TUMOR FACTORS

Several observations have led to the hypothesis that tumor progression and prognosis in patients with colorectal cancer is not based solely on the presence of pro-tumor or absence of anti-tumor factors but rather on the balance between the two. First, the presence of

tumor buds is inversely correlated with the presence of PTL inflammation and intra-epithelial CD8+ TILs<sup>[9,21]</sup>. In MSI-H cancers, where intra-epithelial TILs are abundant, PTL inflammation “encapsulating” the tumor at the invasive front and pushing tumor border are commonly seen, tumor budding is virtually absent<sup>[20]</sup>. When it occurs, tumor budding in the MSI-H lacks the full budding immunophenotype and the cytoplasmic podia which give budding cells a temporal dimension<sup>[20]</sup>. In a previous study on MSS colorectal cancers, we hypothesized that an intense peritumoral inflammatory reaction at the invasive front could be “nipping colorectal cancer in the bud” by specifically targeting budding cells for destruction<sup>[9]</sup>. We recently investigated CD8+ lymphocytes directly positioned in the microenvironment of the tumor buds. We could demonstrate that the ratio of CD8+ lymphocytes to tumor buds (CD8+/tumor budding index) out-performed both tumor budding or CD8+ lymphocytes alone as independent prognostic factors in two independent cohorts<sup>[103]</sup>. Using double immunostaining for CD8+ antibody and pan-cytokeratin, a ratio of 3:1 for CD8+ lymphocytes to tumor buds was a highly favourable phenotypic constellation associated with earlier pT stage, lymph node negativity, low tumor grade and absence of vascular and lymphatic invasion in addition to conferring a prolonged clinical outcome in both stage II and stage III colorectal cancer (Figure 1E and F). Although we cannot allude to the direct functional interaction between CD8+ lymphocytes and tumor buds themselves, the strong circumstantial relationship between the ratio of tumor budding and CD8+ lymphocytes in the microenvironment of budding cells appears, nonetheless, to be a reproducible and independent prognostic factor in colorectal cancer.

## DISCUSSION

The invasive front of colorectal cancer represents a dynamic interface between pro- and anti-tumor factors, which can be visualized as a balance between “attackers” (pro-tumor) and “defenders” (anti-tumor). On the one hand, the infiltrating tumor border configuration and its “cavalry” tumor budding promote progression and dissemination of tumor cells by penetrating the vascular and lymphatic vessels. On the other, the host attempts to fend off this attack by mounting an immune response using its “infantry”, in particular cytotoxic T lymphocytes, to protect vascular and lymphatic channels from invasion by tumor buds. Although evidence shows that both pro- and anti-tumor factors contribute prognostic information in a TNM-independent manner, the ratio of attackers and defenders may better capture the interaction at the invasive front which could translate into more powerful prognostic indicators.

Whereas standard pathology reporting of breast and prostate cancer involves additional prognostic features, such as the BRE and Gleason scores, the ratio of attackers/defenders could be a promising approach for the future development of a prognostic score for patients

with colorectal cancer which could complement TNM stage to improve the clinical management of patients with this disease.

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## Colorectal cancer screening in Europe

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### Abstract

Colorectal cancer (CRC) is the second most frequent malignant disease in Europe. Every year, 412 000 people are diagnosed with this condition, and 207 000 patients die of it. In 2003, recommendations for screening programs were issued by the Council of the European Union (EU), and these currently serve as the basis for the preparation of European guidelines for CRC screening. The manner in which CRC screening is carried out varies significantly from country to country within the EU, both in terms of organization and the screening test chosen. A screening program of one sort or another has been implemented in 19 of 27 EU countries. The most frequently applied method is testing stool for occult bleeding (fecal occult blood test, FOBT). In recent years, a screening colonoscopy has been introduced, either as the only method (Poland) or the method of choice (Germany, Czech Republic).

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**Key words:** Colorectal cancer; Europe; Fecal occult blood test; Screening colonoscopy; Screening programs

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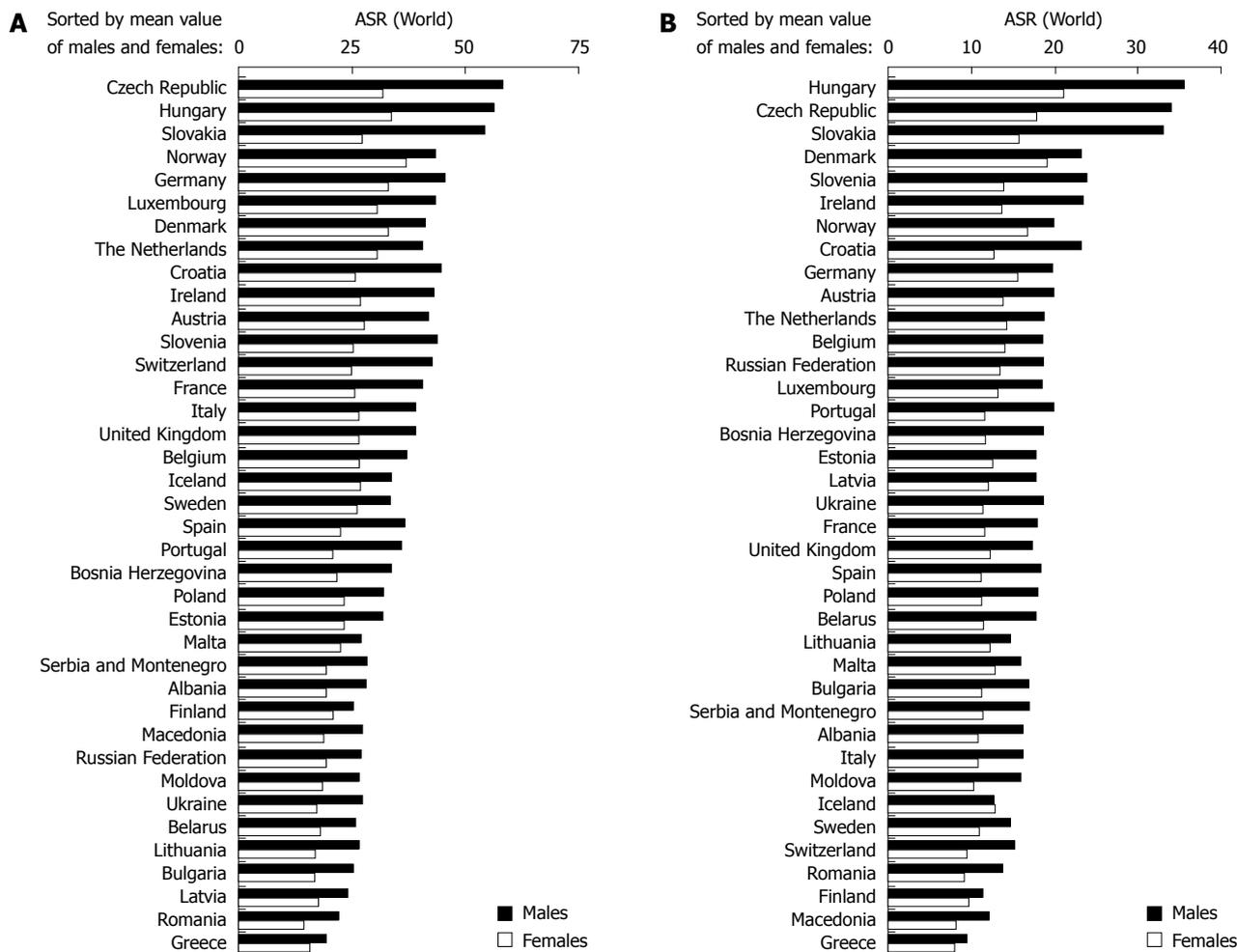
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### INTRODUCTION

Colorectal cancer (CRC) poses a serious health problem in countries with a Westernized lifestyle. Over the last decade, a whole range of new technologies have been introduced in clinical practice to diagnose and treat the disease, with therapeutic modalities extending to advanced stages of the disease. Nevertheless, prevention undoubtedly remains the key to reducing morbidity and mortality. The introduction of national or transnational population-wide screening programs is a priority for the healthcare policy of individual states, and this is also being addressed at the highest level by European Union (EU) administrators. The approach of individual countries to screening programs varies significantly because of differences in health insurance systems and budgets. This summary article focuses on a brief description and comparison of these programs.

### EPIDEMIOLOGY

CRC is the second most frequent malignant disease in developed countries. The incidence of CRC is generally higher for men, and the risk of the disease increases with age, as the majority of cases are diagnosed in patients more than 50 years of age<sup>[1]</sup>. European countries rank highest in the global statistics, both in terms of incidence and mortality. In 1998 to 2002, the incidence of CRC in the USA for men and women was 38.6 and 28.3, respectively; in Europe, it was 38.5 and 24.6 [world age standardization (ASR-W)], as calculated per 100 000 inhabitants<sup>[2]</sup>. However, mortality over the same period of time was much higher in Europe than in the US, both for men and women: in the USA, the figures were 13.5 and 9.2, respectively, while in Europe, they were 18.5 and 10.7 (ASR-W), as calculated per 100 000 inhabitants<sup>[3]</sup>. A detailed comparison of data for European countries is made difficult because of the absence of a unified data



**Figure 1** Epidemiology of colorectal cancer in European countries. A: Incidence in international comparison-European countries; B: Mortality in international comparison-European countries. Adapted from: Ferlay J, Bray F, Pisani P, Parkin DM. GLOBOCAN 2002: Cancer incidence, mortality and prevalence worldwide. IARC Cancer Base No. 5 version 2.0. Lyon: IARC press, 2004. Available from: URL: <http://www-dep.iarc.fr/>, section C15 I-VIII (Detailed). Last accessed on August 8, 2009.

Table 1 Colorectal cancer incidence in European countries in 2006	
Parameter	Incidence
Countries with the highest incidence	> 70/100000 men (ASR-E): Hungary (106), Czech Republic (94.4), Slovakia (87.1), Switzerland (79.1), Germany (70.2) > 45/100000 women (ASR-E): Switzerland (55.6), Norway (51.2), Hungary (50.6), Denmark (48), Czech Republic (46), Germany (45.1)
Countries with the lowest incidence	< 40/100000 men (ASR-E): Albania (13.6), Greece (31), Bosnia Herzegovina (34.6), Republic of Moldova (38.7), Finland (39.2) < 26/100000 women (ASR-E): Greece (21.3), Albania (21.4), Romania (25.1), Spain (25.4)

Adapted from: Ferlay J, Autier P, Boniol M, Heanue M, Colombet M, Boyle P. Estimates of the cancer incidence and mortality in Europe in 2006. *Ann Oncol* 2007; 18: 581-592. ASR-E: European age standard.

source. Not all countries maintain sophisticated population and cancer registers, and it is sometimes necessary to obtain input data by projecting aggregated data. In this outline, figures available from international studies summarizing global and European epidemiologic data have been used<sup>[4,5]</sup>. A detailed comparison of countries within Europe using the ASR-W of incidence and mortality is presented in Figure 1. Most recent epidemiologic data on CRC for 2006 recalculated to the European age standard are given in Tables 1 and 2.

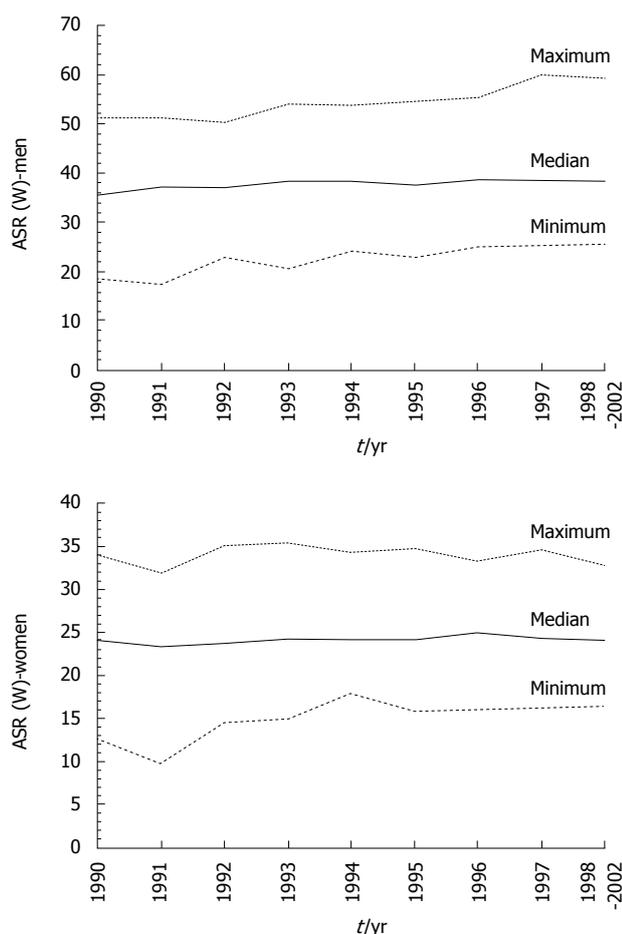
CRC comprises 12.9% of all newly-diagnosed carcinomas in the European population (men 12.8%, women 13.1%) and account for 12.2% of deaths caused by malignancy.

CRC is the second most frequent malignancy, after breast carcinoma (13.5% of all malignancies) and bronchogenic carcinoma (12.1% of all malignancies). It has been estimated that in 2006, 412000 people were diagnosed with CRC in Europe, and 207400 of them die of the disease<sup>[6]</sup>. The average incidence has shown a tendency to increase in recent years (2001-2005), with a year-on-year growth of 0.5%. Available data on time trends of CRC incidence and mortality are shown in Figures 2 and 3. A detailed analysis of individual diagnoses confirms that malignant disease of the colon is the most frequent, accounting for 57% of all cases (> 35 cases/10<sup>5</sup> inhabitants), followed by malignant diseases of the rectum

Table 2 Colorectal cancer mortality in European countries in 2006

Parameter	Mortality
Countries with the highest mortality	> 40/100 000 men (ASR-E): Hungary (54.4), Czech Republic (51), Slovakia (43.3), Croatia (40.7)
	> 20/100 000 women (ASR-E): Hungary (26.7), Slovakia (24.4), Czech Republic (24.1), Denmark (24.1), Norway (21.4)
Countries with the lowest mortality	< 20/100 000 men (ASR-E): Albania (7.3), Greece (15.5), Finland (17.9), Switzerland (19.1), Cyprus (19.3), Bosnia Herzegovina (19.5)
	< 12/100 000 women (ASR-E): Albania (9.9), Greece (10.8), Finland (11.3), Switzerland (11.6)

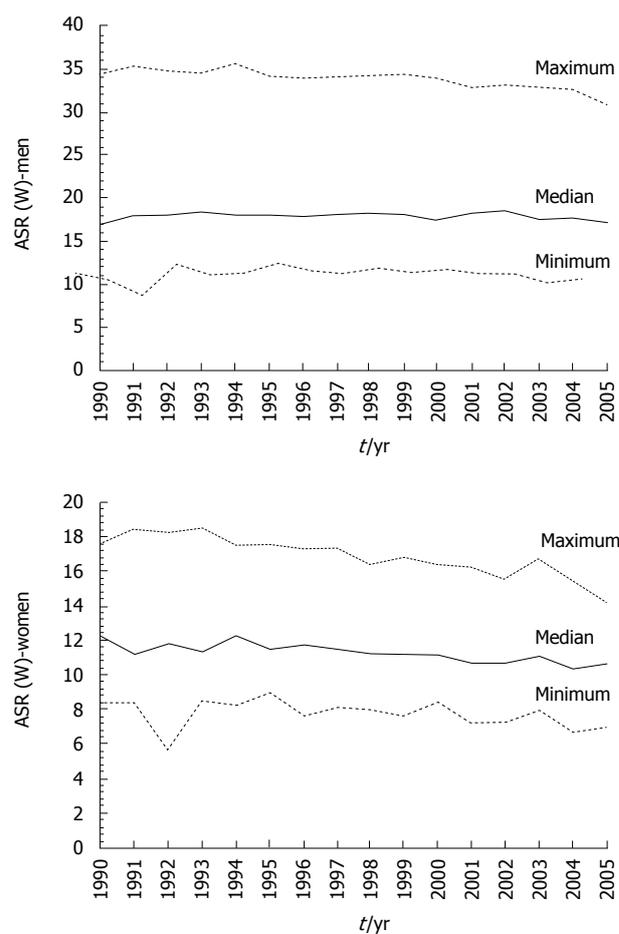
Adapted from: Ferlay J, Autier P, Boniol M, Heanue M, Colombet M, Boyle P. Estimates of the cancer incidence and mortality in Europe in 2006. *Ann Oncol* 2007; 18: 581-592.



**Figure 2 Incidence trends of colorectal cancer in Europe.** Thirty nine cancer registries in 1990-1996, 37 cancer registries in 1997, 96 cancer registries in 1998-2002. Adapted from: Parkin DM, Whelan SL, Ferlay J, Storm H. *Cancer Incidence in Five Continents, Vol. I to VIII.* IARC CancerBase No. 7, Lyon, 2005. Available from: URL: <http://www-dep.iarc.fr/>, section C15 I-VIII (Detailed). Last accessed on August 8, 2009; Curado MP, Edwards B, Shin HR, Storm H, Ferlay J, Heanue M, Boyle P, editors. *Cancer Incidence in Five Continents, Vol. IX.* IARC Scientific Publications No. 160, Lyon: IARC, 2007. Available from: URL: <http://www-dep.iarc.fr/> section C15 IX. Last accessed on August 8, 2009.

and rectosigmoid ( $> 22$  cases/ $10^5$  inhabitants) and tumors of the anus and anal channel ( $> 1.0$  cases/ $10^5$  inhabitants) (Table 3). According to recently published data, CRC-related mortality has stabilized or shown a slight decrease over recent years.

The most extensive population study monitoring the relative survival rate (RSR) is the EURO CARE program<sup>[7]</sup>, which takes registers of patients suffering from malignant diseases as a basis. Data have been gathered and evaluated since 1978. The most recent version, EURO CARE-4,



**Figure 3 Mortality trends of colorectal cancer in Europe.** As available in WHO database, countries with cancer registry (*Cancer Incidence in Five Continents, Vol. IX*). Adapted from: CancerMondial - WHO, International Agency for Research on Cancer, 2008. Available from: URL: <http://www-dep.iarc.fr/>; World Health Organization (2006), mortality database <http://www.who.int/whosis/whosis/>, United Nations, *World Population Prospects, the 2006 revision*. Available from: URL: <http://www-dep.iarc.fr/>. Last accessed on August 8, 2009.

uses comparative analyses of data from the year 1995 to 1999, while data are also available for the years 2000 to 2002<sup>[8]</sup>. Data from the European population carcinoma register are also used in the CONCORD study<sup>[9]</sup>, which focuses on a systematic comparison of statistical data between Europe and Northern America. Apart from these two studies, data from population registers of carcinoma have been published for some European countries. Data available regarding the 5-year RSR show high variability across European countries, with borderline values in the Czech Republic (50%) on the one hand and Germany

**Table 3** Epidemiology of colorectal cancer in the Europe (96 individual cancer registries, 1998-2002)

Parameter incidence	Sex	C18-C21	Individual diagnoses		
			C18	C19-C20	C21
Crude incidence (cases/100.000 inhabitants)	Men	63.9	37.1	26.0	0.8
	Women	53.7	34.8	17.6	1.3
	All	58.6	35.9	21.7	1.1
ASR-E	Men	58.0	33.5	23.7	0.8
	Women	36.8	23.4	12.4	1.0
	All	45.8	27.6	17.3	0.9
ASR-W	Men	38.5	22.0	15.9	0.5
	Women	24.6	15.5	8.4	0.7
	All	30.6	18.3	11.7	0.6
Mean age (yr)	Men	69.1	69.7	68.2	65.4
	Women	71.3	71.9	70.4	67.7
	All	70.1	70.8	69.1	66.8
Ratio (males: females) (based on No. of cases)		1.1:1	1.0:1	1.4:1	0.6:1

Adapted from: Curado MP, Edwards B, Shin HR, Storm H, Ferlay J, Heanue M, Boyle P, editors. Cancer Incidence in Five Continents, Vol. IX. IARC Scientific Publications No. 160, Lyon: IARC, 2007. Available from: URL: <http://www-dep.iarc.fr/> section CI5 IX. Last accessed on August 8, 2009. ASR-W: World age standardization.

(60%) on the other hand<sup>[7-16]</sup> (Table 4). Several studies have confirmed a favorable time trend in the 5-year RSR; however, these results have to be interpreted carefully with respect to the hidden reasons leading to such positive conclusions. Evaluation of survival rate based on clinical studies of CRC is, unfortunately, rather rare, and therefore, it is impossible to make a representative evaluation of this indicator. This fact should be seen as a challenge when improving population registers of malignant diseases.

## SCREENING METHODS

CRC screening focuses on asymptomatic individuals more than 50 years of age. Age is a low (average) risk factor for sporadic CRC, that is, carcinoma in patients with negative family or case history of CRC or chronic inflammatory bowel disease; this type of carcinoma accounts for 70 to 95% of all CRC cases. Three groups of screening methods are currently used as indicated in Table 5.

Guaiac-based fecal occult bleeding test (gFOBT) is at present the most frequently used method in screening programs. It detects the peroxidase reaction of hemoglobin, which causes the detection paper impregnated with guaiac resin to turn blue. Dietetic provisions are necessary to exclude false-positive results. A recent study showed limited sensitivity of this test for both, advanced adenomas (11%) and carcinomas (13%)<sup>[17]</sup>. With the use of gFOBT, a decrease in mortality for CRC by 15 to 33% has been proved<sup>[18]</sup>.

Immunochemical fecal occult bleeding test (iFOBT) reacts exclusively to human hemoglobin, so no dietetic restrictions are necessary. Taking and assessing the stool samples are easier than is the case with gFOBTs, which may explain a higher participation rate in the target group. A wide range of qualitative and quantitative tests is presently available, with varying levels of sensitivity

and specificity. The advantage of quantitative tests is the possibility to set cut-off limits; the most frequently used values are 75 or 100 ng/mL. The disadvantage of iFOBT is its cost; however, the price is now approaching that of gFOBT, particularly for qualitative tests<sup>[19]</sup>.

New screening methods include tests which examine the stool for the presence of abnormal DNA. Studies published to date focused on the characteristics of the test rather than the reduction in CRC incidence or mortality. Generally, these tests have higher sensitivity but lower specificity than gFOBT. The major obstacle to their implementation in screening programs is price<sup>[20]</sup>.

Flexible sigmoidoscopy (FS) is an endoscopic examination with maximum reach to the splenic flexure. On the basis of the information available, this is a promising screening test, although the studies published to date do not show sufficient statistical significance to determine reduction in CRC mortality. The recommended interval varies from 3 to 5 years. The risk of serious complications is 0% to 0.03%<sup>[21]</sup>.

Unlike FS, colonoscopy also detects lesions in the proximal colon. Its biggest advantage is the possibility of removing pathological lesions within a single examination. It is more sensitive in detecting both adenomas and carcinomas, although limited information is available on reducing CRC incidence and mortality, and on the recommended interval between examinations. The risk of serious adverse events is higher than for FS, at 3 to 5 events per 1000 colonoscopies<sup>[22]</sup>. To date, no prospective, randomized, multicenter study has been published supporting a reduction in CRC incidence and mortality with the use of screening colonoscopy. Nevertheless, its implementation in screening programs is one of the most widely discussed topics and the American College of Gastroenterology recommends screening colonoscopy as a preferred screening test<sup>[23]</sup>. On the other hand, no study addressing reductions in the incidence and mortality rates through stool analyses would have been completed without the "gold standard" of colonoscopy.

Computed tomographic colonography (CTC) shows lesions in the colorectum by reconstructing two- and three-dimensional images. To date, no studies have been published assessing reduction in CRC incidence or mortality. The majority of studies have focused on comparing the characteristics of this method with colonoscopy. For larger polyps (over 10 mm), the sensitivity of the methods is comparable; for smaller polyps (less than 5 mm), flat and depressed adenomas, the sensitivity is much higher for optical colonoscopy. Results of studies assessing the effect in terms of reduction in incidence and mortality, cost-effectiveness, and the potential risk of radiation are awaited<sup>[24]</sup>.

Double contrast barium enema shows the entire colorectum, although with significantly lower sensitivity and specificity than colonoscopy or CTC. The percentage of undetected carcinomas is up to 22%. The test is no longer widespread and available, but still has a purpose in countries lacking sufficient resources for other examinations<sup>[25]</sup>.

CRC screening is a complex process which, to function properly, requires the coexistence of a number of factors, such as a functioning invitation-reminder system,

**Table 4** Five-year relative survival rate (RSR) for colorectal cancer for selected European countries

Country	Diagnoses	Assessment period	Five-year RSR (%)	Change in time (%)	Stage-specific estimates
EUROCARE pool <sup>[7]</sup>	C18-C21	1995-1999	53.5	4.2	NA
EUROCARE pool <sup>[8]</sup>	C18-C21	2000-2002	56.2	NA	NA
England & Wales <sup>[10,11]</sup>	C18	1996-1999	47.6 M; 47.4 F	5.6 M; 5.6 F	NA
	C19-C20	1996-1999	48.7 M; 51.3 F	7.4 M; 8.1 F	NA
Germany <sup>[12]</sup>	C18-C21	2000-2002	60.8	NA	85.4 L; 58.1 R; 10.7 M
Finland <sup>[13]</sup>	C18-C20	2000-2004	57.9	2.4	NA
Norway <sup>[14]</sup>	C18-C21	2000-2004	59.2	3.6	NA
Slovenia <sup>[15]</sup>	C18-C21	2000-2004	46.9	8.0	NA
Sweden <sup>[16]</sup>	C18	2000-2002	58.1 M; 59.7 F	1.8 M; 2.6 F	NA
	C19-C21	2000-2002	57.5 M; 59.1 F	2.5 M; -1.7 F	NA

M: Estimate for males; F: Estimate for females; NA: Not available; L: Localized; R: Regional; M: Metastatic; NA: Not available. Numbers in brackets represents source of data available at references section.

**Table 5** Screening methods

Type of method	Method
Stool tests	For presence of occult blood (FOBT) Guaiac-based (gFOBT) Immunochemical (iFOBT) For presence of abnormal DNA
Endoscopic examinations	Flexible sigmoidoscopy (FS) colonoscopy
Radiologic examinations	Computed tomographic colonography (CTC) Double contrast barium enema (DCBE)

media campaigns targeted at the general public, the development of recommendations for general practitioners, patient compliance, sufficient funding, stratification of risks, and last but not least the selection of the most suitable screening test. Of the above described tests, only the fecal occult blood tests meet the WHO criteria for screening. As published recently, most CRC screening strategies lead not only to a reduction in CRC incidence and mortality, but also to better control of the costs of CRC treatment, especially with increased chemotherapy costs for advanced CRC<sup>[26]</sup>.

## GENERAL ONCOLOGY PREVENTATIVE PROGRAMS IN EUROPE

In 1985, the Europe Against Cancer program was initiated, which aimed at a reduction of 15% in the number of deaths caused by tumors (from 1 000 000 to 850 000) by 2000. The program was implemented, thanks to the cooperation of professional and lay public, charities and anti-smoking groups, healthcare media, and healthcare workers. The project focused on three major areas: prevention, screening, and education. Results published show that although the planned goal was not achieved, the mortality due to tumors was reduced by 10% in the EU. In some countries (Austria and Finland), the desired reduction of 15% was achieved, while in others (Portugal and Greece), the mortality reduction was much lower<sup>[27]</sup>. The experience gained in this program served as a basis for the Recommendations of the Council of the EU for screening programs following comprehensive European quality assurance guidelines. In December 2003, these

recommendations were unanimously approved by the health ministers of the individual EU states. European guidelines for quality assurance of breast and cervical cancer screening have been developed by experts and published by the European Commission; quality assurance guidelines for CRC screening are currently under preparation<sup>[28]</sup>.

## CRC SCREENING IN EUROPE

In 2008, the Report on the Implementation of the Council Recommendation on Cancer Screening<sup>[29]</sup>, which provides the most comprehensive available data, was published; giving the definitions of program screening as requiring public responsibility by law or official regulation and supervision in contrast to “wild” screening outside of any program. In program screening, the screening test, the examination interval and the eligible group of persons should be specified. Organized screening should generally include a regional or national team responsible for the implementation, quality assurance and reporting of results. Comprehensive guidelines, rules and a quality assurance structure should be available. Population-based screening requires the identification and personal invitation of each person in the eligible target population (by an office or special agency). According to this report CRC screening is running or being established in 19 of 27 EU countries. The target group contains approximately 136 million individuals suitable for CRC screening (aged 50 to 74 years). Of this number, 43% individuals come from 12 countries where CRC population screening is performed or being prepared on either national or regional levels; 34% come from 5 countries where national population screening has been implemented (Finland, France, Italy, Poland, and United Kingdom). In 7 EU countries, national non-population based screening is carried out, which covers 27% of the target population. In 2007, gFOBT (which in 2003 was the only test recommended by the Council of the European Union) was used as the only screening method in twelve countries (Bulgaria, Czech Republic, Finland, France, Hungary, Latvia, Portugal, Romania, Slovenia, Spain, Sweden, and United Kingdom). Colonoscopy was the only screening method used in Poland. In six countries, two types of tests were used: iFOBT and FS in Italy, and gFOBT and

Table 6 Colorectal cancer screening programs in 2007

	Program		Test type	Screening interval years or times in LT	Age eligible national population	
	Type	Status			Age (yr)	Persons (× 1000)
Austria	NonPB	Natw	FOBT	1 or 2	> 50	2210
	NonPB	Natw	CS	10	> 50	2210
Belgium	No Prog					2880
Bulgaria	NonPB	Natw	FOBT	1	> 31	2340
Cyprus	PB	Natw-plan	FOBT	1 in LT	50	10
	PB	Natw-plan	CS	1 in LT	55	10
Czech Republic	NonPB	Natw	FOBT	2	> 50	3010
Denmark	No Prog					1540
Estonia	No Prog					370
Finland	PB	Natw-roll ong	FOBT	2	60-69	570
France	PB	Natw-roll ong	FOBT	2	50-74	16600
Germany	NonPB	Natw	FOBT	1 and 2	> 50	24500
	NonPB	Natw	CS	10 (2 in LT)	55-74	18800
Greece	NonPB	Natw	FOBT	5	> 50	3180
	NonPB	Natw	CS	5	> 50	3180
Hungary	PB	Natw-pilot	FOBT	2	50-70	2630
Ireland	No Prog					940
Italy	PB	Natw-roll ong	FOBT	2	50-69 (70-75)	13800
	PB	Reg-roll ong	FS	1 in LT	58 or 60	80
Latvia	NonPB	Natw	FOBT	1	> 50	630
Lithuania	No Prog					870
Luxembourg	No Prog					120
Malta	No Prog					120
Netherlands	No Prog					4460
Poland	PB	Natw-roll ong	CS	10	50-65	7500
Portugal	PB	Natw-plan	FOBT	2	50-70	2520
Romania	PB	Natw-plan	FOBT	2	50-74	5800
Slovak Republic	NonPB	Natw	FOBT		> 50	1360
	NonPB	Natw-plan	CS	10	> 50	1360
Slovenia	PB	Natw-plan	FOBT	2	50-69	490
Spain	PB	Reg-pilot	FOBT	2	50-69	210
Sweden	PB	Reg-plan	FOBT	2	60-69	220
UK	PB	Natw-roll ong	FOBT	2	(50) 60-69 (74)	7600
Dual prog/test						-25630
Subtotal						106490
Excluded pop.						29500
Total						135990

PB: Population based; Prog: Program; Natw: Nationwide; Reg: Regional; Plan: Planning; Roll ong: Rollout ongoing; Pilot: Piloting; CS: Colonoscopy; LT: Lifetime. dual prog/test: Individuals entered twice due to screening programs of different implementation or using different screening tests. excluded pop.: Individuals excluded from national target populations due to regional or national variations in the age group targeted for screening, or due to lack of screening programs in some regions of countries with regional implementation status. Adapted from: von Karsa L, Anttila A, Ronco G, Ponti A, Malila N, Arbyn M, Segnan N, Castillo-Beltran M, Boniol M, Ferlay J, Hery C, Sauvaget C, Voti L, Autier P. Cancer screening in the European Union. Report on the implementation of the Council Recommendation on cancer screening - First Report. ISBN 978-92-79-08934-3. European Communities (publ.) Printed in Luxembourg by the services of the European Commission, 2008. Available from: URL: [http://ec.europa.eu/health/ph\\_determinants/genetics/documents/cancer\\_screening.pdf](http://ec.europa.eu/health/ph_determinants/genetics/documents/cancer_screening.pdf). Last accessed on August 4, 2009.

colonoscopy in Austria, Cyprus, Germany, Greece, and Slovak Republic. In the remaining eight states (Belgium, Denmark, Estonia, Ireland, Lithuania, Luxembourg, Malta, and the Netherlands), CRC screening has not been implemented yet. The age limit for the target population varies across EU countries (Table 6). In 2007, it was estimated that a total of 12 million individuals participated in CRC screening.

In the United Kingdom, a screening program was announced in 2004 and initiated in 2006, with the prospect of national coverage in 2009. It has been designed in two stages, with gFOBT examinations at 2-year intervals and colonoscopy for positive tests. In 2007, compliance was 52%. The program is carried out through regional centers

falling under one of five national hubs. The role of general practitioners is less significant here<sup>[30]</sup>.

In France, a screening program was initiated in 2003, based on gFOBT tests at 2-year intervals with colonoscopy for positive results. The role of general practitioners as coordinators is of crucial importance. The major advantage of the French program is its good organization, with a call-recall system comprising central management at national level and individual steps taken by centers in individual departments. Asymptomatic individuals aged from 50 to 74 are mailed gFOBT tests, with a reminder at three-monthly intervals for nonparticipants. Compliance in referred districts achieved 42%, and the overall positive test rate was 2.7%<sup>[31]</sup>.

In Italy, a nation-wide campaign was initiated in 2005; the implementation was entrusted entirely to 21 regional centers, including choice of the testing method. With state financial support, screening has been initiated in 11 regions to date, mostly in the industrial areas of northern Italy. In the Piedmont region, FS is the method of choice, in other regions immunochemical FOBT, with colonoscopy for positive tests. Compliance in iFOBT and FS programs was 44.6% and 51.4%, respectively. Positivity rate of iFOBT was 5.3% at first and 3.9% at repeat screening<sup>[32]</sup>.

In Spain, no screening program has taken place as yet. The main obstacle to its implementation is the highly heterogeneous healthcare system, in terms of organization and insurance coverage in individual self-governing units. Catalonia, for instance, considers implementation of country-wide screening in 2010, while in other regions only limited pilot studies have been held so far.

In Finland, a structured screening program was initiated in 2004. The target population, aged from 60 to 69 years (106 000 individuals), was randomized into two groups. Individuals in the screening group were mailed a gFOBT test at intervals of 2 years. The Finnish program shows a high level of compliance of the target population (70.8%), particularly for females<sup>[33]</sup>.

In the Netherlands, the optimum screening strategy is still being developed. It will be based on the results of studies currently taking place at major academic workplaces, comparing the effect of endoscopic procedures, various types of FOBTs, and fecal DNA analysis.

Poland is the only state at the moment using colonoscopy as the only screening method, without the alternative of FOBT. An opportunistic screening program was initiated in 2000, and by 2005, this had grown to 57 centers across Poland. The program is financed by the Ministry of Health, independent of the overall healthcare system. The target population (asymptomatic individuals aged 55-66 years) is recruited through general practitioners. High emphasis is placed on the quality control of colonoscopies, with complications reported for 0.1% of procedures, and no patient mortality. The advantage of the program is thorough monitoring and evaluation, including monitoring of interval cancers<sup>[34]</sup>.

Germany was the first country to introduce a population screening program (in 1976) based on annual gFOBT for individuals more than 44 years of age. Starting from 2002, it has been offering participants a choice between colonoscopy at 55 years of age and FOBT at annual intervals between 50 and 55 years of age. After 55 years of age, examinations are carried out at 2-year intervals. If the test results are positive, colonoscopy is indicated. Those who undergo a screening colonoscopy with no neoplasia detected at the initial examination are recommended reexamination in 10 years time if the first colonoscopy was carried out before they were 65 years. The positive feature of the screening and data gathering in Germany is the emphasis on staging the disease at the time of its diagnosis. Recent cost analyses have proven that this type of screening is cost-efficient<sup>[35]</sup>.

In the Czech Republic, CRC screening has many years of tradition<sup>[36,37]</sup>. The country was the second in the world to start screening nation-wide, in 2000. In the initial years, gFOBT was the first method offered to asymptomatic individuals more than 50 years of age by their general practitioners at preventative medical checks, followed by colonoscopy if tests were positive. From 2000 to 2008, 1 685 289 gFOBTs were carried out, of which 63 296 were positive (3.76%). In 2006, a central database for online data input was established. Between 2006 and 2008, 17 813 colonoscopies were carried out, indicated as a result of a positive FOBT; carcinoma was diagnosed in 1047 (5.9%) individuals, and 5362 (30.1%) adenomas were removed by endoscopic polypectomy. The participation of the target group, however, was only 20%<sup>[38]</sup>. In order to achieve a higher compliance rate, screening colonoscopy was added to current FOBT screening as an alternative method, in the same intervals as in the German program. Both, gFOBT and iFOBT are offered as well. The implementation of the newly designed program is supported by an intensive media campaign (<http://www.kolorektum.cz/index-en.php>).

The first study which focused on monitoring the effect of colonoscopy screening on reducing CRC incidence and mortality is NordICC (The Nordic-European Initiative on Colorectal Cancer), which is currently underway in northern states of Europe (Norway, Sweden, and Iceland), Poland, and the Netherlands. It will involve a minimum of 66 000 individuals aged 55 to 64 years. Individuals in the screening group will undergo a screening colonoscopy once in a lifetime. The primary objective is to compare incidence and mortality against the control group (with no screening) after 10 years<sup>[39]</sup>.

## CONCLUSION

CRC presents a serious public healthcare issue for the population of Europe. Understandably, the number of countries introducing population screening has been growing constantly. Although epidemiologic data differ in various European countries, implementation of screening programs in accordance with the principles spelled out in the Council Recommendation on Cancer Screening of 2 December 2003 may be expected to have a favorable effect on the burden of this disease in the population. Countries in the EU may benefit from unified policy, knowhow and central oncology registers, while economically less developed countries may draw on special funding for the development of preventative programs. At the same time, varying epidemiologic situations, economic conditions, and different systems of health insurance and organization of healthcare are factors that may limit the implementation of a unified screening program. Therefore, to respond to the needs of the member countries, the EU should consider adopting the recommendation of the World Gastroenterology Organization for CRC screening, possibly even in a modified form<sup>[40]</sup>. This is a cascade concept in which recommendations for individual countries are graded into six levels, depending on the resources available (financial

Table 7 Cascade concept

Level	Average risk	High risk
1	Colonoscopy in 10 years interval, from 50 years of age	Special procedure, for individual groups
2	Colonoscopy once in a lifetime, at 50 years of age	Special procedure, for individual groups
3	Flexible sigmoidoscopy in 5 years interval, from 50 years of age; colonoscopy to follow if positive	Special procedure, for individual groups
4	Flexible sigmoidoscopy once in a lifetime, at 50 years of age; colonoscopy to follow if positive	Special procedure, for individual groups
5	Flexible sigmoidoscopy once in a lifetime, at 50 years of age; colonoscopy to follow only if advanced adenoma is detected	Same as individuals with average risk, if resources are not available for colonoscopy
6	FOBT in annual interval after 50 years of age; if positively tested, colonoscopy or double contrast barium enema	Same as individuals with average risk, if resources are not available for colonoscopy

Adapted from: World Gastroenterology Organization/International Digestive Cancer Alliance. Practice Guidelines: Colorectal cancer screening. Available from: URL: <http://www.worldgastroenterology.org/colorectal-cancer-screening.html>. Last accessed on August 4, 2009.

and professional) (Table 7). In the case of lack of funds, FOBT at intervals of 1 or 2 years for individuals with average risk is a realistic possibility. This open concept best fulfils the simple recommendation by Sydney Winawer, Co-Chair of IDCA (International Digestive Cancer Alliance): "The best screening test is the one that gets done...and gets done well. Do what you can with what you have".

In most European countries, fortunately, the majority of the population is covered by some form of health insurance, meaning that economic aspects need not critically affect the availability of screening programs. Although at the end of 2007, CRC screening was still not running or being established in 8 of 27 EU member states, some of which rank among the most developed economies of the world, additional programs are currently under development. Given the substantial burden of the disease, implementation and continuous improvement in CRC screening programs should remain high on the healthcare agenda in Europe.

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REVIEW

## Progress in researches about focal adhesion kinase in gastrointestinal tract

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### Abstract

Focal adhesion kinase (FAK) is a 125-kDa non-receptor protein tyrosine. Growth factors or the clustering of integrins facilitate the rapid phosphorylation of FAK at Tyr-397 and this in turn recruits Src-family protein tyrosine kinases, resulting in the phosphorylation of Tyr-576 and Tyr-577 in the FAK activation loop and full catalytic FAK activation. FAK plays a critical role in the biological processes of normal and cancer cells including the gastrointestinal tract. FAK also plays an important role in the restitution, cell survival and apoptosis and carcinogenesis of the gastrointestinal tract. FAK is over-expressed in cancer cells and its over-expression and elevated activities are associated with motility and invasion of cancer cells. FAK has been proposed as a potential target in cancer therapy. Small molecule inhibitors effectively inhibit the kinase activity of FAK and show a potent inhibitory effect for the proliferation and migration of tumor cells, indicating a high potential for application in cancer therapy.

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**Key words:** Focal adhesion kinase; Restitution; Survival and apoptosis; Cancer; Inhibitor

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Hao HF, Naomoto Y, Bao XH, Watanabe N, Sakurama K, Noma K, Tomono Y, Fukazawa T, Shirakawa Y, Yamatsuji T, Matsuoka J, Takaoka M. Progress in researches about focal adhesion kinase in gastrointestinal tract. *World J Gastroenterol* 2009; 15(47): 5916-5923 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5916.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5916>

### INTRODUCTION

Focal adhesion kinase (FAK) is a 125-kDa non-receptor protein tyrosine which was originally identified in chicken embryo cells transformed by v-Src<sup>[1]</sup> and BALB/c3T3 fibroblasts<sup>[2]</sup> and was shown to localize in focal adhesions as well. FAK is a non-receptor and non-membrane associated protein tyrosine kinase (PTK), which does not contain Src homology2 (SH2) or SH3 protein interaction domains<sup>[3]</sup>. FAK contains three main domains: a centrally located catalytic kinase domain, a large N-terminal domain comprising the FERM (FAK, ezrin, radixin, moesin) region and a C-terminal domain harboring the focal adhesion targeting<sup>[3-5]</sup>. Growth factors or the clustering of integrins facilitate the rapid phosphorylation of FAK at Tyr-397 in adherent cells and this in turn recruits Src-family PTKs, resulting in the phosphorylation of Tyr-576 and Tyr-577 in the FAK activation loop and full catalytic FAK activation<sup>[3,5]</sup>; while extracellular pressure can activate the FAK in suspended cells<sup>[6,7]</sup>.

FAK is associated with gastrointestinal diseases, here we will review the progresses which have been made in the researches about FAK in the gastrointestinal tract. Research data shows that FAK plays an important role in the restitution, cell survival and apoptosis and carcinogenesis of the gastrointestinal tract. Due to the crucial role of FAK in integrin-mediated signal transduction, which affects the regulation of cell survival, proliferation, spreading and migration, FAK has been proposed to be a potential target in cancer therapy. Antisense oligonucleotides, the entire C-terminal, non-catalytic domain of FAK (FAK related non-kinase-FRNK), siRNA

and small molecule inhibitors can affect and inhibit the activities and expression of FAK in various tumor cells. Small molecule inhibitors targeting FAK have been developed as potential cancer treatment modalities. PF-573228, PF-562271 and NVP-226 (TAE226) have already shown potent inhibitory effect for tumor cell growth *in vitro* and *in vivo*.

## RESTITUTION

After intestinal superficial mucosal injuries such as erosion, ulcerations, inflammatory bowel disease and infection, the repair of epithelial injury in the gastrointestinal tract begins in a process known as restitution<sup>[8]</sup>. The restitution is established through migration of viable epithelial cells from areas adjacent to or just beneath the injured surface to cover the denuded area, independent of cell proliferation and regulated by cytokines and growth factors<sup>[9-14]</sup>. Intestinal epithelial migration, proliferation and differentiation are essential to restitution<sup>[15]</sup>. FAK has been indicated to be involved in the integrin signaling which regulates the migration, proliferation and differentiation of various normal and cancer cells<sup>[16]</sup>. Correspondingly, FAK plays an important role in the mucosal restitution of the intestine.

It is well established that intestinal epithelial cells undertake a specialized phenotype adapted to motility and mucosal healing during mucosal restitution and FAK is involved in the cell signaling which regulates the intestinal epithelial migratory phenotype<sup>[17]</sup>. The disruption of actin stress fiber formation with reduced tyrosine phosphorylation of FAK and FAK in focal adhesions can suppress the repair of gastric mucosal injury and ulcer healing<sup>[18-20]</sup>. FAK plays a critical role in lysophosphatidic acid (LPA)-induced migration, lamellipodia formation and assembly of focal adhesions in intestinal epithelial cells<sup>[21,22]</sup>.

The expression and activation levels of FAK protein are linked to phenotypic changes which affect cell differentiation, function, adhesion and migration in various tissues<sup>[23-29]</sup>. Activated FAK<sup>397</sup> levels vary with differentiation and cell migration in Caco-2 and HT-29 human colon cancer cells<sup>[30]</sup>. The expression level of activated FAK is related to gastric wound healing *in vivo*<sup>[19,31]</sup>. Intestinal epithelial cell motility regulates FAK protein abundance at the mRNA level in both human Caco-2 and rat non-transformed IEC-6 intestinal epithelial cells<sup>[32]</sup>. It has been shown that immunoreactivity to FAK is decreased in cells migrating across matrix protein compared to static Caco-2 cells<sup>[33]</sup> and immunoreactivity to FAK and FAK<sup>397</sup> were lower in epithelial cells at the migrating edge of the ulcer<sup>[34]</sup>.

FAK mediates the mitogenic response to repetitive deformation in intestinal epithelial cells. Two deformation-activated signal pathways that converge upon FAK have been proposed: one is Src- and Rac1-independent-which stimulates FAK-Tyr397 phosphorylation, and the other is Src- and Rac1-dependent, which is required to further activate FAK by phosphorylation at FAK-Tyr576 (within the FAK kinase activation loop)<sup>[28]</sup>. Repetitive deformation stimulates intestinal epithelial motility

across fibronectin, which requires both Src activation and a novel Src-independent FAK-Tyr 925-dependent pathway activating extracellular signal-related kinase (ERK)<sup>[29]</sup>. Smad3-dependent disruption of the transforming growth factor- $\beta$  (TGF- $\beta$ ) signaling pathway impairs the healing of murine intestinal mucosal ulcers, which is followed by altering patterns of activated FAK and ERK immunoreactivity important for cell migration at the ulcer edge<sup>[15]</sup>.

Recently, the relationship between TGF- $\beta$  and FAK has been studied. TGF- $\beta$  was found to enhance FAK protein, mRNA levels and FAK promoter activity in human and rat intestinal epithelial cells<sup>[34]</sup>. TGF- $\beta$  also affected the restitution and proliferation partly mediated through its induction of FAK expression<sup>[35]</sup>. It is considered to play an essential role in embryogenesis, host response to tumors, and the repair response damaging the tissues by immune and non-immune reactions<sup>[36]</sup>.

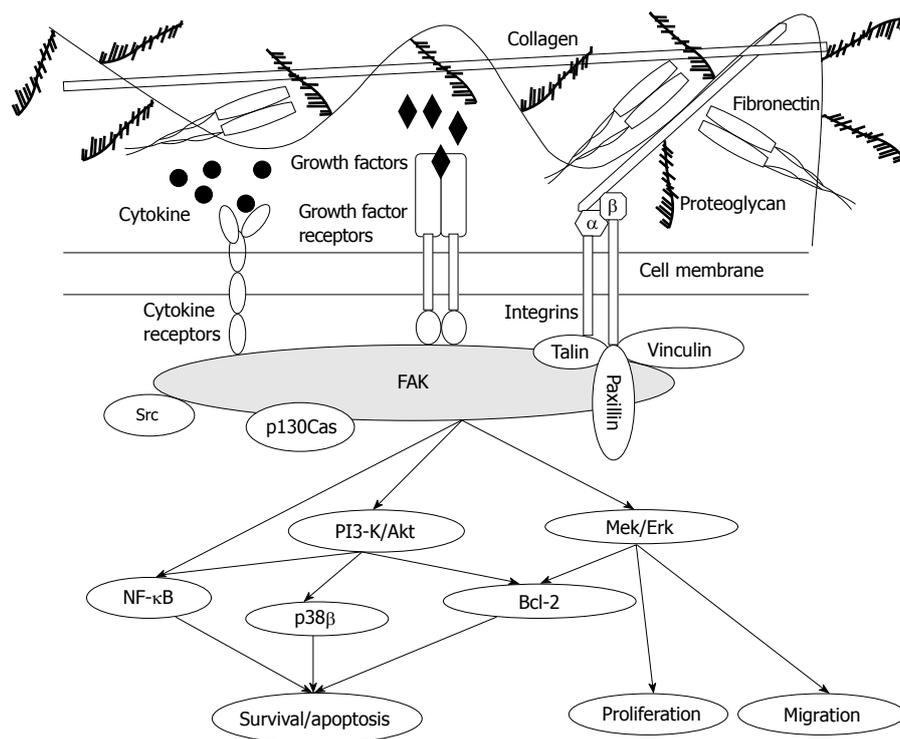
Taken together, the interaction between inflammatory cells, the extracellular matrix, locally released cytokines and growth factors guarantee efficient ulcer healing<sup>[31]</sup>. Tissue injury and wound healing spatially and temporally activate several growth factors and extracellular matrix facilitates the rapid phosphorylation of FAK at Tyr-397 and this in turn recruits Src-family PTKs, resulting in the phosphorylation of Tyr-576 and Tyr-577 in the FAK activated loop and other focal adhesive proteins including talin,  $\alpha$ -actinin, vinculin, paxillin and p130Cas (Figure 1)<sup>[3,5,16,37]</sup>. Activated FAK and cell adhesive protein transduce the signal to the Mek/Erk to down-regulate proliferation, differentiation and migration in the process of restitution. However, the detailed mechanism for the role of FAK in the process of restitution is still unknown.

## SURVIVAL AND APOPTOSIS

Programmed cell death, or apoptosis, is a complex and tightly regulated process that executes crucial roles in tissue homeostasis and repair<sup>[38,39]</sup>. It is well established that the Bcl-2 family of proteins plays a major role in cell survival and apoptosis<sup>[38,40,41]</sup>. Extracellular signals can affect the expression and/or functions of the Bcl-2 family by signaling events to determine if a cell lives or dies<sup>[42]</sup>. FAK is the canonical mediator of such extracellular signals which originate from integrin and growth factors<sup>[5]</sup>. Thus, FAK is related to cell survival and apoptosis in the gastrointestinal epithelium.

The detachment of intestinal epithelial cells from matrix induces apoptosis through the disruption of anti-apoptotic signals transduced by integrin/FAK/Src<sup>[43]</sup>. Induced FAK suppresses apoptosis by activating nuclear factor  $\kappa$ B (NF- $\kappa$ B) signaling in intestinal epithelial cells<sup>[44]</sup>. FAK inhibition in human intestinal epithelial cells produces anoikis while FAK induction in rat intestinal IEC-6 cells suppresses apoptosis<sup>[44,45]</sup>.

Recent studies in the function of FAK in survival and apoptosis of intestinal epithelial cells have focused on integrin/Fak/Src, PI3K/Akt and MEK/Erk pathways



**Figure 1** FAK mediates the extracellular signaling to regulate the proliferation, migration and survival/apoptosis of the cells. NF- $\kappa$ B: Nuclear factor  $\kappa$ B; FAK: Focal adhesion kinase.

which are all presumed to modulate the expression and function of multiple Bcl-2 homologs<sup>[42,44-46]</sup>. Many studies showed that integrin/FAK/Src modulate the PI3K/Akt and MEK/Erk pathways individually or in combination in different cell lines<sup>[5,42,47-55]</sup>. The Bcl-2 family is the central regulator of caspase activation which executed the cell-suicide program and play an anti- or pro-apoptotic role in cell apoptosis<sup>[56]</sup>. Butyrate-induced apoptosis of Caco-2 cells might occur *via* NF- $\kappa$ B activation together with a defective  $\beta$ 1 integrin-FAK-PI3-kinase pathway signaling<sup>[47]</sup>. A study showed that integrins, FAK, PI3-K/Akt-1, MEK/Erk, and p38 isoforms play distinct roles in the regulation of HIEC-6 cell survival and/or death, accompanied by modulating individual Bcl-2 homologs<sup>[46]</sup>.  $\beta$ 1 integrins/Fak/Src signaling down-regulated PI3-K/Akt-1 and MEK/Erk pathways in the suppression of anoikis, which play a role in the survival of differentiated cells, whereas the PI3-K/Akt-1 pathway is crucial for cell survival regardless of the state of differentiation<sup>[45]</sup>.  $\beta$ 1 integrins/Fak/Src signaling translates into integrated, complex regulatory functions by PI3-K/Akt-1 and MEK/Erk in the expression/activity of Bcl-2 homologs, as well as in the specific activation of the pro-apoptotic p38 $\beta$  SAPK isoform, thus determining their own requirement (or not) in the suppression of HIEC (Human Intestinal Epithelial Crypt) apoptosis/anoikis<sup>[42]</sup>.

Extracellular/Fak/Src signaling down-regulates PI3-K/Akt and MEK/Erk and further regulates the expression and activity of Bcl-2, and finally control the survival and apoptosis. PI3-K/Akt also specifically activates the apoptosis/anoikis driving p38 $\beta$  SAPK, and regulates the survival and apoptosis. Besides, extracellular/Fak/Src signaling has a new pathway to control the survival and apoptosis *via* regulating the NF- $\kappa$ B.

## CANCER

FAK is closely associated with cancer. Many studies have shown FAK over-expression in various tumor cells and its expression correlate with increased tumor malignancy. The alteration of FAK function in normal cells causes tumor progression.

FAK has been indicated to over-express at mRNA and protein levels in various tumors including gastrointestinal tumors. As early as in 1993, researchers found increased levels of FAK in 1 of 8 adenomatous tissues, in 17 of 20 invasive tumors, and in all 15 of 15 metastatic tumors, which suggests that FAK over-expression may result in changes in the signaling pathways involved in tumor cell invasion<sup>[57]</sup>. In human colon cancer cells, increased dosage of the FAK may contribute to the elevated protein expression during conversion from adenoma to carcinoma<sup>[58]</sup>. Quantitative realtime RT-PCR of gene expression levels in all gastrointestinal stromal tumors (GIST) indicated that FAK was over-expressed in malignant GIST<sup>[59]</sup>. Immunohistochemical analysis also demonstrated that FAK is over-expressed in colorectal, esophageal, pancreatic and mammary cancers, which indicated that FAK and P-FAK are involved in the carcinogenesis of digestive organs<sup>[60,61]</sup>. Another research group got similar results *via* immunohistochemistry, which showed that high levels of FAK and Src were predictive for recurrence of colorectal cancer<sup>[62]</sup>. The FAK expression level might be a valuable marker for the carcinogenesis and progression of some types of carcinoma<sup>[63,64]</sup>.

An increased expression of FAK is associated with the invasive potential of colon and breast tumors<sup>[65]</sup>. Immunohistochemical analysis of gastric cancer and colorectal cancer showed that the expression of FAK is more significantly associated with carcinogenesis,

differentiation and metastasis, and furthermore FAK may not only be a transformation-linked enzyme but also a progression-linked enzyme<sup>[63]</sup>. FAK over-expression of esophageal squamous cell carcinoma was related to cell differentiation, tumor invasiveness, and lymph node metastasis<sup>[66]</sup>. The expression of gastrin-releasing peptide (GRP) and its cognate receptor critically mediates a GRP-dependent phase of cell motility by phosphorylating FAK at multiple specific sites in colon cancer cells<sup>[30]</sup>. Gastrin can evidently promote invasiveness of Colo320 cells *via* the gastrin-gastrin receptor-FAK signal transduction pathway<sup>[67]</sup>.

Not only the expression level but also the activities of FAK are essential for the motility and invasion of cancer cells. Colon carcinomas exhibited a marked elevation in FAK tyrosine kinase activity and phosphotyrosine content and the catalytic activity of FAK is enhanced by its phosphotyrosine content<sup>[68]</sup>. The amount of total FAK and FAK phosphorylated at Y397 and Y407 correlates closely with the differentiation of human colon cancers<sup>[69]</sup>. The migratory phenotype of colon cancer cells is controlled by the combined activities of Src and FAK, and the recruitment of FAK to adhesive sites results in its phosphorylation by Src and other peripheral tyrosine kinases<sup>[70]</sup>.

The over-expression and elevated activities of FAK are associated with motility and invasion of cancer cells, however the exact mechanism is still unknown. Integrin $\alpha$ 2/FAK/ERK/ $\mu$ -calpain signaling pathway plays a critical role in tumor cell motility and these results would cause the interruption of FAK function at the early stages of colon tumorigenesis<sup>[71]</sup>. In a colon adenocarcinoma, cell proliferation and differentiation can occur concomitantly and these deregulated processes are controlled by autocrine secretion through the ErbB1/ERK1, 2 and FAK pathways<sup>[72]</sup>. The Cholecystokinin-2 receptor regulates the invasion and motility of colon cancer cells, and supports the role of CCK2R in the progression of colon cancer through the activation of FAK<sup>[73]</sup>. EGFR pathway substrate 8 could modulate the expression of FAK *via* mTOR/STAT3, which enable the cells to proliferate and migrate<sup>[74]</sup>. The mechanism of the increasing invasion of colon cancer cells by gastrin17 is probably that gastrin17 makes FAK-Tyr397 phosphorylate and localize to lamellipodia, causing the formation of FAK-Src-p130(Cas)-Dock180 signaling complex when it is bound to its receptor CCK-2 and the activation of Rac<sup>[75]</sup>. The engagement of  $\alpha$ 1-integrins with functional molecular scaffolds using FAK/Src and p130Cas/JNK is involved in human colon cancer cell invasion through the induction and activation of the MMP-2 and MMP-9 matrix metalloproteinases<sup>[76]</sup>. A model has been proposed to indicate how the interaction of FAK and SFKs down-regulate the MAPK/Erk1/2 and PI3K/Akt pathways in the early process of cell adhesion in SW480 colon cancer cells: Integrin engagement induces quick FAK-Y397 autophosphorylation and subsequent translocation of a fraction of FAK in raft compartments, FAK interacts only with Fyn in lipid domains, while it interacts with c-Src and Fyn in non-raft fractions. In parallel, PI3K/Akt signaling

is quickly activated which is dependent on lipid domain integrity, while MAPK/Erk1/2 signaling is activated with longer kinetics which is not dependent on lipid domain integrity. Both signaling pathways contribute to the adhesive process of SW480 cells<sup>[77]</sup>.

These data show the strong relationship between the expression and activity level of FAK and the generation and progression of gastrointestinal tumors, however the exact mechanism needs further studies.

### Inhibitor

Due to the crucial role of FAK in integrin-mediated signal transduction, which affects the regulation of cell survival, proliferation, spreading and migration, FAK has been considered a potential target in cancer therapy. There are many ways to suppress the activity and expression of FAK, thereby inhibiting the growth of tumor cells. The attenuation of FAK expression *via* antisense oligonucleotides induces detachment and apoptosis in tumor cells<sup>[78]</sup>. The entire C-terminal, non-catalytic domain of FAK (FAK related non-kinase-FRNK) is autonomously expressed in some cell types, and has been used as a dominant negative mutant to elucidate FAK function<sup>[79-82]</sup>. Specific short interfering RNA is often used to reduce the expression of FAK. Knockdown of FAK protein through FAK-SiRNA significantly inhibited LPA-induced migration of both IEC-18 and IEC-6 cells<sup>[22]</sup>.

As described earlier in this article, phosphorylation of Tyr-397 at FAK is essential to the phosphorylation of Tyr-576 and Tyr-577 in the FAK activation loop, full catalytic FAK activation, the activity of other adhesive protein and its downstream molecules which all play important roles in integrins or growth factors initiated signaling pathways. So targeting the phosphorylation of FAK seems to be promising for the cancer therapy. Small molecule inhibitors targeting FAK as potential cancer therapies have been developed. Sulindac sulfide (NSAID) and the phenolic antioxidant caffeic acid phenethyl ester were used to reduce the phosphorylation of FAK and cell invasion in human colon carcinoma cells<sup>[83]</sup>. Butyrate treatment results in a significant down-regulation of c-Src and FAK in human colon cancer cells and finally inhibits tumor growth and invasion<sup>[84]</sup>. Exposure of HT-29 cells to 10 mmol/L garcinol inhibited cell invasion and decreased the dose-dependent tyrosine phosphorylation of FAK, which suggests that garcinol reduces cell invasion and survival through inhibiting the downstream signaling of FAK<sup>[85]</sup>.

Recently, compounds PF-573228, PF-562271 and NVP-226 (TAE226) have been generated by two groups. These compounds are ATP analogs and effectively inhibit the kinase activity of FAK<sup>[86,87]</sup>. PF-573228 inhibited phosphorylation of FAK and its downstream effector paxillin, and affected cell migration and adhesion turnover<sup>[86]</sup>. But PF-573228 had little inhibitory effect on the growth and apoptosis of normal and cancer cells possibly because the FAK kinase activity is not essential for cell growth-proliferation mediated through FAK FERM regulation of p53<sup>[88]</sup>.

PF-562271 is a newly developed diaminopyrimidine-type compound that inhibits FAK and Pyk2 and shows a high degree of selectivity in the inhibition of PTKs<sup>[89]</sup>. PF-562271 have inhibited the tumor growth of prostate, pancreatic, colon, glioblastoma, and H460 lung xenotropic tumor models<sup>[89]</sup> and blocked bFGF-stimulated blood vessel angiogenesis as shown in chicken chorioallantoic membrane assays. Low dosage of PF-262271 potently blocked blood vessel sprouting without detectable changes in vascular leakage<sup>[88]</sup>. The oral administration of PF-562271 suppressed the growth and local spread of intratibial tumors and restored tumor-induced bone loss<sup>[90]</sup>. The combination of PF-562271 and sunitib could effectively block the growth and recovery of human hepatocellular carcinoma in a rat xenograft model<sup>[91]</sup>. PF-562271 has since moved to clinical trials, and has shown minimal toxicity along with tumor regression<sup>[92]</sup>.

TAE226 is a novel ATP-competitive tyrosine kinase small-molecule inhibitor designed to target FAK, and can effectively prevent FAK phosphorylation, ERK, S6 ribosomal protein phosphorylation and downstream signal transduction, as determined by decreased AKT. TAE226 inhibits insulin receptor (InsR) and insulin-like growth factor-I receptor (IGF-IR), albeit, 10 fold less potently (IC<sub>50</sub> = 44 nmol/L for InsR and IC<sub>50</sub> = 140 nmol/L for IGF-IR), and is a potent inhibitor of FAK (IC<sub>50</sub> = 5.5 nmol/L)<sup>[87,93]</sup>. TAE226 was shown to induce apoptosis in breast cancer cell lines<sup>[94]</sup>. Furthermore, TAE226 can significantly prolong the survival of animals bearing intracranial glioma xenografts and ovarian tumor cells orthotopic implantation<sup>[86,95]</sup>. TAE226 also showed a potent inhibitory effect of tumor cell growth in gastrointestinal tract. When esophageal adenocarcinoma cells were treated with TAE226, cell proliferation and migration were greatly inhibited with an apparent structural change of actin fiber and a loss of cell adhesion, which suggest that TAE226, a dual tyrosine kinase inhibitor for FAK and IGF-IR, might become a new remedy for Barrett's esophageal adenocarcinoma<sup>[96]</sup>. Furthermore, TAE226 has shown significant inhibitory effects on mTOR signaling and the esophageal cancer cell growth<sup>[97]</sup>. TAE226 can effectively suppress the growth of imatinib-resistant GIST cells, indicating its potential application for treating the imatinib-resistant GISTs<sup>[98]</sup>. So the small molecule inhibitors show a significant promise for cancer therapy.

## CONCLUSION

FAK plays a critical role in the biological processes of normal and cancer cells including the gastrointestinal tract. Research data shows that FAK plays an important role in the restitution, cell survival and apoptosis and carcinogenesis of the gastrointestinal tract, however the exact mechanism needs further studies. FAK is over-expressed in cancer cells and over-expression and enhanced activities of FAK are associated with motility and invasion of cancer cells. So FAK has been proposed as a potential target in cancer therapy. Small molecule inhibitors effectively inhibit the kinase activity of FAK

and show a potent inhibitory effect in the proliferation and migration of tumor cells, indicating a high potential for future application in cancer therapy.

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ORIGINAL ARTICLE

## TRAIL-induced apoptosis of hepatocellular carcinoma cells is augmented by targeted therapies

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were treated with kinase inhibitors and chemotherapeutic drugs. Apoptosis induction and cell viability were analyzed *via* flow cytometry and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

**RESULTS:** TRAIL-R1 and -R2 were profoundly expressed on the HCC cell lines Huh7 and Hep-G2. However, treatment of Huh7 and Hep-G2 with TRAIL and agonistic antibodies only induced minor apoptosis rates. Apoptosis resistance towards TRAIL could be considerably reduced by adding the chemotherapeutic drugs 5-fluorouracil and doxorubicin as well as the kinase inhibitors LY294002 [inhibition of phosphoinositol-3-kinase (PI3K)], AG1478 (epidermal growth factor receptor kinase), PD98059 (MEK1), rapamycin (mammalian target of rapamycin) and the multi-kinase inhibitor Sorafenib. Furthermore, the antiapoptotic BCL-2 proteins MCL-1 and BCL-x<sub>L</sub> play a major role in TRAIL resistance: knock-down by RNA interference increased TRAIL-induced apoptosis of HCC cells. Additionally, knock-down of MCL-1 and BCL-x<sub>L</sub> led to a significant sensitization of HCC cells towards inhibition of both c-Jun N-terminal kinase and PI3K.

**CONCLUSION:** Our data identify the blockage of survival kinases, combination with chemotherapeutic drugs and targeting of antiapoptotic BCL-2 proteins as promising ways to overcome TRAIL resistance in HCC.

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### Abstract

**AIM:** To analyze the effect of chemotherapeutic drugs and specific kinase inhibitors, in combination with the death receptor ligand tumor necrosis factor-related apoptosis inducing ligand (TRAIL), on overcoming TRAIL resistance in hepatocellular carcinoma (HCC) and to study the efficacy of agonistic TRAIL antibodies, as well as the commitment of antiapoptotic BCL-2 proteins, in TRAIL-induced apoptosis.

**METHODS:** Surface expression of TRAIL receptors (TRAIL-R1-4) and expression levels of the antiapoptotic BCL-2 proteins MCL-1 and BCL-x<sub>L</sub> were analyzed by flow cytometry and Western blotting, respectively. Knock-down of MCL-1 and BCL-x<sub>L</sub> was performed by transfecting specific small interfering RNAs. HCC cells

**Key words:** Hepatocellular carcinoma; Apoptosis; Tumor necrosis factor-related apoptosis inducing ligand; BCL-x<sub>L</sub>; MCL-1; 5-fluorouracil; Doxorubicin; Sorafenib; Phosphoinositol-3-kinase; (Mitogen-activated protein kinase)/(extracellular signal regulated kinase) kinase; c-Jun N-terminal kinase

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide. It ranks at third place in the list of malignancies leading to death. Over the past decades the incidence of HCC has increased worldwide, especially in eastern Asia and sub-Saharan Africa<sup>[1,2]</sup>. HCC is clinically characterized by its invasiveness, poor prognosis and limited therapeutic opportunities, mostly due to the high resistance of HCC cells towards chemotherapeutic agents. Today, surgery is considered to be the only curative treatment procedure for most HCC patients<sup>[3]</sup>. However, in many patients, HCC is diagnosed at an advanced or metastasized stage. For the treatment of these patients, the Food and Drug Administration approved the multi-kinase inhibitor Sorafenib in 2007<sup>[4,5]</sup>, which highlights the fact that specific inhibition of survival pathways is an effective treatment option in HCC<sup>[6]</sup>.

Apoptosis is a genetically determined process of controlled cellular suicide<sup>[7]</sup>. Dysregulation of apoptosis is involved in the pathophysiology of liver diseases including hepatocarcinogenesis<sup>[8]</sup>. Resistance of HCC cells to apoptosis is a crucial aspect in cancer treatment because it impairs the efficacy of different therapy regimens<sup>[9]</sup>.

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is a promising anti-tumor agent since it is capable of killing tumor cells *via* receptor-mediated apoptosis<sup>[10,11]</sup>. TRAIL ligates two different types of receptors: (1) death receptors triggering TRAIL-induced apoptosis, and (2) decoy receptors possibly inhibiting the TRAIL death-signaling pathway. Receptors TRAIL-R1 and -R2 contain an intracellular death domain (DD) motif essential for signal transduction. In contrast, TRAIL-R3 (DcR1) and -R4 (DcR2) appear to act as “decoys”, lacking a DD. Due to this fact they are capable of binding the ligand without effecting a death signal. Under certain conditions, a relative TRAIL resistance occurs in cells expressing high levels of DcR1 or DcR2.

Binding of an agonistic ligand or mAb to TRAIL-R1 or -R2 leads to the intracellular formation of a protein complex termed death inducing signaling complex (DISC). DISC formation includes the activation of the apical activator caspase 8, representing the initial point of receptor-related apoptosis signaling.

In addition to this receptor-related extrinsic pathway, there is an intrinsic pathway of apoptosis, which is crucial as a cellular response to DNA damage and oxidative stress. Central organelles for the intrinsic pathway are mitochondria, where a delicate balance between pro- and antiapoptotic BCL-2 proteins decides cell destiny. If DNA damage or other intrinsic triggers occur, proapoptotic BCL-2 proteins and mitochondria are activated. Subsequently, a

multimeric protein complex, designated as an apoptosome, is formed. The apoptosome cleaves caspase 9, which in turn activates the downstream effector caspase 3, where intrinsic and extrinsic pathways of apoptosis converge.

Notably, receptor-mediated caspase 8 activation can promote an activation of mitochondria by cleavage and subsequent activation of the proapoptotic BCL-2 protein, BID<sup>[12]</sup>. The crosstalk between extrinsic and intrinsic apoptosis pathways amplifies a death signal mediated by TRAIL, leading to a more effective execution of apoptosis.

MCL-1 and BCL-x<sub>l</sub> are antiapoptotic members of the BCL-2 family serving as protective factors against several death stimuli. Both proteins were found to be expressed at a high level in different solid tumor entities, including HCC<sup>[13-15]</sup>. Antiapoptotic BCL-2 proteins interact with proapoptotic BCL-2 proteins BAX and BAK, thereby inhibiting the activation of mitochondria. It appears that high expression levels of MCL-1 and BCL-x<sub>l</sub> provide resistance of tumor cells to chemotherapeutic drugs and TRAIL<sup>[16,17]</sup>.

Resistance towards TRAIL can be due to failure at any step in the death signaling cascade. For example, TRAIL resistance can be located at receptor level due to an inappropriate expression or at DISC level mediated by proteins counteracting DISC formation<sup>[18-20]</sup>. Furthermore, an inability to activate mitochondria during apoptosis, due to high expression levels of antiapoptotic proteins (e.g. MCL-1), can cause resistance towards TRAIL<sup>[16,21]</sup>. Finally, antiapoptotic pathways, such as phosphoinositol-3-kinase (PI3K)/Akt signaling, are aberrantly activated in various tumor cells, thus contributing to TRAIL resistance<sup>[22,23]</sup>.

In our study, we investigated whether TRAIL resistance in HCC cells can be overcome by combining TRAIL with chemotherapeutic drugs, inhibitors of survival signaling or targeted therapies against antiapoptotic BCL-2 proteins.

## MATERIALS AND METHODS

### Reagents and cell lines

HCC cell lines, Hep-G2 and Huh7, were purchased from ECACC. Cells were cultured in DMEM (Invitrogen, Karlsruhe, Germany), supplemented with 10% fetal calf serum (FCS, Biochrom, Berlin, Germany), 1% Pen/Strep (PAA laboratories, Pasching, Austria), 1% HEPES and 1% L-Glutamine (Cambrex, Verviers, Belgium). Cells were cultivated at 37°C with a concentration of 5% CO<sub>2</sub>. Transfection experiments were performed in OPTIMEM (Invitrogen).

Reagents were purchased from the following suppliers: recombinant TRAIL (with Enhancer applied in a concentration of 1 µg/mL) and SuperKillerTRAIL (SkTRAIL) from Alexis Biochemicals (SanDiego, CA, USA), goat anti-human IgG F(ab)<sup>2</sup> from Meridian Life Science (Cincinnati, USA), 5-fluorouracil (5-FU), doxorubicin (Doxo) from Sigma (Deisenhofen, Germany), SP600125, AG1478, PD98059, LY294002 and rapamycin (RAPA) from Calbiochem (Schwalbach, Germany). LBY135 was supplied from Novartis (Basel, Switzerland), Sorafenib (BAY 43-9006) from Bayer (Leverkusen, Germany).

**Viability test**

Cell viability was determined by a colorimetric 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HCC cell lines were seeded onto 96-well plates. On day one after seeding, cells were treated as indicated. We added 10  $\mu$ L MTT (5 mg/mL) 48 h after treatment and incubated cells for a further 3 h at 37°C. Next, supernatant was discarded and cells were lysed by adding 100  $\mu$ L 1-propanol to each well followed by shaking plates till complete lysis. Absorbance was measured at 550 nm in a microtiter plate reader. A viability of 1 was defined as the absorbance of untreated cells.

**Coating of microtiter plates**

To ease ligand-receptor interaction with the crosslinking supplement IgG F(ab)<sub>2</sub>, 96-well plates were coated with IgG F(ab)<sub>2</sub> before seeding cells. One hundred microliters of sterile filtered 100 nmol/L sodium bicarbonate buffer (pH 9.2) containing 5  $\mu$ g/mL IgG F(ab)<sub>2</sub> was added to each well and incubated for 2 h at room temperature (RT). After replacement of F(ab)<sub>2</sub> buffer by cell culture media, plates were stored at 4°C. Coated plates were stable for at least 1 wk.

**RNAi and transfection**

To knock-down protein expression, we administered specific small interfering RNA (siRNA) against MCL-1 or BCL-xL. As a control we used siRNA specific for green fluorescent protein (GFP). The following siRNA sequences were applied (MWG Biotech, Ebersberg, Germany): BCL-xL, 5'-gcuuggauaaagaugcaATT-3' (sense) and 5'-uugcaucuuuauccaagcAG-3' (antisense), MCL-1, 5'-aagauacacagcuucucTT-3' (sense) and 5'-gagaacgucugugauacuuTT-3' (antisense), GFP, 5'-ggcuacguccaggagcgcacTT-3' (sense) and 5'-ggugcgcuccuggacguagcTT-3' (antisense). Here, capitals represent deoxyribonucleotides and lower case letters represent ribonucleotides. Huh7 cells were seeded onto 12-well plates and after 24 h transiently transfected in OPTIMEM with Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's protocol. Expression levels were analyzed 24, 48 and 72 h after transfection *via* Western blotting analysis.

**Detection of apoptosis**

HCC cells were seeded onto 12-well plates and treated as indicated 1 d after transfection. Forty eight hours after treatment, cells were washed with cold PBS, collected and resuspended in a hypotonic buffer containing 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100, and 50  $\mu$ g/mL Propidium iodide (PI, Sigma). After 3 h incubation at 4°C, nuclei from apoptotic cells were quantified by fluorometric absorbance cell sorting according to the protocol of Nicoletti *et al*<sup>[24]</sup>.

**Cell lysis and Western blotting**

Cell lysis, SDS-PAGE and Western blotting were performed as described previously<sup>[13]</sup>. Immunodetection of proteins was performed using the following antibodies:

anti-BCL-xL (Labvison/NeoMarkers, Warm Springs Blvd. Fremont, Canada), anti-MCL-1 (Santa Cruz Biotechnology, Heidelberg, Germany) and anti- $\alpha$ -tubulin (Sigma) as loading control.

**Detection of receptor expression**

HCC cells were cultured as described and collected. Five hundred thousand cells for each receptor analysis were transferred to polystyrene tubes, washed twice with PBS and resuspended in PBS containing 0.5% BSA (Sigma). A specific monoclonal antibody to either TRAIL-R1, -R2, -R3, -R4 or unspecific mouse IgG1 as isotype control was applied at 5  $\mu$ g/mL. Cells were incubated for 20 min with gentle rocking at RT. Cells were washed twice in PBS and secondary fluorescein isothiocyanate-conjugated polyclonal goat antibody to mouse IgG1 (1:200 in PBS containing 0.5% BSA) was added, followed by incubation protected from light for 30 min with gentle rocking at RT. Cells were then washed and resuspended in PBS containing 0.5% BSA. Analysis of receptor expression was performed *via* flow cytometry. All antibodies were purchased from Alexis.

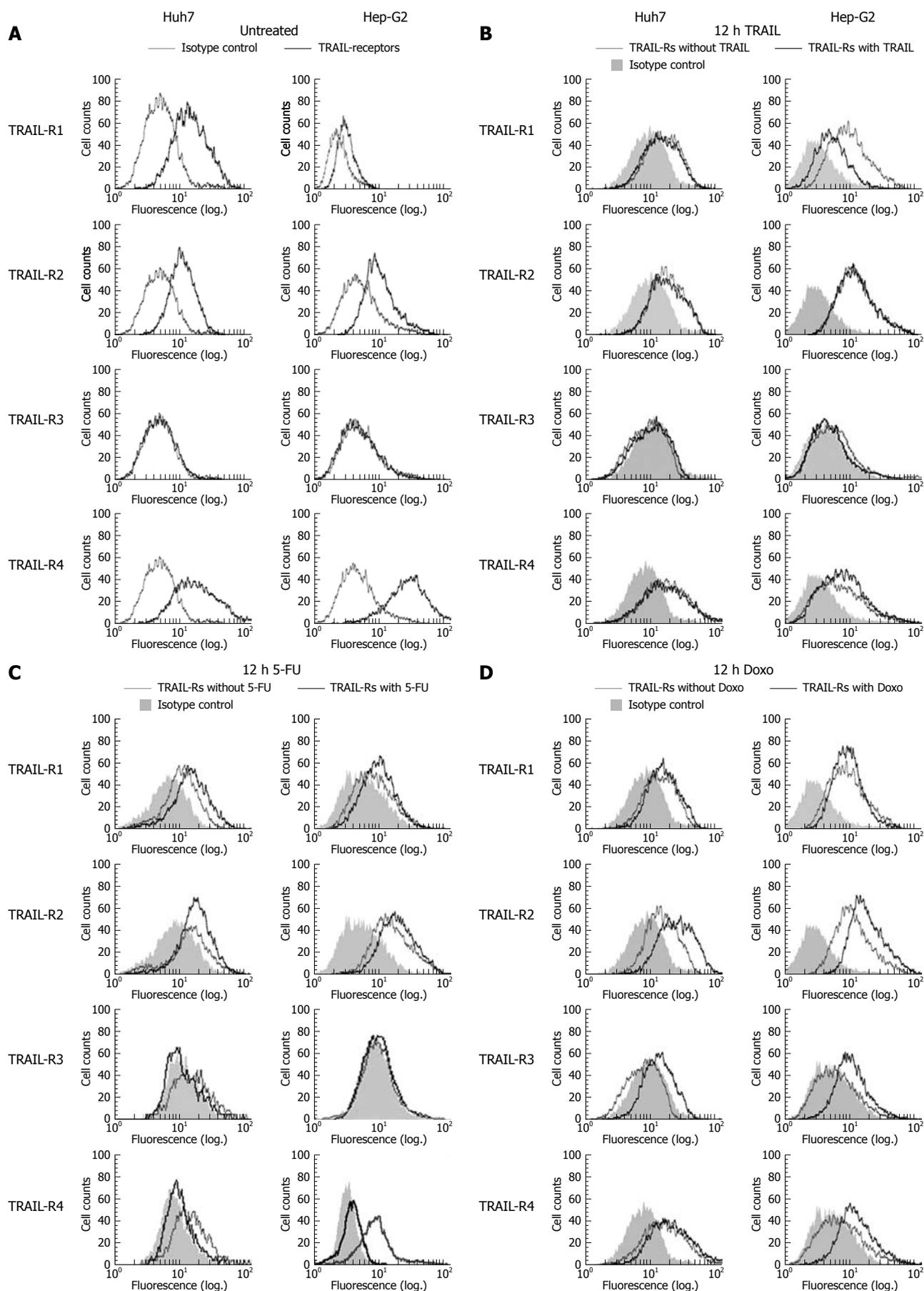
**Statistical analysis**

All results are expressed as mean  $\pm$  SD. Data were analyzed by students *t*-test (paired, two-sided) based on normal data distribution. *P* < 0.05 was considered significant.

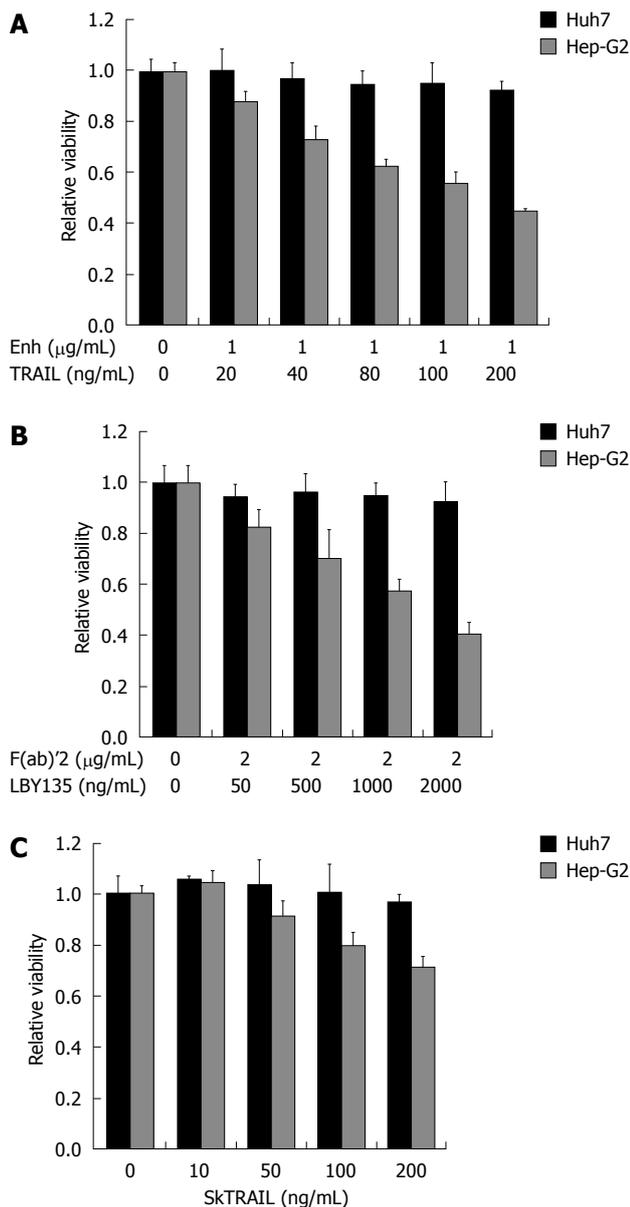
**RESULTS****TRAIL receptor expression in HCC cells upon treatment with TRAIL and chemotherapeutic agents**

It is known that TRAIL resistance can be mediated at the receptor level, either by low expression of TRAIL-R1 and -R2 or by a comparably high expression of TRAIL-R3 and -R4<sup>[25]</sup>. Firstly, we analyzed surface receptor expression of the HCC cell lines Huh7 and Hep-G2. Except for TRAIL-R3, all receptors were found to be expressed: we detected high expression levels of TRAIL-R1, -R2 and -R4 in both cell lines (Figure 1A). Next, we analyzed the expression levels after treatment with TRAIL and consequently the possibility of TRAIL-induced regulation in a feedback manner. After 12 h-treatment with TRAIL, we observed downregulation of TRAIL-R1 and a moderate upregulation of TRAIL-R4 in Hep-G2 cells. In contrast, no changes in receptor expression were detected in Huh7 cells (Figure 1B).

In order to study the effect of chemotherapeutics on TRAIL receptor expression, we treated HCC cells with 5-FU and Doxo, both applied for transarterial chemoembolization in patients with HCC<sup>[26]</sup>. 12 h-treatment with 5-FU resulted in upregulation of TRAIL-R1 and -R2 in both cell lines. In contrast, TRAIL-R3 was downregulated in Huh7 and unaffected in Hep-G2 cells. For TRAIL-R4, we observed a significant downregulation in both Hep-G2 and Huh7 cells (Figure 1C). 12 h-treatment with Doxo resulted in a slight upregulation of TRAIL-R1 in both cell lines. Remarkably, TRAIL-R2 was considerably upregulated. TRAIL-R3 surface expression



**Figure 1** Surface expression of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) receptors on Huh7 and Hep-G2 cells. Flow cytometric analysis of TRAIL receptors was performed using monoclonal mouse IgG1, anti-TRAIL-R1, -R2, -R3, -R4 antibodies and secondary FITC-conjugated polyclonal goat anti mouse-IgG1 antibodies. Unspecific mouse IgG1 antibodies were used as isotype control. Receptor surface expression was analyzed in untreated Huh7 and Hep-G2 cells (A) and 12 h after treatment with 100 ng/mL rec. TRAIL + 1 µg/mL Enhancer (B), 50 µg/mL 5-fluorouracil (5-FU) (C) and 0.5 µmol/L doxorubicin (D). Diagrams are representative of at least two independent experiments. Doxo: Doxorubicin.



**Figure 2** TRAIL-induced apoptosis in hepatocellular carcinoma (HCC) cells. Huh7 and Hep-G2 cells were seeded onto 96-well plates and treated on day one after seeding with different TRAIL compounds. A: Cells were treated for 48 h with rec. TRAIL + 1 μg/mL Enhancer as indicated; B: Plates were coated with crosslinker IgG F(ab)2 for 24 h before seeding of cells. Cells were then treated for 48 h with LBY135 + 1 μg/mL F(ab)2 as indicated; C: Cells were treated for 48 h with SkTRAIL as indicated. Cell viability was analyzed by MTT assay. Viability is shown relative to untreated controls. Assays were performed in six-fold values and are representative of three independent experiments. Values are expressed as mean ± SD. Enh: Enhancer.

was detectable in both cell lines after Doxo treatment. TRAIL-R4 was upregulated in Hep-G2 and unaffected in Huh7 cells (Figure 1D).

#### Sensitivity of HCC cells towards different TRAIL compounds

Next, we determined sensitivity of HCC cells towards TRAIL-induced apoptosis. Firstly, we analyzed the effect of recombinant TRAIL in concentrations from 20 up to 200 ng/mL (combined with Enhancer, 1 μg/mL). Hep-G2 cells were sensitive towards recombinant TRAIL in a dose-dependent manner, whereas Huh7 were resistant

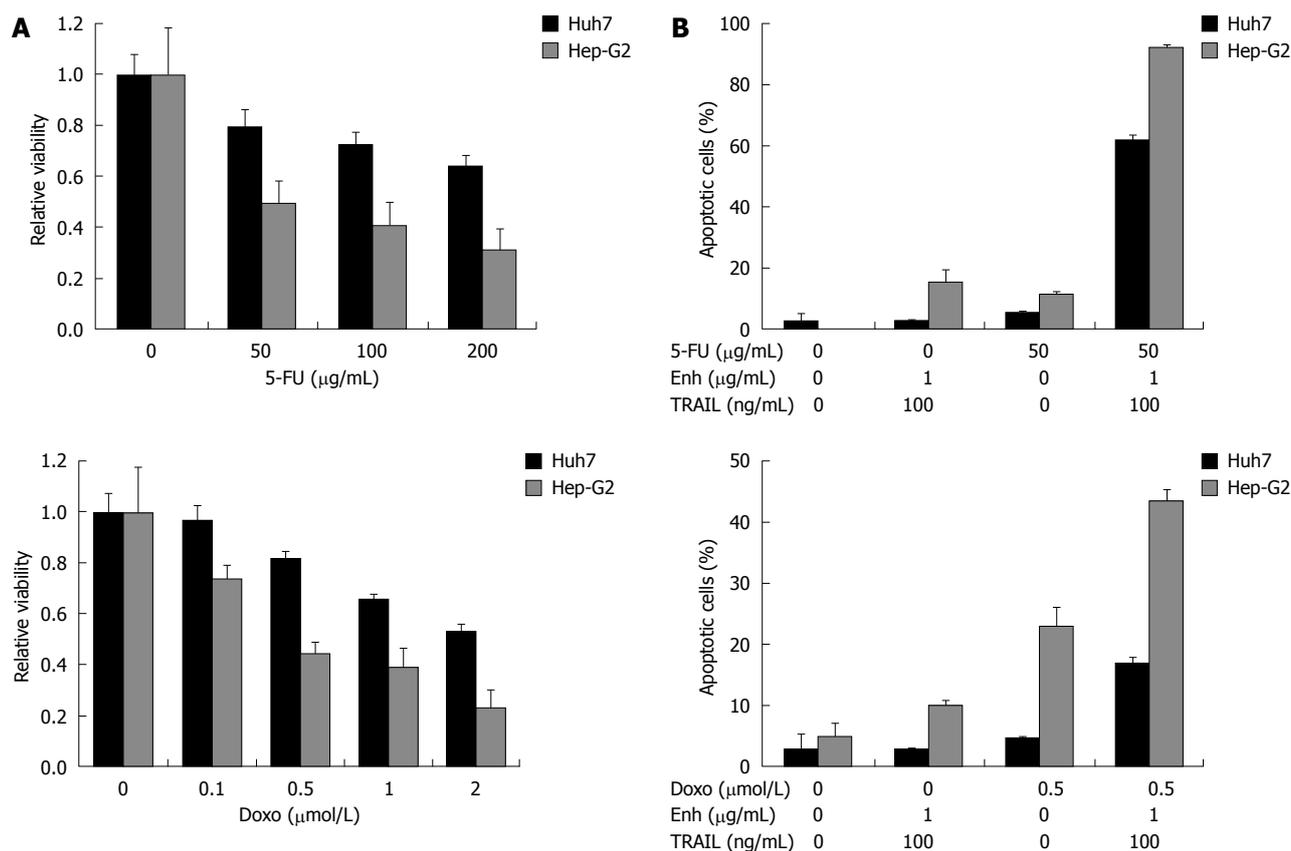
(Figure 2A). Next, we tested LBY135, a chimeric monoclonal antibody targeting TRAIL-R2, in concentrations from 50 to 2000 ng/mL, together with the cross linker F(ab)2 (2 μg/mL). To optimize the interaction between LBY135 and F(ab)2, we coated the plates with the F(ab)2-fragment before seeding the cells. Hep-G2 showed moderate sensitivity towards LBY135-induced apoptosis in a dose-dependent manner, whereas Huh7 cells were resistant (Figure 2B). To discover whether resistance was due to impaired interactions between enhancer or F(ab)2, the ligand and the receptor, we included SkTRAIL in our study. SkTRAIL interacts effectively with TRAIL receptors without additional supplements. Again, Hep-G2 cells revealed a dose-dependent sensitivity to SkTRAIL in concentrations from 10 to 200 ng/mL. In contrast, Huh7 cells were resistant to SkTRAIL (Figure 2C).

#### Treatment of HCC cells with TRAIL in combination with chemotherapy

Next, we analyzed whether HCC cells were sensitized to TRAIL-induced apoptosis by co-treatment with the chemotherapeutic drugs 5-FU and Doxo. As a first step, we analyzed whether the chemotherapeutics induced loss of viability if applied alone: after 48 h treatment of Huh7 and Hep-G2 cells with 5-FU (50, 100 and 200 μg/mL) and Doxo (0.1, 0.5, 1 and 2 μmol/L), we observed a dose-dependent decrease of cell viability (Figure 3A). Next, we applied these agents in concentrations which exhibited less significant cytotoxic effects, in combination with recombinant TRAIL (+ Enhancer 1 μg/mL). 5-FU (50 μg/mL) or TRAIL (100 ng/mL) did not induce apoptosis in Huh7 cells when administered alone. However, combination of 5-FU and TRAIL induced apoptosis in 62% of Huh7 cells. In Hep-G2 cells, TRAIL (100 ng/mL) induced apoptosis in 15% of cells. 5-FU treatment alone triggered apoptosis of 12% of Hep-G2 cells. 5-FU and TRAIL co-treatment of Hep-G2 resulted in 93% apoptotic cells (Figure 3B, upper panel). Next, we tested the combination of Doxo (0.5 μmol/L) and TRAIL (100 ng/mL). Doxo induced apoptosis in less than 5% of Huh7 cells, whereas the combination of Doxo and TRAIL resulted in 17% apoptotic cells. Treatment of Hep-G2 with Doxo alone induced apoptosis in 24% of cells, whereas Doxo and TRAIL in combination led to apoptosis rates of 43% (Figure 3B, lower panel).

#### Treatment of HCC cells with TRAIL in combination with specific kinase inhibitors

Antiapoptotic pathways such as PI3K/Akt, epidermal growth factor receptor (EGFR), [mitogen-activated protein kinase (MAPK)/extracellular signal regulated kinase (ERK) kinase] (MEK)/ERK are well known to be activated in malignant cells, thus contributing to cell cycle progression and tumor growth. Therefore, we analyzed whether inhibition of kinases involved in these pathways could overcome resistance towards TRAIL-mediated apoptosis. Firstly, we applied the multi-kinase inhibitor Sorafenib to inhibit RAF/MEK/ERK signaling, in escalating concentrations (2.5, 5 and 10 μmol/L). A dose-dependent decrease of cell viability in Huh7 and Hep-G2



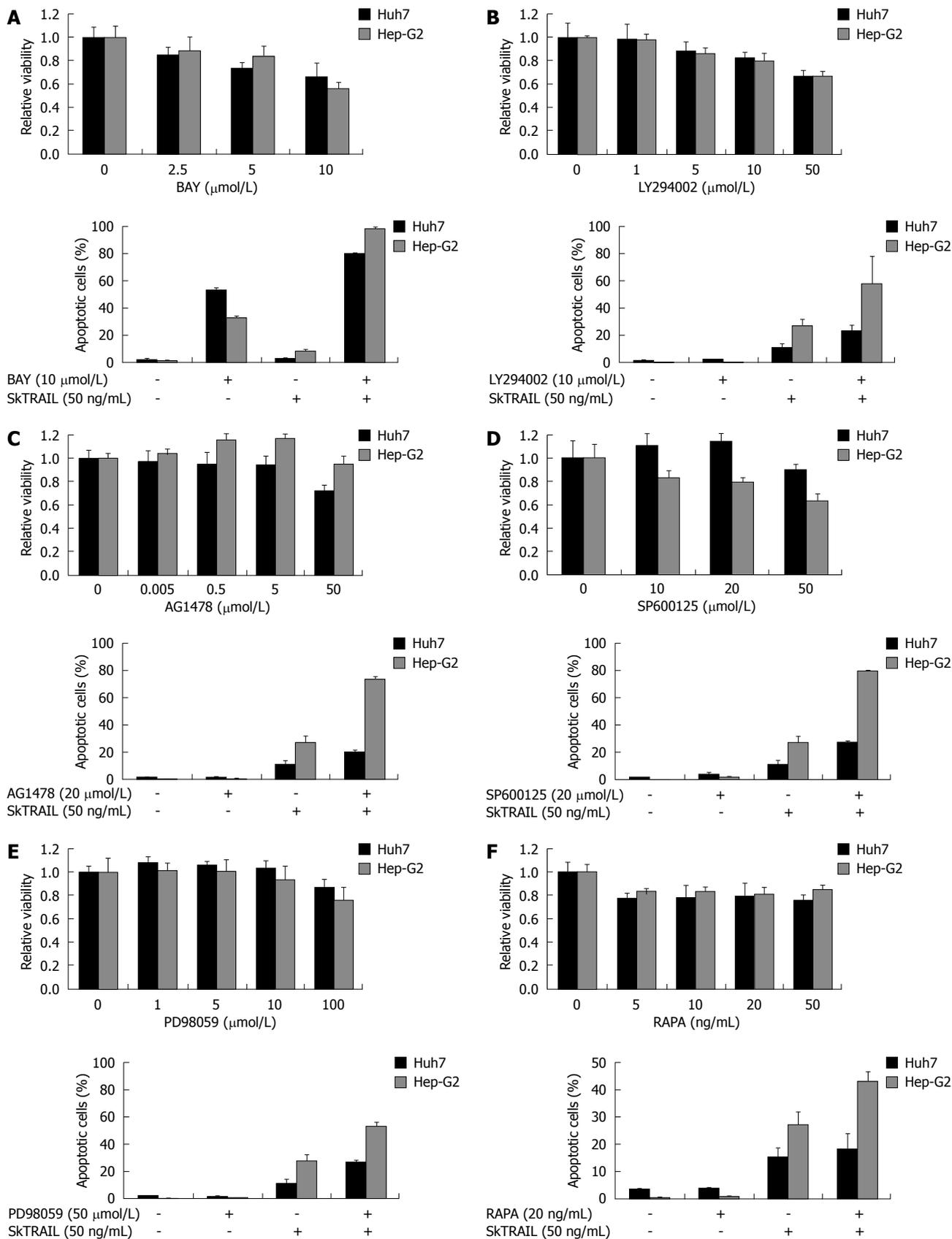
**Figure 3 Treatment of HCC cells with TRAIL in combination with 5-FU and doxorubicin.** Values are expressed as mean  $\pm$  SD. A: Huh7 and Hep-G2 cells were analyzed for cell viability after treatment with the chemotherapeutic agents 5-FU and doxorubicin alone. Cells were seeded onto 96-well plates and treated on day one after seeding. Cells were treated for 48 h with 5-FU (upper left panel) and doxorubicin (lower left panel) as indicated. Cell viability was analyzed by MTT assay. Viability is shown relative to untreated controls. Assays were performed in six-fold values; B: Apoptosis induction in Huh7 and Hep-G2 cells treated with 50  $\mu$ g/mL 5-FU (upper right panel) and 0.5  $\mu$ mol/L doxorubicin (lower right panel) either alone or in combination with 100 ng/mL TRAIL + 1  $\mu$ g/mL Enhancer. Cells were seeded onto 12-well plates, harvested 48 h after treatment and analyzed for apoptosis induction by flow cytometry. Assays were performed in triplicate and are representative of at least two independent experiments.

was observed (Figure 4A, upper panel). In a second step, we analyzed the impact of Sorafenib on TRAIL treatment. In Huh7 cells, Sorafenib (10  $\mu$ mol/L) induced apoptosis rates of 50%. Strikingly, the combination of SkTRAIL (50 ng/mL) and Sorafenib (10  $\mu$ mol/L) induced apoptosis in 80% of the cells. In Hep-G2 cells Sorafenib caused only minor apoptosis rates (33%). However, combination of TRAIL and Sorafenib led to 98% apoptotic cells (Figure 4A, lower panel).

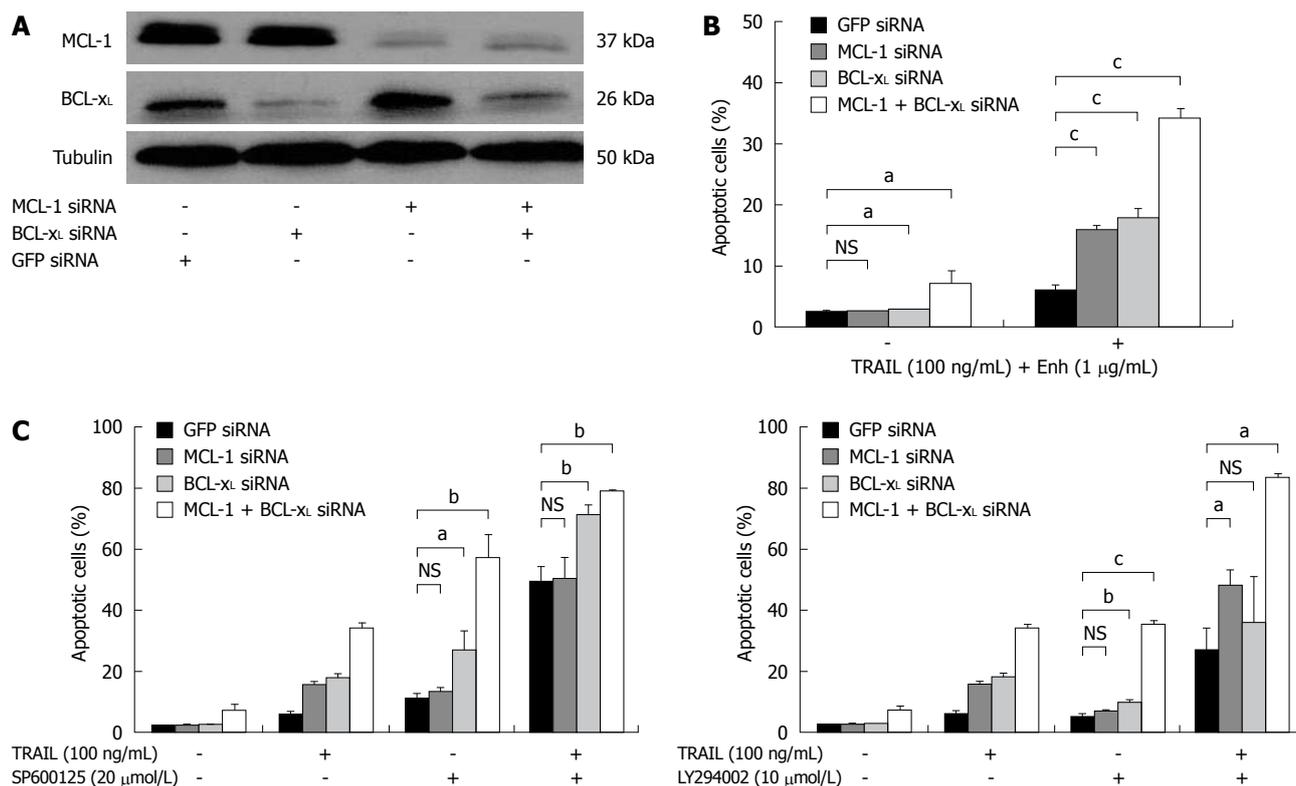
Next, we inhibited the PI3K/Akt pathway by application of the PI3K inhibitor LY294002. A slight decrease of cell viability was observed in Huh7 and Hep-G2 after 48 h treatment in concentrations lower than 50  $\mu$ mol/L (Figure 4B, upper panel). However, combination of LY294002 (10  $\mu$ mol/L) and SkTRAIL (50 ng/mL) doubled apoptosis rates in Hep-G2 cells to 59% compared to SkTRAIL treatment alone. In Huh7, we observed an increased rate of apoptosis after treatment with the combination of LY294002 and SkTRAIL compared to SkTRAIL alone (23% *vs* 11%, respectively. Figure 4B, lower panel). Furthermore, we used AG1478 to inhibit EGFR kinase. Interestingly, inhibition of EGFR kinase increased cell viability in Hep-G2 cells in concentrations up to 5  $\mu$ mol/L. In Huh7 cells, AG1478 caused no significant changes in cell viability when applied in low concen-

trations (Figure 4C, upper panel). AG1478 (20  $\mu$ mol/L) and SkTRAIL (50 ng/mL) co-treatment increased the rate of apoptotic cells to 27% in Huh7 and 74% in Hep-G2 cells (Figure 4C, lower panel). Next, we inhibited the c-Jun N-terminal kinases 1 and 2 (JNK1 and JNK2) with the anthrapyrazolone inhibitor SP600125. We observed increased cell viability in Huh7 cells and a slight, dose-dependent decrease of cell viability in Hep-G2 cells after 48 h of SP600125 treatment (Figure 4D, upper panel). Notably, a high percentage of cells were arrested in the G2 phase 48 h after treatment with the JNK inhibitor (data not shown). Combined treatment with SP600125 (20  $\mu$ mol/L) and SkTRAIL (50 ng/mL) led to 28% apoptosis of Huh7 and to 80% apoptosis of Hep-G2 cells (Figure 4D, lower panel). Next, we included a specific inhibitor of MAP kinase kinase (MEK), PD98059, in our study. Again, a death inducing effect of MEK inhibition alone was only observed when applied in high concentrations of more than 50  $\mu$ mol/L (Figure 4E, upper panel). However, in combination (50  $\mu$ mol/L PD98059 and 50 ng/mL SkTRAIL), a two-fold increase of apoptosis, compared to monotherapy with SkTRAIL, was detectable in Huh7 and Hep-G2 cells (Figure 4E, lower panel).

Finally, we inhibited mammalian target of rapamycin (mTOR) with rapamycin (Sirolimus). Rapamycin alone



**Figure 4 Treatment of HCC cells with TRAIL in combination with specific kinase inhibitors.** Viability of HCC cells treated with kinase inhibitors alone (upper panels). On day one after seeding of Huh7 and Hep-G2 cells onto 96-well plates, cells were treated with multi-kinase inhibitor Sorafenib (A), PI3 kinase inhibitor LY294002 (B), EGFR kinase inhibitor AG1478 (C), JNK inhibitor SP600125 (+ 0.2% DMSO as vehicle) (D), MEK inhibitor PD98059 (E) and mTOR inhibitor rapamycin (RAPA) (F) at the indicated concentrations for 48 h. Cell viability was analyzed by MTT assay. Viability is shown relative to untreated or 0.2% DMSO treated controls, respectively. Assays were performed in six-fold values. Values are expressed as mean  $\pm$  SD. Apoptosis induction in Huh7 and Hep-G2 cells treated with 10  $\mu\text{mol/L}$  Sorafenib (A), 10  $\mu\text{mol/L}$  LY294002 (B), 20  $\mu\text{mol/L}$  AG1478 (C), 20  $\mu\text{mol/L}$  SP600125 (+ 0.2% DMSO as vehicle) (D), 50  $\mu\text{mol/L}$  PD98059 (E) and 20 ng/mL rapamycin (F) in combination with 50 ng/mL SkTRAIL (lower panels). Cells were seeded 1 d before treatment onto 12-well plates, harvested 48 h after treatment and analyzed for apoptosis induction by flow cytometry. Assays were performed in triplicate and are representative of at least two independent experiments. Values are expressed as mean  $\pm$  SD.



**Figure 5** TRAIL-induced apoptosis in Huh7 cells after targeted therapy approaches and knock-down of BCL-xL and MCL-1. **A:** Huh7 cells were transfected with siRNAs (40 nmol/L) specific for MCL-1 and BCL-xL either alone or in combination. SiGFP was used as control. Whole cell lysates were prepared 24 h after transfection. MCL-1 and BCL-xL expression was analyzed by Western blotting.  $\alpha$ -Tubulin expression was used to control equal loading; **B:** 24 h after siRNA transfection, cells were treated for 48 h with 100 ng/mL TRAIL (+ 1 μg/mL Enhancer); **C:** 20 μmol/L SP600125 (left panel) or 10 μmol/L LY294002 (+ 0.2% DMSO as vehicle, right panel), either alone or in combination with 100 ng/mL TRAIL (+ 1 μg/mL Enhancer). Cells were harvested on day two after treatment and analyzed for apoptosis induction by flow cytometry. Assays were performed in triplicate and are representative of at least two independent experiments. Values are expressed as mean  $\pm$  SD. NS: Not significant. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>c</sup> $P < 0.001$ .

only caused a moderate decrease of cell viability (20%) in Huh7 and Hep-G2 cells (Figure 4F, upper panel). Combination of 20 ng/mL rapamycin with 50 ng/mL SkTRAIL resulted in a slight increase of apoptosis rates in Huh7 cells (18% *vs* 15% SkTRAIL alone) and a profound increase of apoptosis in Hep-G2 cells (43% *vs* 27%, Figure 4F, lower panel).

#### Treatment of HCC cells with TRAIL after knock-down of MCL-1 and BCL-xL

The antiapoptotic BCL-2 proteins, MCL-1 and BCL-xL, are profoundly expressed in tissues of human HCC, thus contributing to apoptosis resistance of HCC cells<sup>[13,15,27]</sup>. To analyze the role of antiapoptotic BCL-2 proteins in TRAIL-induced apoptosis, we manipulated their expression in Huh7 cells *via* specific siRNA-mediated knock-down. An effective reduction of MCL-1 and BCL-xL expression levels was observed 24 h after transfection (Figure 5A).

A knock-down of BCL-xL induced significant apoptosis in comparison to mock transfected cells ( $P < 0.05$ ). Knock-down of MCL-1 did not induce significant apoptosis rates. Additionally, combined knock-down of MCL-1 and BCL-xL induced spontaneous apoptosis in 8% of Huh7 cells ( $P < 0.05$ , Figure 5B). Downregulation of either MCL-1 or BCL-xL significantly enhanced susceptibility towards TRAIL-induced apoptosis (17% *vs*

6% and 18% *vs* 6%, respectively,  $P < 0.001$ ). Remarkably, we detected 34% apoptotic cells in Huh7 lacking BCL-xL and MCL-1 expression after treatment with TRAIL ( $P < 0.001$ , Figure 5B). Furthermore, we analyzed whether lack of MCL-1 and BCL-xL expression sensitized cells towards the JNK inhibitor SP600125 and the PI3K inhibitor LY294002. Inhibition of JNK and PI3K showed significantly enhanced anti-tumoral efficacy after knock-down of BCL-xL and MCL-1. In cells lacking BCL-xL expression, apoptosis was induced in 27% *vs* 11% of control cells after treatment with SP600125 (20 μmol/L) ( $P < 0.05$ , Figure 5C). In contrast, cells lacking MCL-1 did not show increased susceptibility to JNK inhibition (14% *vs* 11%, not significant, Figure 5C). Knock-down of MCL-1 and BCL-xL increased SP600125-induced apoptosis rates to 57% ( $P < 0.005$ , Figure 5C, left panel). Additionally, single knock-down of BCL-xL ( $P < 0.001$ ) and double knock-down of MCL-1 and BCL-xL ( $P < 0.001$ ) significantly increased apoptosis after combined treatment of SP600125 with recombinant TRAIL (100 ng/mL). Single knock-down of MCL-1 did not exhibit sensitizing effects (differences not significant, Figure 5C).

Next, we analyzed the effects of MCL-1 and BCL-xL knock-down in combination with the PI3K inhibitor LY294002. We observed a significant sensitizing effect of BCL-xL knock-down on LY294002-induced apoptosis in Huh7 cells ( $P < 0.005$ , Figure 5C, right panel). Knock-

down of MCL-1 did not increase LY294002-induced apoptosis. However, in Huh7 cells lacking both MCL-1 and BCL-x<sub>L</sub>, apoptosis rates increased to 35% after LY294002 treatment ( $P < 0.001$ ). Finally, we found an increased rate of apoptosis after combined treatment of LY294002 (10  $\mu\text{mol/L}$ ) with recombinant TRAIL (100 ng/mL) in cells lacking MCL-1 (48% *vs* 27% of mock transfected Huh7,  $P < 0.05$ ). A moderate sensitizing effect in cells lacking BCL-x<sub>L</sub> was observed (not significant). Importantly, the combined knock-down of MCL-1 and BCL-x<sub>L</sub> caused apoptosis rates of 83%, if cells were treated with a combination of LY294002 and recombinant TRAIL ( $P < 0.05$ , Figure 5C, right panel).

## DISCUSSION

Amongst the various approaches to induce apoptosis in tumor cells, application of the death receptor ligand TRAIL is very promising. Preclinical studies suggest that TRAIL induces apoptosis of tumor cells *in vivo* without lethal toxicities<sup>[28,29]</sup>. A major obstacle for the clinical use of TRAIL is its limited efficacy in monotherapeutic approaches in different tumor entities. Thus, it appears worthwhile to persist in investigating ways to enhance TRAIL's capacity for apoptosis induction. Resistance towards TRAIL can be caused at receptor level by inhibitory proteins and at mitochondrial level by antiapoptotic proteins<sup>[17,18,21]</sup>. For example, a diminished membrane expression of TRAIL-R1 and -R2, as well as reduced caspase 8 levels, mediate TRAIL resistance in myeloma cells<sup>[19]</sup>. In this present study we analyzed different approaches in sensitizing HCC cells to TRAIL-induced apoptosis.

TRAIL receptor expression was similar in the HCC cell lines Huh7 and Hep-G2. After TRAIL treatment, expression patterns changed only slightly in Hep-G2 cells. Strikingly, chemotherapeutic drugs influenced the expression pattern in HCC cells. Upregulation of TRAIL-R1 and profound upregulation of TRAIL-R2 after Doxo and 5-FU treatment in both cell lines might represent a potential mechanism of chemotherapy-mediated TRAIL sensitization. Interestingly, TRAIL-R3 (DcR1) was also upregulated after chemotherapy. This could also represent a mechanism of TRAIL resistance upon chemotherapy, since TRAIL-R3 acts as a decoy receptor. Notably, upregulation of TRAIL receptors in Huh7 cells which express mutated p53 suggests that receptor regulation occurs independently from p53<sup>[30]</sup>.

Several mAbs targeting TRAIL receptors and recombinant TRAIL agonists have already entered clinical trials<sup>[31-33]</sup>. In order to analyze the efficacy of different TRAIL compounds, we included LBY135, a chimeric antibody targeting TRAIL-R2, recombinant TRAIL and SkTRAIL in our study. We demonstrate that crosslinking elements IgG F(ab)<sup>2</sup> are mandatory for LBY135-induced apoptosis. Consistent findings were obtained for recombinant TRAIL, where combination with an enhancer is necessary to induce apoptosis. Comparing the death-inducing capacities of LBY135, TRAIL and SkTRAIL in HCC cells, we assume that TRAIL-R2 plays

a major role, which would be in line with observations in colon and breast cancer<sup>[34]</sup>. In contrast, it has been shown that chronic leukemia cells are selectively sensitive to TRAIL-R1<sup>[35]</sup>. Taken together, it appears likely that a cell type dependency determines the efficiency of TRAIL-mediated apoptosis induction, even if both TRAIL-R1 and -R2 are expressed.

Chemotherapeutic drugs such as Doxo and 5-FU have shown limited efficacy for the treatment of HCC<sup>[20]</sup>. However, anti-tumoral effects have been described for Doxo if administered into the liver *via* chemoembolization<sup>[26,36]</sup>. In our study we aimed to discover whether the combination of TRAIL with chemotherapy exerts anti-tumoral effects in HCC. Importantly, chemotherapy with Doxo or 5-FU increased TRAIL susceptibility in Hep-G2 cells and sensitized Huh7 cells towards TRAIL, opening the possibility of a treatment regime including reduced doses of chemotherapeutic drugs in combination with TRAIL.

The multi-kinase inhibitor Sorafenib has recently been approved for the therapy of advanced HCC. Sorafenib acts by inhibition of the RAF/MEK/ERK pathway and downregulation of MCL-1, leading to a disruption of survival signals in HCC cells<sup>[4,37]</sup>. In combination with TRAIL, Sorafenib profoundly increased apoptosis induction advocating TRAIL as a potential and effective agent for HCC treatment along with Sorafenib.

There is evidence that constitutive activation of various antiapoptotic pathways is a basic principle of tumor growth, cell cycle progression and apoptosis resistance. A well described antiapoptotic pathway is the PI3K/Akt signaling pathway, found activated in several tumor entities, including HCC<sup>[38]</sup>. The PI3K inhibitor LY294002<sup>[39,40]</sup> has already been employed in preclinical studies in combination with TRAIL. Consistent with data for prostate cancer and leukemia cells, our results indicate that blockage of PI3K by LY294002 overcomes resistance towards TRAIL in HCC cells<sup>[22,23]</sup>.

The mTOR, a protein with growing clinical relevance in oncology, is located downstream of PI3K<sup>[41]</sup>. The significant sensitization towards TRAIL in Hep-G2 cells by mTOR inhibition underlines a pivotal role of PI3K/Akt signaling for the resistance of HCC towards TRAIL.

In addition, the MAPK/ERK pathway exerts antiapoptotic effects in cancer cells. The MEK is a key component downstream of Raf serine/threonine kinases<sup>[42,43]</sup>. MEK inhibitors have been described as sensitizing human cancer cells to apoptosis, e.g. after treatment with chemotherapeutic agents<sup>[44,45]</sup>. In this study, we observed no apoptosis induction and only a slight decrease of cell viability after MEK inhibition in HCC cells. However, the combination of MEK inhibition and TRAIL caused a significant increase of TRAIL-induced apoptosis. This observation suggests that an aberrantly activated Raf/MAPK/ERK pathway plays a crucial role for TRAIL resistance in HCC.

Furthermore, we focused on the EGFR, which is an upstream receptor in Ras-Raf-MEK-ERK signaling<sup>[46]</sup>. It has been shown that overexpression of EGFR represents a protective factor against apoptosis stimuli in

HCC<sup>[47,48]</sup>. The combined treatment of TRAIL with the specific EGFR kinase inhibitor AG1478 caused a significant increase of TRAIL-induced apoptosis in HCC cells. Thus, EGFR blockage is another promising approach for TRAIL sensitization of HCC cells.

Recently, it has been shown that JNK inhibition sensitizes HCC cells, but not healthy hepatocytes, towards TRAIL-induced apoptosis<sup>[49]</sup>. In contrast, other results indicate that JNK activation is not relevant for TRAIL-induced apoptosis<sup>[50]</sup>. We found a significantly increased proapoptotic effect of TRAIL if combined with the JNK inhibitor SP600125.

Aberrant activity of survival signaling pathways exerts antiapoptotic effects at least in part *via* triggering of the expression of antiapoptotic proteins, such as antiapoptotic BCL-2 proteins. Importantly, antiapoptotic BCL-2 proteins, such as MCL-1 and BCL-x<sub>L</sub>, have been described as contributing to TRAIL resistance in cancer cells<sup>[51]</sup>. MCL-1 and BCL-x<sub>L</sub> mainly act by directly inhibiting their proapoptotic relatives BAX and BAK, thereby guarding the cell from various death stimuli. In addition, expression of antiapoptotic BCL-2 proteins is a prognostic factor for various tumor entities, e.g. expression of MCL-1 in breast and gastric cancer<sup>[52,53]</sup>. In the liver, MCL-1 has been found to be a key factor for apoptosis regulation<sup>[13,54,55]</sup>. A lack of MCL-1 causes increased rates of apoptosis and a significantly higher susceptibility towards chemotherapeutic treatment in HCC<sup>[54]</sup>. In addition, it has been shown that MCL-1 acts as a key factor for resistance towards TRAIL in leukemia cells<sup>[56]</sup>. In our study, we show that knock-down of MCL-1 or Bcl-x<sub>L</sub> increased TRAIL-induced apoptosis in HCC. Taking into consideration that there is a putative functional redundancy between these two proteins, we performed a double knock-down of MCL-1 and BCL-x<sub>L</sub>. Cells lacking both MCL-1 and BCL-x<sub>L</sub> expression showed profound spontaneous apoptosis, indicating that both proteins contribute to mitochondrial integrity in HCC cells. Importantly, HCC cells lacking MCL-1, BCL-x<sub>L</sub> or both showed an increased apoptosis rate after treatment with TRAIL. In summary, our data suggest a central role of MCL-1 and Bcl-x<sub>L</sub> for the resistance of HCC cells towards TRAIL-induced apoptosis.

Additionally, recent studies have revealed a synergistic effect of PI3K/Akt signaling with MCL-1 and BCL-x<sub>L</sub>, contributing to apoptosis resistance in cancer<sup>[57-59]</sup>. In our study, we treated HCC cells with TRAIL in combination with a PI3K inhibitor and with a knock-down of MCL-1 and BCL-x<sub>L</sub> *via* RNA interference. We found that cells lacking BCL-x<sub>L</sub> or lacking both MCL-1 and BCL-x<sub>L</sub> evolve a significantly increased sensitivity to apoptosis induced by PI3K inhibition. A knock-down of MCL-1 alone did not enhance LY294002-induced apoptosis. Importantly, we observed a profound increase of apoptosis in cells lacking MCL-1 and a rather low increase in cells lacking Bcl-x<sub>L</sub> after combined treatment with PI3K inhibitors and TRAIL. Strikingly, combined knock-down of MCL-1 and BCL-x<sub>L</sub> led to profound induction of apoptosis after treatment with PI3K inhibitors and TRAIL. In summary, our results suggest a major role of PI3K/Akt signaling in resistance towards TRAIL-mediated apoptosis and

emphasize the role of antiapoptotic BCL-2 proteins for TRAIL resistance of HCC cells.

A recent study showed that JNK1 exerts antiapoptotic effects *via* stabilization of MCL-1 in hepatocytes<sup>[60]</sup>. Therefore, we analyzed the role of MCL-1 and BCL-x<sub>L</sub> expression for the apoptosis-inducing capacity of JNK inhibitors and TRAIL. We found profoundly increased apoptosis rates after JNK inhibition in cells lacking BCL-x<sub>L</sub>, whereas cells lacking MCL-1 did not exhibit sensitivity towards JNK inhibition. Strikingly, a combined treatment of a JNK inhibitor and TRAIL caused major rates of apoptosis in cells lacking MCL-1 and BCL-x<sub>L</sub>. Furthermore, we could show that inhibition of JNK is not capable of inducing apoptosis alone unless BCL-x<sub>L</sub> is effectively downregulated in HCC cells, revealing a major role of BCL-x<sub>L</sub> for the resistance of HCC cells towards apoptosis induced by JNK inhibitors.

In conclusion, the application of recombinant TRAIL, as well as that of monoclonal antibodies targeting TRAIL receptors, is an encouraging therapeutic approach in cancer patients. However, resistance to TRAIL treatment is a common phenomenon in many cancer entities. The aim of our study was to provide novel treatment options to overcome resistance of HCC cells towards TRAIL-induced apoptosis. Our results demonstrate that TRAIL is an effective treatment option in HCC if combined with the chemotherapeutic drugs Doxo and 5-FU or kinase inhibitors, such as LY294002, AG1478 and PD98059. In addition, we revealed a pivotal role of the antiapoptotic BCL-2 proteins MCL-1 and BCL-x<sub>L</sub> for HCC resistance towards TRAIL. Further studies are warranted to evaluate the potential of combined treatment approaches and clear the trail for clinical usage.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide. Numerous clinical trials have failed to establish an effective therapy regimen in patients with advanced or metastasized HCC. Thus, new strategies for these patients are mandatory. Defects in apoptosis signaling contribute to resistance of HCC cells towards the death receptor ligand tumor necrosis factor-related apoptosis inducing ligand (TRAIL), which is a promising anti-tumor agent since it is capable of killing tumor cells *via* receptor-mediated apoptosis. New combined treatment regimens have the aim of overcoming resistance towards TRAIL and to make TRAIL an effective treatment option in patients suffering from HCC.

### Research frontiers

Hyperactivation of the PI3K/Akt, EGFR and [Mitogen-activated protein kinase/extracellular signal regulated kinase (ERK) kinase] (MEK)/ERK survival pathways and decreased mitochondrial sensitivity due to overexpression of the BCL-2 proteins MCL-1 and BCL-x<sub>L</sub> are key mechanisms of TRAIL resistance in HCC. Furthermore, resistance towards TRAIL can be located at receptor level, contributing to inefficient treatment of HCC cells with TRAIL compounds.

### Innovations and breakthroughs

Previous articles have demonstrated that TRAIL is an effective treatment option in HCC in a combined setup with sensitizing agents. In this study the authors demonstrate new treatment options for the sensitization of HCC cells towards TRAIL-induced apoptosis by combination of chemotherapeutic drugs doxorubicin (Doxo) and 5-fluorouracil (5-FU) or kinase inhibitors, such as LY294002, AG1478, PD98059 and SP600125 with TRAIL. In addition, the authors reveal a pivotal role of the antiapoptotic BCL-2 proteins MCL-1 and BCL-x<sub>L</sub> for HCC resistance towards TRAIL. The importance of MCL-1 and Bcl-x<sub>L</sub> for mitochondrial integrity has been extensively studied in this study.

## Applications

TRAIL is an effective treatment option in HCC if combined with the chemotherapeutic drugs Doxo and 5-FU or kinase inhibitors, such as LY294002, AG1478, PD98059 and SP600125. These results open the possibility of a treatment regime which includes reduced doses of chemotherapeutic drugs in combination with TRAIL. The authors also revealed a pivotal role of the antiapoptotic BCL-2 proteins MCL-1 and BCL-x<sub>L</sub> for HCC resistance towards TRAIL. Thus, downregulation of these anti-apoptotic proteins alone (e.g. by application of so-called "BH3-only mimetics") is a promising approach for the treatment of HCC patients. "BH3-only mimetics" have already entered clinical trials in cancer patients.

## Terminology

Apoptosis, also described as programmed cell death, is a genetically determined process of controlled cellular suicide characterized by typical morphological changes, e.g. fragmentation of DNA. TRAIL ligates two different types of receptors: (1) death receptors triggering TRAIL-induced apoptosis and (2) decoy receptors possibly inhibiting the TRAIL death-signaling pathway. MCL-1 and BCL-x<sub>L</sub> are antiapoptotic members of the BCL-2 family serving as protective factors against several death stimuli. Antiapoptotic pathways such as PI3K/Akt, EGFR, MEK/ERK are well known as being activated in malignant cells, thus contributing to cell cycle progression and tumor growth

## Peer review

This is an interesting work, elegantly performed and with possible future clinical applications. Although the article contains a wealth of experimental details, making it a difficult reading for the uninitiated, I believe it will be of interest for both clinicians and basic science readers of the *World Journal of Gastroenterology* because of its possible clinical implications

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ORIGINAL ARTICLE

## Diarrhoea-predominant irritable bowel syndrome distinguishable by 16S rRNA gene phylotype quantification

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### Abstract

**AIM:** To study whether selected bacterial 16S ribosomal RNA (rRNA) gene phylotypes are capable of distinguishing irritable bowel syndrome (IBS).

**METHODS:** The faecal microbiota of twenty volunteers with IBS, subdivided into eight diarrhoea-predominant (IBS-D), eight constipation-predominant (IBS-C) and four mixed symptom-subtype (IBS-M) IBS patients, and fifteen control subjects, were analysed at three time-points with a set of fourteen quantitative real-time

polymerase chain reaction assays. All assays targeted 16S rRNA gene phylotypes putatively associated with IBS, based on 16S rRNA gene library sequence analysis. The target phylotypes were affiliated with *Actinobacteria*, *Bacteroidetes* and *Firmicutes*. Eight of the target phylotypes had less than 95% similarity to cultured bacterial species according to their 16S rRNA gene sequence. The data analyses were made with repeated-measures ANCOVA-type modelling of the data and principle component analysis (PCA) with linear mixed-effects models applied to the principal component scores.

**RESULTS:** Bacterial phylotypes *Clostridium cocleatum* 88%, *Clostridium thermosuccinogenes* 85%, *Coprobacillus cateniformis* 91%, *Ruminococcus bromii*-like, *Ruminococcus torques* 91%, and *R. torques* 93% were detected from all samples analysed. A multivariate analysis of the relative quantities of all 14 bacterial 16S rRNA gene phylotypes suggested that the intestinal microbiota of the IBS-D patients differed from other sample groups. The PCA on the first principal component (PC1), explaining 30.36% of the observed variation in the IBS-D patient group, was significantly altered from all other sample groups (IBS-D vs control,  $P = 0.01$ ; IBS-D vs IBS-M,  $P = 0.00$ ; IBS-D vs IBS-C,  $P = 0.05$ ). Significant differences were also observed in the levels of distinct phylotypes using relative values in proportion to the total amount of bacteria. A phylotype with 85% similarity to *C. thermosuccinogenes* was quantified in significantly different quantities among the IBS-D and control subjects ( $-4.08 \pm 0.90$  vs  $-3.33 \pm 1.16$ ,  $P = 0.04$ ) and IBS-D and IBS-M subjects ( $-4.08 \pm 0.90$  vs  $-3.08 \pm 1.38$ ,  $P = 0.05$ ). Furthermore, a phylotype with 94% similarity to *R. torques* was more prevalent in IBS-D patients' intestinal microbiota than in that of control subjects ( $-2.43 \pm 1.49$  vs  $-4.02 \pm 1.63$ ,  $P = 0.01$ ). A phylotype with 93% similarity to *R. torques* was associated with control samples when compared with IBS-M ( $-2.41 \pm 0.53$  vs  $-2.92 \pm 0.56$ ,  $P = 0.00$ ). Additionally, a *R. bromii*-like phylotype was associated with IBS-C patients in comparison to control subjects ( $-1.61 \pm 1.83$  vs  $-3.69 \pm 2.42$ ,  $P = 0.01$ ). All of the above mentioned phylotype specific alterations were independent of the effect of time.

**CONCLUSION:** Significant phylotype level alterations

in the intestinal microbiotas of IBS patients were observed, further emphasizing the possible contribution of the gastrointestinal microbiota in IBS.

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**Key words:** Irritable bowel syndrome; Diarrhoea-predominant irritable bowel syndrome; Intestinal microbiota; Quantitative real-time polymerase chain reaction; 16S ribosomal RNA

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## INTRODUCTION

Irritable bowel syndrome (IBS) is a functional gastrointestinal (GI) disorder with a worldwide prevalence of 10%-20%<sup>[1]</sup>. The main symptoms include abdominal pain or discomfort, diarrhoea, constipation, abdominal bloating, and flatulence. The symptoms are associated with changes in the frequency and form of stool, improved by defecation, and they typically fluctuate with time. Although IBS does not predispose to malignancies, it essentially lowers the patients' quality of life. Multiple interacting mechanisms lie behind IBS aetiology<sup>[2,3]</sup>. These include psychological stress and disturbances, physiological features, such as altered GI motility and visceral hypersensitivity, low-grade inflammation, and bacterial gastroenteritis.

The possible role of the GI microbiota in IBS aetiology (for review, see Parkes *et al.*<sup>[4]</sup>) is supported by low-grade mucosal inflammation in the GI tract of IBS patients<sup>[5,6]</sup>, onset of GI symptoms after a gastroenteritis (generating a subset of patients diagnosed with post-infectious IBS<sup>[7,8]</sup>), and observations suggesting the presence of altered GI microbiota in IBS<sup>[9-12]</sup>. Recently, Gecse *et al.*<sup>[13]</sup> associated the elevated level of non-endogenous colonic serine protease in diarrhoea-predominant IBS patients with increased mucosal permeability and subsequent visceral hypersensitivity. The detected increase in the level of colonic serine protease was suggested to originate from intestinal bacteria. In addition, antibodies to bacterial flagellins A4-Fla2 and Fla-X associated with the *Clostridium* cluster XIVa are elevated in IBS compared to healthy controls<sup>[14]</sup>. The potential role of GI microbiota in IBS is further supported by studies where probiotics have alleviated IBS symptoms (for a review, see Spiller *et al.*<sup>[15]</sup>). In the recent study of Kajander *et al.*<sup>[16]</sup>, a multispecies probiotic was also shown to stabilize the gut microbiota, but the microbial alterations were not specified.

16S ribosomal acid (rRNA) gene based methods have identified almost 900 bacterial phylotypes in the human GI tract with, of which only 18% represent cultured species<sup>[17]</sup>. Richness estimates within an individual's colon extend to 300 phylotypes<sup>[18]</sup>, while a vast variation is introduced by disparities in the phylotype composition between individuals<sup>[18-20]</sup>. The main phyla found in 16S rRNA gene sequencing based studies are *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, and *Verrucomicrobia*<sup>[18,21-23]</sup>.

Using culture-based techniques, the GI microbiota of IBS patients has been characterized to have less lactobacilli and bifidobacteria and an elevated amount of aerobes relative to anaerobes<sup>[24-26]</sup>. Specific divergences have been observed with quantitative real-time polymerase chain reaction (qPCR) assays targeting *Lactobacillus* spp, *Veillonella* spp, *Bifidobacterium* spp, *Clostridium coccoides*, and *Bifidobacterium catenulatum*<sup>[10]</sup>, and with 16S rRNA cloned sequence-based assays targeting phylotypes within the genera *Coprococcus*, *Collinsella*, and *Coproccillus*<sup>[11]</sup>. With a 16S rRNA gene-based phylogenetic microarray analysis targeting over a 1000 human intestinal phylotypes, the faecal microbiota of IBS patients and control subjects could be distinguished by hierarchical cluster analysis and stronger variation in the composition of the microbiota was seen in the IBS patients' profiles<sup>[12]</sup>. Furthermore, a higher degree of temporal instability among IBS patients has been detected with ribosomal RNA-based denaturing gradient gel electrophoresis<sup>[9]</sup>. Mucosal bacteria have also been found to be more abundant in IBS patients than in healthy controls<sup>[27]</sup>.

In this study, we applied a set of eight novel and six previously published<sup>[11]</sup> qPCR assays to the analysis of faecal samples obtained from IBS patients and healthy controls to detect possible aberrations in the GI microbiotas of IBS patients. The design of the novel qPCR assays was based on comparing the 16S rRNA clone libraries of IBS patients and healthy controls, but in this study three time-points per subject during a 6-mo survey were analysed instead of one<sup>[11]</sup>.

## MATERIALS AND METHODS

### Subjects and study design

Faecal samples were collected from 20 IBS patients and 15 healthy control subjects (Table 1) at time-points 0, 3 and 6 mo of a 6-mo follow-up period.

The IBS patients were recruited by experienced physicians and fulfilled the Rome II criteria<sup>[28]</sup>, except for three subjects who reported slightly less than 12 wk of abdominal pain during the preceding year<sup>[29]</sup>. All patients had undergone clinical investigation and endoscopy or barium enema of the GI tract less than a year prior to the study. Exclusion criteria included pregnancy, lactation, organic intestinal disease, other severe systematic disease, antimicrobial medication during the previous 2 mo, previous major or complicated abdominal surgery, severe endometriosis and dementia or otherwise inadequate cooperation capability. Patients with lactose intolerance

**Table 1** Characteristics of IBS patients and control subjects

	IBS-D	IBS-C	IBS-M	Controls
<i>n</i>	8	8	4	15
Age (yr): mean (range)	43.6 (26-60)	48.6 (24-64)	50.8 (31-62)	47 (25-64)
Gender (female/male)	4/4	9/1	3/1	10/5
Predominant bowel habit	Diarrhoea	Constipation	Mixed-type	-

IBS: Irritable bowel syndrome; IBS-D: Diarrhoea-predominant IBS; IBS-C: Constipation-predominant IBS; IBS-M: Mixed symptom-subtype IBS.

were included if they were reported to follow a low-lactose or lactose-free diet. All IBS patients were advised not to make any changes to their medication, including ongoing IBS medication (mainly commercial fibre analogues, laxatives, or antidiarrhoeals). The IBS patients formed the placebo group of a 6-mo probiotic intervention study<sup>[29]</sup>. They received daily a placebo capsule consisting of microcrystalline cellulose, magnesium stearate, and gelatine as the encapsulating material. Consumption of probiotic products was not allowed during the study.

Control subjects devoid of GI symptoms were also recruited and originally age- and gender-matched with the IBS patients as a whole<sup>[26]</sup>. Volunteers with regular intestinal disturbances, lactose intolerance, celiac disease, or antibiotic therapy during the preceding 2 mo of the study were excluded. The faecal samples of the controls and IBS subjects<sup>[9-11,26,29,30]</sup> have been studied previously. The novelty in the present study resides in the eight previously unpublished 16S rRNA phylotype targeting assays, the analysis of several time-points during the 6-mo survey, and in the in-depth statistical analysis of the results.

### Ethics

All participants gave their written informed consent and were told that they could withdraw from the study at any time. The Human Ethics Committee of the Joint Authority for the Hospital District of Helsinki and Uusimaa (HUS) approved the study protocol for the IBS patients. The ethical committee of the Technical Research Centre of Finland (VTT) approved the study protocol for the healthy controls.

### Extraction and purification of DNA from faecal samples

Faecal samples were preserved anaerobically immediately after defecation, stirred and aliquoted, and stored at -70°C within 4 h of delivery. For qPCR analysis, total DNA was isolated from 1 g of faecal material according to Apajalahti *et al.*<sup>[31]</sup>, which included removing the undigested particles from the faecal material by three rounds of low-speed (200 × *g*) centrifugation and collection of the bacterial cells with high-speed centrifugation (30 000 × *g*) at 15°C for 15 min using a Beckman Avanti<sup>TM</sup> centrifuge (Fullerton, CA, USA) with the rotor JA 25.50 or JLA 16.250 rotor, respectively. The bacterial cells were lysed after centrifugation with a combination of freeze-thaw

cycles (freezing for 1 h at -70°C and thawing for 15 min in a 37°C water bath), lysozyme and vortexing with glass beads. DNA concentrations were determined with a NanoDrop ND-1000 Spectrophotometer (NanoDrop products, Wilmington, DE, USA).

### Design of qPCR assays

Divergences detected by comparing the sequence data of 16S rRNA gene clone libraries of healthy controls and symptomatically sub-grouped IBS patients (diarrhoea-predominant IBS, IBS-D; constipation-predominant IBS, IBS-C; and mixed symptom-subtype IBS, IBS-M) were used as the basis for selection of qPCR targets<sup>[11]</sup>. Prior to cloning and sequencing, the faecal microbial genomes had been profiled and fractioned on the basis of genomic guanine-plus-cytosine content<sup>[11]</sup>. Partial 16S rRNA gene sequences encompassing the variable regions V1 and V2 combined from all four sample types were aligned using either the version Beta 2003-08-22 of ARB<sup>[11,32]</sup> or ClustalW 1.83<sup>[33]</sup>. For the ARB alignment, an aligned sequence database (ssu\_jan04\_corr\_opt.arb) was downloaded from the ARB home page (<http://www.arb-home.de>) and the in-house sequences<sup>[11]</sup> were aligned using the ARB-EDIT FastAlign function, followed by manual correction of the alignments with special attention to the ends of the sequences. Finally, the sequences were imported into an existing tree file of the database (Tree-Bacteria) by filtering the data against a sequence of similar length as the imported partial 16S rRNA gene sequences. Regions of the tree where sequences derived from one subject group (healthy *vs* IBS or healthy *vs* IBS subtypes) dominated over the other groups were considered as potentially interesting. In addition, a ClustalW 1.83 alignment (FAST DNA pairwise alignment algorithm option, gap penalty 3, word size 4, number of top diagonals 1 and window size 1) was constructed covering approximately 450 bp from the 5' end of the 16S rRNA gene and visually inspected and cut from the *Escherichia coli* position 430 (universally conserved GTAAA) with BioEdit version 7.0.5.3<sup>[34]</sup>. Distance matrices were calculated from the ClustalW alignment with Phylip 3.66 Dnadist<sup>[35]</sup> using Jukes-Cantor correction. The distribution of sequences into operative taxonomic units (OTUs) was determined using DOTUR<sup>[36]</sup> by applying the furthest neighbour rule option and 98% cut-off for sequence similarity. Uneven distribution of sequences originating from the different sample types within an OTU was used as criteria for qPCR target selection.

Potential primer target sites for specific quantitative analyses were assessed manually from ClustalW 1.83 alignments. Primer 3 online interface<sup>[37]</sup> and mfold 3.3 DNA-folding servers<sup>[38]</sup> were used for optimizing the final primer sequences and secondary structure analyses. The primer specificity against publicly available prokaryotic 16S rRNA sequences was checked with FASTA<sup>[39]</sup> provided by the European Bioinformatics Institute (<http://www.ebi.ac.uk/>) and against in-house 16S rRNA clone library sequences of human faecal origin, using the blastall option of Parallel BLAST<sup>[40]</sup> with Corona hardware

(<http://corona.csc.fi>) maintained by the Finnish IT Center for Science (CSC - Scientific Computing Ltd., Finland). The qPCR primers were synthesized commercially by Oligomer Oy (Helsinki, Finland). The clone sequences used to generate the standard curve in each qPCR assay were classified using The Ribosomal Database Project II Classifier<sup>[41]</sup>. The assays were named according to the most similar 16S rRNA gene sequence of a cultured bacterial species with the similarity percentages below 98% indicated.

### qPCR optimization and conditions

For each assay, the optimal annealing temperature and MgCl<sub>2</sub> concentration were defined using the iCycler iQ Real-Time Detection System (Bio-Rad, Hercules, CA, USA) associated with the iCycler Optical System Interface software (version 2.3; Bio-Rad). Actual samples were run as triplicates with optimized reaction conditions using SYBR Green I chemistry and 25 ng (specific phylotype targeting assays) or 0.5 ng (universal 16S rRNA gene assay) of faecal bacterial DNA. For all assays, the samples were run with different sample groups randomly mixed in the individual runs to minimize the effect of technical deviation between runs. Amplified clonal 16S rRNA genes were used as standards, ranging from 10<sup>2</sup> to 10<sup>7</sup> gene copies per reaction. The reaction mixtures consisted of a 1:75000 dilution of SYBR Green I (Lonza biosciences, Basel, Switzerland), 10 mmol/L Tris-HCl (pH 8.8), 50 mmol/L KCl, 0.1 % Triton X-100, 2-5 mmol/L MgCl<sub>2</sub>, 100 μmol/L each dNTP, 0.5 μmol/L each primer, 0.024 U Dynazyme II polymerase (Finzymes, Espoo, Finland) and 5 μL of either template or water. The amplification involved one cycle at 95°C for 5 min for initial denaturation, followed by 40 cycles of denaturation at 95°C for 20 s, primer annealing at the defined optimal temperatures for 20 s, extension at 72°C for 30 s and a fluorescence detection step at 80-89°C for 30 s. The specificity of the qPCR assays was checked with a reassociation curve analysis after amplification by slow cooling from 95°C to 60°C, with fluorescence collection at 0.3°C intervals for 10 s at each decrement. The qPCR efficiencies were calculated from the standard curves using the equation  $E = (10^{1/k}) - 1$ , where  $E$  and  $k$  stand for efficiency and slope, respectively.

### Statistical analysis

In the raw data from qPCR assays, microbe groups with low abundance were occasionally undetected (below qPCR detection limit). These values may not be truly zero or missing values, but are caused by limitations in the technical accuracy of the qPCR equipment. Therefore, for data analysis, zeros and missing values were imputed with the mean values obtained from the qPCR runs with the same primer pair applied to molecular grade water. If those too were undetected, the minimum of all the detected water runs was used.

After imputing the undetected values, the raw data was transformed to log<sub>10</sub> ratios of relative amount of 16S rRNA gene copies detected *vs* the amount of bacterial

16S rRNA gene copies detected with the universal qPCR assay. Using the ratio will, to some extent, control the sample specific variation due to lab procedures and sample handling affecting the overall bacterial concentration. All the statistical analyses were carried out with these values.

Statistical analyses were made with standard mixed-effect linear models having fixed effects for the time, and the IBS subtype, and a random effect for individual (taking into account the repeated measures from the same subject). In summary, this set up results in a repeated-measures ANOVA-type modelling of the data.

The model selection between whether to use the full model with interaction term between time and group and the age term, or the simpler model without interaction and the age was based on  $F$ -tests. The inference from the estimated models was based on the standard  $F$ -tests and  $t$ -tests.

For multivariate analysis of the data, principal component analysis (PCA) was used to visualize the data sets. Linear mixed-effects models were also applied to the first four principal component scores to quantify potential multivariate effects present in the data.

All the analyses were made with statistical programming language R 2.6.2<sup>[42]</sup> utilizing the package *lme* for mixed-effects linear models<sup>[43]</sup> and contrast for computing the contrasts.

## RESULTS

### Design and optimization of qPCR assays

A total of 14 qPCR assays were designed and optimized (Table 2) for analyzing alterations in the faecal microbiotas of IBS patients sub-grouped according to symptom subtype and healthy controls. The optimized annealing and detection temperatures ranged from 60°C to 67°C and 80°C to 89°C, respectively. For the universal assay, an annealing temperature of 50°C was used. The PCR efficiencies for the optimized qPCR reactions were above 80% with the exception of *Collinsella aerofaciens*-like, *Coprococcus eutactus* 97% and *Spiroplasma chinense* 84% assays.

Non-specific product peaks with a lower melting temperature than the desired product were observed for some of the faecal DNA samples in the reassociation analyses of several assays (*Bacteroides intestinalis*-like, *Butyrivibrio crossotus*-like, *Clostridium coeleatum* 88%, *C. eutactus* 97%, *S. chinense* 84%, *Ruminococcus torques* 91%, *R. torques* 94% and *Slackia faecicanis* 91%). The fluorescence detection temperatures in these assays were set above the melting point of the unspecific products to avoid detecting them.

### Analysis of faecal samples

The log<sub>10</sub> number of bacterial 16S rRNA gene copies detected ranged from 11.71 to 11.93 per gram of faeces (wet weight) and the average relative log<sub>10</sub> numbers of 16S rRNA gene copies detected with phylotype targeting assays in proportion to the universal bacterial assay ranged from -7.34 to -0.72 (Table 3; time-point specific averages are presented in Supplementary Table 1). Target

Table 2 Phylum level classification, primers and assay conditions of qPCR assays

qPCR assay (Phylum)	Primers (5'→3')	Standard	Target size (bp)	MgCl <sub>2</sub> (mmol/L)	Annealing T (°C)	Detection T (°C)	Average PCR efficiency ± SD
<i>Bacteroides intestinalis</i> -like (Bacteroidetes)	F: AGCATGACCTAGCAATAGGTT R: CCTTCTCGTATATCTATCCGGTAT	AM277809	124	3	63	83	87 ± 6
<i>Bifidobacterium catenulatum</i> / <i>Bifidobacterium pseudocatenulatum</i> -like <sup>[11]</sup> (Actinobacteria)	F: ACTCCTCGCATGGGGTGTG R: CCGAAGGCTTGCTCCCGAT	AM277149	275	3	68	87	90 ± 8
<i>Butyrivibrio crossotus</i> -like (Firmicutes)	F: TGCTAATACCGCATAAAAACAGCAGA R: CGCTGGATCAGGCTTTTCG	AM275497	232	4	63	85	82 ± 5
<i>Clostridium cocleatum</i> 88% <sup>[11]</sup> (Firmicutes)	F: AATACATAAGTAACCTGGCRTC R: CGTAGCACTTTTCATATAGAGTT	AM276544	104	4	60	80	88 ± 10
<i>Clostridium thermosuccinogenes</i> 85% (Firmicutes)	F: ACATGCAAGTCGAAACGGAAGTC R: TGCCTCAGAGTTTCTCCATIG	AM275406	373	2	62	81	88 ± 8
<i>Collinsella aerofaciens</i> -like <sup>[11]</sup> (Actinobacteria)	F: CCCGACGGGAGGGGAT R: CTCTGCAGGTACAGTCTTGAC	AM276090	260	4	67	89	75 ± 3
<i>Coprobacillus cateniformis</i> 91% (Firmicutes)	F: CGGACGCGATGCTTCT(A/G)GC R: AACATATCTCCCATGCGGTIG	AM275478	133	4	62	82	93 ± 8
<i>Coprococcus eutactus</i> 97% <sup>[11]</sup> (Firmicutes)	F: AGCTTGCTCCGGCYGATTTA R: CGGTTTTACCAGTCGTTCCAA	AM275825	97	2	63	83	73 ± 5
<i>Ruminococcus bromii</i> -like (Firmicutes)	F: CGAACGGAACGTGTTTGAAAGA R: CAAAACCATGTGGTCCGATAT	AM275413	156	4	62	81	97 ± 6
<i>Ruminococcus torques</i> 91% <sup>[11]</sup> (Firmicutes)	F: TGCTTAACGTATCTTCTTCGGA R: CGGTATTAGCAGTCATTTCTG	AM276624	119	5	62	82	88 ± 5
<i>R. torques</i> 93% <sup>1</sup> (Firmicutes)	F: GACTGCTTTTGAAACTGTCA R: AGGTCGGTTAAGGA	AM275798	396	4	61	83	85 ± 4
<i>R. torques</i> 94% <sup>[11]</sup> (Firmicutes)	F: AATCTTCGGAGGAAGAGGACA R: AACTACACCATGCGGTCCT	AM275522	137	2	65	85	81 ± 6
<i>Slackia faecicanis</i> 91% (Actinobacteria)	F: GAGTAACGCGTGACCGACCTT R: CCCGGAGTACCCGGTATCA	AM276086	75	4	64	86	90 ± 5
<i>Spiroplasma chinense</i> 84% (Firmicutes)	F: ATGGCCCAGTGAAGGTTIG R: CCCAACGAAAAGGTAGGTCA	AM275518	101	4	66	83	79 ± 4
Universal <sup>[61]</sup>	F: TCCTACGGGAGGCAGCAGT R: GGACTACCAGGTATCTAATCCTGTT	<i>B. longum</i> <sup>2</sup>	466	3	50	80	92 ± 6

<sup>1</sup>For the *R. torques* 93% assay the sequence AY305319<sup>[62]</sup> was used for primer design; <sup>2</sup>The *Bifidobacterium longum* DSM 20219T 16S rRNA gene was used as standard in the universal qPCR assay. qPCR: Quantitative real-time polymerase chain reaction.

Table 3 The average relative log<sub>10</sub> amount of the 16S rRNA gene copies detected with qPCR assays in proportion to the universal qPCR results

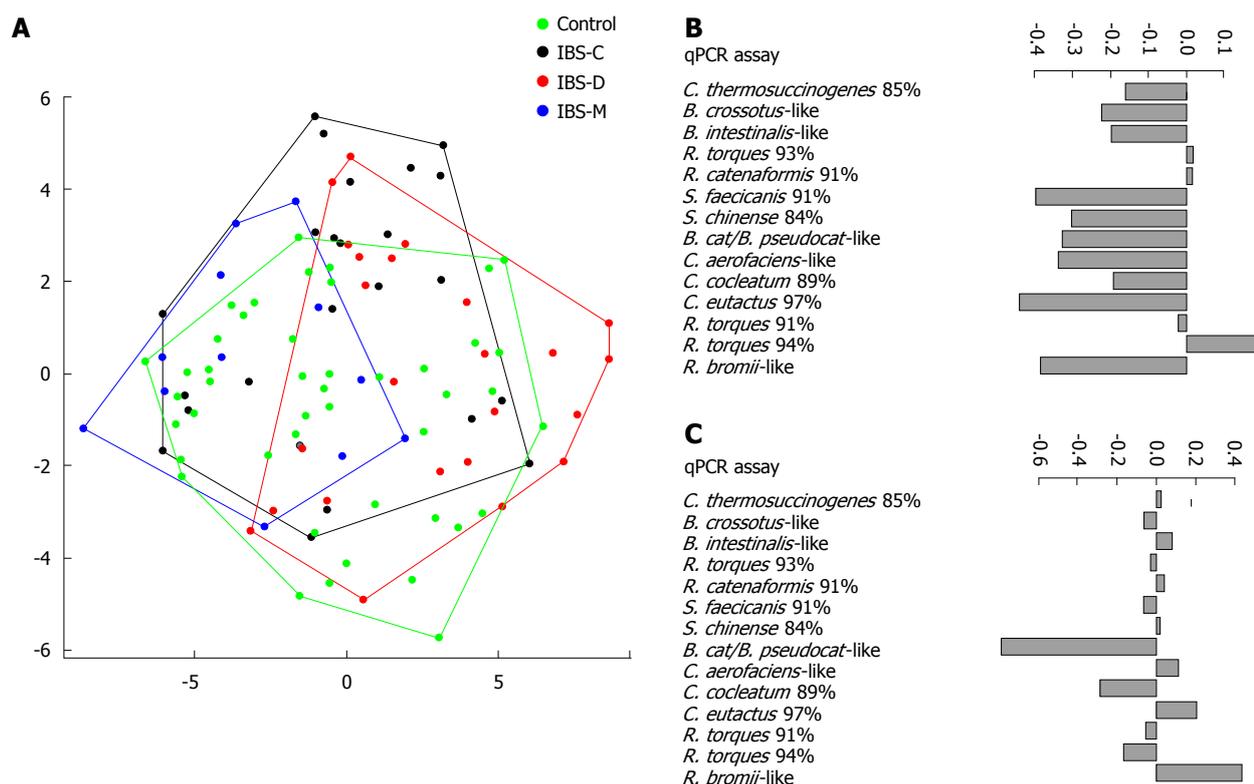
qPCR assay	Control (n = 15)	IBS-C (n = 8)	IBS-D (n = 8)	IBS-M (n = 4)
<i>Bacteroides intestinalis</i> -like	-4.85 ± 1.52 <sup>1</sup> (12)	-4.71 ± 1.42 (5)	-5.8 ± 1.32 (5)	-3.46 ± 1.26 (4)
<i>Bifidobacterium catenulatum</i> / <i>Bifidobacterium pseudocatenulatum</i> -like	-4.1 ± 2.22 (14)	-5.63 ± 2.52 (7)	-5.42 ± 2.63 (5)	-4.4 ± 2.54 (4)
<i>Butyrivibrio crossotus</i> -like	-6.2 ± 2.03 (8)	-6.5 ± 1.97 (3)	-7.34 ± 1.58 (0)	-6.04 ± 2.19 (2)
<i>Clostridium cocleatum</i> 88%	-1.7 ± 1.32 (15)	-2.36 ± 2.35 (8)	-2.69 ± 2.33 (8)	-0.72 ± 0.98 (4)
<i>Clostridium thermosuccinogenes</i> 85%	-3.33 ± 1.16 <sup>a</sup> (15)	-3.7 ± 0.84 (8)	-4.08 ± 0.90 <sup>ab</sup> (8)	-3.08 ± 1.38 <sup>b</sup> (4)
<i>Collinsella aerofaciens</i> -like	-2.45 ± 1.16 (15)	-2.9 ± 2.33 (7)	-4.63 ± 2.35 (7)	-1.73 ± 2.61 (4)
<i>Coprobacillus cateniformis</i> 91%	-4.72 ± 0.77 (15)	-4.41 ± 0.67 (8)	-4.79 ± 0.61 (8)	-4.71 ± 0.25 (4)
<i>Coprococcus eutactus</i> 97%	-5.44 ± 2.53 (9)	-5.91 ± 2.61 (3)	-6.55 ± 2.28 (2)	-4.09 ± 2.69 (3)
<i>Ruminococcus bromii</i> -like	-3.69 ± 2.42 <sup>c</sup> (15)	-1.61 ± 1.83 <sup>c</sup> (8)	-3.4 ± 2.49 (8)	-2.08 ± 1.56 (4)
<i>Ruminococcus torques</i> 91%	-3.13 ± 0.77 (15)	-2.87 ± 1.10 (8)	-2.58 ± 1.09 (8)	-2.83 ± 1.26 (4)
<i>R. torques</i> 93%	-2.41 ± 0.53 <sup>d</sup> (15)	-2.61 ± 0.72 (8)	-2.65 ± 0.59 (8)	-2.92 ± 0.56 <sup>d</sup> (4)
<i>R. torques</i> 94%	-4.02 ± 1.63 <sup>c</sup> (14)	-3.39 ± 1.40 (8)	-2.43 ± 1.49 <sup>c</sup> (8)	-3.82 ± 2.16 (3)
<i>Slackia faecicanis</i> 91%	-5.53 ± 2.26 (8)	-5.6 ± 2.33 (4)	-6.22 ± 2.16 (3)	-4.01 ± 2.28 (4)
<i>Spiroplasma chinense</i> 84%	-5.62 ± 2.04 (9)	-5.36 ± 2.20 (5)	-6.51 ± 1.98 (2)	-5.7 ± 2.22 (2)

The number of subjects with target 16S rRNA gene copies detected above the calculated threshold value in any of the three samples analysed are given in parentheses. <sup>1</sup>Values are presented as averages of log<sub>10</sub>-values ± SD from three time-points (0, 3 and 6 mo). <sup>a</sup>P = 0.04, <sup>b</sup>P = 0.05, <sup>c</sup>P = 0.01, <sup>d</sup>P = 0.00.

bacterial phylotypes were detected from all samples with the *C. cocleatum* 88%, *Coprobacillus cateniformis* 91%, *Clostridium thermosuccinogenes* 85%, *Ruminococcus bromii*-like, *R. torques* 91%, and *R. torques* 93% assays (Table 3).

#### Divergences in the intestinal microbiota in IBS

In a PCA of the 14 phylotype targeting assays and three time-points (0, 3 and 6 mo), the IBS-D group differed from the control group (*P* = 0.01), IBS-M (*P* = 0.00),



**Figure 1** Principal component analysis (PCA) of fourteen 16S rRNA phylotypes quantified from faecal samples of irritable bowel syndrome (IBS) patients and healthy volunteers. **A:** The PCA plot with outermost data points within each sample group is outlined. The control samples are presented in green, the constipation-predominant IBS (IBS-C) in black, the diarrhoea-predominant IBS (IBS-D) in red and the mixed symptom-subtype IBS (IBS-M) in blue. Each time-point is presented as a separate point. To quantify the multivariate differences between the groups, linear mixed-effects models were applied to the first (x-axis) and the second (y-axis) principal component scores, which represent the dominant multivariate changes present in the data; **B:** The bars represent the relative contribution of each quantitative real-time PCR (qPCR) assay to the principal component 1 (PC1). On PC1 the IBS-D samples differed from the control ( $P \leq 0.01$ ), IBS-M ( $P \leq 0.01$ ), and IBS-C ( $P \leq 0.05$ ) samples; **C:** The bars represent the relative contribution of each qPCR assay to the principal component 2 (PC2). On PC2, the IBS-C patients diverged from the control subjects ( $P \leq 0.05$ ) and time-points. In addition, the second time-point (3 mo) diverged significantly from the first (0 mo,  $P \leq 0.01$ ) and the third (6 mo,  $P \leq 0.01$ ) time-points independent of sample group; The height of the bars in graphs in Figure 1B and C reflect the relative magnitude of the contribution and the direction the sign of the contribution (in relation to the other assays and to the axis in Figure 1A). For example, in PC1 (Figure 1B), the largest contributor is the *Coprococcus eutactus* 97% phylotype, while the samples on the right in Figure 1A (mostly IBS-D) tend to have higher concentrations of *Ruminococcus torques* 94% and lower concentrations of phylotypes with bars highly on the negative side. Similarly, on PC2 (Figure 1C) the samples with high PC2 value in the top part of the Figure 1A tend to have higher concentrations of the *Ruminococcus bromii*-like phylotype, and lower concentrations of the *Bifidobacterium catenulatum/Bifidobacterium pseudocatenulatum*-like phylotype. On PC2, the IBS-C patients diverged from the control subjects ( $P \leq 0.05$ ) and time-points. In addition, the second time-point (3 mo) diverged significantly from the first (0 mo,  $P \leq 0.01$ ) and the third (6 mo,  $P \leq 0.01$ ) time-points independent of sample group. qPCR: Quantitative real-time polymerase chain reaction; IBS-C: Constipation-predominant irritable bowel syndrome; IBS-D: Diarrhoea-predominant irritable bowel syndrome; IBS-M: Mixed-subtype irritable bowel syndrome.

and IBS-C ( $P = 0.05$ ) on the first principal component (PC1; Figure 1A and B). The *R. torques* 94% phylotype was unique in being more predominant in IBS-D (Figure 1A and B). On the second principal component (PC2), the IBS-C patients diverged from the control subjects ( $P = 0.03$ ; Figure 1A and C). Time-points were significantly different on PC1 and PC2 (data not shown).

Quantities of *C. thermosuccinogenes* 85%, *R. bromii*-like, *R. torques* 93%, and *R. torques* 94% phylotypes diverged between different IBS symptom subtypes and healthy subjects independent of the effect of time (Table 3). Relatively high levels of the *C. thermosuccinogenes* 85% phylotype were associated with IBS-M patients and control subjects compared with IBS-D patients. The relative amount of *C. thermosuccinogenes* 85% 16S rRNA gene copies detected in proportion to the universal assay were 0.08%, 0.05%, and  $< 0.01\%$  for the IBS-M, control, and IBS-D subjects, respectively. The *R. bromii*-like

phylotype was significantly ( $P = 0.01$ ) more abundant in the IBS-C (relative abundance 2.45%) than in the control (relative abundance 0.02%) subjects' samples and the *R. torques* 93% phylotype was significantly ( $P = 0.00$ ) more abundant in the control (relative abundance 0.39%) than in the IBS-M subjects' samples (relative abundance 0.12%). The lowest amount of *R. torques* 94% phylotypes was quantified in the control samples (relative abundance  $< 0.01\%$ ) significantly differing ( $P = 0.01$ ) from the relative amount detected among the IBS-D patients' samples (relative abundance 0.37%).

Additional time-point dependent divergences between the sample groups were also detected (Supplementary Table 1): The *B. intestinalis*-like and *C. cocleatum* 88% phylotypes were relatively abundant in the IBS-M and control samples, and were detected in lower amounts in the IBS-D patients' samples. The relative amounts of *C. aerofaciens*-like phylotype detected were lowest in samples of the IBS-D patients, whereas the relative

amounts of *R. torques* 91% phylotype were lowest in the control subjects' samples.

## DISCUSSION

The aim of this study was to test the capability of a set of qPCR assays targeting the 16S rRNA gene on a phylotype level to differentiate between IBS symptom subtypes and healthy controls. Eight novel and six previously published<sup>[11]</sup> qPCR assays were used to study faecal samples of 20 IBS patients grouped according to symptom subtype and 15 healthy controls at three time-points (0, 3 and 6 mo). None of the assays have previously been applied to samples from several time-points. The knowledge of putative alterations on phylotype level may be essential in association with health, as has been shown to be the case for *Faecalibacterium prausnitzii* in Crohn's disease<sup>[44,45]</sup>.

In our approach, the selection of faecal bacteria phylotypes for analysis was based on a comparison of clone sequence libraries of IBS patient symptom subtypes and healthy controls<sup>[11]</sup>. The amount of bacterial 16S rRNA genes detected with the universal qPCR was in accordance with previous findings<sup>[46]</sup>. Quantities relative to the amount of bacterial 16S rRNA gene copies detected with the universal bacterial qPCR assay were used in data analyses. The diarrhoea-predominant symptom subtype diverged significantly from the other IBS symptom subtypes and healthy controls in a PCA of all 14 qPCR analyses and three time-points (Figure 1). In addition, *C. thermosuccinogenes* 85%, *R. bromii*-like, *R. torques* 93%, and *R. torques* 94% phylotypes diverged between different IBS symptom subtypes and healthy controls independent of the time-point analysed (Table 3). According to the results presented here and in previous studies<sup>[10-12,47,48]</sup>, grouping of IBS patients based on their main symptom subtype is advisable in future studies.

The *C. thermosuccinogenes* 85% -phylotype represents an uncultured firmicute within the human GI microbiota. It was detected in significantly lower amounts in IBS-D patients' samples in comparison to healthy controls or IBS-C patients. The target sequence of the *C. thermosuccinogenes* 85% -assay has previously been found from human faecal samples in several studies<sup>[11,18,49]</sup> and from human mucosal biopsy samples taken from the caecum, descending and sigmoid colon, and the rectum<sup>[18]</sup>, implying that the phylotype truly represents a human intestinal bacterium. However, the closest isolated strain has negligible similarity according to the 16S rRNA sequence (85% similarity with *Ruminococcus* sp. 16442 strain 16S rRNA sequence).

The *R. bromii*-like phylotype was significantly more abundant in IBS-C patients than in healthy controls samples. *R. bromii* is a common starch degrader of the human intestinal microbiota<sup>[50]</sup>. The amounts of *R. bromii*-related phylotypes have been shown to increase with a diet high in resistant starch<sup>[51]</sup>. In the present study, the possible effect of diet could not be ruled out, but it is more likely that the slowed colonic transit in IBS-C, rather

than a dietary effect, results in a favourable environment for the *R. bromii*-like phylotype associated with IBS-C.

*Ruminococcus torques*, a resident mucin-degrading member of the human GI microbiota<sup>[52]</sup>, has been associated with the mucosa of Crohn's disease patients<sup>[53]</sup>. The specific target sequence of the *R. torques* 94% -assay applied in this study has been found from human faecal samples in several studies<sup>[19,54,55]</sup> and has also been associated with Crohn's disease<sup>[56]</sup>. In the present study, a comparatively higher abundance of *R. torques* 94% phylotype was linked with IBS-D in both the multivariate and assay specific analyses. The *R. torques* 91% phylotype was associated with IBS-D and IBS-M and the *R. torques* 93% phylotype was more abundant in IBS-M than in healthy controls. The target sequences of *R. torques* 91%, 93% and 94% are affiliated with *Lachnospiraceae* as is the 16S rRNA sequence of the strain A4 (DQ789118)<sup>[57]</sup> carrying the IBS associated flagellin Fla2<sup>[14]</sup>.

As a further support to our previous results<sup>[11]</sup>, a significantly lower abundance of the *C. aerofaciens*-like phylotype was associated with the IBS-C and IBS-D symptom subtypes at two of the time-points analysed. *Collinsella aerofaciens* (formerly *Eubacterium aerofaciens*) belongs to the order *Coriobacteriales* within the high G+C Gram-positive *Actinobacteria*. It is a prominent member of the endogenous human intestinal microbiota<sup>[58]</sup> and has previously been connected with a low risk of colon cancer<sup>[59]</sup>.

Significantly lower levels of several 16S rRNA gene phylotypes within the genus *Bacteroides* (*B. ovatus*, *B. uniformis*, and *B. vulgatus*) have previously been discovered among IBS-C patients in comparison to healthy controls, but no effect was seen with the *B. intestinalis*-like phylotype targeting probes<sup>[12]</sup>. All samples analysed in this study have previously been analysed with a *Bacteroides-Prevotella-Porphyrromonas* -group and a *B. fragilis* species-specific qPCR assay<sup>[10]</sup> without detecting any significant divergences. In this study, a *B. intestinalis*-like phylotype was quantified with qPCR and found to be least abundant in the IBS-D patient group and most abundant in the IBS-M patient group at the selected time-points. The seemingly contradictory results might be due to different specificities of the probes and primers used.

The qPCR assays presented here were based on a thorough analysis of IBS associated faecal bacterial 16S rRNA gene sequence data originating from the same samples and both the previously published partial 16S rRNA gene sequences. The qPCR assays detailed here will be valuable in upcoming IBS studies. A more thorough sequencing approach using novel high-throughput sequencing technologies<sup>[23]</sup> on IBS subjects' GI microbiota would be valuable in further investigating IBS-associated alterations within the GI microbiota.

The faecal microbiota of IBS patients has been associated with less temporal stability within individuals<sup>[47]</sup> and more variation between individuals<sup>[12]</sup> compared to that of the healthy controls. Therefore, the results of this study should be further confirmed with independent sample panels including both IBS subjects and healthy

controls. In addition, analyzing mucosal samples, in addition to luminal samples, would be of interest, since the mucosal and faecal microbiotas differ from each other<sup>[60]</sup>. Previously, IBS patients have been shown to have a slightly more abundant mucosal microbiota compared to that of healthy volunteers, but the difference was not statistically significant<sup>[27]</sup>. However, obtaining mucosal samples from IBS patients would require colonoscopy, which is not a regular procedure on IBS patients.

In conclusion, we observed alterations in the GI microbiota of IBS-D subjects with a multivariate analysis and several additional statistically significant differences were detected between the intestinal microbiotas of the different IBS subtypes and healthy controls in assay-specific analyses. Recovering the target bacteria of the *C. thermosuccinogenes* 85% and *R. torques* 94% qPCR assays would be essential for further analysis of their possible role in the human GI tract and their association to IBS. In the future, biomarkers associated to the GI microbiota could aid therapeutic trial follow-up, diagnosis and treatment of IBS patients.

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## COMMENTS

### Background

Irritable bowel syndrome (IBS) is a common gastrointestinal functional disorder that can greatly affect the patient's well being. Multiple interacting mechanisms, including alterations in the intestinal microbiota, are suspected to lie behind IBS aetiology.

### Research frontiers

Alterations in the gastrointestinal microbiota in association to health and disease have become an essential field of research in gastroenterology. For instance, indications of dysbiosis have been detected in relation to Crohn's disease. In this study, assays for analyzing phylotype specific bacterial alterations in association to IBS were developed and applied.

### Innovations and breakthroughs

The authors' results support the hypothesis of intestinal bacteria having a role in IBS, as significant phylotype specific alterations between the faecal microbiotas of IBS symptom subtype groups and healthy controls were detected. Furthermore, the results emphasize the importance of subgrouping IBS patients in future studies.

### Applications

An IBS-associated 16S ribosomal RNA (rRNA) gene sequence library data was used to design the real-time polymerase chain reaction (PCR) assays capable of differentiating IBS symptom subgroups and healthy controls in the test sample panel. The detected altering phylotypes might be useful as targets in diagnostic, therapeutic and host-microbe interaction studies.

### Terminology

The bacterial 16S rRNA gene is constructed from conserved and variable regions according to its phylogenetic origin. It enables the detection and quantification of microbes from environmental samples even when the bacteria cannot be cultivated. Real-time PCR targeting the 16S rRNA gene can be used to quantify bacterial subpopulations of 0.01% from faecal DNA samples.

### Peer review

The authors examined faecal bacterial phylotypes in eight diarrhea-predominant, eight constipation-predominant, four mixed symptom subtype IBS patients, and 15 control subjects with quantitative real-time polymerase chain

reaction assays. They found significant phylotype level alterations in the intestinal microbiotas of IBS patients.

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BRIEF ARTICLE

## Ketoprofen, peginterferon 2a and ribavirin for genotype 1 chronic hepatitis C: A phase II study

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### Abstract

**AIM:** To evaluate the safety of adding ketoprofen to pegylated-interferon (PEG-IFN) with or without ribavirin and the effect on viral kinetics, STAT1 activity and expression of 2'-5'-oligoadenylate synthetase (2'-5'OAS) in genotype 1 chronic hepatitis C in a phase II study.

**METHODS:** Forty-five patients were studied: fifteen were randomized to PEG-IFN plus ribavirin (PR), 16 to PEG-IFN plus ketoprofen and 14 to PR and ketoprofen. The

molecular study of IFN-dependent signal transduction was conducted in 9 patients from each group.

**RESULTS:** The combination of ketoprofen and PEG-IFN with or without ribavirin was safe and well tolerated. An early activation of STAT1 was observed in ketoprofen-treated patients, but this activation was less sustained over time. Conversely, ketoprofen plus PEG-IFN and ribavirin induced an early and sustained increase of 2'-5'OAS transcription starting 24 h after the first dose until the 36th wk. These data are consistent with the clinical results, showing a better sustained virological response and a lower relapse rate in patients receiving ketoprofen plus PEG-IFN and ribavirin.

**CONCLUSION:** The addition of ketoprofen to the standard therapy of chronic hepatitis C should be explored in larger randomized clinical studies.

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**Key words:** Liver; Viral hepatitis; Chronic hepatitis C; Clinical pharmacology; Non-steroidal antiinflammatory drugs

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### INTRODUCTION

The treatment of chronic hepatitis C virus (HCV) infection has dramatically improved over the last 20 years. Interferon  $\alpha$  (IFN $\alpha$ ) was one of the first agents used to treat this infection, but its efficacy in terms of sustained virological response (SVR, i.e. sustained absence of HCV from serum up to 6 mo after stopping therapy)

was extremely poor<sup>[1]</sup>. Strategies to overcome this limitation have included increasing the duration of therapy or adding ribavirin (an antiviral drug) to the treatment regimen<sup>[2,3]</sup>. The introduction of a “pegylated” preparation substantially increased the antiviral activity of IFN $\alpha$ . Today, pegylated-interferon (PEG-IFN) in combination with ribavirin is the standard treatment for patients with chronic hepatitis C<sup>[4]</sup>. Large international controlled clinical trials have demonstrated that this combination therapy yields SVR rates in 54%-63% of treated HCV-infected patients<sup>[5-7]</sup>. However, patients infected with HCV genotype 1 are particularly resistant to antiviral treatment, as demonstrated by the lower SVR rates observed in this patient subset, ranging from 42% to 52%<sup>[5-7]</sup>. Thus, a substantial proportion of patients remain unresponsive to antiviral treatment.

IFN signaling pathways are activated by binding of IFN to its specific receptor, which induces autophosphorylation of protein tyrosine kinases Tyk-2 and Jak-1 on tyrosine residues, thus activating signal transducer and activator of transcription (STAT1 and STAT2) proteins. Activated STATs translocate to the nucleus where they activate the transcription of IFN-inducible genes, such as 2'-5'-oligoadenylate synthetase (2'-5'-OAS)<sup>[8]</sup>. Non-steroidal antiinflammatory drugs (NSAIDs) have been demonstrated to amplify the IFN signaling pathways and to enhance the anti-viral effect of IFN<sup>[9-14]</sup>. Furthermore, it has recently been found that acetylsalicylic acid suppresses HCV expression in a hepatoma cell line containing HCV subgenomic replicon<sup>[15]</sup>. However, clinical studies evaluating the use of NSAIDs in combination with standard IFN $\alpha$  in patients with chronic HCV infection have given conflicting results<sup>[16-20]</sup>, although results with ketoprofen have generally been encouraging<sup>[17-21]</sup>.

A rational approach to combination therapies for patients with chronic HCV infection demands a detailed knowledge of how the different drugs affect viral kinetics and IFN intracellular signaling. Therefore, we conducted a pilot phase II study to evaluate the effect of ketoprofen plus PEG-IFN with or without ribavirin compared with PEG-IFN plus ribavirin (PR) on viral kinetics, STAT1 activity and expression of the IFN-dependent gene 2'-5'-OAS in patients with chronic hepatitis C. We also assessed the safety and tolerability of these treatment schedules. In order to minimize the influence of HCV viral variability on our results, only patients infected with HCV genotype 1 were included.

## MATERIALS AND METHODS

### Patients

Treatment-naïve patients aged 18 to 65 years with chronic HCV infection genotype 1a or 1b were eligible for enrollment if they had: elevated alanine aminotransferase (ALT) levels within the previous 6 mo, a positive test for serum HCV-RNA and a liver biopsy specimen consistent with chronic hepatitis C obtained in the previous 12 mo. Patients were excluded if they had neutropenia (neutrophil count  $< 1.5 \times 10^9$  cells/L), thrombocytopenia (platelet count  $< 70 \times 10^9$  cells/L), anemia (hemoglobin

level  $< 12.0$  g/dL in women and  $< 13.0$  g/dL in men) or a medical condition that would be clinically worsened by anemia, serum creatinine levels more than 1.5 times the upper limit of normal, evidence of liver disease due to causes other than chronic HCV infection, human immunodeficiency virus positivity, esophageal varices, decompensated liver disease, organ transplant, severe or poorly controlled psychiatric disease (especially depression), malignant neoplastic disease, severe cardiac or chronic pulmonary disease, history of peptic disease, autoimmune disease (except controlled thyroid disease), seizure disorder, alcohol or drug dependency within 1 year of study entry, clinically significant comorbid conditions; and, if female, pregnancy or unwilling to use contraception.

### Study design

This was an open-label, randomized, phase II, pilot study. Patients eligible for the study were randomized into three groups: (1) PR group: patients received subcutaneous PEG-IFN $\alpha$ 2a 180  $\mu$ g/wk plus oral ribavirin 800 mg/d for 48 wk; (2) PEG-IFN plus ketoprofen (PK) group: patients received subcutaneous PEG-IFN $\alpha$ 2a 180  $\mu$ g/wk for 48 wk plus oral ketoprofen 200 mg twice daily for the first 4 wk and then 200 mg/d for the next 20 wk; (3) PEG-IFN plus ribavirin and ketoprofen (PRK) group: patients received subcutaneous PEG-IFN $\alpha$ 2a 180  $\mu$ g/wk plus oral ribavirin 800 mg/d for 48 wk plus oral ketoprofen 200 mg twice daily for the first 4 wk and then 200 mg/d for the next 20 wk.

After 24 wk, treatment was withdrawn in patients who did not experience a decrease from baseline in viral load of  $\geq 2$  log<sub>10</sub> IU/mL or a negative qualitative serum HCV-RNA. A safety assessment was conducted in these patients between 4 and 8 wk after their last dose of study drug.

After the 48-wk study there was a 24-wk treatment-free follow-up period. Virological response was defined as a negative test for qualitative serum HCV-RNA. An end of treatment response (ETR) was defined as undetectable levels of HCV-RNA at the end of treatment (week 48) and SVR was defined as undetectable levels of HCV-RNA at the end of follow-up (24 wk after treatment cessation). Relapsers were defined as patients who obtained an ETR, but relapsed after completion of treatment and tested HCV-RNA positive at the end of follow-up.

PEG-IFN $\alpha$ 2a and ribavirin were kindly supplied by Roche S.p.A., Monza (MI), Italy and ketoprofen by IBI (Istituto Biochimico Italiano) S.p.A., Aprilia (LT), Italy. All subjects gave written informed consent before entering the study. The study protocol and patient-informed consent forms were approved by the Institutional Ethics Committee of Azienda Ospedaliero-Universitaria di Bologna, Policlinico S.Orsola-Malpighi, Bologna, Italy (registration number: 58/2002/U) and the study was conducted according to the ethical guidelines of the Declaration of Helsinki.

### Study procedures

All patients underwent liver biopsy within 12 mo before entry. Each liver biopsy was scored according to the

histological activity index proposed by Knodell *et al*<sup>[22]</sup>. After the screening evaluations, laboratory parameters were monitored and recorded at regular intervals throughout the 48-wk study period and a physical examination was conducted at the end of treatment. Adverse events and serious adverse events were recorded throughout the study and up to the end of follow-up period.

Serum samples were collected from each patient before treatment (on day -1), at baseline (day 0), at 24 and 48 h after the first dose and at 1, 2, 4, 12, 24, 36, 48, 60 and 72 wk after the beginning of treatment. Quantitative HCV-RNA serum levels were assessed in all samples using a branched DNA assay (Versant<sup>®</sup> RNA 3.0 assay; Siemens, Milano, Italy). Virological response was assessed on weeks 1, 2, 4, 12, 24, 36, 48 and 72 by means of Transcription Mediated Amplification technique (Versant<sup>®</sup> HCV-RNA Qualitative Assay; Siemens, Milano, Italy; low limit of detection: 6 HCV IU/mL).

### RNA extraction and real-time quantitative polymerase chain reaction (RTQ-PCR)

Peripheral blood mononuclear cells (PBMCs) were collected from patients at 0, 24 and 48 h and at 1, 2, 4 and 36 wk after the beginning of treatment. Total RNA was extracted from PBMCs using an "RNeasy Protect Mini Kit" (Qiagen, GmbH, Hilden, Germany). Full length cDNAs were synthesized from 1 µg total RNA using the "Thermoscript RT-PCR system" kit (Invitrogen), according to the manufacturer's instructions.

Comparative RTQ-PCR was performed with the "Platinum SYBR Green qPCR SuperMix-UDG" kit (Invitrogen) and analyzed on Mx3000P apparatus from Stratagene.

A 0.2 µmol/L concentration of the following primers was used: for 2'-5'OAS forward 5'-ATTGACAGTGCT GTTAACATCATCC-3' and reverse 5'-GTGAGTTATG GAACACGACGAG-3'; for GAPDH forward 5'-GAAG GTGAAGGTCGGAGTC-3' and reverse 5'-GAAGAT GGTGATGGGATTTTC-3'. RTQ-PCR conditions used to amplify 2'-5'OAS and GAPDH cDNAs were: 95°C for 5 min, followed by 40 cycles comprising 30 s at 95°C, 30 s at 60°C and 1 min at 72°C. In order to check for DNA contamination, amplification of total RNA before cDNA synthesis was performed in parallel with amplification of the cDNA. Reactions were run in triplicate, and a mean value of the three samples was calculated. 2'-5'OAS mRNA levels were expressed as the relative amount of product adjusted for the level of GAPDH, using the Mx3000P software (Stratagene) and employing a comparative Ct ( $\Delta\Delta C_t$ ) value method. Dissociation curves were generated to ensure that a single amplicon had been produced.

### Western blotting

PBMCs were lysed with Ripa buffer containing 50 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 0.1% Nonidet P-40, 1 mmol/L EDTA, 2 mmol/L phenylmethylsulfonyl fluoride, 1 µg/mL aprotinin, 1 µg/mL of leupeptin and phosphatase inhibitors. Protein concentrations were measured

Table 1 Baseline characteristics of enrolled patients (mean ± SD)

	PR group (n = 15)	PK group (n = 16)	PRK group (n = 14)	P-value
Age (yr)	45 ± 12	48 ± 12	42 ± 10	NS
Sex (M/F)	9/6	9/7	9/5	NS
HCV-RNA × 10 <sup>3</sup> IU/mL	429 ± 578	1261 ± 1073	1555 ± 1322	0.01
HCV-RNA > 700 × 10 <sup>3</sup> IU/mL (%)	4 (27)	8 (50)	10 (71)	0.02
ALT (U/L)	82 ± 45	85 ± 33	87 ± 49	NS
Median total HAI <sup>1</sup> (range)	8 (3-14)	10 (6-13)	8 (3-10)	NS
Median grading (range)	7 (2-11)	9 (5-10)	7 (3-9)	NS
Median fibrosis (range)	1 (1-3)	1 (1-3)	1 (0-3)	NS

<sup>1</sup>HAI: Histological activity index according to Knodell *et al*<sup>[22]</sup>. PR: PEG-IFN $\alpha$ 2a plus ribavirin; PK: PEG-IFN $\alpha$ 2a plus ketoprofen; PRK: PEG-IFN $\alpha$ 2a plus ribavirin and ketoprofen; M: Male; F: Female; HCV: Hepatitis C virus; ALT: Alanine aminotransferase; NS: Not significant.

using a colorimetric assay (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins were electrophoresed on SDS-PAGE. Proteins were transferred to PVDF membrane (Immobilon-PVDF, Millipore) and treated with 1:300 specific primary antibody anti-STAT1 (Santa Cruz Biotechnology, Inc., CA, USA) and its activated form (Biosource). After incubation with peroxidase-coupled secondary antibodies (Santa Cruz Biotechnology), the sheets were visualized by ECL kits (Amersham, GE Healthcare Life Science, Germany). Antibody anti-actin was purchased from Santa Cruz Biotechnology.

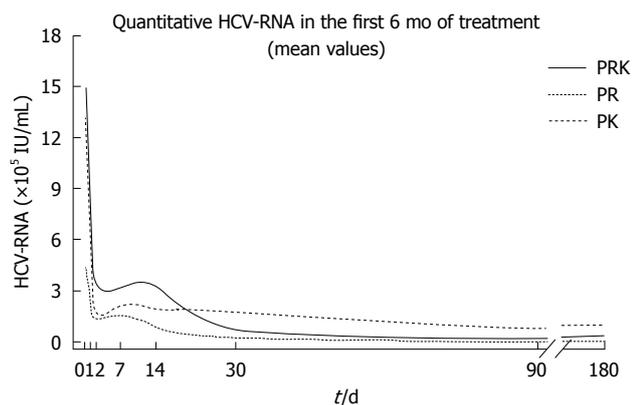
### Statistical analysis

This was a pilot study and therefore no formal sample size calculation was performed. Data were analyzed on an intention-to-treat basis. Patients who dropped out of the trial for any reason were classified as non responders. The safety population comprised all patients who received at least one dose of study drugs.  $\chi^2$  test and Mann-Whitney *U* test were used to compare quantitative and qualitative variables between the groups, respectively. Differences in virological response rates between treatment groups were analyzed using Fisher's exact test and/or Yates corrected  $\chi^2$  test, as necessary. A *P* < 0.05 was considered to be significant. Data analysis was carried out using the SPSS for Windows version 11.0.1. Plots of the viral load were carried out using SigmaPlot version 9.0.

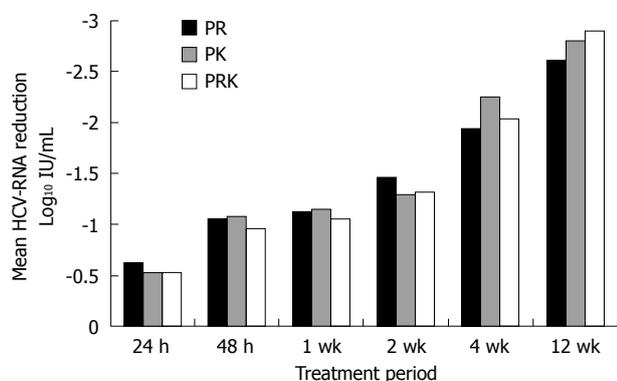
## RESULTS

Forty-five patients were enrolled: 15 were randomized to the PR group, 16 to the PK group and 14 to the PRK group.

The baseline characteristics of the three treatment groups are reported in Table 1. Patients were comparable with respect to age, gender, serum ALT levels and histological features. Overall, 4 out 15 (27%) of PR patients, 3 out 16 of PK (19%) and 2 out of 14 (14%) of PRK



**Figure 1** Mean hepatitis C virus (HCV)-RNA levels during the first 24 wk of treatment in patients with HCV genotype 1 infection receiving PEG-IFN $\alpha$ 2a plus ribavirin (PR) or PEG-IFN $\alpha$ 2a plus ketoprofen (PK) or PEG-IFN $\alpha$ 2a plus ribavirin and ketoprofen (PRK).



**Figure 2** Mean reduction from baseline in log<sub>10</sub> HCV-RNA levels in patients with HCV genotype 1 infection receiving PR or PK or PRK.

patients had a liver fibrosis score equal to 3. The viral load was significantly different between the three groups, being significantly lower in the PR group ( $P = 0.01$ ).

**Viral kinetics and antiviral activity**

Mathematical modeling of the decline in HCV-RNA serum levels revealed a triphasic response in all treatment groups (Figure 1). The rapid first phase (1-2 d) was similar in the three groups, although HCV-RNA levels were consistently higher in the PRK group during this time. A second, or “shoulder” phase was observed between 2 and 7-14 d, where the decline in HCV-RNA levels was faster in the PR group compared with both PK and PRK groups. In particular, the PR group showed a slight and progressive reduction of viremia over time after week 1. In the third phase (week 2 onwards), the decrease in HCV-RNA levels was slowest in the PK group while it was more rapid in the PRK group. In this latter group, HCV-RNA levels declined to those observed in the PR group by study end.

The mean log<sub>10</sub> reduction from baseline in HCV-RNA levels over the course of the first 12 wk of treatment is shown in Figure 2. At week 4, the mean log<sub>10</sub> decrease from baseline was comparable between the three groups (-1.95 in the PR group, -2.25 in the PK group and -2.05

**Table 2** Virological response rates by study group  $n$  (%)

	PR group ( $n = 15$ )	PK group ( $n = 16$ )	PRK group ( $n = 14$ )	$P$ -value
Virological response rate				
Week 1	0	0	1 (7.1)	NS
Week 2	1 (6.7)	1 (6.3)	2 (14.3)	NS
Week 4	5 (33.3)	2 (12.5)	3 (21.4)	NS
Week 12	10 (66.7)	7 (43.8)	7 (50)	NS
Week 24	11 (73.3)	11 (68.8)	10 (71.4)	NS
ETR	11 (73.3)	11 (68.8)	10 (71.4)	NS
Relapse rate	4/11 (36.4)	6/11 (54.5)	2/10 (20)	NS
SVR	7 (46.7)	5 (31.3)	8 (57.1)	NS

ETR: End of treatment response; SVR: Sustained virological response.

**Table 3** Baseline characteristics of the patients enrolled in the molecular study (mean  $\pm$  SD)  $n$  (%)

	PR group ( $n = 9$ )	PK group ( $n = 9$ )	PRK group ( $n = 9$ )	$P$ -value
Age (yr)	42 $\pm$ 13	54 $\pm$ 9	42 $\pm$ 12	0.04
Sex (M/F)	5/4	4/5	5/4	NS
HCV-RNA $\times 10^3$ IU/mL	263 $\pm$ 232	1032 $\pm$ 878	1742 $\pm$ 1392	0.01
HCV-RNA $> 700 \times 10^3$ IU/mL	1 (27)	4 (44)	7 (78)	0.02
ALT (U/L)	70 $\pm$ 21	80 $\pm$ 27	100 $\pm$ 57	NS
ETR	7 (78)	5 (56)	6 (67)	NS
SVR	5 (56)	3 (33)	5 (56)	NS

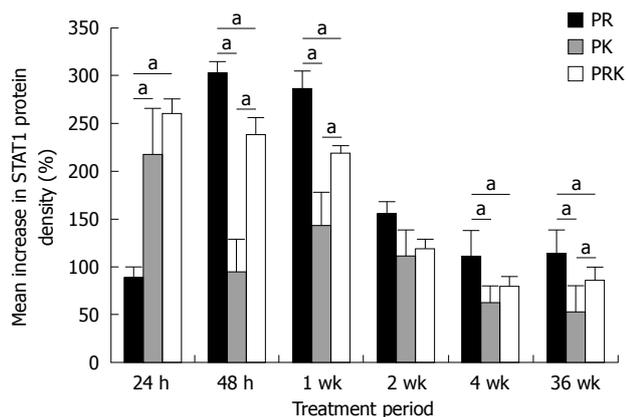
in the PRK group). As shown in Table 2, some patients had undetectable HCV-RNA levels as early as 7-14 d after starting treatment. The PR treatment group displayed an earlier virological response when compared with the other two groups (Table 2).

An ETR was obtained in 11/15 patients (73%) in the PR group, 11/16 (69%) in the PK group and 10/14 (71%) in the PRK group. During the treatment-free follow-up period, the relapse rate was lower in the PRK group than in both the other two groups, but the differences were not statistically significant. A SVR was obtained in 7 patients in the PR group (47%), in 5 in the PK group (31%) and in 8 in the PRK group (57%). No association was found between baseline viremia and SVR in any treatment group. However, in the subgroup of patients with high baseline viremia ( $> 700 \times 10^3$  IU/mL), 6/10 (60%) of the PRK group achieved a SVR compared to a quarter (25%) of the PR group and 3/8 (37.5%) of the PK group.

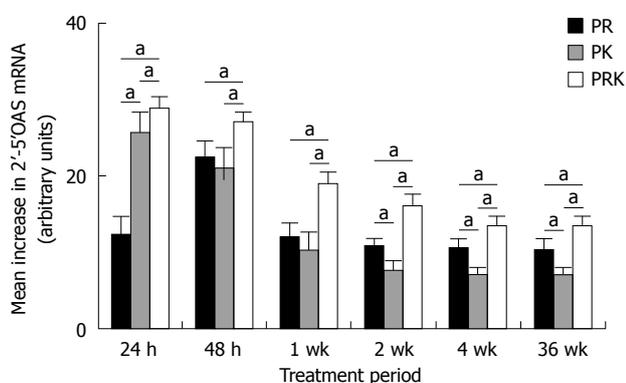
**Modulation of IFN signaling**

The first 9 randomized patients in each group were enrolled in the molecular study of IFN-dependent signal transduction. Their characteristics are described in Table 3.

A time course analysis of STAT1 activity over 36 wk by means of densitometric analysis was performed. STAT1 activity was consistently up-regulated, as shown by an increase from baseline in mean STAT1 density as early as 24 h after the first drug administration. Nevertheless, a difference between the three treatment groups can be seen in Figure 3. At 24 h, the increase from baseline in STAT1 density was significantly higher in both ketoprofen-



**Figure 3** Mean percentage increase from baseline in STAT1 protein density in patients with HCV genotype 1 infection receiving PR or PK or PRK. Data are presented as the mean ( $\pm$  SD) percentage increase over basal activity in STAT1, assessed by densitometric analysis. <sup>a</sup> $P < 0.05$ .



**Figure 4** 2'-5'-oligoadenylate synthetase (2'-5'OAS) induction in patients with HCV genotype 1 infection receiving PR or PK or PRK. Data are presented as the mean ( $\pm$  SD) fold increase over basal activity in 2'-5'OAS mRNA levels, assessed by RTQ-PCR. <sup>a</sup> $P < 0.05$ .

treated groups as compared with patients of the PR group. However, in PK patients STAT1 activation not only peaked earlier, but it also decreased more rapidly, while in the PRK group a strong STAT1 activation was still present after 1 wk. In contrast, in PR patients the activation of STAT1 peaked at 48 h, 24 h later than in both PK and PRK patients, but was still maintained at higher levels after 1 wk. A steady state was gained between 2 to 36 wk. Thus, the activation of STAT1 in the presence of ribavirin was slower and peaked later but was sustained longer as compared with that observed in the presence of ketoprofen. No relationship was found between STAT1 and viral load. There was no significant difference in STAT1 activity between patients with a SVR and non responders (data not shown).

The analysis of 2'-5'OAS mRNA levels by RTQ-PCR (Figure 4) showed that the presence of ketoprofen increased the transcription rate of this gene, especially early in treatment (24 h after the first dose). The addition of ketoprofen resulted in an early upregulation of 2'-5'OAS mRNA level and permitted the maintenance of this transcript at significantly higher levels in the PRK

**Table 4** Side effects occurring during 48 wk of treatment *n* (%)

	PR group ( <i>n</i> = 15)	PK group ( <i>n</i> = 16)	PRK group ( <i>n</i> = 14)	<i>P</i> -value
Anemia	8 (53)	7 (44)	11 (79)	NS
Neutropenia	9 (60)	7 (44)	10 (71)	NS
Arthro-myalgia	4 (27)	4 (25)	5 (36)	NS
Headache	3 (20)	3 (19)	2 (14)	NS
Fatigue	5 (33)	5 (31)	4 (29)	NS
Flu-like symptoms	4 (27)	4 (25)	2 (14)	NS
Psychiatric disorders	5 (33)	4 (25)	4 (29)	NS
Gastrointestinal disorders	6 (40)	3 (19)	4 (29)	NS
Pruritus	4 (27)	1 (6)	0	NS
Insomnia	2 (13)	2 (12.5)	2 (14)	NS

**Table 5** Time course evaluation of hemoglobin and creatinine serum levels during treatment (mean  $\pm$  SD)

	PR group ( <i>n</i> = 15)	PK group ( <i>n</i> = 16)	PRK group ( <i>n</i> = 14)
Hemoglobin (g/dL)			
Baseline	14.5 $\pm$ 1.3	14.2 $\pm$ 1.9	14.9 $\pm$ 1.5
Week 12	12.2 $\pm$ 1.4	12.5 $\pm$ 1.7	12.2 $\pm$ 1.6
Week 24	12.2 $\pm$ 1.6	12.6 $\pm$ 1.8	11.9 $\pm$ 1.7
Week 48	11.6 $\pm$ 1.4	13.1 $\pm$ 1.8	12.0 $\pm$ 1.6
Creatinine (mg/dL)			
Baseline	0.97 $\pm$ 0.13	0.82 $\pm$ 0.22	1.01 $\pm$ 0.07
Week 12	0.87 $\pm$ 0.11	0.82 $\pm$ 0.16	0.92 $\pm$ 0.12
Week 24	0.86 $\pm$ 0.15	0.79 $\pm$ 0.16	0.91 $\pm$ 0.15
Week 48	0.44 $\pm$ 0.15	0.80 $\pm$ 0.17	0.92 $\pm$ 0.15

group compared with the PR and PK groups, from the beginning of treatment until week 36. Finally, 2'-5'OAS mRNA levels were significantly higher in patients with a SVR than in non responders at each time point and in each group (data not shown).

### Safety

The type and frequency of adverse events were similar in the three treatment arms and are summarized in Table 4. Anemia, defined as hemoglobin level  $< 12.0$  g/dL in women and  $< 13.0$  g/dL in men, and neutropenia, defined as neutrophil count  $< 1000/\text{mm}^3$  were the most common side effects. However, only one patient in the PRK group met the hemoglobin criterion for ribavirin dose reduction (hemoglobin level  $< 10$  and  $\geq 8.5$  g/dL), while the dose of PEG-IFN was reduced because of neutropenia (neutrophil count of  $< 750$  and  $\geq 500/\text{mm}^3$ ) in one patient each in the PR and in PK group. The latter developed a pneumonia requiring antibiotic treatment. No patient experienced renal dysfunction. In particular, in no case did creatinine serum levels exceed the upper limit of normal range (1.2 mg/dL) during treatment. Time course evaluation of hemoglobin and creatinine serum levels during treatment in the three groups is reported in Table 5.

One patient each in the PR group and the PK group withdrew prematurely from the study. The patient in the PR group dropped out after 1 mo because of severe depression and the patient in the PK group dropped out after 6 mo because of poor compliance.

## DISCUSSION

The objective of this phase II study was to assess the safety of ketoprofen in combination with PEG-IFN $\alpha$ 2a with or without ribavirin in treatment-naïve patients with HCV genotype 1 infection, and the effect of these regimens on viral kinetics and IFN $\alpha$  signaling modulation. Our results showed that ketoprofen was safe and well tolerated. In particular, gastrointestinal-related adverse events were mild and did not lead to dose reduction or to premature treatment discontinuation in any patient. Given the importance of managing the tolerability of HCV antiviral treatment, our observation that the addition of ketoprofen to PEG-IFN and ribavirin is as well tolerated as the standard regimen is reassuring.

The kinetics of viral decay during treatment showed a triphasic response that was more evident in patients receiving ketoprofen, independent of the use of ribavirin. Following the first phase of rapid decline in HCV-RNA levels, patients receiving ketoprofen showed a pronounced “shoulder phase” starting 2 to 14 d after initiation of therapy. It has recently been suggested that the triphasic decline in HCV-RNA levels occurs only in patients in whom a majority of hepatocytes are infected before therapy<sup>[23]</sup>. Thus, the higher baseline viremia of both ketoprofen groups could help to explain the differences in viral kinetics between the groups receiving ketoprofen and the PR group. Interestingly, in the third and final phase, the HCV-RNA levels were lower in the two groups receiving ribavirin than in the PK group. This enhanced response to treatment in ribavirin recipients supports the hypothesis that ribavirin not only improves the anti-HCV immune response, but also had a mutagenic effect against HCV<sup>[23,24]</sup>. However, it should be pointed out that in both the PK and PRK groups, ketoprofen was administered only during the first 24 wk of treatment. Thus, the similarity between the PRK and PR groups in HCV-RNA levels during the final phase could be attributable to the absence of ketoprofen in the PRK group during this time.

As far as the IFN $\alpha$  signaling modulation is concerned, the activation of STAT1 occurred very early after treatment initiation in the two groups receiving ketoprofen, being evident after 24 h from the start of treatment. However, there was a rapid decline in STAT1 activation thereafter, particularly in the PK group. In contrast, the PR group exhibited the greatest activation after 48 h and this activation was more sustained over time compared with the ketoprofen-containing regimens. The mechanisms responsible for these differences in the control of IFN-induced responses probably include down-regulation and degradation of receptors<sup>[15]</sup>. Moreover, it has been demonstrated that both ribavirin and NSAIDs act synergistically with IFN- $\alpha$  in induction of STAT1 activation<sup>[13,25]</sup>, yet there was lower STAT1 activation in the PRK group compared with the PR group. Thus, an antagonistic effect between ketoprofen and ribavirin cannot be excluded.

On the other hand, our data demonstrated an early and sustained increase of 2'-5'OAS transcription in the PRK group compared with the PR group, suggesting that the addition of ketoprofen to the conventional combination therapy induces early activation of the IFN $\alpha$

pathway, followed by a better activation of the IFN $\alpha$ -dependent intracellular pathway.

Even if this study was not designed to assess antiviral efficacy, the clinical results are consistent with the molecular data. At baseline, the PR group had a significantly lower mean HCV viral load than both the PK and PRK groups. Thus, the proportion of patients with low viral load ( $\leq 700\,000$  IU/mL) was significantly higher in the PR group (73%) than in the PK (50%) or in the PRK group (29%). It is well known that in genotype 1 patients, baseline viral load is the best prognostic factor for response to antiviral treatment<sup>[5-7,26]</sup>. Thus, it was not surprising that patients in the PR group obtained a better virological response at week 4. Nevertheless, the SVR rate observed in the PRK group (57%) was better than that observed in PR (47%) and in PK (31%). Furthermore, among patients with high viral load ( $> 700\,000$  IU/mL), those of the PRK group obtained the better SVR (60% *vs* 25% and 38% in the PR and PK groups, respectively). It should be pointed out that our study was initiated before the optimal dose of ribavirin for patients with HCV genotype 1 (i.e. 1000 mg/d or 1200 mg/d according to body weight) was determined<sup>[7]</sup>. However, even if the dose of ribavirin utilized in this study was suboptimal and might have influenced the SVR, both groups receiving ribavirin had the same dose regimen.

In conclusion, considering that the use of ketoprofen does not add further side effects, is associated with better viral kinetics and early activation of the IFN signaling pathway, and in combination with PR improves virological response rates, this pilot study suggests the exploration of the clinical efficacy of this three-drug combination in well-designed randomized clinical trials. We conclude that such studies are warranted since, in this era of development of new drugs for HCV, the clinical use of novel compounds up till now has been hampered by toxicity issues and rapid promotion of drug-resistant HCV viruses<sup>[27]</sup>.

## COMMENTS

### Background

The current standard treatment for chronic hepatitis C with pegylated-interferon (PEG-IFN) and ribavirin is effective in approximately 50%-60% of patients, so that a substantial proportion of patients remain unresponsive. A rational approach to develop alternative therapeutic strategies for patients with chronic hepatitis C virus (HCV) infection demands a detailed knowledge of how the different drugs affect viral kinetics and IFN intracellular signaling. Non-steroidal antiinflammatory drugs (NSAIDs) have been demonstrated to amplify the IFN signaling pathways and to enhance the anti-viral effect of IFN. This phase II study evaluated the effect of ketoprofen (a NSAID) plus PEG-IFN with or without ribavirin compared with PEG-IFN plus ribavirin (PR) on viral kinetics, STAT1 activity and expression of the IFN-dependent gene, 2'-5'-oligoadenylate synthetase (2'-5'OAS), in patients with genotype 1 chronic hepatitis C.

### Research frontiers

The results of this pilot study support the proposal of an evaluation of the clinical efficacy of the addition of ketoprofen to the standard PR treatment for chronic hepatitis C in well-designed randomized clinical trials.

### Innovations and breakthroughs

This is the first study to report both molecular and clinical data about the use of ketoprofen in association with PEG-IFN $\alpha$  and ribavirin in chronic hepatitis C. The authors found that the addition of ketoprofen to the conventional combination therapy is associated with better viral kinetics and early activation of the IFN $\alpha$  signaling pathway, thus improving virological response rates.

## Applications

The results may stimulate further experimental and clinical investigations regarding the role of NSAIDs in association with IFN-based therapy in the context of HCV-related liver diseases.

## Terminology

IFN signaling pathways are activated by binding of IFN to its specific receptor, which induces autophosphorylation of protein tyrosine kinases Tyk-2 and Jak-1 on tyrosine residues, thus activating signal transducer and activator of transcription (STAT1 and STAT2) proteins. Activated STATs translocate to the nucleus where they activate the transcription of IFN-inducible genes, such as 2'-5'OAS.

## Peer review

The authors postulate that a larger trial should be done with this 3 drug combination, compared to standard of care. While a small study, there is useful data.

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## Education-based approach to addressing non-evidence-based practice in preventing NSAID-associated gastrointestinal complications

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in the education-based study that recorded data from 3728 patients. The specialists overestimated the risk of GI complications with NSAIDs, underestimated the GI safety profile of coxibs, but were aware of the risk factors and of the current prevention strategies. Proton pump inhibitors were co-prescribed with NSAIDs in > 80% of patients with and without risk factors. The educational program had little impact on prescribing habits.

**CONCLUSION:** Specialists are informed of advances in NSAID-associated adverse effects and have high rates of GI-prevention therapy. Our educational program did not alter these rates.

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**Key words:** Nonsteroidal anti-inflammatory agents; Education; Gastrointestinal diseases; Adverse effects; Cyclooxygenase 2 inhibitors; Proton pump inhibitors

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### Abstract

**AIM:** To evaluate an evidence-based educational program for improving strategies for prevention of non-steroidal anti-inflammatory drug (NSAID)-associated gastrointestinal (GI) complications.

**METHODS:** Four hundred and fifty-six specialists replied to a questionnaire that covered issues related to NSAID-induced adverse effects. They also collected data from their last five consecutive patients before and after they had attended an evidence-based seminar on GI prevention strategies.

**RESULTS:** Four hundred and forty-one of 456 specialists (96.7%) participated in the survey, and 382 (83.7%)

### INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are prescribed extensively worldwide, with at least 20% of the adult population using them for at least 1 mo per year<sup>[1]</sup>. NSAID use is associated with a wide range of side effects, the most usual being those involving the gastrointestinal (GI) tract. Research in this field has progressed considerably, especially since the commercialization of cyclooxygenase 2 inhibitors (coxibs), and the amount of information published is impressive. Recent data derived from studies of the side effects associated with coxibs and traditional NSAIDs have received a good deal of attention in scientific and non-scientific

publications. Based on existing and new information, scientific organizations, regulatory agencies and influential journals have made recommendations regarding GI prevention strategies with NSAIDs<sup>[2-4]</sup>. These indicate that patients requiring NSAIDs should be evaluated for the presence of GI and cardiovascular (CV) risk factors, and should undergo prevention therapy when found to be at risk. Patients with a high risk of CV complications are to avoid coxibs and/or NSAIDs. Patients with GI risks should receive either a coxib, or concomitant therapy with misoprostol or a proton pump inhibitor (PPI). In all cases, the minimum effective dose and the shortest possible administration time should be a joint objective. In the light of all this knowledge, it remains unclear whether or not information has been translated into clinical practice.

Recent evidence indicates that prescribing patterns are far from appropriate; a trend that has become more pronounced since the withdrawal of rofecoxib from the market<sup>[5]</sup>. Reports suggest that, in Europe, up to 80% of patients with one or two risk factors for GI complications do not receive the appropriate prevention strategies<sup>[6,7]</sup> and that, in the United States, the lack of GI protection has grown since 2004<sup>[5]</sup>. In addition, some patients who are not at risk receive unnecessary therapy or receive inadequate and ineffective drugs that compromise their health and increase the cost of NSAID treatment<sup>[8,9]</sup>. The reasons for the lack of or inappropriate use of prevention strategies are unclear, but conflicting results or variations in their interpretation and divergences in the recommendations that they support<sup>[2-4,10,11]</sup> may have contributed to the confusion.

However, the aforementioned data and conclusions have been obtained at primary care level, therefore, the present study was designed firstly to evaluate the level of knowledge regarding NSAID-associated adverse effects among specialists treating patients with rheumatic diseases, and secondly, to evaluate whether an educational program based on a review of current evidence produced an improvement in the pattern of patient management.

## MATERIALS AND METHODS

The study was approved by the Regional Institutional Review Board of Aragón and carried out during 2006 to 2007. First, we conducted a nationwide survey of 456 specialists distributed across the country, and among those who had previously participated in similar studies and had delivered good-quality data, and that represented the most frequently occurring specialties among patients suffering from musculoskeletal conditions. Physicians received a letter from one of the researchers (Lanas A) explaining the purpose of the study, voluntary nature of participation, the confidentiality of the information provided, and absence of commercial purposes of the study. Physicians were questioned about issues related to NSAIDs and their adverse effects, with a special focus on research over the previous 4-5 years that may have affected clinical practice directly. Table 1 summarizes the main questions.

The educational program was designed to answer the objective of the study, which was to evaluate the impact of an evidence-based seminar on clinical practice, and to describe current practice in the prevention of GI adverse effects in the specialized setting. Only physicians who participated in the initial survey were invited to participate in the educational program. Firstly, doctors sent data of their last five consecutive patients visited in the office (phase I). These patients had to be at least 18 years old and taking or having been prescribed NSAIDs at the time of the visit. None of the data collected revealed the identity of the subjects. The responses were transmitted anonymously to one of the researchers (Sobreviela E), who entered the information into a database without including any data that could identify physicians or patients. Between 2 and 4 mo later, these specialists attended small group seminars that lasted approximately 2 h, and were based on current evidence in the field and received literature related to NSAID prevention strategies. These educational programs reviewed the main adverse effects associated with NSAIDs and coxibs, as well as risk factors, therapy-specific risks, the pros and cons of available prevention strategies, and treatment options for the cases most frequently encountered in clinical practice. The different therapeutic options, depending on the presence/absence of GI and CV risk factors<sup>[12,13]</sup>, were also reviewed and discussed. All these seminars were given by the same two investigators (Lanas A and Esplugues JV). Between 3 and 4 mo after the seminar, the same physicians again sent data of their last five consecutive patients, with the same inclusion criteria as described above (phase II of the educational program-based study).

### Statistical analysis

Data were analyzed using SAS software v.8.02 for Windows (SAS Institute, Cary, NC, USA). For categorical variables, absolute frequencies and percentages were obtained; for continuous variables, mean  $\pm$  SD, median, percentiles 25-75, maximal and minimum values and 95% CI were obtained. Significance related to categorical variables was obtained using the  $\chi^2$  test or Fisher's exact test. Comparisons reached statistical significance at  $P < 0.05$ .

## RESULTS

### Physician survey sub-study

Of a total of 456 invited physicians, 441 (96.7%) returned valid questionnaires. Those that responded had a mean  $14 \pm 8.6$  years of professional activity. Three hundred and seventy-four (84.8%) were members of one or more scientific societies, and 189 (42.9%) were aware that their respective societies had published guidelines or recommendations for the management of NSAIDs. Two hundred and eighty (63.4%) were orthopedic surgeons, 116 (24.7%) were rheumatologists, and 45 were other types of specialists (10.2%).

Only 24 (5.7%) doctors responded that NSAID use was not associated with GI toxicity; 368 (88.2%), a substantial majority, stated that NSAID use was associated with GI, renal, CV, or liver damage. A total of 207 (50.2%)

**Table 1** A summary of the main questions (from a total of 22) assessing physicians' knowledge of current evidence in the field of NSAID use and adverse effects

NSAID use is associated with adverse effects. Which of the following do you believe is not associated with NSAID use?
What is the expected annual incidence of upper GI complications in patients taking NSAIDs, as reported in the most recent large outcome studies?
The occurrence of dyspepsia in patients who take NSAIDs has been reported to be less than 25% (true or false)
NSAIDs may induce GI complications in the lower GI tract (true or false)
Which of the following factors do you believe is/are risk factors for GI complications in patients who take NSAIDs? (list)
Which of the following NSAIDs do you believe is more toxic to the GI tract? (list)
Concerning COX-2 selective inhibitors, for each of the following, indicate whether the statement is true or false:
They are not as effective as traditional NSAIDs in the treatment of OA or RA
The use of these compounds is associated with a 50% reduction in the risk of GI complications compared to NSAIDs
The concomitant use of low-dose aspirin reduces or eliminates the GI benefit of these compounds when compared to NSAIDs
The use of these compounds has been associated with an increased risk of CV events
In high-risk patients, the combination of NSAIDs plus a PPI is safer than a coxib alone
Concerning gastroprotective agents, indicate for each of the following statements whether they are true or false:
H2-RAs are effective in the prevention of gastric ulcers, duodenal ulcers, and GI complications
PPIs are effective in the prevention of gastric ulcers, duodenal ulcers, and GI complications
Misoprostol is effective in the prevention of gastric ulcers, duodenal ulcers, and GI complications
Which of the following agents has been proved to be effective in the treatment or prevention of NSAID-induced dyspepsia? (list)

NSAID: Nonsteroidal anti-inflammatory drug; GI: Gastrointestinal; CV: Cardiovascular; PPI: Proton pump inhibitor; H2-RAs: H2 receptor antagonists.

**Table 2** Responses to the question, "Which of the following factors do you believe is/are risk factors for GI complications in patients who take NSAIDs?" *n* (%)

	Rheumatologists	Orthopedic surgeons	Others	Total
History of peptic ulcer	115 (99.1)	275 (98.2)	21 (100.0)	411 (98.6)
History of complicated peptic ulcer	116 (100.0)	275 (98.2)	21 (100.0)	412 (98.8)
Age > 65 yr	114 (98.3)	229 (81.8)	18 (90.4)	361 (86.6)
Concomitant use of low-dose aspirin for CV prevention	114 (98.3)	228 (81.4)	19 (90.4)	361 (86.6)
Concomitant use of anticoagulants	112 (96.5)	247 (88.2)	20 (95.3)	379 (90.9)
<i>Helicobacter pylori</i> infection	103 (88.8)	257 (91.8)	19 (90.4)	379 (90.9)
Smoking	87 (75.00)	223 (79.6)	13 (61.7)	323 (77.5)
Dyspepsia history	73 (62.9)	250 (89.3)	19 (90.4)	342 (82.0)
Alcohol	105 (90.5)	257 (91.8)	20 (95.3)	382 (91.6)
High dose of NSAIDs	113 (97.4)	275 (98.2)	21 (100.0)	409 (98.1)

overestimated the overall rate of upper GI complications in NSAID users, and 261 (63.0%) stated that NSAID use could lead to complications of the lower GI tract. The two symptoms that doctors considered to be the most frequently reported by patients in relation to NSAID therapy were epigastric pain (67.1%) and heartburn (54.8%). The frequency of dyspepsia as an adverse effect of NSAIDs was underestimated by 45.2% of respondents. As summarized in Table 2, most identified the risk factors for GI complications in NSAID users; there were no differences between the responses of rheumatologists and orthopedists, which were the two main specialties represented by the participants. Indomethacin (61.9%), piroxicam (34.0%), diclofenac (18.5%) and ketorolac (11.0%) were considered to be the most gastrototoxic agents, while coxibs, paracetamol and metamizol were considered to be the safest for the GI tract.

When questioned about coxibs, 93 (22.5%) of the specialists believed them to be less effective than NSAIDs, but 84.6% said they were safer for the GI than NSAIDs were. However, 43.9% of the specialists stated that coxibs were more toxic for the GI tract than a combination of NSAID + PPI. Furthermore, 211 (52.2%) reported that concomitant low-dose aspirin reduced the GI benefit of coxibs, and 394 (94.7%) considered coxibs to be toxic to

the CV system; a proportion that fell to 72.7% ( $P = 0.140$ ) when the same question was asked about NSAIDs.

Over half of the physicians (56.1%) reported that histamine H2 receptor antagonists (H2-RAs) were effective in preventing ulcers and ulcer complications in NSAID users; almost all (98.5%) reported the same effect with PPIs. Responding about GI prevention therapy habits with NSAIDs, 217 (52.4%) took this precaution on a routine basis, 45.9% only when risk factors were present, and 5.3% only when patients were receiving long-term NSAID therapy. H2-RAs (44.6%), misoprostol (41.2%) and PPIs (94%) were considered to be effective for the prevention and treatment of NSAID-induced dyspepsia.

### **Effects of the educational program on patient management**

**Demographics and characteristics of patients:** Of 456 invited participants, 382 (83.7%) submitted information regarding 3728 patients over the two phases (1732 in phase I - before the evidence-based seminar, and 1722 in phase II - after the seminar). Two hundred and seventy-four patients were excluded for the following reasons: 43 were under the age of 18 years, and 231 lacked an NSAID prescription. Table 3 summarizes the main characteristics of the patients included in the study. No statistical

**Table 3** Characteristics of patients included in the educational program of the study<sup>1</sup> *n* (%)

Variable	Phase I ( <i>n</i> = 1732)	Phase II ( <i>n</i> = 1722)
Age (mean ± SD)	61.06 ± 13.37	60.81 ± 13.89
Female	1038 (60.4)	980 (57.6)
History of ulcer	238 (13.7)	307 (17.8)
History of ulcer bleeding	61 (3.5)	69 (4.0)
ASA use	167 (9.6)	168 (9.8)
CV history	203 (11.7)	205 (11.9)
Increased blood pressure	845 (48.8)	810 (47.0)
Anticoagulant use	126 (7.3)	120 (7.0)
Corticosteroid use	162 (9.3)	190 (11.0)
History of dyspepsia	782 (45.1)	766 (44.5)

<sup>1</sup>No statistical differences were found between patients enrolled in the two phases. Phase I: Before physicians attended the evidence-based seminar; Phase II: After the seminar; ASA: Aspirin.

**Table 4** Prescription of NSAIDs to patients in each of the two study phases of the educational program *n* (%)

Drug therapy	Phase I		Phase II	
	Before visit	After visit	Before visit	After visit
No NSAID therapy	718 (41.45)	162 (9.35)	653 (37.92)	190 (11.03)
NSAID therapy	1014 (58.55)	1570 (90.65)	1069 (62.08)	1532 (88.97)
Aceclofenac	146 (8.43)	248 (14.32) <sup>b</sup>	148 (8.59)	202 (11.73) <sup>b</sup>
Celecoxib	45 (2.60)	100 (5.77) <sup>b</sup>	35 (2.03)	116 (6.74) <sup>b</sup>
Diclofenac	229 (13.22)	271 (15.65)	238 (13.82)	270 (15.68)
Etoricoxib	16 (0.92)	46 (2.66) <sup>b</sup>	18 (1.05)	79 (4.58) <sup>b</sup>
Ibuprofen	281 (16.22)	432 (24.94) <sup>b</sup>	297 (17.25)	406 (23.58) <sup>b</sup>
Indomethacin	63 (3.64)	62 (3.58)	73 (4.24)	75 (4.36)
Ketorolac	15 (0.87)	25 (1.44)	28 (1.63)	31 (1.80)
Meloxicam	71 (4.10)	234 (13.51) <sup>b</sup>	101 (5.87)	215 (12.49) <sup>b</sup>
Piroxicam	74 (4.27)	75 (4.33)	64 (3.72)	64 (3.72)
Other NSAIDs (includes naproxen)	19 (1.10)	22 (1.27)	16 (0.93)	28 (1.63)
Analgesics				
Paracetamol	137 (7.91)	120 (6.93)	136 (7.90)	122 (7.08)
Metamizol	35 (2.02)	28 (1.62)	53 (3.08)	26 (1.51)
Total	1732 (100)		1722 (100)	

<sup>b</sup>*P* < 0.001 *vs* before the visit.

differences were found between patients referred to in the two phases.

**NSAID treatment:** In both phases, ibuprofen (16.2% and 17.25% in phases I and II, respectively), diclofenac (13.2% and 13.8%) and aceclofenac (8.4% and 8.6%) were the three most frequently prescribed NSAIDs. Coxib prescription was low (3.5%). There was a statistically significant (*P* < 0.0001) increase in prescription rates of aceclofenac, celecoxib, ibuprofen, meloxicam and etoricoxib after the visit with the specialist, but this increase was similar in both phases (Table 4). The main reasons for prescribing NSAIDs was the diagnosis of osteoarthritis [1015 (63.24%) in phase I and 987 (61.96%) in phase II] or rheumatoid arthritis [148 (9.22%) and 186 (11.68%) in phases I and II, respectively]. In phase I, NSAID therapy was terminated in 15.98% of patients following the visit to the specialist, a similar percentage

**Table 5** Risk factors (RFs) of patients reported by doctors in the educational program according to either a non-restrictive or a restrictive definition<sup>1</sup> *n* (%)

Number of RFs	Non-restrictive		Restrictive	
	Phase I <sup>2</sup>	Phase II <sup>3</sup>	Phase I <sup>2</sup>	Phase II <sup>3</sup>
0	347 (20.03)	352 (20.44)	961 (55.48)	891 (51.74)
1	660 (38.11)	598 (34.73)	558 (32.22)	573 (33.28)
2	517 (29.85)	536 (31.13)	176 (10.16)	213 (12.37)
> 2	208 (12.01)	236 (13.70)	37 (2.14)	45 (2.61)
Total	1732 (100)	1722 (100)	1732 (100)	1722 (100)

<sup>1</sup>A non-restrictive definition of risk factors for NSAID-related complications included age > 60 years, history of dyspepsia, history of either complicated or non-complicated ulcer, concomitant therapy with NSAIDs and low-dose aspirin, or anticoagulants or corticosteroids. A restrictive definition of risk factors included age ≥ 70 years, history of complicated or non-complicated ulcer, concomitant therapy with NSAIDs and low-dose aspirin, or anticoagulants or corticosteroids; <sup>2</sup>In Phase I, the specialists received an anonymous questionnaire regarding data and prescriptions for their last five consecutive patients; <sup>3</sup>In Phase II, the process was repeated 4-5 mo later after specialists had attended an evidence-based seminar that reviewed current evidence on NSAID-related issues, with a focus on GI prevention strategies in NSAID users.

to that reported in phase II (17.77%). The duration of NSAID therapy after the visit was also similar in both phases. Most treatments were prescribed for a short duration (< 30 d) (74.8% and 72.04% in phases I and II, respectively). No significant differences were found between the two phases.

**NSAID treatment and dyspepsia:** In phase I, 1129 (66%) of 1710 patients who were seen had suffered or were suffering GI symptoms prior to the visit, and 65.6% of the 1129 were receiving NSAID therapy; a higher proportion than those who did not have symptoms before the visit 256/581 (45.6%) (*P* < 0.0001). After the visit, physicians increased the prescription of NSAIDs to a similar rate (88.8% and 94%) in both groups of patients (*P* = 0.0006 *vs* before the visit). Similar percentages were observed in phase II, and no differences were observed between the phases.

Among the patients with GI symptoms, 57.1% in phase I and 60.1% in phase II were undergoing treatment for symptom relief before the visit to the specialist, and about one-third of them were being treated with a PPI. After the visit to the specialist, almost all these patients with symptoms were prescribed PPI therapy (*P* < 0.0001). No differences were found between the two phases.

**Risk factors and prevention strategies:** The number of patients with risk factors depends on the definition of these factors. The two most prevalent risk factors were age and a history of dyspepsia. We present data for a restrictive definition (age > 70 years and excluding history of dyspepsia) and for a non-restrictive definition of those risk factors (e.g. age > 60 years and history of dyspepsia; Table 5). Very few patients with risk factors were switched from a traditional NSAID to a coxib alone; 54 (3.1%) *vs* 129 (7.4%) (*P* < 0.0001) before and after the visit to the specialist in phase I, and 42 (2.4%) *vs* 155

**Table 6** Proportion of patients on NSAID therapy that received concomitant therapy with a PPI or misoprostol after the medical visit, according to the number of RFs *n* (%)

Number of RFs	Non-restrictive		Restrictive	
	Phase I	Phase II	Phase I	Phase II
0	268/347 (77.2)	283/352 (80.4)	782/961 (81.4)	728/891 (81.7)
1	536/660 (81.2)	499/598 (83.4)	471/558 (84.4)	504/573 (87.9)
2	453/517 (87.6)	456/536 (85.1)	151/176 (85.8)	168/213 (78.9)
> 2	175/208 (84.1)	201/236 (85.2)	28/37 (75.7)	39/45 (86.7)

(9.0%) in phase II ( $P < 0.0001$ ). No differences between phases I and II were observed after the visit, although we observed a trend toward an increase in coxib prescription in phase II ( $P = 0.09$ ).

The most widely used strategy for prevention of GI complications in Spain is concomitant therapy with PPIs. In both phases of the study, physicians prescribed appropriate gastroprotection therapy for over 80% of patients with risk factors, with little therapeutic benefits observed after the educational program (Table 6). The study also reveals a similar pattern of gastroprotection prescription rates among patients without GI risk factors.

A sub-analysis of data in high-risk patients (defined as those with previous ulcer bleeding or those who were being treated with anticoagulants) showed that very few of these patients were prescribed NSAIDs without PPIs [11/163 (8.4%) in phase I, and 6/158 (4.08%) in phase II].

## DISCUSSION

We found that the majority of specialists who treat patients with rheumatic disease are aware of recent evidence concerning the adverse effects associated with traditional NSAIDs and coxibs. The study also revealed that gastroprotection-related prescribing rates by the specialists among at-risk patients receiving NSAIDs were high, and that an educational program aimed at influencing prescription patterns had little impact.

The first step in the process of implementing prevention strategies in NSAID-treated patients at risk of GI complications is to be familiar with the risk factors. We observed that the specialists who treat patients with different rheumatic conditions are well informed of these factors, which may explain the high rates of preventative prescriptions observed in our study population. Other recent findings in the field, such as the increased risk of CV events with coxibs and traditional NSAIDs, are also well known and may explain the use of short-term courses of treatment with these compounds.

Previous studies have reported that most patients on NSAIDs with one or more risk factors for GI complications were not prescribed prevention-related treatment<sup>[6,7]</sup>. This was not the case in our patient population, of which a high proportion showed risk factors and received concomitant prescription of NSAIDs with gastroprotective agents,

specifically PPIs. The reasons for this discrepancy are not clear, but previous studies were based on a primary care database and not on prescribing data obtained from specialists, who may be more aware of risk factors and strategies to reduce their impact. In addition, this study provides more recent data on prescribing habits than the above-mentioned studies<sup>[6,7]</sup>, which did report a tendency towards more appropriate prescribing rates with time.

This study differs from those carried out in the United States<sup>[5]</sup>, in one aspect specifically: the most prevalent prevention strategy in the current study was the concomitant prescription of PPIs and NSAIDs, while the prescribing rate of coxibs was low, in agreement with sales data for these compounds across Europe<sup>[14]</sup>. This difference between practices in the United States and Europe<sup>[15]</sup> may reflect a widespread belief among the participants in the current study that adding a PPI to a NSAID confers greater upper GI protection than administration of a coxib alone, a belief that is not based on evidence<sup>[16]</sup>.

Also of interest is our finding that the PPI prescribing rate was high among patients whose NSAID treatment was discontinued after a visit to the specialist, and in patients with no risk factors. Even considering the non-restrictive framework for risk factors, which includes a history of dyspepsia as justification for prescribing gastroprotectants, > 20% of patients from the overall study population who had no GI risk factors were being prescribed preventative therapies. This excess of PPI concomitant therapy is not intrinsically inappropriate, given that it may reduce the risk of complications in patients with a low risk of GI, but it does significantly increase the cost of NSAID therapy by an estimated 80%<sup>[17]</sup>. Even in a market in which generics are prescribed and promoted widely, this added expense is not to be disregarded. Furthermore, although PPI treatment is considered to be relatively safe<sup>[18]</sup>, the long-term treatment with these type of drugs is associated with some adverse effects, including an increased risk of GI infections, pneumonia and even hip fracture<sup>[19-22]</sup>. Finally, according to current guidelines, implementing unnecessary prevention strategies is incorrect medical practice.

The other major finding of our study was the failure of our educational program to have any real effect on prescribing habits, although the effect achieved may have been so small because of the baseline circumstances. We observed a minor and statistically insignificant increase in prescribing rates for the safest NSAIDs, including coxibs, and for gastroprotectants among patients running the highest risk of GI adverse effects; a change that would appear to be a result of the educational program. On the other hand, we saw no effect on declining prescribing rates for gastroprotectants among patients without GI risk factors. The shift towards an evidence-based approach to practice seems a challenging task.

A recent study demonstrated that intervention consisting of a combination of education and computer alerts improved gastroprotection<sup>[23]</sup> in at risk patients prescribed NSAIDs, but still the rates were far from being optimal. On the other hand, educational programs may lead to short-term improvements in our knowledge,

but the impact on clinical practice is modest, especially in the mid- to long-term<sup>[24]</sup>. As suggested previously<sup>[25]</sup>, successful educational tools are costly because they require regular feedback and reinforcement. In any case, our study suggests that a high proportion of specialists are well informed about the latest advances in the NSAID field and implement appropriate prevention therapies in at-risk patients, which suggests that continuing medical education is the key to progress.

Our study had some limitations. The information was not obtained from a database but from the records of the participating physicians. Therefore, it was possible that their records differed from actual practice. However, high PPI prescribing rates have been observed in other studies<sup>[26]</sup> and reflect the marked decrease in national rates of upper GI bleeding over the last 5 years. In addition, the survey results concerning the degree of knowledge were in accordance with the clinical practice reported. Another limitation was that the study involved only one time point of observation after completion of the educational program, which did not allow the short- or long-term effects of the program to be analyzed. Finally, the data obtained cannot be extrapolated to clinical practice at the primary care level, which accounts for a major part of NSAID prescribing rates. In fact, the concomitant prescribing rate of gastroprotectants in NSAID users before visiting the specialist (which may well reflect practices in primary care) was much lower than that observed after the visit. Obviously, if a continued drop in GI complications among NSAID users is the goal, prevention strategies should be implemented at all levels of care. Further research should be carried out at the level of primary care to detect areas for improvement and to design improved educational programs by which GI complications in NSAID users may be prevented. Finally one potential limitation of the study is the validity of our conclusions outside Spain. While some data may be country-specific (e.g. prescription rates of PPI), we believe that other aspects of the study can probably be extrapolated (e.g. usefulness of the educational approach, awareness of the specialist on the medical advance,) and therefore be of interest in other areas.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Advances in medical sciences may not be rapidly translated into medical practice. Also contradictory results reported in the literature may difficult appropriate medical care. Advances in the understanding of adverse effects of nonsteroidal anti-inflammatory drugs (NSAIDs) and prevention strategies have been enormous in the last 5 years.

### Research frontiers

It is not know whether specialists treating patients rheumatic diseases are aware of advances in the field of NSAID-associated adverse effects and whether these advances have been translated into medical practice. It has been tested whether specialists confronted with scientific evidence incorporate this into medical practice and modify prescription patterns to these patients.

## Innovations and breakthroughs

The study shows that specialists dealing with patients suffering from rheumatic diseases and prescribing NSAIDs in Spain are aware of the recent advances in the NSAID field, identify the main gastrointestinal risk factors and of the current available prevention strategies. The study detects inappropriate use of prevention strategies in patients being prescribed with NSAIDs. An evidence based seminar of the prevention strategies carried out with these specialist do not change their prescription patterns.

## Applications

The study was carried in one European country and it is unclear whether the data can be extrapolated to other countries, where prescription patterns have shown to be different. Nevertheless, the study shows other aspects that can be applied to other areas and countries: (1) Knowledge of evidence by the specialist is no automatically translated into clinical practice; (2) Modification of clinical practice based on scientific evidence needs a complex intervention.

## Terminology

Evidence based clinical practice refers to medical care which is applied to patients based on studies with sufficient scientific quality that have been published in peer-review and that have been accepted by the scientific community (guidelines, expert reports, scientific societies, etc.) as appropriate.

## Peer review

This is a well presented approach to evaluation of an evidence-based educational program for improving NSAID-associated prevention strategies.

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BRIEF ARTICLE

## Hyperphosphatemia after sodium phosphate laxatives in low risk patients: Prospective study

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### Abstract

**AIM:** To establish the frequency of hyperphosphatemia following the administration of sodium phosphate laxatives in low-risk patients.

**METHODS:** One hundred consecutive ASA I-II individuals aged 35-74 years, who were undergoing colonic cleansing with oral sodium phosphate (OSP) before colonoscopy were recruited for this prospective study. Exclusion criteria: congestive heart failure, chronic kidney disease, diabetes, liver cirrhosis, intestinal obstruction, decreased bowel motility, increased bowel permeability, and hyperparathyroidism. The day before colonoscopy, all the participants entered a 24-h period of diet that consisted of 4 L of clear fluids with sugar or honey and 90 mL (60 g) of OSP in two 45-mL doses, 5 h apart. Serum phosphate was measured before and after the administration of the laxative.

**RESULTS:** The main demographic data (mean  $\pm$  SD) were: age, 58.9  $\pm$  8.4 years; height, 163.8  $\pm$  8.6 cm; weight, 71  $\pm$  13 kg; body mass index, 26  $\pm$  4; women, 66%. Serum phosphate increased from 3.74  $\pm$  0.56 to 5.58  $\pm$  1.1 mg/dL, which surpassed the normal value (2.5-4.5 mg/dL) in 87% of the patients. The highest serum phosphate was 9.6 mg/dL. Urea and creatinine remained within normal limits. Post-treatment OSP se-

rum phosphate concentration correlated inversely with glomerular filtration rate ( $P < 0.007$ ,  $R^2 = 0.0755$ ), total body water ( $P < 0.001$ ,  $R^2 = 0.156$ ) and weight ( $P < 0.013$ ,  $R^2 = 0.0635$ ).

**CONCLUSION:** In low-risk, well-hydrated patients, the standard dose of OSP-laxative-induced hyperphosphatemia is related to body weight.

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**Key words:** Bowel preparation; Colonic cleansing; Colonoscopy; Hyperphosphatemia; Laxatives; Sodium phosphate; Preoperative evaluation; Dehydration

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### INTRODUCTION

The widespread use of colonoscopy for early detection of colorectal pathology has increased the use of osmotic laxatives for colonic cleansing. Among these, oral sodium phosphate (OSP) is the preparation of choice because is the best tolerated given the small volume in which it is administered, and it results in better colonic cleansing<sup>[1]</sup>. Under normal conditions, phosphate is absorbed in the small intestine and eliminated by the kidney as calcium phosphate<sup>[2]</sup>. Several complications associated with the use of OSP have been reported in recent years, especially hyperphosphatemia and acute and chronic renal failure. There is evidence linking these complications to conditions that increase the absorption of phosphate or render its renal elimination difficult. Although many of these complications are facilitated by dehydration and inadequate selection of patients when indicating the laxative<sup>[3-5]</sup>, some patients without such conditions also have been reported<sup>[6]</sup>. Most of the information comes from retrospective studies or case reports.

In this prospective clinical trial, we investigated the

frequency of hyperphosphatemia in low-risk ASA I - II patients<sup>[7]</sup>, who were chosen to avoid high-risk patients. To avoid dehydration, we administered 4 L of clear liquids. The main aim was to identify the percentage of patients with hyperphosphatemia following the administration of OSP for video colonoscopy, and before anesthesia induction. A secondary objective was to establish the frequency of dehydration and hypocalcemia.

## MATERIALS AND METHODS

### Patients and methods

This study was approved by the Institutional Review Board. From May to December 2007, 100 consecutive patients who underwent elective colonoscopy were enrolled. Inclusion criteria were: 18-75 years of age, ASA I and II physical status, written informed consent, and colon cleansing with OSP.

Individuals with congestive heart failure, chronic kidney disease, diabetes, liver cirrhosis, intestinal obstruction, decreased bowel motility, increased bowel permeability (Crohn's disease, ulcerative or ischemic colitis) and hyperparathyroidism were prevented from entering this trial. These conditions were ruled out in the pre-anesthetic evaluation by medical history and anamnesis. Those patients who refused to participate were also excluded. Patients who had not undergone colonic cleansing were also excluded.

All the participants received full information regarding the study protocol and procedures in the pre-anesthetic interview and signed the informed consent to participate. Vital parameters were measured and laboratory tests, including hematocrit, hemoglobin concentration, and serum osmolality, phosphate,  $\text{Ca}^{2+}$ , electrolytes, creatinine and urea were carried out.

Forty-eight hours before the test, a fiber- and dairy-free diet (without fruit and vegetable products) was prescribed, and from 20 to 26 h before the study, 4 L of clear liquids (tea, coffee, infusions, jelly, broth and drained juices, or isotonic drinks<sup>[8]</sup>) with sugar or honey (on demand) were administered up to 2 h before the test.

The day before colonoscopy, all the participants were given 90 mL (60 g) of OSP (fosfo-dom<sup>®</sup>; Laboratorio Dominguez S.A, Buenos Aires, Argentina) diluted in 400 mL of water in two divided doses, administered 5 h apart (17:00 pm and 22:00 pm) on the day before colonoscopy. Ten micrograms metoclopramide were also administered 1 h before the laxative.

The day after colonic cleansing and immediately before starting anesthesia with propofol and sevoflurane, blood pressure and heart rate were measured and a second venous sample was drawn and sent to the laboratory to assess hematocrit, hemoglobin, and serum osmolality, phosphate,  $\text{Ca}^{2+}$ , electrolytes, creatinine and urea. The results obtained were compared with those obtained at baseline.

The following formulas were used to calculate plasma volume, total body water and glomerular filtration rate: Plasma volume (PV) (Beaumont formula)<sup>[9]</sup> %PV:

Table 1 Demographic data (mean  $\pm$  SD)

Demographic data	
Age (yr)	58.9 $\pm$ 8.4
Height (cm)	163.8 $\pm$ 8.6
Weight (kg)	71 $\pm$ 13
BMI	26 $\pm$ 4
Sex (%)	66% women, 34% men
TBW	36.8 $\pm$ 8.63
GFR	95.25 $\pm$ 21.27

BMI: Body mass index; TBW: Total body water; GFR: Glomerular filtration rate.

$100/(100 - \text{HCT1}) \times 100 (\text{HCT1} - \text{HCT2})/\text{HCT2}$ .  
HCT = hematocrit.

Total body water (TBW; L) (Watson formula)<sup>[10]</sup>:  
Male:  $\text{TBW} - \text{W} = 2.447 - (0.09156 \times \text{age}) + (0.1074 \times \text{height}) + (0.3362 \times \text{weight})$ ; Female:  $\text{TBW} - \text{W} = -2.097 + (0.1069 \times \text{height}) + (0.2466 \times \text{weight})$ .

Glomerular filtration rate (GFR; mL/min) (Cockcroft-Gault equation)<sup>[11]</sup>:  $(140 - \text{age}) \times \text{weight kg} (\times 0.85 \text{ if female})/\text{creatinine} \times 72$ .

### Statistical analysis

All data are expressed as mean  $\pm$  SD. The Student *t* test was used to analyze normally distributed variables. A univariate linear correlation model that considered post-treatment OSP serum phosphate as a dependent variable was also performed. STATA<sup>®</sup> version 8.0 (StataCorp LP, <http://www.stata.com>) statistical software was used to carry out the statistical analysis.  $P < 0.05$  was considered as statistically significant.

## RESULTS

The main demographic data (mean  $\pm$  SD) were: age, 58.9  $\pm$  8.4 years; height, 163.8  $\pm$  8.6 cm; weight, 71  $\pm$  13 kg; body mass index (BMI), 26  $\pm$  4; women, 66% (Table 1). The main laboratory data (mean  $\pm$  SD) are shown in Table 2. Serum phosphate (mg/dL; mean  $\pm$  SD) increased from a basal value of 3.74  $\pm$  0.56 to 5.58  $\pm$  1.1 after OSP ( $P = 0.001$ ). Hyperphosphatemia appeared in 87% of the patients. The highest serum phosphate was 9.6 mg/dL. Post-OSP serum phosphate had a significant inverse correlation with GFR ( $P < 0.007$ ,  $R^2 = 0.0755$ , Figure 1A), TBW ( $P < 0.001$ ,  $R^2 = 0.156$ , Figure 1B), and weight ( $P < 0.013$ ,  $R^2 = 0.0635$ , Figure 1C). No correlation was observed between post-OSP serum phosphate and creatinine, height or BMI. The prevalence of hyperphosphatemia increased in parallel and steadily with stage of chronic renal disease according to the National Kidney Foundation classification<sup>[12]</sup>, which approached 80% for stage 1, 88% for stage 2, and 100% for stage 3.

After OSP,  $\text{Ca}^{2+}$  decreased significantly ( $P = 0.001$ ), although the difference was not clinically relevant. Pre- and post-OSP urea and creatinine levels remained within normal limits.

Plasma volume decreased by 3.65% after OSP. This represents a dehydration of  $< 1.46\%$ , which was not

**Table 2** Laboratory data, creatinine values and arterial pressure

		Mean	SD	Min	Max	P
Na <sup>+</sup> (mmol/L)	Pre	139.26	2.05	135.00	146.00	NS
	Post	139.72	2.95	133.00	146.00	
Cl <sup>-</sup> (mmol/L)	Pre	104.88	2.68	98.00	111.00	NS
	Post	104.46	3.64	95.00	120.00	
K <sup>+</sup> (mmol/L)	Pre	4.46	0.39	3.50	5.80	0.001
	Post	3.62	0.46	2.40	5.10	
PO <sub>4</sub> (mg/dL)	Pre	3.74	0.56	2.60	5.70	0.001
	Post	5.58	1.10	2.50	9.60	
Ca <sup>2+</sup> (mmol/L)	Pre	1.14	0.10	0.76	1.37	0.001
	Post	1.04	0.12	0.50	1.28	
Hto (%)	Pre	40.28	3.13	31.70	48.30	0.070
	Post	41.18	4.00	27.10	50.90	
Urea (mg/dL)	Pre	32.57	9.93	15.00	68.00	0.001
	Post	21.36	7.53	6.00	43.00	
Osm (mosm/kg)	Pre	291.03	5.35	277.00	304.00	0.002
	Post	288.56	6.01	274.00	307.00	
Creatinine (mg/dL)	Pre	0.87	0.193	0.40	1.50	NS
	Post	0.87	0.190	0.50	1.40	
AP <sub>S/D</sub> (mmHg)	Pre	125/78	14/10	90/60	170/100	NS
	Post	128/74	29/12	80/40	185/100	

AP<sub>S/D</sub>: Arterial pressure sistolic/diastolic; NS: Not significant.

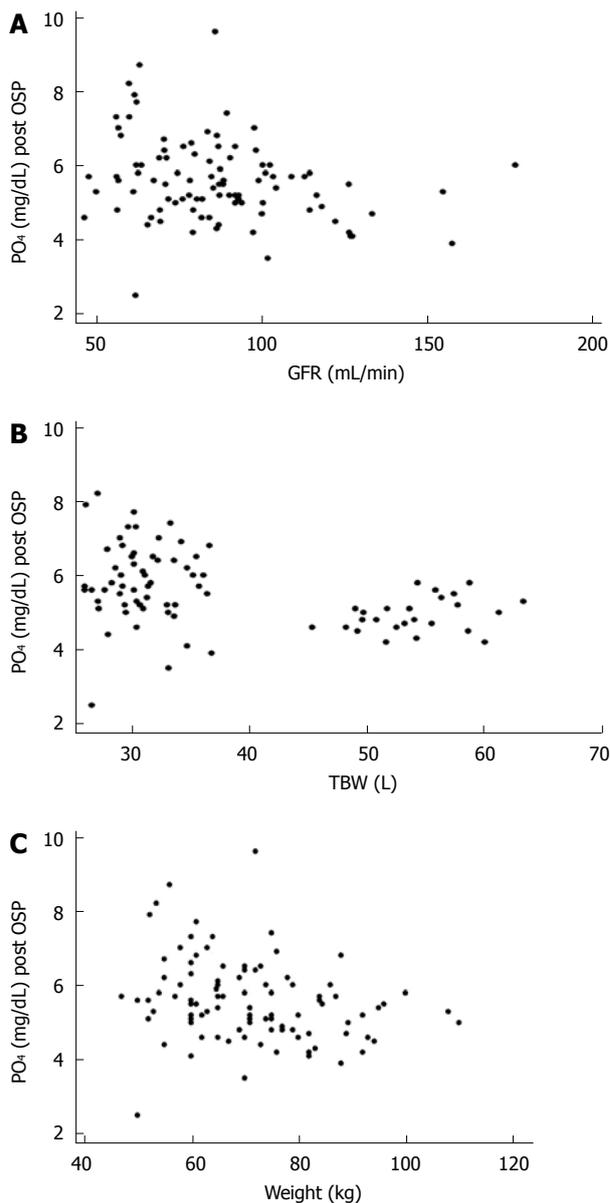
significant<sup>[13,14]</sup>. There was a decrease in serum osmolality (Tables 1 and 2). There was a low incidence (4%) of hypotension (arterial pressure reduction  $\geq 30\%$ ) after colonic cleansing (Table 2).

## DISCUSSION

The osmotic effect of OSP causes dehydration<sup>[15]</sup>, an average loss of 3-4 L of fluids is estimated during colonic cleansing with 60 g OSP<sup>[16,17]</sup>. In support of these data, increases in the concentration of hemoglobin<sup>[18]</sup>, hematocrit and serum osmolality<sup>[15]</sup> have been reported. Several authors have stated that maintaining appropriate hydration is possible to dilute the urine, and reduce its calcium and phosphate concentration<sup>[19,20]</sup>. In consequence, the risk of calcium phosphate crystalluria and precipitation in the renal tubules is diminished<sup>[16,21]</sup>. Sanders *et al*<sup>[18]</sup> have corroborated the efficiency of intravenous hydration (average 2 L) during colonic cleansing for surgery, but this requires a hospital stay and makes ambulatory procedures difficult. Markowitz *et al*<sup>[21]</sup> has suggested that patients must be encouraged to drink eight cups of fluids (1920 mL) and Rex *et al*<sup>[19]</sup> have promoted taking 3.6 L of clear fluids.

Following the 1999 American Society of Anesthesiologists recommendations for all interventions that require general anesthesia or sedation, oral fluid intake is allowed up to 2 h before colonoscopic evaluation<sup>[22]</sup>. The rationale for the preoperative fasting is to reduce the content and acidity of the stomach, thus avoiding the risk of aspiration pneumonitis at induction of anesthesia<sup>[23,24]</sup>.

Since the seminal studies of Beaumont in 1833<sup>[25]</sup>, it is widely known that emptying of clear liquids is passive, without the need for gastric motility, and is completed in  $< 60$  min<sup>[26]</sup>. Clear fluids have a washing and dragging effect that allows the gastric content to move easily



**Figure 1** Correlation among phosphorus post-oral sodium phosphate (OSP) with glomerular filtration rate (GFR), total body water (TBW), and weight. A: Between phosphorus post-OSP and GFR; B: Between phosphorus post-OSP and GFR and TBW; C: Between phosphorus post-OSP and weight.

to the duodenum<sup>[27]</sup>. Patients with 2 h fasting with clear liquids (i.e. no liquid intake for 2 h before colonoscopy) had less volume and gastric acidity than those with complete 8 h fasting<sup>[28-32]</sup>. These results also have been reported in children<sup>[33-36]</sup>.

The absence of fluid intake before surgery favors the development of hypotensive reactions during anesthesia induction, as well as dehydration, hypoglycemia and a strong sensation of thirst and hunger that leads to irritability, especially in older patients and infants<sup>[8,37]</sup>. Clear liquid intake not only diminishes the risk of aspiration pneumonia and notably improves patient wellbeing, but it also facilitates adequate hydration.

To evaluate the changes produced by the administration of OSP, it was vital to avoid dehydration. We encouraged patients to freely take 4 L of clear liquids during colonic preparation, up to 2 h before the test. This did not

lead to a significant incidence of dehydration and hypotension, which was reinforced by no significant modifications in haemoglobin and hematocrit. The average reduction in PV was 3.65%, which represented dehydration of < 1.46%, which was not significant<sup>[13,14]</sup>. Besides, in contrast to Gutierrez Santiago's study<sup>[15]</sup>, we observed a decrease in the average osmolality. Only 4% of the patients developed hypotension, a degree of blood pressure reduction of 20%-30%. These results support the efficiency of this oral hydration regime for avoiding dehydration.

At the onset of our study, the suggested interval between doses was 5-10 h, and we used a 5-h interval. As 28% of the phosphate taken is retained by the body for up to 18 h<sup>[16,38]</sup>, recent studies have recommended longer intervals between doses<sup>[4]</sup>.

The maximum safe dose of sodium phosphate is 90 mL<sup>[39]</sup>. Several studies on the adverse effects of high doses of OSP have suggested that these should be avoided<sup>[2,3]</sup>, as is the case with their association with phosphate enemas<sup>[40-42]</sup>. If the recommended dose of 60 g (90 mL) is surpassed, or if the interval between doses is < 5 h, severe hyperphosphatemia could develop<sup>[2,19,39,43-46]</sup>.

Many authors make reference to the fact that administering laxatives and sodium phosphate enemas<sup>[40,41]</sup> leads to a slight though statistically significant increase in phosphorus and a decrease in calcium concentration<sup>[2,19,21,47-49]</sup>, due to intestinal absorption<sup>[2]</sup>. However, they have also suggested that well-hydrated adults who have normal renal function tolerate the amount of phosphate loading without showing significant adverse effects<sup>[6,4,49-52]</sup>. This trend was confirmed in our study, with a maximum registered plasma phosphate level of 9.6 mmol/L and a minimum calcium level of 0.5 mmol/L.

The serious electrolyte disturbances reported have appeared in patients in whom sodium phosphate was contraindicated: inflammatory colonic diseases (Crohn's disease, ulcerative colitis)<sup>[53,54]</sup>, delayed intestinal transit (megacolon, obstruction), and in conditions with intestinal vascular alteration (congestive heart failure, ischemic colitis)<sup>[49]</sup>. It also has been reported in patients with impaired renal function<sup>[55-57]</sup>, or who receive drugs that affect kidney perfusion (diuretics, angiotensin-converting enzyme inhibitors and angiotensin receptor blockers). To avoid the administration of OSP in the sub-clinical kidney disease, measurement of urea and creatinine is recommended<sup>[43]</sup>. Hyperphosphatemia has been observed in patients with dehydration, ascites<sup>[4]</sup> or vomiting<sup>[45]</sup>.

Fine *et al.*<sup>[57]</sup> have found that the mortality rate was 33%, and that the risk of death was high if serum phosphate increased beyond 32.69 mg/dL (10.56 mmol/L). Most of the deaths reported in the literature have been caused by arrhythmia or heart attack associated with electrolyte changes and dehydration<sup>[4]</sup>. Fatal cases have been observed among patients with a history of renal damage<sup>[41,43,50]</sup>, ischemic colitis<sup>[50]</sup>, cirrhosis<sup>[45]</sup>, and in elderly patients with normal renal function<sup>[42,58]</sup>. Azzam *et al.*<sup>[43]</sup> and Wexner *et al.*<sup>[5]</sup> have described high levels of phosphate and kidney damage in patients without previous kidney pathology.

Gutierrez-Santiago *et al.*<sup>[15]</sup> have found an increase in

phosphatemia in 57% of patients, while Lieberman *et al.*<sup>[51]</sup> have found it in 25%. Both studies were retrospective and they did not specify the patient's clinical condition. In our study in low-risk patients, we found an increase in phosphate in a significant percentage (87%). The average increase of serum phosphate was 1.84 mg/dL, which was less than that reported by Tan *et al.*<sup>[59]</sup> (3.09-3.18 mg/dL). The maximum plasma phosphate value registered was 9.6 mg/dL (3.1 mmol/L), which was twice the normal concentration. This result shows that OSP used as laxative is not free of complications, even in low-risk patients. These values do catch our attention because the careful selection of patients anticipated a much lower incidence. It is possible to assume that the wide hydration plan and careful selection of participants avoided reaching the values described by Fine *et al.*<sup>[57]</sup>.

All of the patients had normal urea and creatinine values before and after colonic cleansing. We linked the phosphate values with the TBW and GFR, and both showed a negative linear correlation with the increase in phosphate. We observed that the lower the GFR and TBW, the higher the chance of developing hyperphosphatemia. These parameters describe the relationship of weight with a specific function, which shows that the increase in phosphatemia has a negative linear correlation with weight. We avoided dehydration and there was no renal impairment, therefore, these findings contribute towards the concept that hyperphosphatemia is the result of an excessive dose of laxative, as suggested by Rex *et al.*<sup>[4]</sup>.

Tan *et al.*<sup>[2]</sup> have stated that the decrease in plasma calcium associated with OSP-induced hyperphosphatemia is the result of the binding of calcium to the high phosphate level, and thus, the tubular deposition that induces kidney damage. Gutierrez-Santiago *et al.*<sup>[15]</sup> have observed hypocalcemia in 36% of patients. In our study, the decrease in calcium concentration developed in 29% of the patients, but none had symptoms related to hypocalcemia.

The reported OPS-induced hypernatremia is the result of intestinal sodium absorption and can worsen due to dehydration<sup>[2,15]</sup>. We did not observe an increase of plasma sodium in our patients, which suggests that the hydration level achieved with this diet was appropriate.

The sodium and potassium exchange across the colonic epithelium can generate hypokalemia, which is accentuated by renal potassium loss induced as a consequence of the volume contraction-associated secondary aldosteronism<sup>[2,15]</sup>. The decrease in potassium in our sample coincided with that observed by Rex *et al.*<sup>[4]</sup>. It appeared in 4% of the patients and reached 2.4 mmol/L in one case.

Unlike previous studies by other investigators, we did not observe changes in plasma chloride values in our patients<sup>[4]</sup>.

The results in this study show that, in low-risk, well-hydrated patients, hyperphosphatemia following standard OSP doses is related to weight. This is the reason why we believe that, in low-weight patients, lower doses of the laxative should be administered. We consider that further studies are necessary to establish the adequate dose according to weight.

Oral hydration with 4 L of clear liquids during colonic preparation has proven its efficacy in avoiding dehydration.

The possibility of achieving high phosphate levels in low-risk, well-hydrated patients is certainly alarming, especially given the fact that few medical professionals currently take this possibility into account. These discoveries emphasize the need to carry out an adequate hydration and selection of patients to avoid administration of OSP to those individuals at risk of developing hyperphosphatemia or renal failure.

## COMMENTS

### Background

Colon cleansing is used widely for colonoscopic exploration and colonic and gynecological surgery. Oral sodium phosphate (OSP) solution is the osmotic laxative most commonly used for this purpose. It is known that OSP can induce severe hyperphosphatemia and hypocalcemia due to excessive absorption of phosphates, and there have been reports of deaths and irreversible dialysis-requiring renal insufficiency.

### Research frontiers

Hyperphosphatemia after OSP develops in patients with conditions that increase its intestinal absorption (ulcerative colitis, Crohn's disease, ischemic colitis), in conditions in which its elimination is difficult (kidney disease, dehydration, aging), or after OSP overdose (> 60 g). These findings have come from case reports and some rare retrospective studies. No prospective studies have investigated the prevalence of hyperphosphatemia in low-risk patients.

### Innovations and breakthroughs

This was a prospective study that was carried out in low-risk patients. Even though, the authors avoided the conditions that are known to facilitate hyperphosphatemia such as dehydration (inducing oral intake of 4 L of clear liquids) and the diseases described above, 87% of the patients had high serum phosphate levels. None of them developed symptoms of hypocalcemia, and there was no evidence of renal impairment. Hyperphosphatemia was related inversely to body weight. These results highlight the importance of being cautious with the administration of OSP in patients with contraindications and promoting aggressive oral hydration.

### Applications

Taking into account the results of this study, the authors recommend: performing preoperative evaluation aimed at avoiding administration of OSP laxatives to patients at risk; reducing the dose of OSP in patients with low weight; and avoiding dehydration with an adequate oral intake of clear liquids. Additional studies are necessary to establish the appropriate dose adjusted to body weight.

### Terminology

Hyperphosphatemia: serum phosphate levels above normal (2.5-4.5 mg/dL). Hypocalcemia: ionized calcium levels below normal values (1.0-1.35 mmol/L).

### Peer review

This paper presented provides reliable information on the side effects of OSP in low-risk patients. The conclusions addressed are useful for managing patients' prescribed OSP for colon cleansing.

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BRIEF ARTICLE

## Carcinoma of the middle bile duct: Is bile duct segmental resection appropriate?

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selected patients with no adjacent organ invasion and resection margin is negative.

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**Key words:** Bile duct cancer; Segmental resection; Pancreaticoduodenectomy

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### Abstract

**AIM:** To compare survival between bile duct segmental resection (BDSR) and pancreaticoduodenectomy (PD) for treating distal bile duct cancers.

**METHODS:** Retrospective analysis was conducted for 45 patients in a BDSR group and for 149 patients in a PD group.

**RESULTS:** The T-stage ( $P < 0.001$ ), lymph node invasion ( $P = 0.010$ ) and tumor differentiation ( $P = 0.005$ ) were significant prognostic factors in the BDSR group. The 3- and 5-year overall survival rates for the BDSR group and PD group were 51.7% and 36.6%, respectively and 46.0% and 38.1%, respectively ( $P = 0.099$ ). The BDSR group and PD group did not show any significant difference in survival when this was adjusted for the TNM stage. The 3- and 5-year survival rates were: stage I a [BDSR (100.0% and 100.0%) vs PD (76.9% and 68.4%) ( $P = 0.226$ )]; stage I b [BDSR (55.8% and 32.6%) vs PD (59.3% and 59.3%) ( $P = 0.942$ )]; stage II b [BDSR (19.2% and 19.2%) vs PD (31.9% and 14.2%) ( $P = 0.669$ )].

**CONCLUSION:** BDSR can be justified as an alternative radical operation for patients with middle bile duct in

### INTRODUCTION

Extrahepatic bile duct cancer can be classified into hilar, proximal, middle and distal bile duct cancer (DBD) according to the portion of the involved bile duct. Hilar and proximal bile duct cancers (PBD-1) are classified into 5 types as described by Bismuth and Corlette<sup>[1]</sup>. This classification of extrahepatic bile duct cancers is due to the differences in the operative methods that are employed for cancers involving different portions of the bile duct. According to numerous reports, most surgeons consider bile duct resection with liver parenchyma resection to be the standard operation for hilar cholangiocarcinoma<sup>[2-4]</sup>. Pancreaticoduodenectomy (PD) is performed for DBD. Bile duct cancers confined to the middle bile duct (MBD) are rare because bile duct cancers have a tendency to spread along the bile duct wall. This is the reason why PD is usually undertaken for treating most MBD cancers. Yet there is still debate as to the appropriate operative procedure for the bile duct cancers that do not extend above the confluence or below the upper border of the pancreas. PD or bile duct segmental resection (BDSR) is performed at the surgeon's discretion. Clinicopathological studies on hilar and distal cholangiocarcinomas have been done by many authors<sup>[2-11]</sup>, and the results of BDSR for MBD have

been reported, but most of these studies involved a very limited number of cases, and the number of cases is not enough to justify BDSR as a standard treatment.

In the current study, a clinical review of patients who received BDSR (negative resection margins) with lymph node (LN) dissection for the Bismuth type I PBD-1 and also the patients with MBD was performed. We compared survival between BDSR group and PD for treating DBD.

## MATERIALS AND METHODS

Between November 1995 and May 2007, 379 patients underwent surgical procedures that were performed for treating extrahepatic cholangiocarcinomas at Samsung Medical Center. One hundred nine patients who underwent concomitant liver resection and 76 patients who underwent palliative procedures were excluded, and only the cases with bile duct adenocarcinomas that had been confirmed by pathologic assessment were included. There were 45 patients who underwent BDSR for PBD-1 or MBD (BDSR group), and 149 patients who underwent PD for DBD (PD group). A retrospective analysis was performed *via* a review of the medical records and by conducting telephone interviews. The clinicopathologic factors of the BDSR group that we analyzed were age, gender, the preoperative carbohydrate antigen 19-9 (CA19-9) level, serum bilirubin levels both at the time of admission and prior to surgery, preoperative biliary drainage, transfusion, the site of cancer, the extent of LN dissection [D1 (LN #12) or D2 (LN #12, #8, #13)], tumor size, histologic differentiation, TNM stage, LN metastasis and perineural invasion. To find the exact location of the tumor, ERCP or PTC and recently MRCP was done before surgery. Before surgery, biliary and GB CT scans were performed to see if there was invasion to adjacent tissues or organs. Angiography was used to detect any evidence of vascular invasion, but after 1999, we used the CT scan to rule out any vascular invasion. In the BDSR group, all patients underwent frozen section for both bile duct resection margins and to be confirmed as free from carcinoma intraoperatively and on permanent pathology. The tumor stage was based on the 6th edition of the American Joint Committee on Cancer cancer staging<sup>12</sup>. All patients were monitored at 6 mo intervals by measuring CA19-9 levels and performing a CT scan for 3 years and were checked annually at 6 mo intervals. Four patients (8.8%) in the BDSR group were lost to follow up.

### Statistical analysis

Survival of the BDSR and the PD groups was calculated by the Kaplan-Meier method, and the log-rank test was used to analyze differences. Only variables that were statistically significant on univariate analysis were included in the multivariate analysis, which was performed using a Cox proportional hazard regression model. Survival analysis of the PD group was done by the Kaplan-Meier method and comparison of survival between the BDSR group and the PD group was done by log-rank tests.  $\chi^2$  tests and a Mann-Whitney *U*-test were used for comparing

the postoperative complications and the length of the hospital stay between the 2 groups. *P* values less than 0.05 were considered statistically significant.

## RESULTS

### Clinical characteristics of the groups of patients who underwent BDSR for PBD-1 and MBD

Clinical characteristics of the groups of patients who underwent BDSR for PBD-1 and MBD are shown in Table 1. There were 45 patients who underwent BDSR for PBD-1 or MBD. There were 30 (66.7%) males and 15 (33.3%) females. The median age of the patients was 65 years (range: 37-76 years). BDSR with LN dissection and hepaticojejunostomy was done for all these patients, with negative proximal and distal bile duct resection margins being achieved in all 45 patients. The complications arising during the immediate postoperative period (within 1 mo) were analyzed. There were 9 patients with wound infections, pancreatitis, bile leakage and delayed gastric emptying. Dissection of the superior border of the pancreas was done due to the close distal resection margin in all 3 patients who showed postoperative pancreatitis. Of 45 patients, D2 LN dissection (LN #12, #8, #13) was done in 37 (82.2%) patients and D1 dissection (LN #12) was done in 8 (17.8%) patients. LN metastasis was present in 13 (28.8%) patients and perineural invasion was present in 32 (71.1%) patients. Nine (20%) patients had T1 lesions, 33 (73.3%) patients had T2 lesions and 3 (6.7%) patients had T3 lesions with invasion into the gallbladder without liver or pancreas invasion. Nine (20%) patients were stage I a (T1N0M0), 23 (51.1%) patients were stage I b (T2N0M0), and 13 (28.9%) patients were stage II b (T1-3N1M0). There were no stage II a (T3N0M0) patients. The median follow-up period was 25 mo (range: 4-104 mo) (Table 1).

Among the 45 patients, 3 patients (6.6%) underwent additional adjuvant treatment. Two patients with stage I b each had chemoradiation and radiation. One patient with stage II b had chemoradiation. There was no evidence of recurrence in all three patients. The recurrence rate was 44.4% ( $n = 20$ ). The stage specific recurrence rate was as follows: 24.4% ( $n = 11$ ) for stage I b and 20.0% ( $n = 9$ ) for stage II b. There were 12 locoregional recurrences, 5 liver metastases and 3 peritoneal carcinomatoses. Eleven patients underwent palliative treatment and 9 patients refused to go under extra treatment. The 3- and 5-year survival rates of the BDSR group were 51.7% and 36.6%, respectively. The median survival was 25 mo (mean: 31.27 mo). Univariate analysis showed cellular differentiation ( $P = 0.005$ ), the T stage ( $P < 0.001$ ), the LN status ( $P = 0.010$ ) and the TNM stage ( $P = 0.012$ ) to be significant factors that influenced patient survival (Table 1, Figure 1). However, there were no statistically significant independent risk factors that influenced patient survival on multivariate analysis.

### Comparison of survival between the BDSR group and the PD group

There were 149 patients in the PD group. There were

**Table 1** Univariate analysis of the predictors for survival of the 45 patients who underwent radical BDSR for PBD-1 or MBD disease

Characteristic	Number of patients	Median survival time (mo)	Survival rate (%)		P-value
			3-yr	5-yr	
Overall	45	25	51.7	36.6	
Age (yr)					0.466
≤ 65	25	42	56.4	38.7	
> 65	20	33	45.5	34.1	
Gender					0.314
Male	30	35	47	28.2	
Female	15	42	58.2	48.5	
CA19-9 (U/mL)					0.519
≤ 35	19	42	51.6	25.8	
> 35	19	55	61.5	49.2	
No data	7				
Bilirubin at admission (mg/dL)					0.368
≤ 1.6	11	NR	53.3	53.3	
> 1.6	34	42	50.5	34.1	
Bilirubin at operation (mg/dL)					0.149
≤ 1.6	17	55	65.5	43.6	
> 1.6	28	29	43.7	31.9	
Preoperative biliary drainage					0.632
No	10	35	40	0.0	
Yes	35	42	53.8	42.3	
Location					0.547
MBD	34	42	51.4	44.1	
PBD-1	11	52	51.9	26	
LN dissection					0.997
D1	8	42	58.3	29.2	
D2	37	52	51.2	42.6	
Transfusion					0.832
No	36	42	51.2	39.4	
Yes	9	33	47.6	23.8	
Size (cm)					0.892
≤ 2.0	26	35	47	39.2	
> 2.0	19	52	56.6	33.9	
T stage					< 0.001
T1	9	NR	100	100	
T2	33	35	49.6	31.5	
T3	3	16	0.0	0.0	
LN stage					0.010
N0	32	52	63.1	42.6	
N1	13	25	19.2	19.2	
TNM stage					0.012
I a	9	NR	100	100	
I b	23	42	55.8	32.6	
II b	13	25	19.2	19.2	
Cell differentiation					0.005
Well	15	NR	87.5	57.5	
Moderate	26	33	41.6	33.3	
Poor	4	22	0.0	0.0	
Perineural invasion					0.180
No	13	NR	71.1	71.1	
Yes	32	35	47.7	29.8	

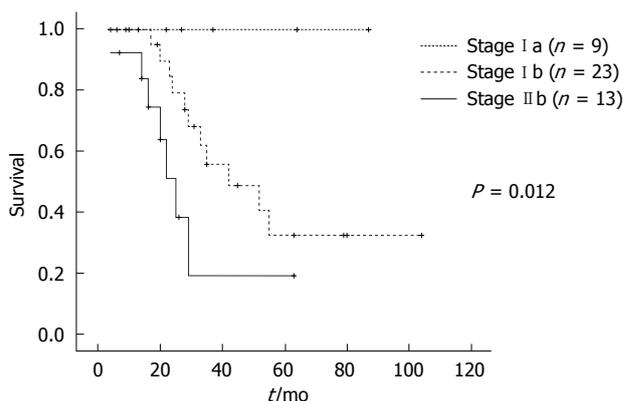
BDSR: Bile duct segmental resection; CA19-9: Carbohydrate antigen 19-9; MBD: Middle bile duct cancer; PBD-1: Proximal bile duct cancers; LN: Lymph node; NR: Not reached.

102 (68.5%) males and 47 (31.5%) females. The median age of the patients was 60 years (range: 31-78 years). Whipple's procedure was done in 97 (65.1%) patients, pylorus preserving PD was done in 45 (30.2%) patients and total pancreatectomy was done in 7 (4.7%) patients. The median follow-up was 21.9 mo (range: 0.4-108.5 mo). During the follow-up period, 88 (59.1%) patients had

**Table 2** The clinical characteristics of the BDSR group were compared with the PD group *n* (%)

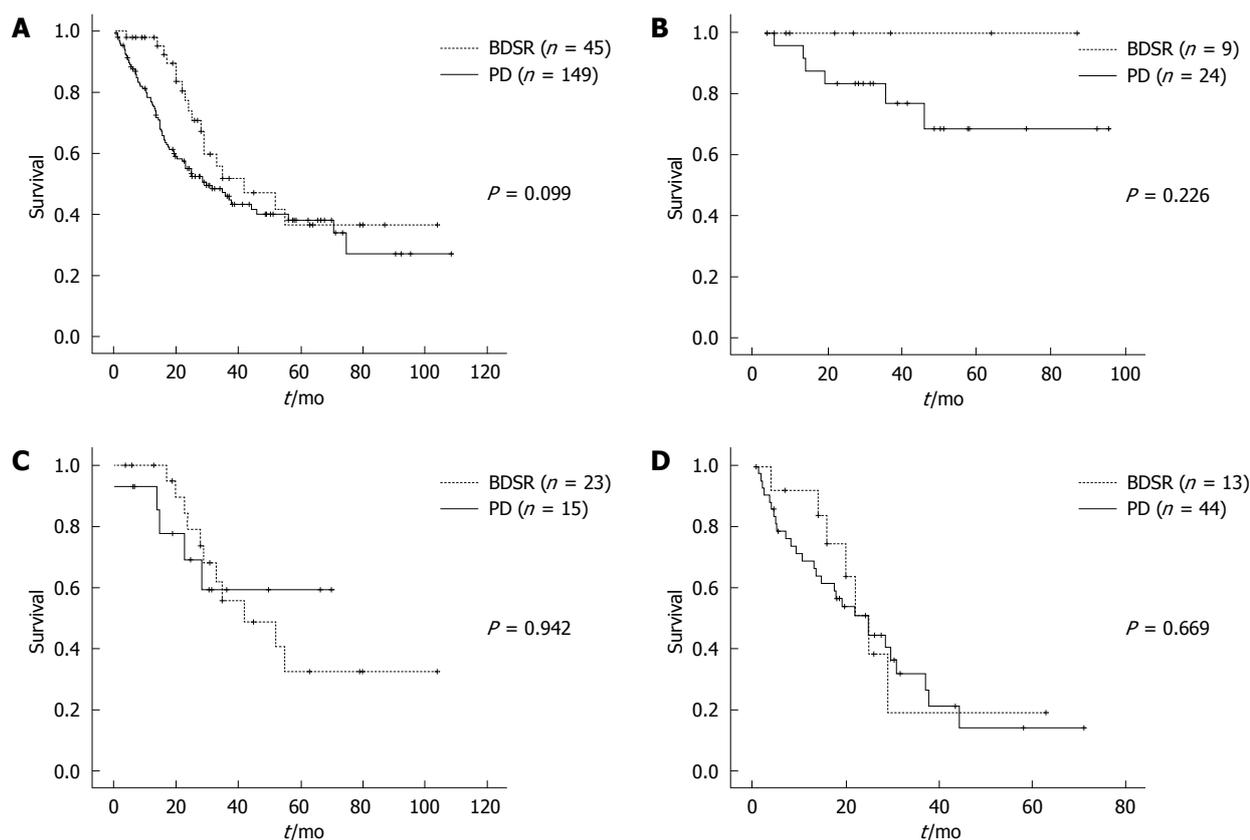
Characteristics	BDSR group	PD group
Total patients	45	149
Age (median, yr)	65 (37-76)	60 (31-78)
Gender		
Male	30 (66.7)	102 (68.5)
Female	15 (33.3)	47 (31.5)
Median follow-up (mo)	25.0 (4.0-104.0)	21.9 (0.4-108.5)
T stage		
T1	9 (20.0)	32 (21.4)
T2	33 (73.3)	20 (13.4)
T3	3 (6.7)	90 (60.5)
T4	0 (0.0)	7 (4.7)
N status		
N0	32 (71.2)	101 (67.8)
N1	13 (28.8)	48 (32.2)
TNM stage		
I a	9 (20.0)	24 (16.1)
I b	23 (51.1)	15 (10.1)
II a	0 (0.0)	58 (38.9)
II b	13 (28.9)	44 (29.5)
III	0 (0.0)	8 (5.4)

PD: Pancreaticoduodenectomy.



**Figure 1** Survival according to the TNM stage of 45 patients who underwent bile duct segmental resection (BDSR) for proximal bile duct cancers (PBD-1) or middle bile duct cancer (MBD) disease.

recurrences and 78 (52.3%) patients died of cancer-related causes. The survival of the BDSR group was compared with the group of patients who underwent PD for their DBD; these patients are known to have similar clinical characteristics as patients with PBD-1 or MBD (Table 2). The survival rate, postoperative complications and length of the postoperative hospital stay were the factors analyzed. The 3- and 5-year overall survival rates were 51.7% and 36.6% in the BDSR group, and 46.0% and 38.1% in the PD group (*P* = 0.099) (Figure 2A). The stage specific survival rates were compared between the BDSR group and the PD group. The 3-year and 5-year survival rates of the patients with stage I a disease were 100.0% and 100.0%, respectively, in the BDSR group, and 76.9% and 68.4%, respectively, in the PD group (*P* = 0.226) (Figure 2B). The 3-year and 5-year survival rates of the patients with stage I b disease were 55.8% and 32.6%, respectively, in the BDSR group, and 59.3% and 59.3%,



**Figure 2 Comparison of survival between the BDSR group and the pancreaticoduodenectomy (PD) group.** A: The 3-year and 5-year overall survival rates were 51.7% and 36.6%, respectively, for the BDSR group and 46.0% and 38.1%, respectively, for the PD group; B: The 3-year and 5-year survival rates of the patients with stage I a disease were 100.0% and 100.0%, respectively, for the BDSR group and 76.9% and 68.4%, respectively, for the PD group; C: The 3-year and 5-year survival rates of the patients with stage I b disease were 55.8% and 32.6%, respectively, for the BDSR group and 59.3% and 59.3%, respectively, for the PD group; D: The 3-year and 5-year survival of patients with stage II b disease were 19.2% and 19.2%, respectively, for the BDSR group and 31.9% and 14.2%, respectively, for the PD group.

respectively, in the PD group ( $P = 0.942$ ) (Figure 2C). The 3-year and 5-year survival rates of patients with stage II b disease were 19.2% and 19.2%, respectively, in the BDSR group, and 31.9% and 14.2%, respectively, in the PD group ( $P = 0.669$ ) (Figure 2D). Therefore, the BDSR group and the PD group did not show any significant difference in survival when this was adjusted for the TNM stage. Sixty one (40.9%) patients in the PD group experienced postoperative complications (pancreatic fistula, bleeding, delayed gastric emptying, wound infection, intraabdominal abscess, *etc.*). There were significantly less postoperative complications in the BDSR group (20.0% *vs* 40.9%, respectively,  $P = 0.01$ ). The length of the postoperative hospital stay was significantly shorter in the BDSR group (mean: 16.62 d *vs* 28.35 d, respectively,  $P = 0.035$ ).

## DISCUSSION

Classification of extrahepatic cholangiocarcinoma according to its anatomic location (proximal, middle, distal) was first proposed by Longmire<sup>[13]</sup> in 1976. There still exists some debate about the classification of MBD carcinoma since cholangiocarcinomas are very rarely located only in this section of the bile duct without infiltration into the proximal or distal bile duct. So there have been other classifications of the cholangiocarcinomas such as

that proposed by Nakeeb *et al.*<sup>[14]</sup> which results in a simpler classification of intrahepatic, hilar and distal cholangiocarcinoma. Jarnagin *et al.*<sup>[15]</sup> also proposed a 2-category system of proximal and distal bile duct cancer, with MBD cancer being in a separate category. Such interest in the classification of cholangiocarcinomas is due to the difference in surgical treatment according the different anatomic locations. Intrahepatic and perihilar cholangiocarcinomas are usually treated by liver resection<sup>[16-18]</sup>. In contrast, distal cholangiocarcinoma requires PD for complete tumor resection. Then what about tumors confined to the MBD? Different surgical treatments are possible for MBD cancers according to the distance between the hilum or the upper border of the pancreas, and also according to the experience and preference of the surgeon. For tumors with negative bile duct margins and no infiltration into vascular structures, BDSR is sometimes undertaken, albeit for only a small proportion of these tumors.

The most important issues for BDSR for PBD-1 or MBD is the radial margin, involvement of the portal vein or hepatic artery and LN dissection<sup>[10]</sup>. T1 and T2 tumors, and T3 tumors with infiltration only into the gallbladder and without vascular invasion, were included in this study. T3 or T4 tumors with vascular, liver or pancreas invasion require extended surgery due to the fact that R0 resection is impossible with BDSR in these

cases. Sufficient radical dissection of the LNs, and especially the LNs around the superior mesenteric artery (SMA), may be another issue. According to the general rules for surgical and pathological studies on cancer of the biliary tract by the Japanese Society of Biliary Surgery, in the case of PBD-1 LNs #12 (a1, a2, b1, b2, c, p1, p2, h) are classified as group 1 and LNs #8 (a, p) and #13 are classified as group 2. For MBD, LNs #12 (b1, b2, c) are classified as group 1 and LNs #12 (a1, a2, c, p1, p2, h), #8 (a, p) and #13 (1) are classified as group 2. Since the LNs around the SMA and #17 are classified as group 3, radical resection LN dissection (D2) is possible with BDSR.

There have been occasional reports of BDSR for treating extrahepatic cholangiocarcinoma in selected patients<sup>[7,8,19-22]</sup>. In the series from the Memorial Sloan Kettering Cancer Center<sup>[7]</sup>, only 13% of patients (6 of 45) were amenable to bile duct excision alone, while this figure was 8% (3 of 34) in the Veterans Hospital study<sup>[22]</sup>. But in most of these reports, risk factor evaluation was not possible due to the limited number of BDSR cases. In this study, 28.8% of the cases had LN metastases, and the LN status was a significant risk factor that affected survival. Furthermore, the cell differentiation, the T stage and the TNM stage were significant factors that affected the outcomes. It has been reported that the frequency of LN metastasis in DBD ranged from 30% to 68%<sup>[7,8,11,14,20,23]</sup>, and negative LN metastasis was a useful predictor of a favorable outcome for patients with DBD<sup>[7,8,14,23]</sup>. However, perineural invasion did not prove to be statistically significant, unlike the other studies that reported the risk factors for survival from cholangiocarcinoma in other parts of the biliary tree<sup>[24,25]</sup>.

In the present study, 3 patients underwent BDSR due to the lesion being close to the upper border of pancreas. All 3 patients developed postoperative pancreatitis. Two of these patients developed local recurrence in the remnant distal bile duct at 9 mo and 31 mo, respectively. Although the 2 cases mentioned were categorized as recurrences, they could also have been considered remnant bile duct cancer when the slow-growth of cholangiocarcinoma was taken into account. For a tumor that is close to the upper border of pancreas and resected bile margin reveals to be free of carcinoma, PD should be considered as a treatment option since the pattern of tumor spread along the periductal tissue can be assumed and it should secure sufficient distal bile duct resection margin.

A comparison of survival would be most accurate between 2 groups that received BDSR or PD for carcinoma confined to the proximal or MBD. But this was not feasible due to the small number of patients included in this category. So a different approach was selected in this study, and comparisons were made with the group of patients who received PD for DBD, which is known to have similar clinical characteristics with its proximal counterparts. Although several authors have reported that MBD had a worse prognosis than hilar or distal bile duct cancer<sup>[26-28]</sup>, other authors did not concur<sup>[8,29]</sup>. According to previous reports, the curative resection rates for DBD

have ranged from 56% to 100%<sup>[7,8,14,19-22]</sup>. However, the 5-year survival rate for DBD is not always high, with some reports showing a range of 14%-47%<sup>[7,8,11,14,19-23]</sup>. In the present study, the overall 3-year and 5-year survival rates of the BDSR group were 51.7% and 36.6%, respectively, which is similar to other published reports<sup>[7,8,11,14,19-23]</sup>. The stage specific overall survival between BDSR group and PD group was not statistically significant. The BDSR group had a significantly shorter postoperative length of hospital stay and fewer complications compared to the PD group. This comparison may not be so meaningful when taking into consideration that BDSR is a far less extensive and complicated operative method (fewer anastomoses). Thus, BDSR can be safely applied to patients with bile duct resection margin negative PBD-1 or MBD tumor rather than performing more extensive surgery such as PD.

In conclusion, to achieve a cure, the surgeon must obtain histologically negative margins on both the proximal and distal bile ducts and all tumor-bearing nodal tissue must be removed. BDSR with LN dissection can be an alternative treatment and may be justified in preference to a more radical operation for patients with PBD-1 or MBD when there is no pancreas, liver, vascular invasion and the both bile duct resection margins are negative.

## COMMENTS

### Background

Although radical bile duct segmental resection (BDSR) is performed by many surgeons in selected cases, there are scant clinical studies on the adequacy of this procedure.

### Research frontiers

To validate the adequacy of radical BDSR, the authors compared survival between a radical BDSR group and a pancreaticoduodenectomy group for treating distal bile duct cancers.

### Innovations and breakthroughs

Clinicopathological studies on hilar and distal cholangiocarcinomas have been done by many authors, and the results of BDSR for middle bile duct (MBD) have been reported, but most of these studies involved a very limited number of cases, and the number of cases is not enough to justify BDSR as a standard treatment.

### Applications

The surgeon must obtain histologically negative margins on both the proximal and distal bile ducts and all tumor-bearing nodal tissue must be removed. BDSR with lymph node dissection can be an alternative treatment and may be justified in preference to a more radical operation for patients with MBD.

### Peer review

Even if it is a retrospective study, the data reported are interesting and well documented.

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S- Editor Wang YR L- Editor O'Neill M E- Editor Zheng XM

BRIEF ARTICLE

## Percutaneous transgastric computed tomography-guided biopsy of the pancreas using large needles

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**CONCLUSION:** Pancreatic biopsy can be obtained by a transgastric route using a large needle as an alternative method, without complications of peritonitis or bleeding.

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**Key words:** Biopsy; Computed tomography; Pancreas; Stomach

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### Abstract

**AIM:** To assess the safety, yield and clinical utility of percutaneous transgastric computed tomography (CT)-guided biopsy of pancreatic tumor using large needles, in selected patients.

**METHODS:** We reviewed 34 CT-guided biopsies in patients with pancreas mass, of whom 24 (71%) had a direct path to the mass without passing through a major organ. The needle passed through the liver in one case (3%). Nine passes (26%) were made through the stomach. These nine transgastric biopsies which used a coaxial technique (i.e. a 17-gauge coaxial introducer needle and an 18-gauge biopsy needle) were the basis of this study. Immediate and late follow-up CT images to detect complications were obtained.

**RESULTS:** Tumor tissues were obtained in nine pancreatic biopsies, and histologic specimens for diagnosis were obtained in all cases. One patient, who had a rare sarcomatoid carcinoma, received a second biopsy. One patient had a complication of transient pneumoperitoneum but no subjective complaints. An immediate imaging study and clinical follow-up detected neither hemorrhage nor peritonitis. No delayed procedure-related complication was seen during the survival period of our patients.

### INTRODUCTION

The diagnosis of a pancreatic mass detected by abdominal imaging can be difficult, and therapeutic decisions are based on the ability to diagnose or exclude malignancy<sup>[1]</sup>. Although most neoplasms are ductal adenocarcinomas, imaging modalities can not reliably be used to diagnose other malignant or benign conditions which may have different treatment options and prognoses<sup>[2]</sup>. Therefore, tissue diagnosis is often needed before surgery.

Computed tomography (CT)-guided biopsy for tissue diagnosis is well established<sup>[3-5]</sup>. In a prospective analysis of 125 procedures, CT-directed biopsy for pancreatic lesions had an accuracy of 95.2%<sup>[3]</sup>. An occasional limitation of the axial CT guidance of such interventional procedures is the presence of intervening vital structures which cannot be avoided even by using a gantry tilt technique<sup>[4]</sup>. Brandt *et al*<sup>[5]</sup> stated that there were no complications with fine, 21-gauge needle passage through the gastrointestinal tract. Fine needle biopsy for pancreatic lesions had an accuracy of 85%, whereas a large needle (16-19 gauge) had an accuracy of 92%<sup>[5]</sup>. Therefore, in daily practice, large needle biopsy could reduce the repeat biopsy rate. One experimental study in rabbits reported that the transgastric route with an 18-gauge cutting needle could

be used for pancreas biopsy, without apparent peritonitis or bleeding<sup>[6]</sup>. However, only a few studies have reported on the technique of large needle, transgastric-route biopsy of pancreatic lesions in humans<sup>[7,8]</sup>.

Since the initial description in 1992, tissue diagnosis under endoscopic ultrasonography (EUS) guidance has emerged as an important modality for evaluating patients with pancreatic lesions<sup>[9-11]</sup>. EUS-guided trucut needle biopsy using a 19-gauge needle, performed through the patient's stomach, is safe and accurate<sup>[11]</sup>. This procedure, however, is limited to selected patients who are willing to undergo endoscopy. Therefore, a transgastric approach using a large needle for CT-guided biopsy of the pancreatic lesion can be an alternative method of choice.

The aim of the present study was to assess the safety, yield, and clinical utility of percutaneous transgastric CT-guided biopsy in patients with pancreatic masses.

## MATERIALS AND METHODS

We reviewed the medical records of 34 consecutive CT-guided biopsies of a solid pancreatic mass from one institution (Taipei Veterans General Hospital, Taipei, Taiwan) over a 4-year period. All the patients were unwilling or failed to undergo EUS-guided biopsy. The indication for biopsy of these pancreatic masses was to obtain a diagnosis of malignancy from tissue before the patients received adjuvant chemotherapy or radiotherapy. Those patients with a resectable mass and scheduled for surgery were not included.

We analyzed the records of these biopsy procedures, and 24 had a direct path to the mass which did not pass through a major organ (71%). In one case (3%), the needle passed through the liver. Nine passes (26%) in eight patients were made through the stomach. These nine CT-guided biopsies performed with a transgastric approach were the basis of this study. The patients were one woman and seven men, with an average age of 65 years (range, 35-78 years).

Magnetic resonance imaging (MRI) or CT images before biopsy were reviewed by two experienced radiologists in a joint meeting, and the interpretation reached consensus. The proper pass route and the biopsy site were evaluated (Figure 1A). All patients were hospitalized and fasted overnight before the procedure. Biopsies were monitored with the patient in the supine position and performed on a CT scanner (Siemens, Germany). The reference scan was obtained first, and an opaque marker placed on the patient's abdomen (Figure 1B). The opaque marker consisted of several parallel segments of angiographic catheter. On the reference image, the accessible pass route and the distance were measured. The coaxial technique was applied with a 17-gauge coaxial introducer needle (Allegiance Health Corporation, McGaw Park, IL, USA) and an 18-gauge Temno biopsy needle (Allegiance Health Corporation, McGaw Park, IL, USA). The coaxial introducer needle penetrated the stomach wall as perpendicularly as possible, with its tip stopped on the edge of the target lesion (Figure 1C). Two to four strips of tissue were obtained in each procedure (Figure 1D).

An immediate follow-up CT image was obtained in all patients to detect possible complications. After each procedure, fasting was not necessary if there were no abnormal findings in follow-up CT images or complaints by the patients. All patients were observed in hospital until their condition was stable. Any delayed procedure-related complication was recorded on the patient's chart and by follow-up images.

## RESULTS

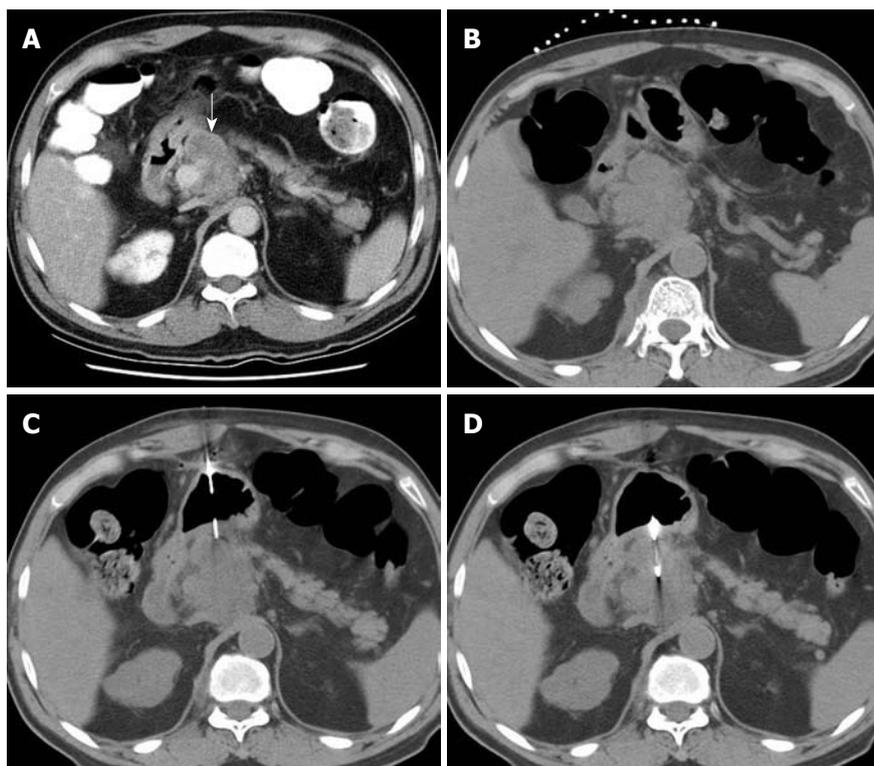
Table 1 shows basic data on our patients who underwent CT-guided transgastric biopsy. Nine biopsies of eight pancreas masses were successfully performed. The tumor sizes ranged from 20 to 75 mm (mean 48.5 mm). The tumor locations were the pancreatic body (62%) and pancreatic head (38%). Histologic diagnoses were obtained in all nine biopsies. There were five adenocarcinomas, one squamous cell carcinoma, one poorly differentiated carcinoma, and one sarcomatoid carcinoma. Because sarcomatoid carcinoma in the pancreas is very rare, our initial biopsy specimen was classified as atypical cells. A second biopsy of the same mass was then obtained and the specimen showed similar histologic findings. Our pathologist then revised the report as a sarcomatoid carcinoma.

One patient had transient, minimal pneumoperitoneum, but no abdominal pain or peritoneal signs were noted. The condition resolved after the patient fasted for 1 d, and was confirmed by an erect chest film. No patient had internal hemorrhage or peritonitis, confirmed by imaging study or during clinical follow-up. There were no late complications, such as tumor spread, in the follow-up images in our patients who had a survival of 1 to 19 mo.

## DISCUSSION

In this study, percutaneous puncture of a pancreatic mass was restricted to patients having advanced disease and who were not candidates for laparotomy. Histologic diagnosis was required in all patients scheduled for chemotherapy, radiotherapy, or both. Ihse *et al*<sup>[12]</sup> suggested that biopsy is not mandatory if the clinical suspicion of cancer is high and the surgical team has documented low postoperative mortality and morbidity rates.

Although percutaneous fine needle aspiration biopsy is well established in evaluating pancreatic masses<sup>[6]</sup>, this technique requires experienced cytopathologists for tissue diagnosis. The amount of aspirated material is often suboptimal for multiple histopathologic examination or certain analyses required to detect endocrine tumors of the pancreas<sup>[13]</sup>. Recently, Li *et al*<sup>[8]</sup> reported a successful diagnosis in 69 of 80 patients (86%) suspected of having pancreatic lesions using an 18-20-gauge cutting needle automated biopsy gun, with no serious complications. In our study, pancreatic biopsies were performed with an 18-gauge biopsy gun. A final histological diagnosis from pancreatic masses can be obtained with this method. In contrast, a correct diagnosis from percutaneous fine needle aspiration with limited tissue specimen may be difficult.



**Figure 1** A 67-year-old man with a pancreatic mass who had no safe route for approaching the target lesion during biopsy. A: Contrast-enhanced axial computed tomography (CT) scan shows a mass lesion (white arrow) in the pancreatic head; B: The patient was in the supine position, with opaque catheters placed on the abdominal wall as reference lines; C: Noncontrast CT image shows a 17-gauge coaxial introducer needle perpendicularly penetrating the gastric wall and the needle tip positioned at the edge of the target lesion; D: Noncontrast CT image shows an 18-gauge biopsy needle tip in the pancreatic mass.

**Table 1** Basic data of transgastric CT-guided biopsy in eight patients with pancreatic masses

Patient	Age/Gender	Target site (Pancreas)	Size <sup>1</sup> (mm)	Histology	Complication	Follow-up
1	74/M	Body	20	Adenocarcinoma	No	Expired, 8 mo later
2	67/M	Body	25	Adenocarcinoma	No, TP	Expired, 2 mo later
3	78/M	Body	30	Adenocarcinoma	No	Expired, 2 mo later
4	76/M	Head	75	Squamous cell carcinoma	No	Expired, 6 mo later
5	77/M	Head	48	Adenocarcinoma	No	Expired, 1 mo later
6	35/M	Body	50	Poorly differentiated carcinoma	No	Expired, 6 mo later
7	48/F	Body	40	Adenocarcinoma	No	Expired, 19 mo later
8	67/M	Head	20	Sarcomatoid carcinoma <sup>2</sup>	No	Expired, 6 mo later

<sup>1</sup>Greatest diameter; <sup>2</sup>Biopsy was performed twice. TP: Transient pneumoperitoneum; M: Male; F: Female; CT: Computed tomography.

In our study, we found that 29% of consecutive biopsies had no direct route for approaching the pancreatic mass without passing through a major organ. Nine passes were made through the stomach and one through the liver. The incidence of an indirect biopsy route was lower than that of previous CT-guided biopsies (40%) and higher than that of ultrasound-guided biopsies (24%)<sup>[5]</sup>. It is generally accepted and has been clinically proved that, for pancreas biopsy, a fine needle crossing the gastrointestinal tract, rather than through the liver<sup>[5]</sup>, is safe and will not result in complications<sup>[5]</sup>. One experimental study in rabbits reported that the transgastric route with an 18-gauge cutting needle could be used without apparent peritonitis and bleeding<sup>[6]</sup>. The EUS-guided trucut needle (19-gauge) biopsy of the pancreas, all performed transgastrically, has been claimed to be safe and accurate<sup>[11]</sup> in selected patients who agreed to undergo endoscopy.

The biopsy-related complication rate using fine needle is low (0.5% to 3%) and acute pancreatitis is the most frequent complication<sup>[14,15]</sup>. Zech *et al*<sup>[7]</sup> reported only one complication in 57 patients who developed acute

pancreatitis after a large core-needle biopsy of the pancreas. In the present study, all nine transgastric biopsies which penetrated both the anterior and posterior stomach walls were performed with 17-gauge coaxial transducer needles and then an 18-gauge biopsy gun. There were no immediate complications such as peritonitis or bleeding. One patient had transient pneumoperitoneum, which resolved after overnight fasting. Late complications, including tumor spread, were not found in follow-up images. However, our study was limited by a relatively short period of follow-up during the survival period of our patients.

The hole made by a gastrostomy catheter is larger than that caused by a biopsy needle. Some authors have reported no untoward effects after the removal of a 10 or 14 French catheter used for percutaneous gastrostomy<sup>[16]</sup>. The muscular layers run in three directions- oblique, circular and longitudinal- from the inner to the outer stomach wall. This arrangement is considered to be the reason why the stomach wall can be punctured without peritoneal leakage<sup>[17]</sup>.

In our study, the stomach was usually empty and

partly collapsed after overnight fasting. It is much more difficult to penetrate the stomach wall when it is “flaccid”. There are two tricks to piercing the stomach wall. The first is to keep the biopsy needle as perpendicular as possible to the gastric wall. If the biopsy needle was tangential, it would slide over rather than penetrate the gastric wall. The second is to advance the needle forcibly and quickly when penetrating the gastric wall. If the needle was advanced slowly, it would tent the gastric wall rather than piercing it. If the stomach wall is tented and can not be punctured, the needle should be withdrawn a little and then advanced again.

We conclude that percutaneous transgastric biopsy of the pancreas in selected patients with a combination of a 17-gauge introducer needle and an 18-gauge biopsy gun can be safe and has a high successful rate.

## COMMENTS

### Background

It is reasonable to obtain a histological diagnosis before treating patients who have pancreatic masses and are unsuitable or unwilling to undergo surgery. As the pancreas is a deep seated organ surrounded by other vital structures, it is a challenge for the physician to obtain an adequate specimen for histological examination. Endoscopic ultrasound-guided biopsy of pancreatic masses has been proved to be a safe and effective method. However, if the hospital has no such facilities or patients are unwilling or intolerant of the procedure, computed tomography (CT)-guided biopsy is an alternative method. In some cases, penetration of other vital organs is unavoidable when approaching the pancreatic mass. In this article, the authors clarified the safety and efficacy of percutaneous transgastric biopsy of pancreatic masses.

### Research frontiers

The stomach has three muscle layers and can be punctured without evidence of leakage. Percutaneous gastrostomy, either by endoscopy or fluoroscopy guidance, has been widely performed for a long time. Although only the anterior wall is punctured during gastrostomy and a catheter is put in place to block the hole, the authors think it would be safe to pierce both the anterior and posterior wall of the stomach using a large biopsy needle. An experimental study in rabbits also showed that it was safe to perform transgastric biopsy.

### Innovations and breakthroughs

Eight patients received 9 CT-guided transgastric biopsies of pancreatic masses located at the pancreas head or body without passing through vital organs. All procedures went smoothly. Only one patient had transient pneumoperitoneum which completely resolved the next day. Although this is a small series, their study shows that it is feasible to perform transgastric biopsy of a pancreatic lesion using a large needle.

### Applications

Besides pancreatic lesions, there are other types of pathology in the upper abdomen, such as enlarged lymph nodes or loculated fluid. Transgastric biopsy of lymph nodes or aspiration or drainage of fluid could be performed safely.

### Terminology

CT-guided biopsy uses CT scanning to perform a biopsy. When facing a deep seated lesion, or lesion blocked by gas, such as a lung nodule, CT scanning can provide better resolution and clearly shows the biopsy needle reaching the target.

### Peer review

As the authors stated in the introduction of the manuscript, endoscopic

ultrasonography guided biopsy of the pancreas is the gold standard to obtain sample tissue for histological diagnosis of pancreatic mass. Percutaneous transgastric CT-guided biopsy for patients with pancreatic mass should be considered an alternative.

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BRIEF ARTICLE

## Clinicopathological and prognostic analysis of 429 patients with intrahepatic cholangiocarcinoma

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cirrhosis. Multivariate analysis indicated that radical resection, lymph node metastases, macroscopic tumor thrombi and size, and CA19-9 were associated with prognosis.

**CONCLUSION:** Surgical radical resection is still the most effective means to cure ICC. Certain laboratory tests (such as CA19-9) can effectively predict the survival of the patients with ICC.

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**Key words:** Intrahepatic cholangiocarcinoma; Diagnosis; Pathology; Surgery; Survival

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### Abstract

**AIM:** To understand the clinicopathological characteristics and treatment selections and improve survival and provide valuable information for patients with intrahepatic cholangiocarcinoma (ICC).

**METHODS:** We retrospectively evaluated 5311 liver cancer patients who received resection between October 1999 and December 2003. Of these, 429 (8.1%) patients were diagnosed with ICC, and their clinicopathological, surgical, and survival characteristics were analyzed.

**RESULTS:** Upper abdominal discomfort or pain (65.0%), no symptoms (12.1%), and hypodynamia (8.2%) were the major causes for medical attention. Laboratory tests showed 198 (46.4%) patients were HBsAg positive, 90 (21.3%) had  $\alpha$ -fetoprotein > 20  $\mu$ g/L, 50 (11.9%) carcinoembryonic antigen > 10  $\mu$ g/L, and 242 (57.5%) carbohydrate antigen 19-9 (CA19-9) > 37 U/mL. Survival data was available for 329 (76.7%) patients and their mean survival time was 12.4 mo. The overall survival of the patients with R0, R1 resection and punching exploration were 18.3, 6.6 and 5.6 mo, respectively. Additionally, CA19-9 > 37 U/mL was associated with lymph node metastases, but inversely associated with

### INTRODUCTION

Patients with intrahepatic cholangiocarcinoma (ICC) are typically at an advanced pathological stage at the time of diagnosis, and are therefore associated with very poor prognosis. The incidence of ICC is increasing worldwide. The cause for this increase remains unknown and may be related to predisposing genetic and environmental factors<sup>[1,2]</sup>. The incidence rate of ICC is approximately 0.5-2.0/100 000 in males and slightly lower in females. In Europe and North America, ICC accounts for 10%-25% of liver cancers in males and even a higher proportion in females<sup>[1]</sup>. The etiology and pathogenesis of ICC are not known and remain to be defined, although many potential factors may contribute to it. For example, chronic biliary tract infection is generally recognized as the most common risk factor for ICC. A multidisciplinary synthetic therapy combining surgical resection with chemotherapy is the most widely used treatment protocol. Surgical resection is the therapeutic aspect with a capacity of curing ICC, while chemotherapy is mainly used for the

patients with unresectable or recurrent disease. Moreover, no conclusion has been reached as to whether adjuvant chemotherapy is effective in the control of ICC<sup>[3]</sup>. This may be because there are no standard chemotherapeutic protocols for ICC. Recently, Gemcitabine or Gemcitabine-based treatment has been a preferable choice to treat some ICC patients. Whether the patients with unresectable and non-metastatic ICC should be given liver transplantation treatment remains controversial, although the effect of liver transplantation for these patients was much better than that of palliative treatment<sup>[4]</sup>.

ICC often shows higher malignant grades and poorer prognosis than those of hepatocellular carcinoma (HCC). The 5-year survival rate of ICC is still less than 5%<sup>[5]</sup>. As a result, improving patients' survival with early detection and more aggressive treatment of ICC has been a focus of our research. Since ICC is a relatively rare neoplasm, to date, very few large-scale studies have been reported. In the current study, we have retrospectively assessed 429 cases of ICC that have undergone surgical treatment in the Eastern Hepatobiliary Surgery Hospital in Shanghai, China. We statistically evaluated the clinical characteristics, pathology, treatment, and prognosis of these patients to determine whether these parameters could contribute to a better prediction of patient survival.

## MATERIALS AND METHODS

### Patients

The study was approved by our institutional review board, and an informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. We retrospectively surveyed a total of 5311 patients with primary liver cancer who underwent surgical treatment in our hospital between October 1999 and December 2003. The pathological diagnoses of these patients included HCC, ICC, or mixed liver neoplasm. As a result, we obtained 429 cases of ICC from the total cases (8.1%). Clinicopathological characteristics for these patients were retrieved, including age, gender, the existence of cholelithiasis, chronic viral hepatitis, tumor size, number of lesions, existence of satellite lesions, lymph node metastases, extrahepatic metastases, cirrhosis, pathology (grade), tumor invasion, some routine tumor marker expressions [ $\alpha$ -fetoprotein (AFP), carbohydrate antigen 19-9 (CA19-9), and carcinoembryonic antigen (CEA)], surgical procedures, and survival data. For surgical procedures, R0 resection was defined as the *en bloc* resection with all margins histologically free of tumor, while R1 resection was defined as one in which the tumor mass was removed but section margins may not necessarily be tumor-free. Other patients underwent exploratory laparotomy for unresectable lesions. All patients were graded according to International Union Against Cancer (UICC) TNM classification, 1997 version. We attempted to follow all 429 patients, but only 329 were available for data analysis. The lost follow-up data in the 100 patients may be due to their death, loss of contact, or other unknown reasons.

### Statistical analysis

Statistical calculations and analyses were performed using SPSS11.0 software. Overall survival rate was plotted by the Life Table method. The univariate and multivariate predictors of prognosis were determined using univariate Cox regression analysis and the Cox proportional hazard model, respectively (Backward). The following variants were taken into account: age, gender, curative resection, lymph node metastases, number of intrahepatic lesions, satellite lesions, extrahepatic metastases, macroscopic tumor thrombi, pathology, cirrhosis, tumor size, encapsulation, microscopic tumor thrombi, tumor invasion, hepatitis B Virus (HBV) infection, AFP, CEA, and CA19-9. The Wilcoxon (Gehan) test was used to evaluate pair-wise comparisons between groups. The association between CA19-9 expression and clinicopathological parameters was analyzed using the  $\chi^2$  test and a logistic regression model.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Clinical features

The 429 ICC patients consisted of 301 men and 128 women, with ages ranging from 22 to 81 years, with a median age of 52 years. The main clinical manifestations included upper abdominal discomfort or pain (65.0%), an asymptomatic presentation (12.1%), hypodynamia (8.2%), abdominal distension (4.0%), jaundice (3.0%), nausea (2.8%), lower back pain (2.6%), abdominal mass, emaciation, and other symptoms (2.3%). Laboratory evaluations showed that 198 patients (46.4%) were HBsAg positive, 1 (1/321, 0.3%) was hepatitis C virus positive, 90 (21.3%) had AFP  $> 20$   $\mu\text{g/L}$ , 50 (11.9%) CEA  $> 10$   $\mu\text{g/L}$ , and 242 (57.5%) CA19-9  $> 37$  U/mL, as detected with an electrochemiluminescence immunoassay (Table 1).

Furthermore, 285 (66.4%) patients had only a single tumor mass, while additional 144 (33.6%) had multiple lesions. Tumor sizes were between 1.5 cm and 20 cm, with a mean size of  $7.1 \pm 3.8$  cm. Macroscopic satellite lesions were found in 99 cases, of these, 40 cases had  $\leq 3$  lesions and 59 cases had more than 3 lesions. In addition, there were 47 cases of macroscopic tumor thrombi with 32 intravascular thrombi (27 portal vein thrombi and 5 hepatic vein thrombi), 10 cases of bile duct thrombi, and 5 cases of concurrent thrombi. Lymph node metastases were found in 88 (20.5%) cases. Tumors metastasizing to lymph nodes at the porta hepatis and hepatoduodenal ligament accounted for 59.1% (52/88) while retroperitoneal metastases accounted for 27.2% (24/88). Extrahepatic metastases usually invaded into the diaphragm, abdominal wall, omentum, stomach, or duodenum (Table 1). TNM classifications are shown in Table 1.

### Surgical procedures and complications

All patients were preoperatively assessed and their operability was evaluated using computed tomography (CT), magnetic resonance imaging (MRI), or both. As a

**Table 1 Clinicopathological characteristics of 429 ICC patients**

	No. of cases	Total No. of cases	Percentage (%)
Gender		429	
Male	301		70.2
Female	128		29.8
Age (yr)		429	
< 53	220		51.3
≥ 53	209		48.7
Cholelithiasis		429	
No	383		89.3
Yes	46		10.7
Pathology T		429	
T1	11		2.5
T2	159		37.1
T3	112		26.1
T4	147		34.3
Pathology N		429	
N0	341		79.5
N1	88		20.5
Pathology M		429	
M0	408		95.1
M1	21		4.9
Pathology stage		429	
I	11		2.5
II	126		29.4
III	133		31.0
IV	159		37.1
Maximum tumor diameter (cm)		429	
≤ 5	145		33.8
> 5, ≤ 10	186		43.4
> 10	76		17.7
Diffuse type	22		5.1
Macroscopic satellite lesions		429	
No	330		76.9
≤ 3	40		9.3
> 3	59		13.8
Macroscopic tumor thrombi		429	
No	382		89.0
In blood vessel	32		7.5
In bile duct	10		2.3
In both	5		1.2
Serum HBsAg and HBcAb		427	
HBsAg (+)	198		46.4
HBsAg (-) and HBcAb (+)	60		14.0
HBsAg (-) and HBcAb (-)	169		39.6
Serum AFP (μg/L)		422	
No	332		78.7
> 20, ≤ 1000	70		16.6
> 1000	20		4.7
Serum CEA (μg/L)		420	
No	370		88.1
> 10, ≤ 100	36		8.6
> 100	14		3.3
Serum CA19-9 (U/mL)		421	
No	179		42.5
> 37, ≤ 507	143		34.0
> 507	99		23.5

ICC: Intrahepatic cholangiocarcinoma; AFP: α-fetoprotein; CEA: Carcino-embryonic antigen; CA19-9: Carbohydrate antigen 19-9.

result of preoperative assessment, 319 (74.3%) received R0 liver resection, 76 (17.7%) received R1 liver resection, and 34 (7.9%) received the exploratory laparotomy. Liver resection was performed using finger fracture and clamp crushing with intermittent Pringle's maneuver under room temperature. In all 395 patients (including R0 and

R1 resections), 237 underwent partial hepatectomy (172 tumors located within two or fewer segments and 65 within three or more segments), 51 segmentectomy or bisegmentectomy, 8 trisegmentectomy, 55 left hepatectomy, 26 right hepatectomy and 18 extended hepatectomy. Fifty-four patients also received common bile duct exploration for cholelithiasis or thrombus resection, 12 patients received Roux-en-Y cholangiojejunostomy, and 19 patients received resection of invading tissues or of organs surrounding liver. Thirty-five patients underwent lymph node dissection, among them 25 patients with and 10 patients without lymph nodes metastasis. Thirty-four patients were excluded from liver resection due to intrahepatic or extrahepatic metastasis and hepatic duct system invasion by tumor metastases or metastatic lymph node.

Five (1.2%) patients died within 1 mo after surgery, 3 of them died of hepatic failure, 1 died of intraperitoneal hemorrhage, and 1 died of adult respiratory distress syndrome (ARDS). Twenty-six (6.1%) patients had surgical complications, i.e. biliary leakage (13 cases), infection of pneumonia, subphrenic or, incision infection (7 cases), bleeding (4 cases), ARDS (1 case), and intestinal obstruction (1 case).

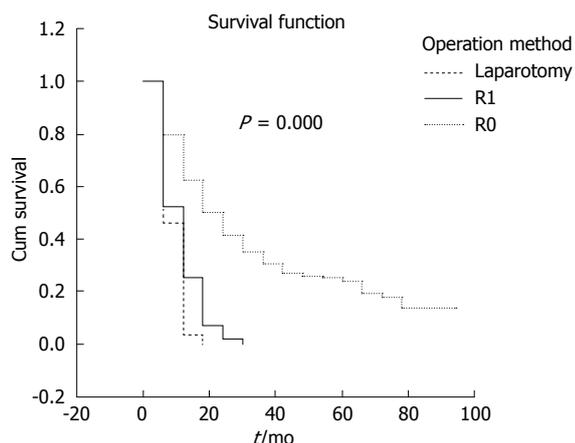
### Pathological features

After surgery, tumors were inspected macroscopically and microscopically, and the data indicated that poorly differentiated tumors accounted for 62.0%, while moderately and well differentiated tumors accounted for 36.7% and 1.3%, respectively. Microscopic tumor thrombi were found in 34.7% of the patients, and 89.4% of tumors did not have a pseudocapsule. One hundred and forty-six patients had cirrhosis in the liver, and of these 92 cases had small-nodule liver cirrhosis. Moreover, bile duct stones were observed in 10.7% (46/429) of patients.

### Prognosis and prognostic factors

The longest follow-up period is 8 years, but only 329 (76.7%) patients were available for data analysis, the rest patients were lost to follow-up after operation. Most the reasons for the lost follow-up is unknown but may be due to lost contact, death, or unspecified causes. Among these 329 patients, the mean survival time was 12.4 mo with 1-, 3- and 5-year survival rates of 50.9%, 22.2%, and 17.4%, respectively. The overall survival period for the patients with R0 resection was 18.3 mo with 1-, 3-, and 5-year survival rates of 62.5%, 30.2%, and 23.6%, respectively. The overall survival for the patients with R1 resection and punching exploration were only 6.6 and 5.6 mo. The overall survival in patients who received R0 resection was significantly higher than those who received R1 resection or punching exploration ( $P = 0.000$ , Figure 1 and Table 2).

Furthermore, the data from the univariate analysis found that prognostic factors included radical resection, lymph node metastases, satellite lesions, extrahepatic metastasis, tumor size, number of tumor lesions, and expression of CEA and CA19-9. The multivariate analysis further confirmed that radical resection, lymph node metastases, macroscopic tumor thrombi, tumor size, and



**Figure 1** The overall survival of patients with intrahepatic cholangiocarcinoma (ICC) after surgery.

Surgical procedures	n	Ratio (%)	Median survival time (mo)	Survival rate (%)			P value
				1-yr	3-yr	5-yr	
R0 <sup>a</sup>	319	74.3	18.3	62.5	30.2	23.6	0.000
R1 <sup>b</sup>	76	17.7	6.6	25.4	0	0	
Exploratory laparotomy	34	7.9	5.6	3.6	0	0	
Total	429	100	12.4	50.9	22.2	17.4	

<sup>a</sup>R0 vs R1 or exploratory laparotomy, *P* = 0.000; <sup>b</sup>R1 vs exploratory laparotomy, *P* = 0.360.

	Regression coefficient	Standard error	P value	Relative risk	95% CI
Curative resection	0.658	0.173	0.000	1.931	1.375-2.713
Lymph node metastases	0.432	0.218	0.048	1.540	1.004-2.361
Macroscopic tumor thrombi	0.455	0.206	0.027	1.576	1.053-2.360
Tumor size	0.159	0.080	0.046	1.173	1.003-1.372
CA19-9	0.191	0.085	0.024	1.210	1.025-1.428

CA19-9 were prognosis factors (Table 3). In addition, Chi-square tests showed that CA19-9 was associated with gender, age, tumor size, HBsAg positivity, and liver cirrhosis (Table 4). The logistic regression analysis revealed that CA19-9 was associated with lymph node metastases and inversely with liver cirrhosis (Table 5).

## DISCUSSION

### Risk factors of ICC

A recent review<sup>[2]</sup> showed the acknowledged risk factors in only a few cases of cholangiocarcinoma, which seem to be associated with chronic inflammation of the biliary epithelium (such as cholangiolithiasis, parasitic infection, intrahepatic biliary stones, and viral infection<sup>[6-10]</sup>). Primary sclerosing cholangitis is the most common known predisposing condition for cholangiocarcinoma in Western

**Table 4** Association of CA19-9 with clinicopathological parameters of the patients

	CA19-9 expression		P value
	> 37 U/mL	≤ 37 U/mL	
Gender			0.032
Male	159	135	
Female	83	44	
Age (yr)			0.040
< 53	112	101	
≥ 53	130	78	
Surgical procedures			0.106
R0	174	139	
R1	44	32	
Exploratory laparotomy	24	8	
Lymph node metastasis			0.082
Yes	182	148	
No	57	30	
Macroscopic satellite lesions			0.229
No	184	140	
≤ 3	20	20	
> 3	38	19	
Extrahepatic metastases			0.185
Yes	227	179	
No	15	6	
Macroscopic tumor thrombi			0.440
Yes	218	157	
No	24	22	
Microscopic tumor thrombi			0.192
Yes	142	120	
No	83	53	
Tumor differentiation			0.221
Well	4	1	
Moderate	85	52	
Poorly	126	105	
Number of lesions			0.843
Single	160	120	
Multiple	82	59	
Tumor size (cm)			0.002
≤ 5	69	72	
> 5, ≤ 10	120	64	
> 10	37	39	
Diffuse type	16	4	
HBsAg			0.049
Yes	101	92	
No	141	87	
Cirrhosis			0.000
Yes	68	80	
No	174	99	

**Table 5** Logistic regression analysis in relationship between CA19-9 expression, lymph node metastases, and liver cirrhosis

	Regression coefficient	Standard error	P value	Relative risk	95% CI
Lymph node metastases	0.637	0.295	0.031	1.891	1.060-3.374
Cirrhosis	-0.539	0.230	0.019	0.584	0.372-0.915
Constant	0.336	0.146	0.021	1.340	

countries<sup>[2]</sup>. In follow-up, or in examination of tissue specimens of cholangiocarcinoma, primary sclerosing cholangitis was found to account for 8%-40% of cholangiocarcinoma. In the current study, 198 patients were HBsAg-positive, accounting for 46.4% of cases, which is significantly higher than the estimated 10% HBV

carrier rate in Chinese population. This data indicate that HBV infection may be one of the risk factors for ICC. Moreover, an additional 60 patients were found to be serum positive for anti-HBc antibody, although they were negative for HBsAg, which is indicative of a past HBV infection. Combining HBsAg and anti-HBc expression, our study population had 60.4% patients with HBV or a history of HBV infection. However, it is unclear how HBV infection contributes to development of ICC. The association of cirrhosis with cholangiocarcinoma development may illuminate HBV infection as a risk factor for cholangiocarcinoma. HBV infection causes the majority of liver cirrhosis in Asian countries. Although other studies showed that hepatitis C virus infection was a risk factor for ICC<sup>[8-9]</sup>, our study did not confirm it because of very low infection rate (0.3%) in our patients.

### Diagnosis

Initial and early diagnosis of ICC could be very difficult to achieve due to the wide range of differential diagnoses. Features identified in CT or MRI evaluations are not typical for ICC, as minimal contrast may occur after enhancement. Therefore, some tumor markers, such as CA19-9, CEA and AFP, may add to the differential diagnoses or diagnostic guide for ICC, although these biomarkers may not be specific for ICC. In the current study, elevated CA19-9, CEA and AFP occurred in 57.5%, 11.9% and 21.3% of the patients, respectively, and 70.9% patients were found to express at least one of these markers. Previous studies did report expression of these biomarkers in association with ICC<sup>[11-13]</sup>; however, due to lack of a large number of patients, the exact rate of positive expression of these markers remained unrevealed until the information reported in this current study.

Nevertheless, it is well known that detection of AFP expression is routinely used for early diagnosis of HCC, and given the high infection rate of HBV in the Chinese population, HCC should be first considered in a patient with elevated AFP. In the present study, 23 patients exhibited an increased AFP ( $> 200 \mu\text{g/L}$  but  $\leq 1000 \mu\text{g/L}$ ), while highly increased AFP ( $> 1000 \mu\text{g/L}$ ) was found in 20 patients, accounting for 5.4% and 4.7% of cases, respectively. Therefore, ICC should also be taken into account for patients with elevated AFP. In addition, for patients with high levels AFP but negative in CA19-9 and CEA, ICC should also be considered before operation.

Furthermore, Positron Emission Computed Tomography (PET)/CT could be an alternative method for differential diagnoses of ICC, as it is superior to the enhanced CT in the diagnosis of extrahepatic or lymph node metastases<sup>[14]</sup>.

### Relationship between CA19-9 levels and clinical features

CA19-9 or known as sialylated Lewis antigen is a blood tumor marker and was discovered in patients with colon cancer and pancreatic cancer in 1981<sup>[15]</sup>. Previous studies found that CA19-9 expression was also prevalent in ICC<sup>[2]</sup>. In the current study, CA19-9 ( $> 37 \text{ U/mL}$ ) was found in 57.5% of ICC patients. Further analyses found

that CA19-9 positivity was significantly associated with gender, age, tumor size, cirrhosis, and HBsAg expression, while logistic regression analysis indicated that expression of CA19-9 was significantly associated with cirrhosis and lymph node metastases. ICC patients with CA19-9 ( $> 37 \text{ U/mL}$ ) presented a higher incidence of lymph node metastases. Other studies demonstrated association of positive CA19-9 and lymph node metastases of gastric and colorectal cancers<sup>[16-20]</sup>.

In addition, our study revealed that CA19-9 ( $> 37 \text{ U/mL}$ ) rate was lower in cirrhosis patients with positive HBsAg. The underlying mechanism for this remains unknown and needs further investigations. However, Schöniger-Hekele *et al*<sup>[21]</sup> reported that the combined elevation of CA19-9 and CA 125 was useful for diagnosis of the advanced fibrosis or cirrhosis. Their observation is definitely not compatible with the results of this current study.

### Surgical resection and prognosis

To date, surgical resection is still the primary and most effective means to cure ICC. Nevertheless, the selection methods used to determine a patient's suitability for surgery will directly affect the patient's chances of survival. In this study, the mean survival of patients receiving R0 resection was 18.3 mo, whereas the mean survival rate for patients with R1 resection was only 6.6 mo, indicating that radical resection is the most important factor in prolonging patient survival. Comparing R1 resection and exploratory laparotomy, the former exhibited a slightly better prognosis; however, this is not statistically significant ( $P = 0.36$ ).

Several other studies<sup>[22-27]</sup> showed that the 1-year survival rate of patients receiving R0 resection was between 61% and 83%, and the 5-year survival rate was between 22% and 63% (Table 6), indicating that their survival rates were much higher than those of our patients. Besides the different patient population and severity of the diseases, we proposed that this might be due to the different surgical methodology. For example, segmental resection is extensively used in Western countries, while non-anatomic resection is primarily used in China. The former is a more curative procedure owing to wider resection margins. The low rate of radical resection may be due to the invasion of local and portal hepatic ducts by ICC. Lymph node metastases and distant metastasis were often observed in patients with ICC.

However, it remains debatable whether extended radical operation in combination with lymph node dissection could improve survival rates. Some studies have reported that 1- and 3-year survival rates were 94% and 82%, respectively, after extended hepatectomy (including vessel resection and reconstruction) in patients with solitary tumors but without vascular invasion or extrahepatic or lymph node metastases<sup>[28]</sup>. However, rather than positive effects, increased morbidity was observed in patients with extended surgery that included anatomic hepatic resection, vessel resection and reconstruction, and extended lymph node dissection<sup>[29]</sup>.

Table 6 Comparison of post-operative survival after R0 resection

Author	No. of total	No. of R0	Ratio (%)	Survival (%)		
				1-yr	3-yr	5-yr
Ohtsuka <i>et al</i> <sup>[22]</sup> , 2003	50	34	68	61.6	37.6	22.5
Morimoto <i>et al</i> <sup>[23]</sup> , 2003 <sup>1</sup>	51	35	68.6	68.2	44.1	32.4
Nakagawa <i>et al</i> <sup>[24]</sup> , 2005	53	44	83.0	66.2	38.3	26.3
Lang <i>et al</i> <sup>[25]</sup> , 2006	54	30	55.5	83	58	48
DeOliveira <i>et al</i> <sup>[26]</sup> , 2007	44	34	77.3	NR	NR	63
Konstadoulakis <i>et al</i> <sup>[27]</sup> , 2008	72	54	75	80	49	25
Our current study	429	319	74.3	62.5	30.2	23.6

<sup>1</sup>Two cases of death were excluded. NR: Not reported.

### Prognostic factors

The present study showed that favorable prognostic factors for ICC are: radical resection, no metastasis of lymph nodes, a small tumor diameter, no macroscopic tumor thrombi, and low levels of CA19-9. Among these favorable factors, radical resection, no metastasis of lymph nodes, and a small tumor diameter are consistent with previous studies<sup>[22,24,30,31]</sup>. This study also showed that macroscopic tumor thrombi and CA19-9 expression were prognostic factors for ICC. In addition, ICC with CA19-9 (> 37 U/mL) exhibited a higher grade of malignancy and prevalence of lymph node metastases. Ohtsuka *et al*<sup>[32]</sup> also reported that CA19-9 was a prognostic factor of ICC. Other studies demonstrated that macroscopic tumor thrombus is a key factor for poor prognosis of hepatocellular carcinoma<sup>[33-35]</sup>. As the incidence of macroscopic tumor thrombus is relatively low in ICC (only 11% in our current study), it could be easily missed, especially studies with a small sample size.

### Liver transplantation

Originally, the prognosis of ICC patients who received liver transplantation treatment was not satisfactory. In particular, Pascher *et al*<sup>[36]</sup> reported that 5-year survival rate reached 29% in a study, but did not exceed 18% in other four studies. However, most recent studies showed an improving 5-year survival rate between 33% and 42%<sup>[4,37]</sup>. Multivariate analysis revealed that single tumor, tumor-free margins, no lymph node metastasis, no jaundice, or no perineural invasion, and early TNM stage were associated with better prognosis<sup>[4,38,39]</sup>. Nevertheless, due to restricted resources of liver donors and poor prognosis after liver transplantation, it is still controversial whether the patients with unresectable and non-metastasis ICC should undergo liver transplantation. Further studies are needed to determine the criteria for selecting the patients who can benefit from liver transplantation. In addition, the effectiveness of adjuvant radiotherapy and chemotherapy both before and after transplantation remains to be defined.

In conclusion, our present study demonstrated that hepatitis B infection, CA19-9, CEA, and AFP are associated with ICC development. CA19-9 levels are associated with lymph node metastases, but inversely with cirrhosis. Radical resection (R0) is the key prognostic factor for ICC. Future studies should focus on evaluation of the molecule-targeted

therapy, and whether it can efficiently control this deadly disease so as to improve the survival of the patients.

## COMMENTS

### Background

Incidence of intrahepatic cholangiocarcinoma (ICC) is increasing worldwide and its prognosis is very poor. Thus, further studies on its clinical characteristics for early detection and on surgical treatment for better prognosis are urgently needed.

### Research frontiers

Early detection of ICC could focus on defining clinical characteristics and biomarker study. Surgery with radical resection always is the key factor to improve the survival of the patients. The effectiveness of chemotherapy is currently limited and novel approaches are needed.

### Innovations and breakthroughs

This study demonstrated that carbohydrate antigen 19-9 (CA19-9) is commonly elevated in ICC and associated with lymph node metastases, but inversely associated with liver cirrhosis, indicating that CA19-9 could further be evaluated for early detection and prognosis of ICC. In addition, hepatitis B virus infection is associated with cholangiocarcinoma and increased  $\alpha$ -fetoprotein (AFP) levels may also be considered for ICC, although AFP is a routinely used biomarker for hepatocellular carcinoma.

### Applications

This study provides an initial assessment of ICC and further studies are needed to confirm the findings, which can apply to future early detection, prediction of prognosis, treatment election, and differential diagnosis of ICC.

### Peer review

This is an interesting paper, with a large number of patients involved, which might be of benefit for future studies of ICC.

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## Neoadjuvant chemoradiotherapy for resectable esophageal carcinoma: A meta-analysis

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### Abstract

**AIM:** To compare neoadjuvant chemoradiotherapy and surgery with surgery alone for resectable esophageal carcinoma.

**METHODS:** We used MEDLINE and EMBASE databases to identify eligible studies and manual searches were done to ensure no studies were missed. Trial validity assessment was performed and a trial quality score was assigned.

**RESULTS:** Eleven randomized controlled trials (RCTs) including 1308 patients were selected. Neoadjuvant chemoradiotherapy significantly improved the overall survival compared with surgery alone. Odds ratio (OR) [95% confidence interval (CI),  $P$  value], expressed as neoadjuvant chemoradiotherapy and surgery vs surgery alone, was 1.28 (1.01-1.64,  $P = 0.05$ ) for 1-year survival, 1.78 (1.20-2.66,  $P = 0.004$ ) for 3-year survival, and 1.46 (1.07-1.99,  $P = 0.02$ ) for 5-year survival. Postoperative mortality increased in patients treated by neoadjuvant chemoradiotherapy (OR: 1.68, 95% CI: 1.03-2.73,  $P = 0.04$ ), but incidence of postoperative complications was similar in two groups (OR: 1.14, 95% CI: 0.88-1.49,  $P = 0.32$ ). Neoadjuvant chemoradiotherapy lowered the local-regional cancer recurrence (OR: 0.64, 95% CI: 0.41-0.99,  $P = 0.04$ ), but incidence of distant cancer recurrence was similar (OR: 0.94, 95% CI: 0.68-1.31,  $P = 0.73$ ). Histological subgroup analysis indicated that esophageal squamous cell carcinoma did not benefit from neoadjuvant

chemoradiotherapy, OR (95% CI,  $P$  value) was 1.16 (0.85-1.57,  $P = 0.34$ ) for 1-year survival, 1.34 (0.98-1.82,  $P = 0.07$ ) for 3-year survival and 1.41 (0.98-2.02,  $P = 0.06$ ) for 5-year survival.

**CONCLUSION:** Neoadjuvant chemoradiotherapy can raise the survival rate of patients with esophageal adenocarcinoma.

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**Key words:** Esophageal carcinoma; Neoadjuvant chemoradiotherapy; Randomized controlled trial; Meta-analysis

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Jin HL, Zhu H, Ling TS, Zhang HJ, Shi RH. Neoadjuvant chemoradiotherapy for resectable esophageal carcinoma: A meta-analysis. *World J Gastroenterol* 2009; 15(47): 5983-5991 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5983.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5983>

### INTRODUCTION

Esophageal carcinoma (EC) is the sixth commonest cause of tumor-related death around the world<sup>[1]</sup>. It is endemic in Asia, southern and eastern Africa, and northern France<sup>[2,3]</sup>. North America and many western European countries are low-incidence regions, but the nearly 6-fold increase in the incidence of esophageal adenocarcinoma (EAC) in the past three decades and the corresponding 7-fold increase in mortality are remarkable. Surgery has always been considered as the standard treatment for patients with resectable esophageal cancer, but the effectiveness of surgery alone was unsatisfactory and the median survival of patients treated by surgery alone rarely exceeded 18 mo<sup>[4]</sup>. So clinicians always make efforts to seek for new treatment strategies to prolong the survival time of patients with

EC. Recently neoadjuvant chemoradiotherapy plus surgery has been studied widely, but opinions vary among clinicians as to the therapeutic effect of the new method, and the outcomes of randomized controlled trials (RCTs) were not consistent. Published meta-analyses did not reach a consensus, some of which was short of enough RCTs or adopted unpublished data. The current study aims to perform a meta-analysis to compare neoadjuvant chemoradiotherapy plus surgery with surgery alone for resectable EC by enough eligible published RCTs to date.

## MATERIALS AND METHODS

Computerized bibliographic and manual searches were done to identify all eligible published literature between 1980 and 2008. MEDLINE and EMBASE were the primary source of RCTs, with the following key words: esophageal cancer, surgery, radiotherapy, chemotherapy, neoadjuvant chemoradiotherapy and RCT. Manual searches were performed by reviewing articles and abstracts cited in the published meta-analysis and RCTs.

The eligible studies must meet the following inclusion criteria: (1) It must be a prospective RCT which compares neoadjuvant chemoradiotherapy plus surgery with surgery alone in the initial management of resectable EC; (2) Outcomes must include survival data; (3) There was no statistical significance in factors such as sex, age, type of pathology, tumour stage between the two groups; and (4) Studies were analyzed by intention-to-treat patients. Trials were not excluded because of cancer histology (squamous cell carcinoma or adenocarcinoma) or language of publication. Unpublished reports, abstracts and theses were excluded. This meta-analysis was performed according to the QUOROM statement<sup>[5]</sup>.

All data were abstracted by three independent researchers and the methodological qualities of all RCTs were assessed by three aspects: blinding, randomization and handling withdrawals and dropouts<sup>[6]</sup>. If researchers had discrepancies in assessing RCTs, a consensus was reached by discussion.

Outcomes including 1-year survival, 3-year survival, 5-year survival, postoperative mortality, incidence of postoperative complication, incidence of local-regional cancer recurrence and incidence of distant cancer recurrence were analyzed. In two trials<sup>[7,8]</sup> we used the Kaplan-Meier estimate of the 1-year survival and 3-year survival in the two groups and the data for the 5-year survival was obtained from another trial<sup>[9]</sup> in the same way. The remaining data were directly available in the corresponding RCTs. Evaluation of therapeutic effectiveness, including survival rate and incidence of recurrence, was performed in all patients who were enrolled in these trials, but for postoperative events, data were calculated only based on the number of patients who underwent surgery as the denominator. Sensitivity analyses were performed to identify the effect of histological subtype (squamous cell carcinoma or adenocarcinoma) and

scheduling of neoadjuvant chemoradiotherapy (concurrent or sequential) on survival.

Data were analyzed by RevMan 4.2.10.  $\chi^2$  tests were used to assess heterogeneity of study results and a planned cut-off for significance of  $P \leq 0.05$ . If  $P > 0.05$ , we used a fixed effect model, otherwise we used a random effect model. The odds ratios (OR) among the frequency of events in both neoadjuvant chemoradiotherapy plus surgery group (CRT group) and surgery alone group (S group) was calculated and these OR are presented as a point estimate with 95% confidence intervals (CI) and  $P$  values in parentheses. The significance level was set at 5%. Funnel plot analysis did not suggest publication bias against negative trials.

## RESULTS

### Features of RCTs

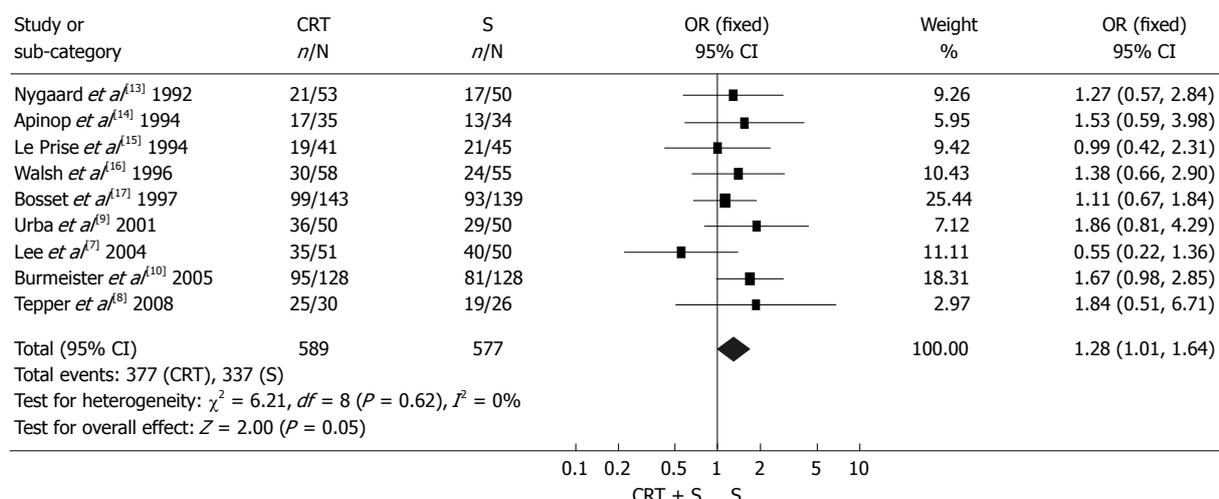
Eleven randomized studies were identified from 1980 to 2008 and the main features of the trials included in the meta-analysis are shown in Table 1<sup>[7-17]</sup>. All studies were published literature. Nine countries including Australia, United States of America, China, France, Ireland, Japan, Korea, Norway and Thailand were involved in the RCTs.

The studies were carried out from 1983 to 2002 and the literatures were published between 1992 and 2008. Because double blinding can not be performed due to the inherent difficulty of the design of the trial (e.g. chemotherapy and radiotherapy) and the method of randomization was not reported in most trials, the RCT quality scores ranged from 1 to 3 (5-point scale) and the average was 2.3<sup>[5,6]</sup>. Of these 11 studies, seven were restricted to patients with esophageal squamous cell carcinoma (ESCC) only, one was restricted to patients with EAC only, and the remaining three trials enrolled patients with either ESCC or EAC. The 11 RCTs included 1308 patients, 659 of whom received neoadjuvant chemoradiotherapy before surgery, and the remaining 649 patients received surgery alone. Nearly all the patients in the S group underwent surgery, yet there were more patients in the CRT group who had not completed the planned treatment regimen for various causes such as side effects of chemotherapy or metastasis of cancer before surgery. The tumor stage of the most patients in the 11 studies ranged from I-III (1987 UICC), but more advanced tumor stage (IVa) was also seen in two RCTs<sup>[9,11]</sup>. In addition, tumor stages were classified in the RCT by Le Prise *et al*<sup>[15]</sup> according to the 1978 American Joint Committee on Cancer, which was not a TNM staging. Finally tumor stage was not reported in the RCT by Walsh *et al*<sup>[16]</sup>.

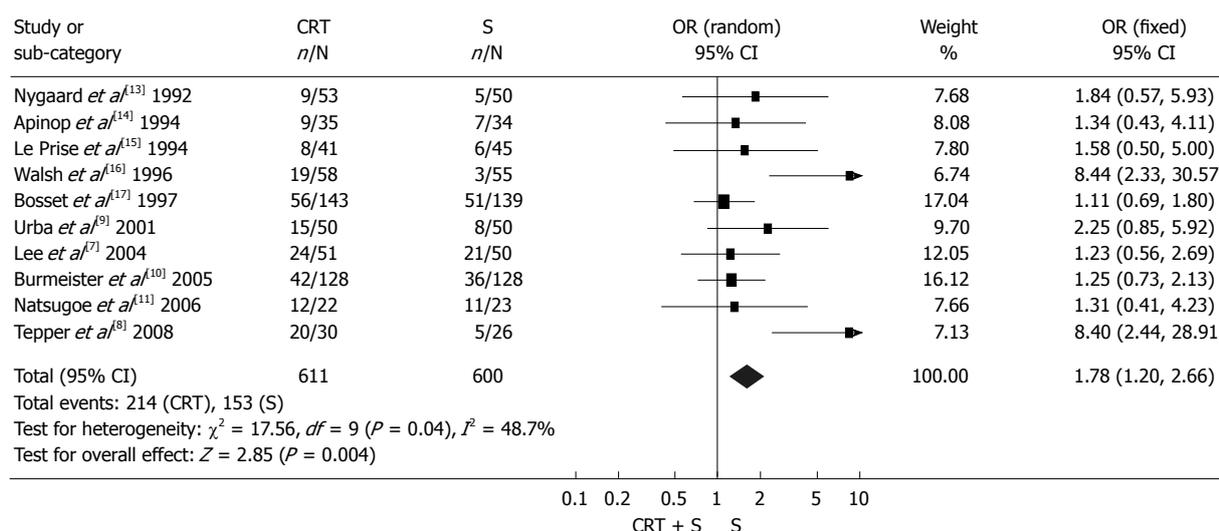
### Survival rate

The effect of neoadjuvant chemoradiotherapy on survival rate is shown in Figure 1. Obviously, there was statistical significance in survival rate between the two groups. OR (95% CI,  $P$  value), expressed as neoadjuvant chemoradiotherapy plus surgery *vs* surgery alone, was 1.28

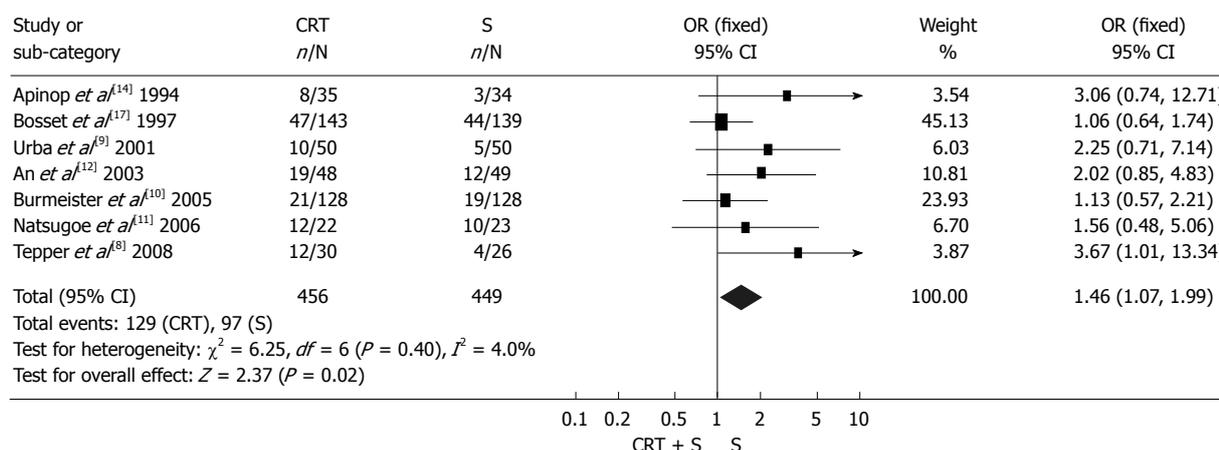
**A** Review: Neoadjuvant chemoradiotherapy for resectable esophageal carcinoma: A meta-analysis  
 Comparison: CRT group *vs* S group  
 Outcome: 1-yr survival



**B** Review: Neoadjuvant chemoradiotherapy for resectable esophageal carcinoma: A meta-analysis  
 Comparison: CRT group *vs* S group  
 Outcome: 3-yr survival



**C** Review: Neoadjuvant chemoradiotherapy for resectable esophageal carcinoma: A meta-analysis  
 Comparison: CRT group *vs* S group  
 Outcome: 5-yr survival



**Figure 1** Postoperative survival rate in neoadjuvant chemoradiotherapy and surgery compared with surgery alone. A: One-year survival; B: Three-year survival; C: Five-year survival. CRT + S: Neoadjuvant chemoradiotherapy and surgery; S: Surgery; OR: Odds ratio; CI: Confidence interval.

Table 1 Features of all trials included in the meta-analysis

Country	Year of RCT published	SCC or AC	Schedule of radiotherapy	Schedule of chemotherapy	Concurrent or sequential	Time of surgery
Norway	1992	SCC	35 Gy 1.75 Gy/d 5 d/wk for 4 wk	Cisplatin: 20 mg/m <sup>2</sup> D1-5, D15-19 Bleomycin: 10 mg/m <sup>2</sup> D1-5, D15-19	Sequential	Not report
Thailand	1994	SCC	40 Gy 2 Gy/d 5 d/wk for 4 wk	Cisplatin: 100 mg/m <sup>2</sup> D1, 29 FU: 1000 mg/m <sup>2</sup> D1, D29-32	Concurrent	4 wk after completion of chemotherapy
France	1994	SCC	20 Gy 2 Gy/d D8-19	Cisplatin: 100 mg/m <sup>2</sup> D1,21 FU: 600 mg/m <sup>2</sup> D2-5, D22-25	Sequential	D42
Ireland	1996	AC	40 Gy 2.67 Gy/d	Cisplatin: 75 mg/m <sup>2</sup> D7 FU: 15 mg/kg D1-5	Concurrent	8 wk after CRT
France	1997	SCC	37 Gy 3.7 Gy/d 5 d/wk for 2 wk	D1-5, 8-12, 15-19 Cisplatin: 80 mg/m <sup>2</sup> D0-2	Sequential	2-4 wk after CRT
USA	2001	SCC and AC	45 Gy 1.5 Gy <i>bid</i> D1-5, 8-12, 15-19	Cisplatin: 20 mg/m <sup>2</sup> D1-5, 17-21 FU: 300 mg/m <sup>2</sup> D1-21	Concurrent	D42
China	2003	SCC	36 Gy 3 Gy/d D21-24, 28-31, 35-38	Vinblastine: 1 mg/m <sup>2</sup> D1-4, 17-20 Cisplatin: 25 mg/m <sup>2</sup> D2-5, D22-25 FU: 1000 mg/m <sup>2</sup> D1-5	Sequential	3 wk after CRT
Korea	2004	SCC	45.6 Gy 1.2 Gy <i>bid</i> D1-28	500 mg/m <sup>2</sup> D21-25 Cisplatin: 60 mg/m <sup>2</sup> D1, 21 FU: 1000 mg/m <sup>2</sup> D2-5	Concurrent	3-4 wk after completion of radiotherapy
Australia	2005	SCC and AC	35 Gy 2.33 Gy/d 5 d/wk for 3 wk	Cisplatin: 80 mg/m <sup>2</sup> D1 FU: 800 mg/m <sup>2</sup> D2-5	Concurrent	3-6 wk after completion of radiotherapy
Japan	2006	SCC	40 Gy 2 Gy/d 5 d/wk for 4 wk	Cisplatin: 7 mg/m <sup>2</sup> FU: 350 mg/m <sup>2</sup> 5 d/wk for 4-6 wk	Concurrent	35-40 d after CRT
USA	2008	SCC and AC	50.4 Gy 1.8 Gy/d 5 d/wk for 5.5 wk	Cisplatin: 100 mg/m <sup>2</sup> D1,29 FU: 1000 mg/m <sup>2</sup> D1-4, D29-32	Concurrent	3-8 wk after CRT

SCC: Squamous cell carcinoma; AC: Adenocarcinoma; CRT: Chemoradiotherapy.

Table 2 Survival rate estimates of patients with EC by schedule of chemoradiotherapy

Schedule of CRT	Overall survival	No. of studies	No. of patients		OR (95% CI)	P
			CRT + S	S		
Sequential	1 yr	3	237	234	1.12 (0.77, 1.64)	0.56
	3 yr	3	237	234	1.24 (0.82, 1.88)	0.31
	5 yr	2	191	188	1.24 (0.81, 1.91)	0.32
Concurrent	1 yr	6	352	343	1.41 (1.03, 1.94)	0.03
	3 yr	7	374	366	2.12 (1.20, 3.76)	0.01 <sup>1</sup>
	5 yr	5	265	261	1.72 (1.10, 2.71)	0.02

<sup>1</sup>Random effects model was used. EC: Esophageal carcinoma; CRT + S: Neoadjuvant chemoradiotherapy and surgery; S: Surgery; OR: Odds ratio; CI: Confidence interval.

(1.01-1.64,  $P = 0.05$ ) for 1-year survival, 1.78 (1.20-2.66,  $P = 0.004$ ) for 3-year survival and 1.46 (1.07-1.99,  $P = 0.02$ ) for 5-year survival. Subgroup analysis showed that there was no survival benefit from neoadjuvant chemoradiotherapy in EC patients when chemotherapy and radiotherapy were given sequentially. On the contrary, EC patients benefited from concurrent chemoradiotherapy. The corresponding OR (95% CI,  $P$  value) is shown in Table 2. Moreover, patients with ESCC did not get any survival benefit from neoadjuvant chemoradiotherapy and corresponding OR (95% CI,  $P$  value) is shown in

Table 3 Survival rate estimates of patients with ESCC for neoadjuvant chemoradiotherapy compared with surgery alone

Overall survival	No. of studies	No. of patients		OR (95% CI)	P
		CRT + S	S		
1 yr	6	368	368	1.16 (0.85, 1.57)	0.34
3 yr	7	390	391	1.34 (0.98, 1.82)	0.07
5 yr	5	293	295	1.41 (0.98, 2.02)	0.06

ESCC: Esophageal squamous cell carcinoma.

Table 3. In addition, another subgroup analysis indicated that the 3-year survival in CRT group was significantly higher than that of S group in patients of the USA and Europe, but in patients of Asia, it was a pessimistic result (Table 4).

### Morbidity after surgery

The resection rate in patients treated with surgery alone was markedly higher than that in patients treated with preoperative chemoradiotherapy (OR: 0.36, 95% CI: 0.24-0.54,  $P < 0.00001$ ), but patients treated with preoperative chemoradiotherapy were more likely to obtain a complete resection (R0 resection), which was defined as gross disease removed with negative margins (OR: 2.16, 95% CI: 1.58-2.97,  $P < 0.00001$ ) (Figure 2). Mortality

Review: Neoadjuvant chemoradiotherapy for resectable esophageal carcinoma: A meta-analysis  
 Comparison: CRT group vs S group  
 Outcome: R0 resection rate

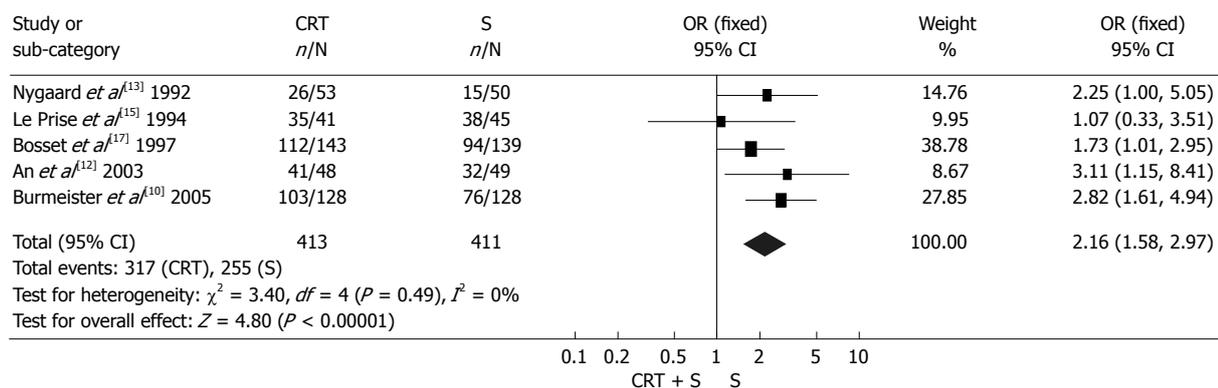


Figure 2 R0 resection rate in neoadjuvant chemoradiotherapy and surgery compared with surgery alone.

Review: Neoadjuvant chemoradiotherapy for resectable esophageal carcinoma: A meta-analysis  
 Comparison: CRT group vs S group  
 Outcome: Postoperative mortality

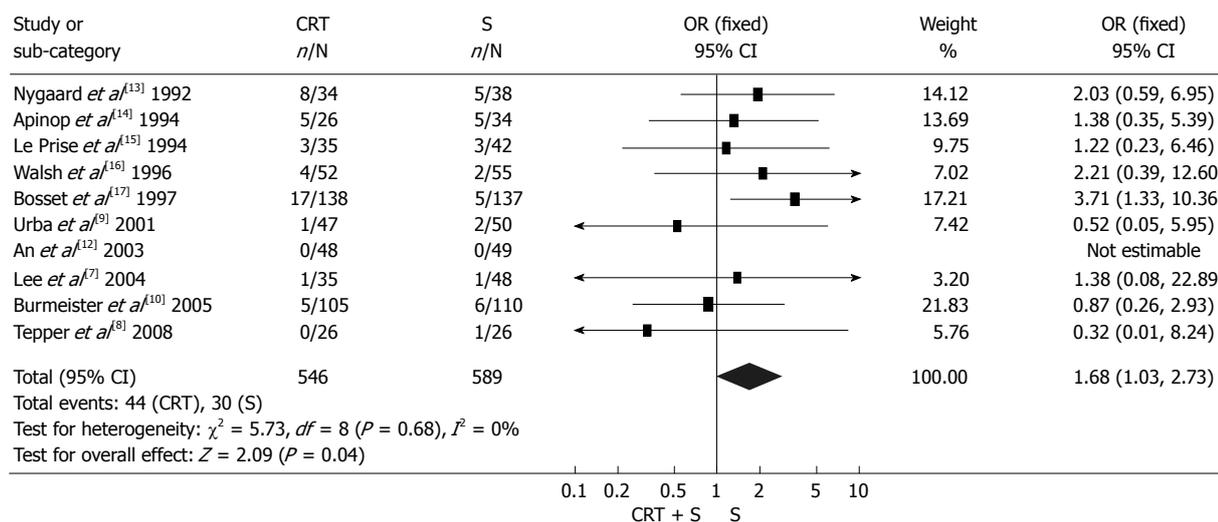


Figure 3 Postoperative mortality in neoadjuvant chemoradiotherapy and surgery compared with surgery alone.

Population	No. of studies	No. of patients		OR (95% CI)	P
		CRT + S	S		
USA	2	80	76	3.74 (1.77, 7.88)	0.0005
Europe	4	295	289	1.59 (1.09, 2.33)	0.02
Asia	3	108	107	1.27 (0.72, 2.24)	0.40

after surgery varied from 0% to 23.5% in CRT group (the highest mortality was in the RCT by Nygaard *et al*<sup>[13]</sup>) and from 0% to 14.7% in S group (the highest mortality was in the RCT by Apinop *et al*<sup>[14]</sup>). Mortality after surgery in CRT group was higher than that in S group (OR: 1.68, 95% CI: 1.03-2.73,  $P = 0.04$ ) (Figure 3). But if the RCT by Nygaard *et al*<sup>[13]</sup> or the RCT by Bosset *et al*<sup>[17]</sup> were excluded, there was no significant difference between the two groups and corresponding OR (95% CI,  $P$  value) was 1.66 (0.99-2.79,  $P = 0.06$ ) and 1.30 (0.75-2.28,  $P = 0.35$ ).

The postoperative complications included nonfatal and fatal complications. There was no significant difference between the two groups (OR: 1.14, 95% CI: 0.88-1.49,  $P = 0.32$ ) (Figure 4).

**Effect on recurrence**

First treatment failure was defined as unequivocal histological or radiological evidence of tumor recurrence for the first time after surgery wherever the tumor relapsed. Seven RCTs provided related data on tumor recurrence. The patients treated by preoperative chemoradiotherapy had lower incidence of local recurrence (OR: 0.64, 95% CI: 0.41-0.99,  $P = 0.04$ ) (Figure 5A), but the two groups had no significant difference in distant recurrence (OR: 0.94, 95% CI: 0.68-1.31,  $P = 0.73$ ) (Figure 5B).

**DISCUSSION**

Our meta-analysis indicated that patients treated by neo-

adjuvant chemoradiotherapy had more survival benefit compared with patients treated by surgery alone, including 1-year survival, 3-year survival and 5-year survival. But subgroup analysis demonstrated patients with ESCC could not benefit from neoadjuvant chemoradiotherapy. The meta-analysis performed by Fiorica *et al*<sup>[18]</sup> suggested that neoadjuvant chemoradiotherapy plus surgery significantly lowered the 3-year mortality compared with surgery alone, but there was no statistical significance between the two groups if all RCTs including patients with EAC were excluded. Another meta-analysis performed by GebSKI *et al*<sup>[19]</sup> demonstrated that both patients with ESCC and patients with EAC benefited from neoadjuvant chemoradiotherapy and corresponding OR (95% CI, *P* value) were 0.84 (0.71-0.99, *P* = 0.04) and 0.75 (0.59-0.95, *P* = 0.02). Since the former *P* value approached 0.05, our conclusion should be cautiously done. Thus we presume that only patients with EAC could benefit from neoadjuvant chemoradiotherapy. Another subgroup indicated patients in Europe and the USA benefited from neoadjuvant chemoradiotherapy; however, patients in Asia did not. This result indicated that different population had different response to neoadjuvant chemoradiotherapy and this may be associated with ethnic difference.

Though patients receiving neoadjuvant chemoradiotherapy had higher survival than patients treated by surgery alone, our meta-analysis showed that the incidence of surgery-related death was higher in the CRT group. Moreover, some patients lost the chance of surgery for the metastasis of tumor or some patients died before surgery for the aggravation of disease. Neoadjuvant chemoradiotherapy made local tissue harder and easier to bleed and as a result fatal postoperative complications such as anastomotic leakage and respiratory insufficiency increased. This may account for the higher postoperative mortality of patients in CRT group. However, sensitivity analysis showed no significant difference between the two groups after excluding the RCT by Nygaard *et al*<sup>[13]</sup>, performed between 1983 and 1988, which was the earliest one among all the RCTs included in this meta-analysis. Probably there was no effective treatment for severe postoperative complications due to the relative undeveloped medical conditions at that time. In fact, the postoperative mortality in the RCTs published after 2000 was significantly lower than those published before 2000. In a word, it is possible that the postoperative mortality of patients receiving neoadjuvant chemoradiotherapy is not significantly higher than that of patients treated by surgery alone under present medical conditions.

Outcomes of our meta-analysis revealed that patients treated by surgery alone had more possibility to undergo the scheduled surgery, however, the rate of complete resection in CRT group was higher than that in S group. This may account for the lower incidence of local recurrence in CRT group, which was another result of this meta-analysis. In the 11 RCTs, only part of patients in CRT group can obtain clinical relief or pathological response. Two RCTs<sup>[12,14]</sup> compared the survival time

between the patients who obtained clinical relief (including complete response and partial response) and the patients who failed to obtain clinical relief (including stable disease and disease progression), and found that the former was markedly greater than the latter. So, if some biological molecules can predict the response of EC patients to neoadjuvant chemoradiotherapy, EC patients will suffer from less physical miseries. Furthermore, this could avoid waste and enhance targeted treatment. Some studies<sup>[20-23]</sup> have indicated that Hsp27, DNA-PKcs, ERCC1 and c-erbB-2 were potential biological molecules, which were related to the response of EC patients to neoadjuvant chemoradiotherapy. Studies in this field are meaningful and promising.

One study reported that the effect of sequential chemoradiotherapy was superior to that of concurrent chemoradiotherapy in treating lung cancer<sup>[24]</sup>. In EC, however, outcome was exactly opposite to those obtained from lung cancer. This meta-analysis showed that EC patients only benefited from preoperative concurrent chemoradiotherapy. Concurrent chemoradiotherapy may work by inhibiting the growth of local tumor and micrometastasis, moreover concurrent chemotherapy can increase the sensitivity of tumor to radiotherapy.

Some studies<sup>[25-27]</sup> indicated that 40%-75% of patients with resectable EC (T1-3N0-1M0) judged according to clinical examination or surgery had subclinical metastasis or tumor had already invaded the adjacent organs or tissues. Accurate tumor staging is crucial to the prognosis of EC patients receiving surgery. Therefore, further measures should be taken to improve the accuracy of tumor staging. Currently, endoscopic ultrasound (EUS) is the most accurate method for staging EC for T and N stage<sup>[28]</sup>. Although helical computed tomography still appears insensitive for the identification of T4 or metastatic involvement of celiac lymph node disease in esophageal cancer, EUS with fine needle aspiration and FDG-PET [fluorine 18-labeled fluorodeoxyglucose (FDG) positron emission tomography (PET)] can make up for this shortcoming<sup>[29]</sup>. Part of patients in RCTs<sup>[9,11]</sup> included in this meta-analysis had metastasis of non-regional lymph nodes. Investigators considered those lymph nodes could be included in the radiation port and should be resected at surgery<sup>[9]</sup>. In fact, this condition belongs to IVa according to TNM staging. We suggest that EC patients (IVa) should not give up the chance of surgery, and they will benefit from neoadjuvant chemoradiotherapy plus surgery too.

In conclusion, this meta-analysis showed that patients with ESCC did not benefit from preoperative concurrent chemoradiotherapy and patients with EAC may be the real beneficiaries of the treatment protocol. Compared with patients treated by surgery alone, patients receiving neoadjuvant chemoradiotherapy more likely obtained complete resection and had lower local cancer recurrence. Neoadjuvant chemoradiotherapy was connected with a little higher mortality after surgery. But it did not increase the incidence of postoperative complications. In addition, patients in Europe and the USA more likely benefited

Review: Neoadjuvant chemoradiotherapy for resectable esophageal carcinoma: A meta-analysis  
 Comparison: CRT group vs S group  
 Outcome: Complication after surgery

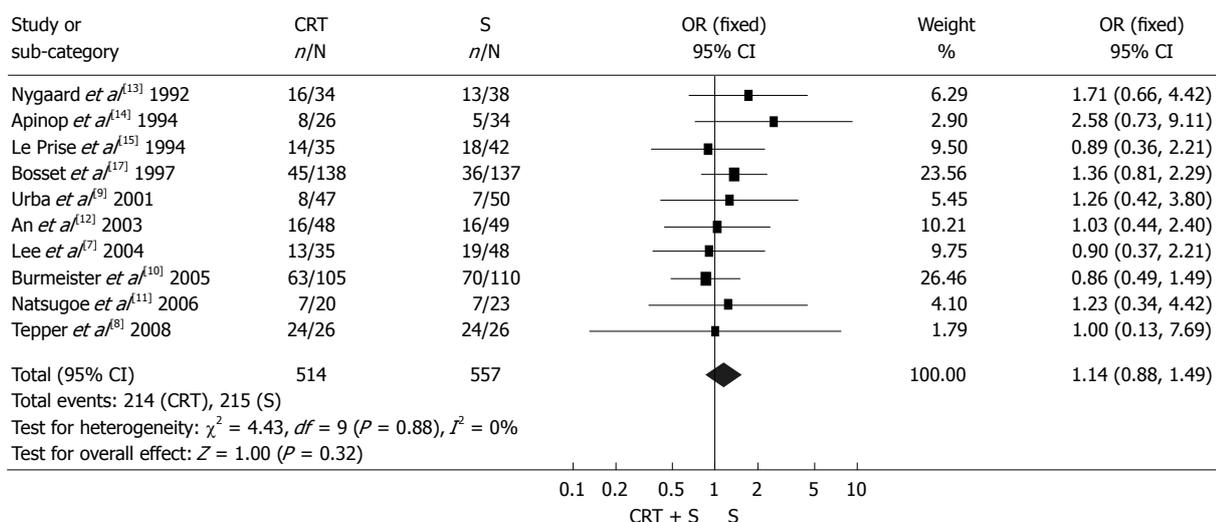
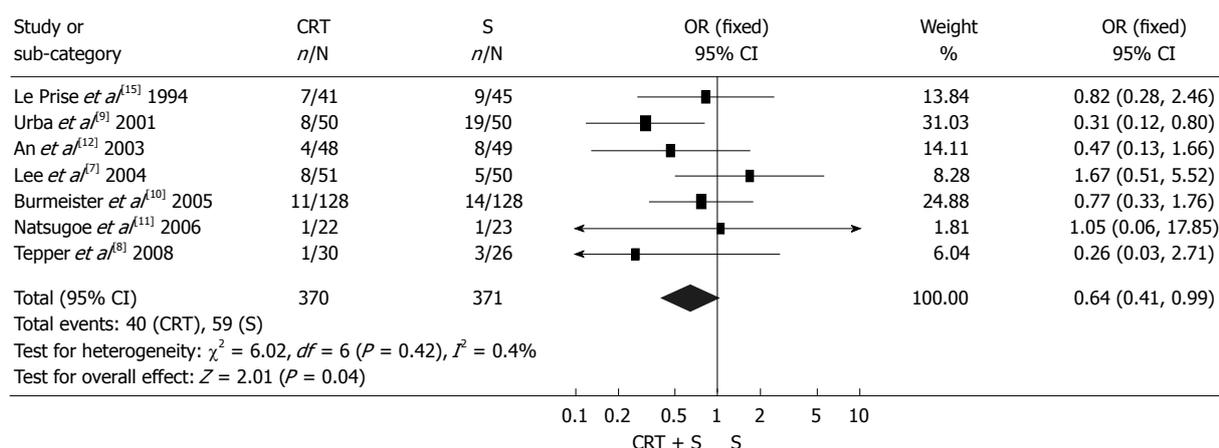


Figure 4 Incidence of postoperative complication in neoadjuvant chemoradiotherapy and surgery compared with surgery alone.

**A** Review: Neoadjuvant chemoradiotherapy for resectable esophageal carcinoma: A meta-analysis  
 Comparison: CRT group vs S group  
 Outcome: Local-regional cancer recurrence



**B** Review: Neoadjuvant chemoradiotherapy for resectable esophageal carcinoma: A meta-analysis  
 Comparison: CRT group vs S group  
 Outcome: Distant cancer recurrence

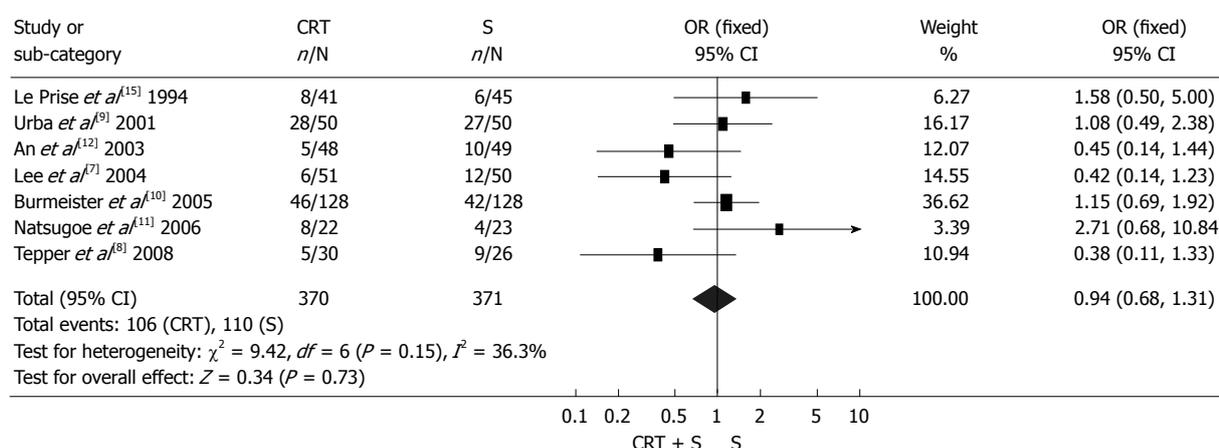


Figure 5 Cancer recurrence after surgery in neoadjuvant chemoradiotherapy and surgery compared with surgery alone. A: Incidence of local-regional cancer recurrence; B: Incidence of distant cancer recurrence.

from neoadjuvant chemoradiotherapy than those in Asia, and this is worth of further studies.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Esophageal carcinoma (EC) is one of the major malignant diseases worldwide. Surgery alone cannot obtain satisfactory effects in patients with EC.

### Research frontiers

Neoadjuvant chemoradiotherapy has been a hotspot for EC treatment research. Several related randomized controlled trials (RCTs) have been published, but opinions vary among clinicians as to the therapeutic effect of the new method. It remains uncertain whether patients with resectable EC can benefit from neoadjuvant chemoradiotherapy.

### Innovations and breakthroughs

Several meta-analyses on the neoadjuvant chemoradiotherapy for EC have been published so far, some of which lacked adequate RCTs or used unpublished data. In this study, the authors collected relatively comprehensive data and all the data were from the published literature. It was found that patients with esophageal squamous cell carcinoma did not benefit from neoadjuvant chemoradiotherapy, while patients with esophageal adenocarcinoma (EAC) were the real beneficiaries. In addition, the authors analyzed the impact of geographical differences on the efficacy of the treatment protocol and found that patients in Europe and the USA more likely benefited from neoadjuvant chemoradiotherapy than those in Asia.

### Applications

Results of this study indicate that neoadjuvant chemoradiotherapy is an effective treatment protocol, which is beneficial to patients with EAC in Europe and the USA.

### Terminology

Neoadjuvant chemoradiotherapy: Chemotherapy and radiotherapy are given to patients with cancer before surgery.

### Peer review

This work is a meta-analysis including 11 randomized prospective studies that analyze the advantages of the use of the neoadjuvant chemoradiotherapy vs surgery alone in the treatment of the EC. The results are interesting and suggest that neoadjuvant chemoradiotherapy is beneficial to patients with EAC.

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BRIEF ARTICLE

## Effect of severe acute pancreatitis on pharmacokinetics of Da-Cheng-Qi Decoction components

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sorption of DCQD components in rats and their pharmacokinetic parameters.

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**Key words:** Severe acute pancreatitis; Da-Cheng-Qi Decoction; Pharmacokinetics; Components

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### Abstract

**AIM:** To investigate the effect of severe acute pancreatitis (SAP) on pharmacokinetics of Da-Cheng-Qi Decoction (DCQD) components in rats.

**METHODS:** Rats were divided into SAP group and sham-operation group as a control group ( $n = 6$ ). Rhein, chrysophanol, rheochrysidin, magnolol, hesperidin and naringin in DCQD were quantified in rat serum by high performance liquid chromatography tandem mass spectrometry for studying their pharmacokinetics.

**RESULTS:** Early absorption of each DCQD component was tended to degrade in SAP group after treatment with DCQD by gavage. The  $C_{max}$  (chrysophanol,  $P = 0.0059$ ; rheochrysidin,  $P = 0.0288$ ; magnolol,  $P = 0.0487$ ; hesperidin,  $P = 0.0277$ ; naringin,  $P = 0.0023$ ) and AUC (rhein,  $P = 0.0186$ ; chrysophanol,  $P = 0.0013$ ; magnolol,  $P = 0.001$ ; hesperidin,  $P = 0.0081$ ; naringin,  $P = 0.0272$ ) of DCQD component were obviously lower in SAP group than in control group. The  $T_{1/2\alpha}$  of chrysophanol and rheochrysidin ( $P = 0.0467$  and  $0.0005$ , respectively) and  $T_{max}$  of chrysophanol and rheochrysidin ( $P = 0.0101$  and  $0.0037$ , respectively) lasted longer in SAP group than in control group.

**CONCLUSION:** SAP can significantly impact the ab-

### INTRODUCTION

Acute pancreatitis, occurring suddenly and usually resolving after a few days of treatment, may become life-threatening if severe complications take place. Fulminant acute pancreatitis is more dangerous<sup>[1]</sup>. Severe acute pancreatitis (SAP), characterized by intricate mechanism, variant symptoms, grave prognosis and multiple complications, seriously threatens the life of patients and brings a heavy burden to the society, families and economy. Each year, about 210 000 patients with acute pancreatitis in the United States are admitted to hospitals<sup>[2]</sup>. Additionally, neither standard treatment nor other medications are available for SAP patients at present<sup>[3]</sup>. SAP, similar to Yangming Fushi syndrome (YMFSS) according to the traditional Chinese medicine, has been treated with purgative herbals throughout China for more than three decades<sup>[4-6]</sup>.

Da-Cheng-Qi Decoction (DCQD), a famous preparation of traditional Chinese medicine used in treatment of digestive diseases, is composed of *Dabuang* (Caulis Fibraureae), *Houpu* (Cortex Magnoliae Officinalis), *Zhishi* (immature bitter orange) and *Mangxiao* (Natrii Sulphas). It has been reported that DCQD can restore gastrointestinal function by facilitating motility, relieving enteroparalysis and evacuating "dry stool"<sup>[7]</sup>, prevent bacterial translocation and counteract with endotoxin, regulate  $Ca^{2+}$ - $Mg^{2+}$ -ATPase in the pancreatic acinar cells<sup>[8]</sup>. SAP

can be treated with Chinese herbal decoctions based on the above mechanism. However, no studies are available on the pharmacokinetics of such decoctions in acute pancreatitis. According to the theory “syndrome and treatment pharmacokinetics”, YMFSS should influence the pharmacokinetics of DCQD<sup>[9]</sup>, but it has not been proved experimentally up to date.

Thus, we quantified the DCQD components absorbed in rats with SAP characterized by YMFSS and studied the influence of SAP on the pharmacokinetics of DCQD components<sup>[10]</sup>.

## MATERIALS AND METHODS

### Animals

Male clean-grade, healthy Sprague-Dawley rats, weighing  $320 \pm 25$  g, at the age of  $90 \pm 5$  d, were used in this study. The rats were handled according to the University Guidelines and the Animal Ethics Committee Guidelines of the Animal Facility of the West China Hospital, maintained in air-conditioned animal quarters at  $22 \pm 2^\circ\text{C}$  with a relative humidity of  $65\% \pm 10\%$ , acclimatized to the facilities for 10 d, and then fasted for 24 h with free access to water prior to experiments.

### Materials, chromatographic and HPLC-mass spectrometry conditions

The structures of rhein, chrysophanol, rheochrysidin, magnolol, hesperidin, naringin and ibuprofen (internal standards) are presented in Figure 1. Reference standards for these components of DCQD and the internal standard (IS) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol with a chromatographic grade was obtained from Tedia Company Inc. (USA). Acetic acid and ethyl acetate were bought from Chongqing Chemistry Co. Ltd. (Chongqing, China). Ammonium acetate, sodium hydroxide and hydrochloric acid (analysis grade) were purchased from Chengdu Kelong Chemical Reagent Factory (Chengdu, China). All aqueous solutions and buffers were prepared with deionized water from a Millipore RiosTM-16 water purifier (Millipore, Billerica, MA, USA)<sup>[11]</sup>.

High performance liquid chromatography tandem mass spectrometry (HPLC-TMS) system, consisted of a SIL-HTc autosampler and a LC-10ADvp pump, was provided by Shimadzu (Kyoto, Japan). API3000 triple-quadrupole LC-MS system was purchased from Applied Biosystems (Foster City, CA, USA). The system was controlled with Analyst 1.4.2 software. Separation was performed on a YMC-Pack ODS-A C18 column ( $5 \mu\text{m}$ ,  $150 \text{ mm} \times 4.6 \text{ mm}$ , YMC, Kyoto, Japan) and a C<sub>18</sub> guard column ( $5 \mu\text{m}$ ,  $4.0 \text{ mm} \times 2.0 \text{ mm}$ , Phenomenex Inc., Torrance, CA, USA). The mobile phase is consisted of methanol-water (92:8, v/v) at a flow rate of 0.3 mL/min. The column was maintained at ambient temperature and the injection volume was 80  $\mu\text{L}$ .

A mass spectrometer was operated using an electrospray source configured to the negative ion mode and quantification was performed by multiple reaction moni-

toring (MRM). Production mass spectra of the analytes are shown in Figure 1 where [M-H]<sup>-</sup> of each analyte was selected as the precursor ion, and the most abundant or specific fragment ion was selected as the production in MRM acquisition. Instrumental parameters were optimized for each analyte by infusing the corresponding standard solution at a flow rate of 5  $\mu\text{L}/\text{min}$ , using a syringe pump integrated into the API 3000 mass spectrometer. Nitrogen was used as a curtain, and auxiliary gas and air were used as a nebulizer gas. Electrospray conditions for the 6 major DCQD components and IS were curtain gas (6.0 L/min), ion-spray voltage (-4500 V), nebulizer gas (6.0 L/min), auxiliary gas (7.0 L/min), turbo temperature ( $4^\circ\text{C}$ ), respectively. Optimized mass spectrometry parameters for each DCQD compound and IS are listed in Table 1<sup>[11]</sup>.

Six calibration standards were prepared by spiking 200  $\mu\text{L}$  of blank plasma with 100  $\mu\text{L}$  of each working solution to obtain the plasma concentrations for rhein and rheochrysidin (5000, 3750, 2500, 1250, 625, 312.5, 156, 78.13, 39.1 and 19.53 ng/mL), and for chrysophanol, naringin, hesperidin and magnolol (879, 586, 390, 195, 97.7, 48.8, 24.4, 12.2, 6.1 and 3.1 ng/mL). Quality control (QC) samples were prepared to obtain plasma concentrations for rhein and rheochrysidin (3750, 625, 156 and 39.1 ng/mL) and for chrysophanol, naringin, hesperidin and magnolol (586, 97.7, 24.4 and 6.1 ng/mL). The spiked plasma samples (standard and QC samples) were pretreated and detected in each analytical batch along with the unknown samples<sup>[11]</sup>.

### Assay validation

Blank and spiked rat plasma chromatograms were compared to evaluate the selected method (Figure 2). Calibration curves were plotted from the peak area ratio of each analyte to IS *v* plasma concentrations using a 1/*c*<sup>2</sup> weighted linear least-squares regression model. The lower limit of quantification was set at the concentration of the lowest non-zero calibration standard ( $S/N \geq 10:1$ ) that could be measured with an acceptable accuracy and precision ( $\leq 20\%$  for both parameters). Intra- and inter-day precisions were determined by assessing the measured results of QC samples at low, medium and high concentrations (Table 1). Accuracy was determined as the difference in percentages between the mean and nominal concentrations detected (Table 1). Extraction recoveries of the 6 analytes were determined by comparing the peak areas obtained from rat plasma samples with those from the unextracted standard solutions at the same concentration (Table 1). Bench-top stability of the 6 analytes in rat plasma was determined by assessing the QC samples after stored for 2 and 4 h at room temperature. Freeze-thaw stability was detected after two cycles and long-term stability was determined by assessing the QC samples stored at  $-30^\circ\text{C}$  for 14 d. QC samples were prepared, injected and reinjected after the samples were maintained in the autosampler at  $8^\circ\text{C}$  for 12 d. Stability of the analytes was detected by comparing the measured results with those of freshly prepared samples at the same concentration<sup>[11]</sup> (Table 2).

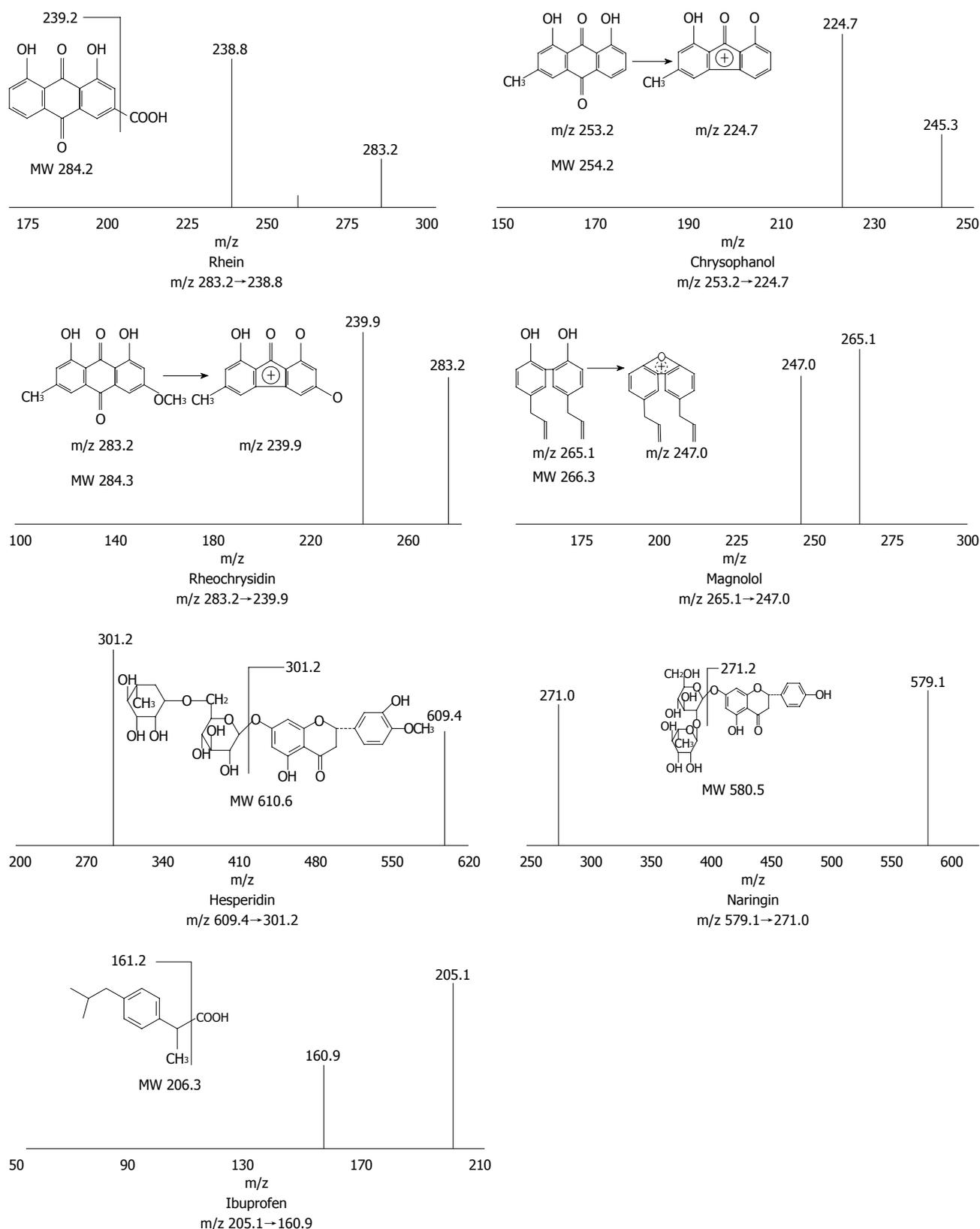


Figure 1 Product ion mass spectra (ESI-) and ion structures of the six major Da-Cheng-Qi Decoction (DCQD) components and internal standards.

### Induction of acute pancreatitis in rats

Acute pancreatitis was induced in rats. The animals were anesthetized with ethyl ether as previously described<sup>[12]</sup>.

### Preparation of Chinese drugs

*Dabuang*, *Houpu*, *Zhishi* and *Mangxiao* were purchased

from Chengdu Green Herbal Pharmaceutical Co. Ltd. (Chengdu, China) and authenticated by Professor Yang Song (Department of Pharmacognosy, Sichuan University, China). DCQD was routinely prepared with 6.0 g of *Dabuang*, 6.0 g of *Houpu*, 6.0 g of *Zhishi* and 6.0 g of *Mangxiao*. For crude drugs, the spray-dried DCQD was

Table 1 Parameters of the 6 major DCQD components in rat plasma QC samples (% ,  $n = 6$ )

	Spiked amount (ng/mL)	Intra-day		Inter-day		Extract	
		RSD	Ac	RSD	Ac	Recovery	RSD
Rhein	39.1	3.76	104.60	3.53	107.94	104.60	3.76
	156	5.55	100.32	5.52	106.87	100.32	5.55
	625	5.31	101.07	5.56	105.36	101.07	5.31
	3750	4.24	101.24	6.23	98.37	101.24	4.24
Chrysophanol	6.1	2.37	99.4	4.46	98.67	99.39	2.36
	24.4	4.38	104.17	4.88	101.14	104.17	4.37
	97.7	1.64	96.66	3.33	98.09	96.66	1.64
	586	3.78	103.98	4.44	101.13	103.98	3.78
Rheochrysidin	39.1	5.69	97.70	5.72	100.78	97.69	5.68
	156	3.33	99.89	4.13	101.14	99.89	3.33
	625	3.22	105.92	3.73	104.37	105.92	3.22
	3750	4.75	103.07	4.84	103.49	103.07	4.75
Magnolol	6.1	5.51	102.13	5.07	106.33	102.13	5.51
	24.4	2.39	104.78	5.79	99.86	104.78	2.39
	97.7	5.51	107.81	4.92	106.23	102.47	5.51
	586	3.77	105.97	5.33	105.29	105.97	3.77
Hesperidin	6.1	4.12	95.96	4.82	98.49	95.96	4.12
	24.4	5.83	99.52	5.10	100.29	99.52	5.83
	97.7	4.21	100.14	3.95	99.72	100.16	4.21
	586	2.77	97.13	4.91	98.52	97.13	2.76
Naringin	6.1	2.66	95.66	4.26	99.29	95.67	2.657
	24.4	2.82	102.46	3.95	103.37	102.45	2.82
	97.7	3.81	100.78	3.96	100.66	100.78	3.81
	586	5.24	98.27	5.59	99.32	98.27	5.24

DCQD: Da-Cheng-Qi Decoction; QC: Quality control.

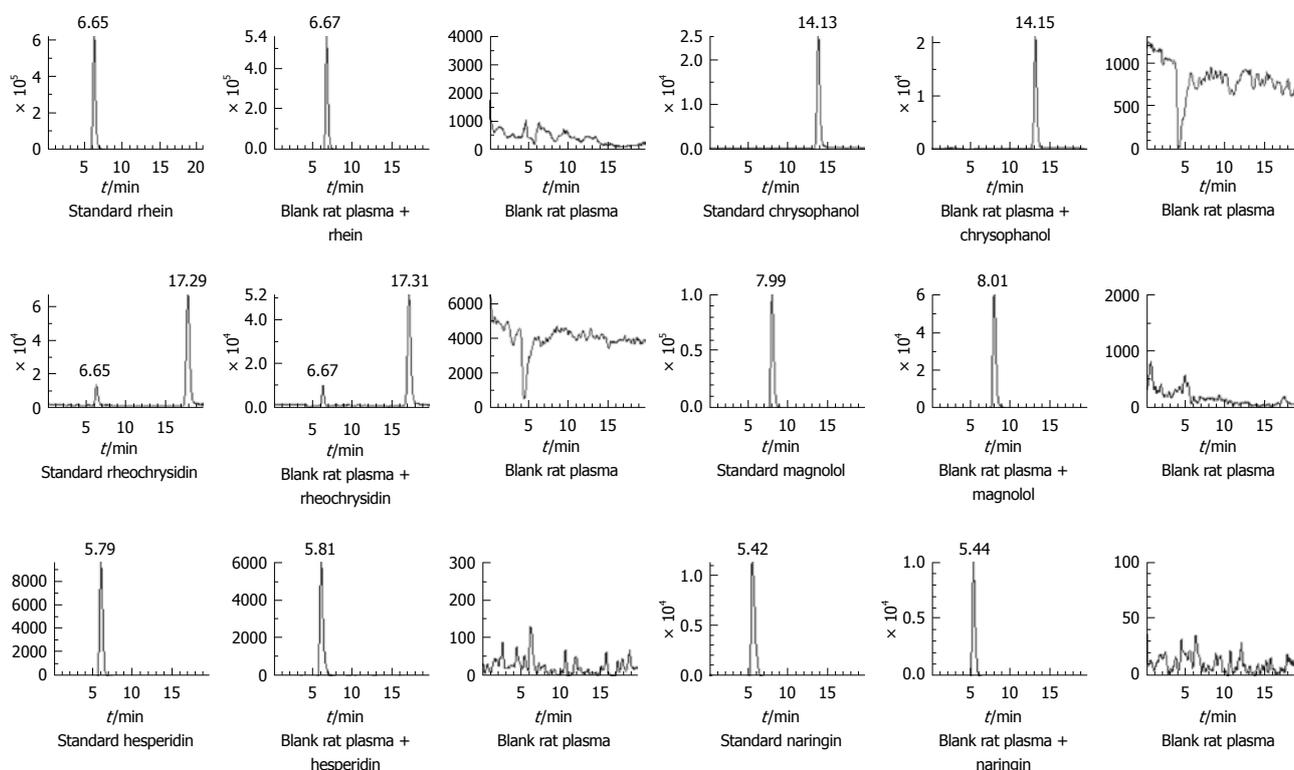


Figure 2 HPLC-TMS showing the six major DCQD components in plasma of rats in two groups.

reconstituted in water to a concentration of 1 g/mL. The contents of the six DCQD components were measured as previously described<sup>[13]</sup>. The crude DCQD preparation was administered through the duodenum of rats at a dosage of 20 g/kg. The voucher specimens were kept in our laboratory.

### *In vivo study*

Rats were randomly divided into SAP group and sham-operation group as a control group ( $n = 6$ ). Rats were given DCQD 2 h after operation. Blood sample (300  $\mu$ L) was collected into a heparinized eppendorf tube *via* the tail vein before and after (10, 15, 20, 30, 45 min and 1,

Table 2 Stability of the 6 major DCQD compositions in rat plasma QC samples (% , n = 3)

	Spiked amount (ng/mL)	Bench-top bias		Long-term bias		Freeze-thaw bias		Extract bias		Autosampler bias	
		2 h	4 h	7 d	14 d	1	2	8 h	24 h	6 h	12 h
Rhein	39.1	-1.32	-4.57	-1.15	-4.27	-1.15	-2.54	6.06	-4.54	-5.25	-5.33
	156	-2.50	-5.39	-2.36	-0.43	-2.36	0.43	2.28	2.48	-1.29	-1.07
	625	5.28	-1.21	1.60	-2.82	1.60	-2.93	-0.90	3.40	-0.48	-0.27
	3750	2.06	5.52	-4.46	0.09	-4.46	-0.17	1.50	1.32	-1.54	-3.60
Chrysophanol	6.1	-0.67	1.00	2.42	3.54	2.42	0.90	4.73	3.19	0.73	2.36
	24.4	5.04	4.62	-2.74	-2.61	-2.74	-6.40	-2.82	-4.42	-3.39	-3.52
	97.7	-0.32	5.80	-5.39	0.27	-5.39	-1.52	-2.34	0.00	-3.41	0.00
	568.0	-0.72	-2.00	2.01	-2.13	2.01	-2.47	-2.92	-0.97	0.63	2.42
Rheochrysidin	6.1	1.59	-2.59	-4.11	-3.78	-4.11	-5.18	-3.93	-6.27	-6.33	-5.10
	24.4	4.09	-0.65	0.00	-4.03	0.00	-1.27	-0.43	3.24	-0.42	-3.18
	97.7	-0.86	-4.45	-0.38	0.59	-0.38	-2.43	-0.58	-0.32	0.38	-2.49
	568.0	2.80	-5.52	-3.40	-2.09	-3.40	-2.18	2.04	1.16	-1.66	-3.66
Magnolol	6.1	-1.58	1.33	-5.23	-4.34	-5.23	-3.09	-2.72	-2.14	-5.18	-4.60
	24.4	5.76	0.84	0.42	-2.49	0.42	0.28	-1.95	1.53	1.25	-0.83
	97.7	1.82	1.17	1.48	-1.10	1.48	0.90	4.23	1.47	-3.21	1.35
	568.0	-2.02	-3.03	-1.60	-4.43	-1.60	-2.21	0.23	-5.01	-3.82	-6.14
Hesperidin	6.1	-4.29	4.85	-0.48	-1.27	-0.48	-3.08	1.01	2.34	-3.98	-1.54
	24.4	-2.03	-3.11	0.41	0.00	0.41	-1.36	1.46	1.46	-4.21	4.76
	97.7	3.42	-1.69	0.11	0.39	0.11	1.37	2.67	2.33	2.70	5.12
	568.0	-3.00	-0.81	-4.73	-2.25	-4.73	-3.55	3.75	0.62	-2.76	0.23
Naringin	6.1	-1.65	2.70	-1.96	1.63	-1.96	-4.90	-1.73	-0.49	2.61	-2.83
	24.4	3.95	4.90	-4.56	-2.73	-4.56	-2.73	1.04	2.86	-4.69	-6.12
	97.7	0.07	2.03	-5.11	-0.96	-5.11	1.58	-4.46	-2.31	-0.45	0.93
	568.0	-4.10	1.68	-4.52	-1.45	-4.52	-4.81	1.20	-3.06	-2.08	-1.56

2, 4, 8, 12 h) DCQD was given. After centrifugation at 3000 r/min for 15 min, the plasma samples were stored at -80°C for analysis.

Rats in SAP and control groups were fed with laboratory rodent chow by gavage. Concentration of DCQD components in plasma was measured by HPLC-TMS. Concentration-time curves were plotted for various components from DCQD.

#### Assay procedure

HPLC-TMS for simultaneous determination of the six components has been validated in our laboratory<sup>[11,14]</sup>. Plasma samples were spiked with the IS (ibuprofen), acidified by HPLC and extracted twice using ethyl acetate. The HPLC-TMS system was operated under MRM modes using electrospray ionization in the negative ion mode.

#### Data collection and analysis

Data collection, peak integration and calibration were performed with Analyst 1.4.2 software. Calibration curves were plotted according to the peak area ratio of analytes to ISs, and the linear regression between plasma concentration and peak area ratio was weighed by  $1/x^2$ . Concentrations of QC and unknown samples were measured by interpolation from the calibration curves. Drug and statistics software programmed by the Chinese Pharmacological Society was used to process the plasma concentration data and compartment model fitting and then all the pharmacokinetic parameters were figured out. The results were expressed as mean  $\pm$  SD. The pharmacokinetic parameters of each DCQD component were compared with statistical software PEMS3.1 and

the difference was compared by sample pairing and *t*-test. *P* < 0.05 was considered statistically significant.

## RESULTS

#### Rhein in rats after a single dose of DCQD by gavage

The mean plasma concentration of rhein was obviously higher, the peak time ( $T_{max}$ ) of rhein was significantly shorter while the  $T_{1/2\alpha}$  was significantly higher, and the clearance rate (CL/F) and AUC of rhein were obviously lower in SAP group at each time point than in control group within 12 h after treatment with DCQD, suggesting that acute pancreatitis can impact the absorption, distribution and elimination of rhein in rats (Figure 3, Table 3).

#### Rheochrysidin in rats after a single dose of DCQD by gavage

The mean plasma concentration of rheochrysidin was significantly higher, the  $T_{max}$  of rheochrysidin was significantly shorter, and the  $T_{1/2\alpha}$  was significantly higher in SAP group at each time point than in control group within 12 h after treatment with DCQD, demonstrating that acute pancreatitis can affect the absorption distribution and excretion of rheochrysidin in rats (Figure 3, Table 3).

#### Chrysophanol in rats after a single dose of DCQD by gavage

The mean plasma concentration of chrysophanol was obviously lower, the  $T_{max}$  of chrysophanol was significantly longer, the  $C_{max}$  and AUC of chrysophanol were significantly lower, the  $T_{1/2\alpha}$  was significantly higher, and the CL/F was lower in SAP group than in control group

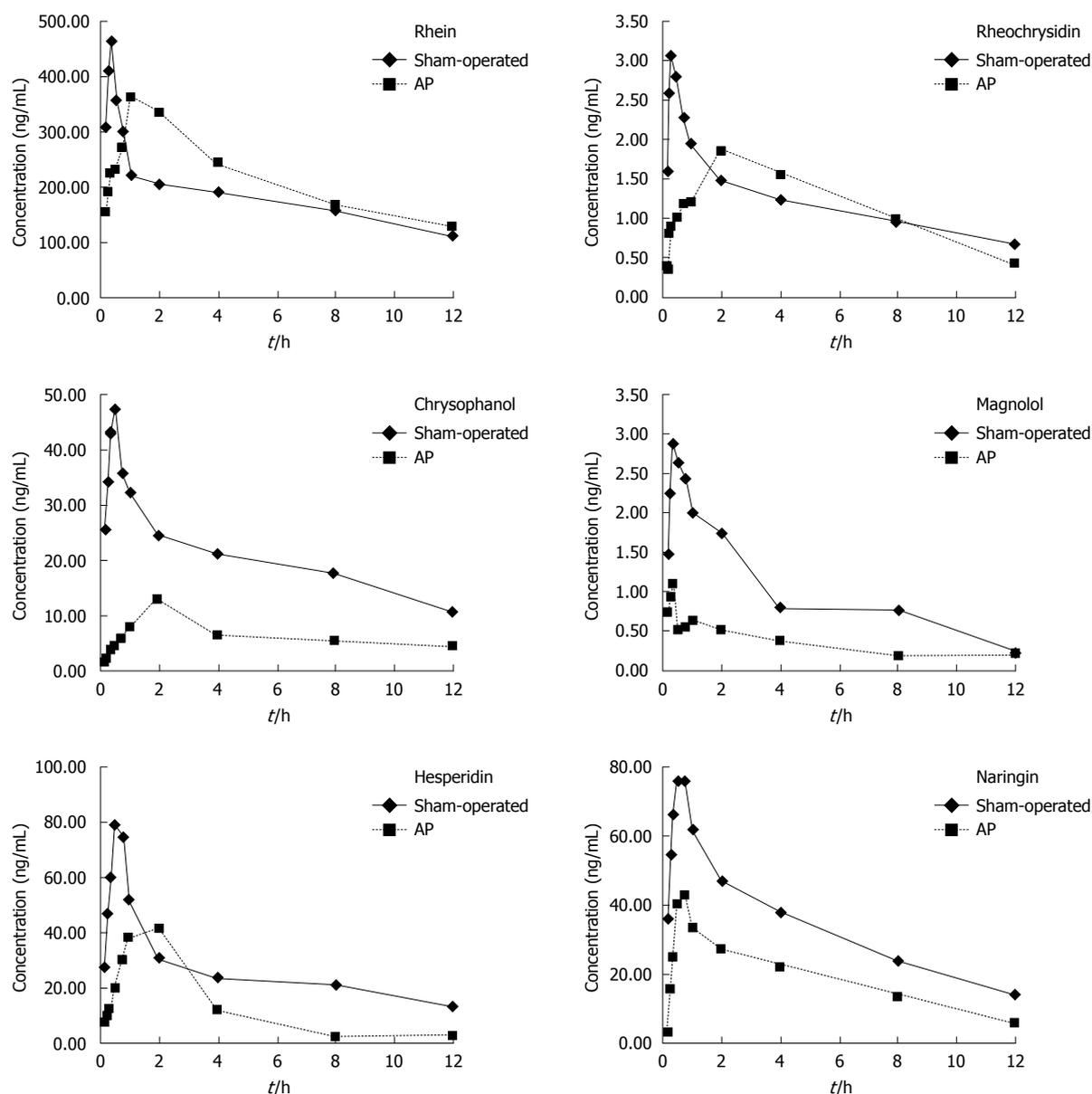


Figure 3 Plasma concentration-time curves for DCQD components in rats of the two groups ( $n = 6$ ).

with no difference in the mean retention time (MRT) between the two groups within 12 h after treatment with DCQD, suggesting that SAP can affect the absorption, distribution, and elimination of chrysophanol in rats (Figure 3, Table 3).

#### **Magnolol in rats after a single dose of DCQD by gavage**

The mean plasma concentration of magnolol was obviously lower, the  $C_{max}$  and AUC of magnolol were obviously lower while the  $T_{1/2\alpha}$ , MRT and  $T_{max}$  were similar between the two groups within 12 h after treatment with DCQD, suggesting that SAP can significantly affect the bioavailability of magnolol (Figure 3, Table 4).

#### **Hesperidin in rats after a single dose of DCQD by gavage**

The mean plasma concentration of hesperidin was obviously higher, the  $T_{max}$  of hesperidin was significantly longer, the  $C_{max}$  and AUC of hesperidin were significantly lower in

SAP group at each time point in control group within 12 h after treatment with DCQD, showing that acute pancreatitis can impact the absorption and distribution and pharmacokinetics of hesperidin in rats (Figure 3, Table 4).

#### **Naringin in rats after a single dose of DCQD by gavage**

The mean plasma concentration of naringin and the  $C_{max}$  and AUC of naringin were obviously lower in SAP group at each time point than in control group within 12 h after treatment with DCQD, revealing that acute pancreatitis can impact the absorption, distribution and bioavailability of naringin in rats (Figure 3, Table 4).

## DISCUSSION

In the present study, the early absorption of each DCQD component tended to degrade in SAP group, the  $C_{max}$  and AUC of DCQD components such as chrysophanol,

Table 3 Twelve-hour pharmacokinetic parameters of DCQD components in rats of the two groups ( $n = 6$ )

	$T_{1/2\alpha}$ (h)	CL/F (L·h per kg)	AUC <sub>(0-∞)</sub> (μg/L per hour)	MRT <sub>(0-t)</sub> (h)	$T_{max}$ (h)	$C_{max}$ (μg/L)
Rhein						
Sham	0.33 ± 0.13	4.03 ± 1.38	4720 ± 1514	8.86 ± 0.62	0.36 ± 0.11	510 ± 283
AP	4.367 ± 2.33	0.133 ± 0.06	2870 ± 563	7.82 ± 3.37	1.75 ± 1.25	479 ± 126
<i>t</i>	4.2721	6.9106	2.8054	0.7513	2.7242	0.2448
<i>P</i>	0.0016	0	0.0186	0.4698	0.0214	0.8116
Rheochrysidin						
Sham	0.354 ± 0.302	0.356 ± 0.14	16.047 ± 6.08	4.189 ± 0.463	0.569 ± 0.26	3.86 ± 1.09
AP	1.464 ± 0.449	0.328 ± 0.109	16.63 ± 5.06	4.34 ± 0.29	1.5 ± 0.55	2.58 ± 0.58
<i>t</i>	5.0247	0.3866	0.179	0.6929	3.7597	2.5511
<i>P</i>	0.0005	0.7072	0.8615	0.5041	0.0037	0.0288
Chrysophanol						
Sham	0.38 ± 0.27	24.32 ± 9.65	461.3 ± 188.7	8.49 ± 0.93	0.59 ± 0.24	53.02 ± 21.9
AP	0.89 ± 0.48	0.095 ± 0.035	115.8 ± 34.89	10 ± 5.1	1.33 ± 0.52	17.59 ± 10.5
<i>t</i>	2.2683	6.0994	4.3965	0.7088	3.165	3.4855
<i>P</i>	0.0467	0.0001	0.0013	0.4947	0.0101	0.0059

Table 4 Twelve-hour pharmacokinetic parameters of DCQD components in rats of the two groups ( $n = 6$ )

	$T_{1/2\alpha}$ (h)	AUC <sub>(0-∞)</sub> (μg/L per hour)	MRT <sub>(0-t)</sub> (h)	$T_{max}$ (h)	$C_{max}$ (μg/L)
Magnolol					
Sham	1.58 ± 1.06	24.89 ± 9.87	6.6 ± 1.85	0.71 ± 0.25	3.47 ± 2.13
AP	1.11 ± 0.48	5.739 ± 1.888	9.202 ± 2.27	0.428 ± 0.282	1.491 ± 0.596
<i>t</i>	1.0821	4.6204	2.2156	1.8329	2.2431
<i>P</i>	0.3046	0.001	0.0511	0.0967	0.0487
Hesperidin					
Sham	0.45 ± 0.25	479.39 ± 225.94	5.62 ± 2.45	0.67 ± 0.13	89.38 ± 25.02
AP	0.69 ± 0.36	162.98 ± 69.76	4.93 ± 2.233	1.25 ± 0.59	53.7 ± 22.026
<i>t</i>	1.3413	3.2953	0.5133	2.3141	2.5744
<i>P</i>	0.2095	0.0081	0.6189	0.0432	0.0277
Naringin					
Sham	1.47 ± 1.57	623.24 ± 332.55	6.43 ± 2.1	0.64 ± 0.24	88.23 ± 23.66
AP	1.1 ± 0.7	267.68 ± 53.65	7.12 ± 1.96	0.83 ± 0.13	45.13 ± 9.59
<i>t</i>	0.5272	2.5852	0.5659	1.732	4.0511
<i>P</i>	0.6095	0.0272	0.5839	0.1139	0.0023

magnolol, hesperidin and naringin were obviously lower in SAP group than in control group, suggesting that lack of an effective blood volume and a systematic inflammatory response to organ damage in SAP rats would affect the distribution, metabolism and excretion of DCQD components<sup>[15]</sup>.

No significant difference was found in  $T_{1/2\alpha}$  and  $T_{max}$  of DCQD components such as magnolol and naringin between the two groups, which may be due to the way of modeling experiments. Rats were anaesthetized with ethyl ether and recovered 10 min later with free activity. Two hours after treatment with DCQD, the rats became conscious and maintained normal physiology, indicating that influence of anesthesia on physiology and pharmacokinetics in rats can be ignored<sup>[16]</sup>.

However, the  $T_{1/2\alpha}$  and  $T_{max}$  of rhein, rheochrysidin and chrysophanol were longer in SAP group in control group. In addition, the absorption of DCQD components was greatly affected by variant molecular constitutions and lower pH of SAP rats *in vitro*.

In summary, SAP can obviously impact the absorption and pharmacokinetic parameters of DCQD containing rhein, chrysophanol, rheochrysidin, magnolol, hesperidin and naringin in rats.

## COMMENTS

### Background

Severe acute pancreatitis (SAP), characterized by intricate mechanism, variant symptoms, grave prognosis and multiple complications, seriously threatens the life of patients and brings a heavy burden to the society, families and economy. Additionally, either standard treatment or other medications for SAP is available at present. In China, clinical and experimental researches on Da-Cheng-Qi Decoction (DCQD) have shown that DCQD is a valid prescription for the treatment of SAP.

### Research frontiers

SAP, similar to Yangming Fushi Syndrome (YMFSS) according to the traditional Chinese medicine, has been treated with purgative herbals throughout China for more than three decades. However, no studies are available on the pharmacokinetics of DCQD components in rats with acute pancreatitis.

### Innovations and breakthroughs

According to the theory "syndrome and treatment pharmacokinetics" in traditional Chinese medicine, YMFSS should influence the pharmacokinetics of DCQD, which has, however, not been proved experimentally up to date. This is the first study to report the effect of acute pancreatitis on the pharmacokinetics of DCQD components in rats.

### Applications

Acute pancreatitis was found to have certain effects on the pharmacokinetics of DCQD components in rats, showing that DCQD can be used in treatment of SAP.

### Peer review

The authors investigated the effect of acute pancreatitis on the pharmacokinetics of DCQD components in rats, which may contribute to the treatment of SAP.

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CASE REPORT

## Complete response to radiation therapy of orbital metastasis from hepatocellular carcinoma

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### Abstract

The incidence of hepatocellular carcinoma (HCC) is increasing in the United States, and 50%-75% of patients with HCC will develop metastatic disease. Orbital metastases from HCC are extremely rare. We report the case of a 52-year-old male with known metastatic HCC, who presented with severe proptosis and diplopia. An orbital mass was identified on magnetic resonance imaging (MRI) and confirmed to have hypermetabolic activity on positron emission tomography/computed tomography. He received a palliative course of external beam radiation therapy to the right orbit. Intensity modulated radiation therapy (IMRT) was used to allow sparing of critical normal tissues in close proximity to the tumor. One month after completion of IMRT to 58 Gray in 30 fractions delivered over 6 wk, the patient had a complete clinical, radiologic (MRI) and symptomatic response. The patient continues to have local control in the orbit 1.7 years after therapy completion. All critical normal structures were kept below the tolerance dose using IMRT, and no toxicities were observed.

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**Key words:** Hepatocellular carcinoma; Eye neoplasms;

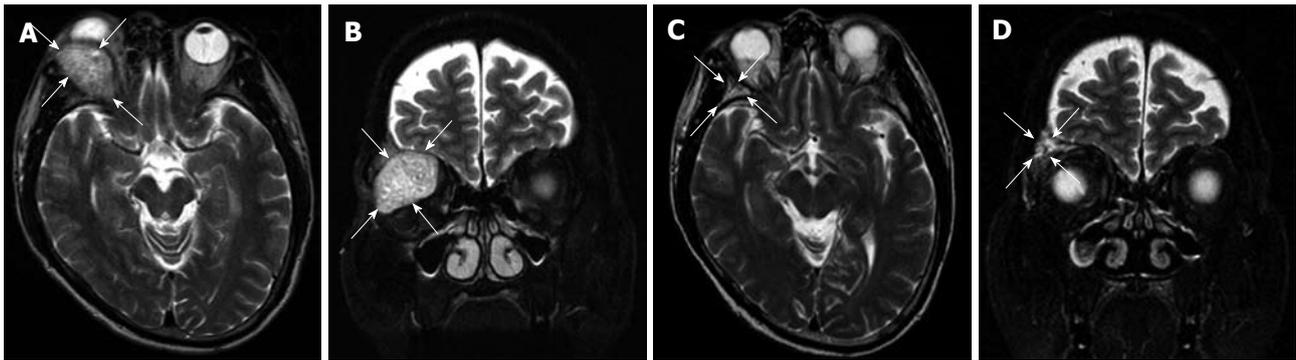
### INTRODUCTION

According to Surveillance, Epidemiology, and End Results data, the incidence of hepatocellular carcinoma (HCC) has been steadily increasing since the mid-1980s and thus presents an increasing health problem in the United States. The average, age-adjusted incidence rates for liver and intrahepatic bile duct cancer, two-thirds of which are HCC, rose from 3.2 per 100 000 persons in 1985 to 6.4 per 100 000 persons in 2005<sup>[1,2]</sup>. The incidence is 3-4 times higher in men than in women and is highest in the Asian population<sup>[2]</sup>. The prognosis for patients diagnosed with HCC is dismal with 5-year survival rates of 3%-5%<sup>[3]</sup>.

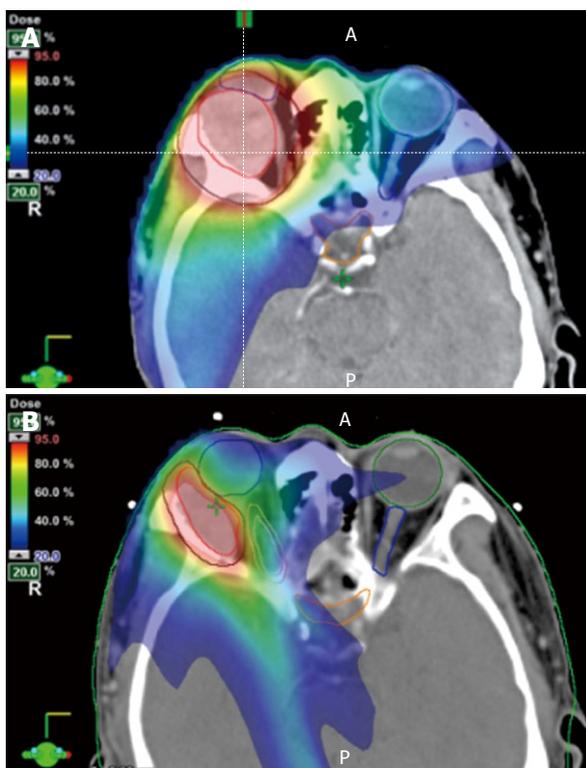
Approximately 50%-75% of patients with HCC will develop metastases during the course of their disease<sup>[4,5]</sup>. The most common sites of metastatic disease are the regional lymph nodes and lung. Less common sites of metastases include bone, brain, adrenal glands, and skin<sup>[4,6-9]</sup>. The orbit has been reported as a site of metastasis from HCC only 14 times in the literature, and information on the palliative response of this highly symptomatic condition with radiation therapy has been very sparse. We report a case of a patient with an orbital metastasis from HCC, who achieved a complete clinical and radiographic response to intensity modulated radiation therapy (IMRT).

### CASE REPORT

A 52-year-old Asian male with a history of hepatitis C but no known cirrhosis presented with elevated liver enzymes



**Figure 1** Axial (A) and coronal (B) T2 weighted MRI of the brain demonstrating the large soft tissue mass in the superior and lateral aspect of the right orbit (white arrows) prior to radiation treatment and resolution of mass on follow-up MRI 12 mo after treatment on axial (C) and coronal images (D).



**Figure 2** Three-dimensional contours for treatment planning demonstrate the gross target volume (GTV) encompassing the tumor (red contour) and the planning target volume (PTV, black contour) on initial (A) and boost (B) CT images. Doses from the initial 7 field intensity modulated radiation (IMRT) plan (A) are represented as color wash according to the scale shown in the figures, representing the range of 95% through 20% of dose coverage. The 7 field IMRT plan for the coned-down field (B) shows even tighter dose coverage and greater sparing of the right optic nerve (pink contour).

while being treated for polycythemia. High resolution triphasic spiral computed tomography (CT) scan of the abdomen demonstrated a well encapsulated, heterogeneous mass in the right lobe of the liver measuring 8.2 cm × 8.8 cm. Imaging characteristics were typical for HCC and  $\alpha$ -fetoprotein was 30 ng/mL. Percutaneous biopsy confirmed HCC in a background of cirrhosis. Exploratory laparotomy with intraoperative ultrasound suggested the need for extended hepatectomy for a curative approach but the volume of the future liver remnant was less than 30%. As such, surgical resection was not completed at

that time. Selective right transarterial chemoembolization, followed by portal vein embolization (PVE) of segments 4-8 was undertaken to promote atrophy of the tumor-bearing liver and hypertrophy of the future liver remnant. Four weeks following PVE, resection was again attempted but metastasis was identified in the future liver remnant. No extrahepatic disease was noted. He was subsequently treated with radioembolization using Yttrium-90.

Shortly after the Yttrium-90 therapy, he developed rapidly progressive diplopia and proptosis of the right eye. Physical examination showed severe proptosis, conjunctival hyperemia, excessive tearing, impaired vision, and limited extraocular muscle movement of the right eye. Magnetic resonance imaging (MRI) of the brain and orbits identified an extraconal 3.7 cm × 3.3 cm × 3.7 cm enhancing mass arising from the floor of the right anterior cranial fossa with extension to the right orbit, resulting in mass effect on the superior and lateral rectus muscles and globe (Figure 1). Positron emission tomography (PET)/CT confirmed a right orbital mass with a peak standardized uptake value (SUV) of 2.8.

He received a course of palliative radiation therapy to a dose of 40 Gray (Gy) in 20 fractions to the right orbit using 6 MV photons and IMRT (Figure 2A). The total gross tumor volume (GTV) was 15.8 cm<sup>3</sup>. A CT scan 10 d after treatment completion showed no response. MRI 1 mo after treatment completion showed the tumor to be decreased from the initial 3.7 cm × 3.3 cm × 3.7 cm to 3.7 cm × 2.8 cm × 3.7 cm. Clinical improvement in the proptosis was also observed. An IMRT boost to the right orbit of an additional 18 Gy in 10 fractions was then delivered (Figure 2B). The total volume of the GTV had decreased to 8.8 cm<sup>3</sup>. The total dose delivered was 58 Gy in 30 fractions over 86 d. The dose to the critical normal structures was kept below the tolerance dose and is presented in Table 1.

One month after receiving 58 Gy to the orbit, the patient reported complete resolution of the diplopia. On examination, his right extraocular muscles functioned normally and proptosis was no longer present. Follow-up PET/CT 1 mo after treatment completion showed decreased fluoro-deoxyglucose activity (peak SUV = 2.2) within the superolateral aspect of the right orbit. MRI of the brain 3, 12 and 17 mo after treatment demonstrated

**Table 1** Critical structures mean dose for original and boost IMRT plans

Structure	Original mean dose (Gy) (2 Gy/fx)	Boost mean dose (Gy) (1.8 Gy/fx)	Total dose (Gy)
Chiasm	6.98	3.55	10.52
Left eye	15.93	1.42	17.34
Left optic nerve	13.3	1.96	15.26
Right eye	30.7	3.96	34.65
Right optic nerve	34.09	6.46	40.55

IMRT: Intensity modulated radiation therapy; Gy: Gray.

complete resolution of the lesion (Figure 1). The patient is alive with controlled orbital disease 20 mo after the initial diagnosis of the orbital metastasis and 26 mo after his original HCC diagnosis. As a result of progression in the liver following Yttrium-90 therapy, he is currently receiving Sorafenib with effective systemic disease control.

## DISCUSSION

Orbital metastases are uncommon and account for 3%-7% of all orbital neoplasms<sup>[7,10,11]</sup>. The most common symptoms of orbital metastases include pain, proptosis, decreased vision or blindness, diplopia, displacement of the globe, exophthalmos, and occasionally enophthalmos. HCC very rarely metastasizes to the orbits, and only a total of 14 case reports of orbital metastases from HCC have been described (Table 2)<sup>[4-15]</sup>. Orbital metastases are usually associated with advanced disease and early mortality. The average survival after occurrence of the orbital metastases is approximately 10 mo<sup>[11]</sup>, although the prognosis ultimately depends on the systemic tumor burden<sup>[5]</sup>.

Among the 14 reported cases of orbital metastases from HCC (Table 2)<sup>[4-15]</sup>, only 4 received primary radiation as the palliative treatment in doses ranging from 30 to 54 Gy, and all showed a response. However, specific details of the radiation planning and treatment delivery in this challenging location are not available. Long-term effects of treatment are poorly understood because only one of the patients survived longer than 1 year after treatment<sup>[11,13]</sup>.

Our patient received a higher dose of radiation than used in the above case reports, and was the first to use IMRT for treatment of orbital HCC metastasis. The orbit is among the most challenging regions for radiation therapy because of the close proximity of dose-limiting critical normal structures, including the brain, ocular structures, and optic chiasm, that can all develop significant complications. IMRT enabled highly conformal treatment delivery and resulted in a durable complete radiologic response.

Our findings are in contrast to the common belief that HCC is a radioresistant tumor. Such reports frequently had to rely on older, less targeted radiation therapy techniques that were unable to spare normal tissues, thus severely limiting the deliverable radiation dose to the tumor to avoid serious toxicity to normal structures. This low tumor dose was not adequate to achieve a significant tumor response<sup>[16]</sup>. Our case shows that dose escalation to 58 Gy, enabled by IMRT, can afford effective local control in

**Table 2** Case reports of orbital metastases from hepatocellular carcinoma

Author	Gender	Age (yr)	Treatment	Survival
Gupta <i>et al</i> <sup>[10]</sup>	M	45	None	NA
Loo <i>et al</i> <sup>[8]</sup>	F	71	Transcranial orbitotomy	3 mo
Schwab <i>et al</i> <sup>[12]</sup>	M	19	Anterior orbitotomy with biopsy	2 wk (moribund state)
Wakisaka <i>et al</i> <sup>[9]</sup>	M	58	Left frontotemporal craniotomy	11 mo
Lubin <i>et al</i> <sup>[22]</sup>	M	69	3000 cGy in 2 wk	NA
Zubler <i>et al</i> <sup>[5]</sup>	M	64	4000 cGy over 8 wk + chemotherapy	3 mo
Srinivasan <i>et al</i> <sup>[4]</sup>	F	76	None	NA
Scolyer <i>et al</i> <sup>[23]</sup>	M	77	None	NA
Font <i>et al</i> <sup>[13]</sup>	F	79	Palliative RT	3 yr
Kim <i>et al</i> <sup>[6]</sup>	F	56	None	2 mo
Machado-Netto <i>et al</i> <sup>[11]</sup>	M	57	Megestrol acetate and Gemcitabine	15 mo
Hirunwiwatkul <i>et al</i> <sup>[7]</sup>	F	74	NA	2 mo
Tranfa <i>et al</i> <sup>[14]</sup>	M	85	Anterior orbitotomy with excisional biopsy	NA
Phanthumchinda <i>et al</i> <sup>[15]</sup>	F	29	5400 cGy in 4 wk	NA

NA: Not available; RT: Radiation therapy; F: Female; M: Male.

HCC. Our observations are supported by studies showing a dose-response relationship for treatment of metastases from HCC. In a retrospective review by Park *et al*<sup>[17]</sup>, 91% of patients with intraabdominal lymph node metastases from HCC treated to  $\geq 50$  Gy<sub>10</sub> had an objective response compared to 65% of patients treated to lesser doses. Recent studies have also shown excellent local control and improved survival with the use of higher doses for primary HCC, delivered with conformal radiation therapy to the partial liver, that were previously intolerable<sup>[18,19]</sup>.

The tumor response in our patient is also characterized by a protracted time course. Symptoms improved slowly, and not until a treatment break and re-imaging 1 mo after a dose of 40 Gy, was the radiologic response evident. Such a slow response pattern may also have led to the conclusion that HCC is not a radio-sensitive tumor. However, the GTV reduction after a 5 wk break following 40 Gy allowed us to further escalate the dose to the orbital tumor, while effectively sparing the sensitive normal structures, especially the right optic nerve (Figure 2B). In conjunction with the highly conformal IMRT delivery, this interval tumor reduction and dose escalation resulted in a durable complete clinical and radiologic response.

Our case also illustrates the importance of 3-dimensional (3D) volumetric analysis of tumor imaging instead of diameter-based measurements for the assessment of tumor response. In the repeat CT for boost planning after 40 Gy, 3D tumor volumetry, determined by tumor delineation on each imaging slice and computation of the volume, demonstrated a reduced tumor volume from 15.8 to 8.8 cm<sup>3</sup>, a change of 44%. However, the diagnostic brain MRI 1 wk previously showed a decrease from 23.7 to 20.1 cm<sup>3</sup> in MRI diameter-based tumor volume

calculation, a change of 15%. Other studies have also shown that diameter-based measurements overestimate tumor size during and after radiation therapy compared to 3D volumetry. This is likely related to the irregular tumor configurations and non-linear tumor shrinkage, that are not adequately assessed by current gold standard diameter-based measurements<sup>[20]</sup>. The high precision of refined 3D volumetry-based measurements, which are easily obtained from treatment planning systems, can overcome the challenge of irregular tumor configuration<sup>[21]</sup>.

In conclusion, because of the increasing incidence and improvement in systemic treatment of primary HCC, the prevalence of symptomatic metastases from HCC will likely increase. Radiation therapy is an excellent treatment option for palliation of challenging metastatic sites, including the orbit, but higher doses than the typical 30 Gy in 10 fractions may be required. With targeted radiation techniques, such as IMRT, that enable sparing of normal critical structures, and 3D volumetric assessment of the response, tumor volume-adapted dose escalation to optimal tumoricidal dose levels can provide durable effective palliation of debilitating symptoms.

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CASE REPORT

## Superior mesenteric artery syndrome in a diabetic patient with acute weight loss

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### Abstract

Superior mesenteric artery (SMA) syndrome is an uncommon disease resulting from compression of the third portion of the duodenum from the superior mesenteric artery. This disease shares many common manifestations with diabetic gastroparesis, including postprandial fullness, nausea, vomiting, and bloating. Therefore, it is often overlooked in diabetic patients. Here, we report a 41-year-old man with poorly controlled diabetic mellitus who developed SMA syndrome due to rapid weight loss. The diagnosis was confirmed by computed tomography and an upper gastrointestinal series. His condition improved after parenteral nutrient, strict sugar control, and gradual weight gain.

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**Key words:** Diabetes mellitus; Superior mesenteric artery syndrome; Gastroparesis

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### INTRODUCTION

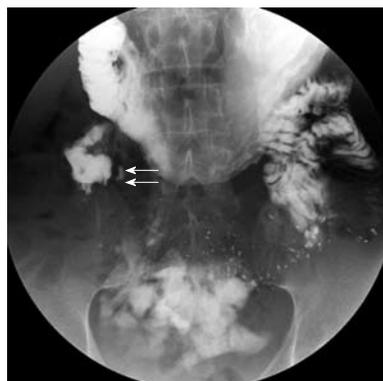
Superior mesenteric artery (SMA) syndrome, also known as Wilkie's syndrome or cast syndrome, is an uncommon disease resulting from superior mesenteric artery compression of the third portion of the duodenum. The clinical manifestations include postprandial fullness or pain, nausea, vomiting, and anorexia due to duodenal obstruction<sup>[1]</sup>. However, the symptoms are similar to those of diabetic gastrointestinal complications. Therefore, SMA syndrome could be misdiagnosed as diabetic gastroparesis. The delayed diagnosis of SMA syndrome might result in malnutrition, electrolyte imbalance, dehydration, and even death.

### CASE REPORT

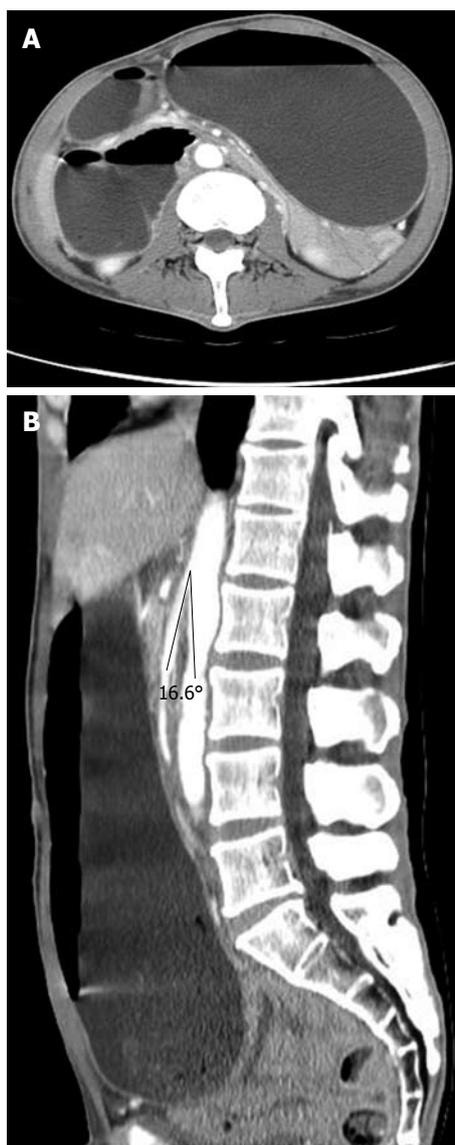
This 41-year-old male was diagnosed type 2 diabetes mellitus four years ago, but the disease was poorly controlled. He did not take any oral antidiabetic agent or insulin therapy for one year. He visited our emergency room complaining of abdominal discomfort and vomiting 30 min after meals for 1 wk. The associated symptoms included general weakness, bilateral lower leg numbness, and a gradual bodyweight loss of 26 kg over the last 3 mo. Physical examination showed a distended abdomen and positive succussion splash sign. His glycemic control was poor and glycosylated hemoglobin (HbA1c) was 11.4%. The abnormal hematologic and biochemical findings included mild anemia (Hgb: 11.7 g/dL) and hypokalemia (K: 3.3 mEq/L). The plain abdomen revealed dilated duodenal bulb and distended stomach with air-fluid level (Figure 1). The gastroduodenoscopy showed a distended stomach with much gastric residue. Therefore, a proximal small bowel obstruction was tentatively diagnosed. To achieve the final diagnosis, we arranged a series of examinations. The upper gastrointestinal series demonstrated a sharp cut-off at the 3rd portion of the duodenum (Figure 2). Compression of the third portion of the duodenum, an



**Figure 1** Abdominal X-ray showing a distended stomach with air fluid level in the stomach and duodenal bulb. The "Double bubble sign" was consistent with high small bowel obstruction.



**Figure 3** Upper gastrointestinal series showing an abrupt cut-off (short arrows) at the third portion of the duodenum.



**Figure 2** Computed tomography (CT) scan showing distended stomach and 2nd portion of duodenum (A); The angle between aorta and superior mesenteric artery (SMA) was 16.6° (B).

aortomesenteric distance of 4.1 mm (normal: 10-28 mm) and a reduction of the aortomesenteric angle 16.6° (normal: 25°-60°) were noted by computed tomography (CT) scan<sup>[2]</sup> (Figure 3). After inserting a nasogastric tube, it drained over 3000 mL turbid, green fluid. His

symptoms improved after nasogastric tube drainage and kept the left lateral decubitus position. We gave him total parenteral nutrition as a nutrition supply and controlled his sugar with an insulin pump for 2 wk. After 2 mo, his bodyweight increased from 44 to 50 kg and he returned to oral intake without subsequent symptoms.

## DISCUSSION

Diabetes mellitus is one of the most common chronic disease of the world and the prevalence of diabetes mellitus is over 10% in Taiwan<sup>[3]</sup>. Gastroparesis is reported in 5% to 12% of diabetic patients. The cardinal symptoms include postprandial fullness, nausea, vomiting, and bloating. Treatment for gastroparesis is prokinetics including metoclopramide, domperidone, and erythromycin<sup>[4]</sup>. However, SMA syndrome can cause the same symptoms as diabetic gastroparesis.

To our knowledge, only two studies have reported diabetic patients with SMA syndrome and all of them had bodyweight loss<sup>[5,6]</sup>. The average bodyweight loss was 29.6 kg (16-50 kg). One of the reported cases received an exploratory laparotomy and the other received intravenous nutrition treatment. In our report, the patient also had a gradually weight loss of 26 kg and improved after medical treatment. The bodyweight loss is a manifestation of the new diagnosis or the poor control of the diabetic patient. It might result in the delayed or missed diagnosis of the SMA syndrome as diabetic gastroparesis.

SMA syndrome is a disease of duodenal obstruction. Weight loss results in loss of the mesenteric fat pad and the superior mesenteric artery compresses duodenum. Bodyweight loss with superior mesenteric artery syndrome, including eating disorders, cardiac cachexia, HIV patients, hereditary motor and sensory neuropathy, have been reported<sup>[7-10]</sup>. The radiographic studies used to establish diagnosis include an upper gastrointestinal series, computed tomography (CT), CT angiography, conventional angiography, abdominal sonography, and magnetic resonance angiography (MRA)<sup>[2,11-13]</sup>. The prone or left lateral decubitus position is effective in the acute status. Conservative treatment with adequate fluid and electrolyte supply is necessary after nasogastric tube placement. Enteral jejunal tube feeding and parenteral nutrition are useful to increase bodyweight. Surgery is indicated when

the conservative treatment fails<sup>[1]</sup>. Laparoscopic duodeno-jejunoscopy has been successful in the cases with SMA syndrome<sup>[14]</sup>.

In conclusion, diabetic patients with gastrointestinal symptoms and bodyweight loss should be considered for SMA syndrome, despite the gastroparesis is the most common etiology. Computed tomography and upper gastrointestinal series are the reliable tools for diagnosis. Adequate nutrition supply is a useful treatment and the aim is bodyweight gain and symptom relief. Surgery is indicated when conservative treatment fails.

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# Aorto-esophageal fistula: A case misdiagnosed as esophageal polyp

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## Abstract

Aorto-esophageal fistula (AEF) is a rare and fatal disorder. It is also a life-threatening cause of massive upper gastrointestinal hemorrhage. Thoracic aortic aneurysm is the most common cause of AEF. Management of a patient with this disorder requires rapid diagnosis and immediate intervention, which is considered the best way to save the patient's life. We report a case of AEF misdiagnosed as esophageal polyp.

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**Key words:** Aorto-esophageal fistula; Aortic aneurysm; Gastrointestinal bleeding

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## INTRODUCTION

Aorto-esophageal fistula (AEF) is a rare and fatal disorder, although it was first described by Dubrueil in 1818. It is also a life-threatening cause of massive upper gastrointestinal hemorrhage. Thoracic aortic aneurysm is

the most common cause of AEF. Prompt diagnosis and emergent intervention are substantial to improve the survival of the patients. We report a case of an AEF due to a descending aorta pseudoaneurysm presenting as an esophageal polyp.

## CASE REPORT

A 62-year-old female patient was admitted to our emergency department for an episode of vomiting bright red blood, with melena and dizziness. Her initial vital sign was normal when she was in emergency. She had a history of intermittent slight chest pain for 2 years. A gastroscopy was performed which revealed a 4 cm fusiform polyp in the mid-esophagus (Figure 1). It was also diagnosed as esophageal polyp with endoscopic ultrasound. The patient was advised to take further examinations, but she refused. During the following 2 years, she received gastroscopy twice (Figure 2) which showed an orifice-like lesion on the top surface of the polyp. Barium radiography reported a 2cm filling defect in the same location (Figure 3). This patient had a history of rib fracture 20 years ago, but no hypertension or diabetes.

A subsequent enhanced computed tomography (CT) documented a descending aortic pseudoaneurysm that compressed the mid-esophagus (Figure 4). The gastroscopy taken after hospitalization documented the same result as before, without fresh blood or clot on the orifice. Twelve days later, she suddenly experienced a shock for her second hematemesis. Blood pressure dropped down to zero. Angiography was performed until her hemodynamic status was stabilized after blood transfusion and hemostasis. The contrast extravasated from the descending aorta rupture with a diameter of about 0.5 cm (Figure 5), which established a diagnosis as descending aortic pseudoaneurysm. A covered endovascular stent grafting was placed immediately. After confirmed to be in good condition by CT, she was discharged on the 10th postoperative day and has remained healthy since then.

## DISCUSSION

AEF, which constitutes approximately 10% of aorto-enteric fistulas, is associated with a high morbidity and mortality. Thoracic aortic aneurysm is the most common cause of AEF, other causes include carcinoma, trauma (including iatrogenic trauma), foreign body ingestion

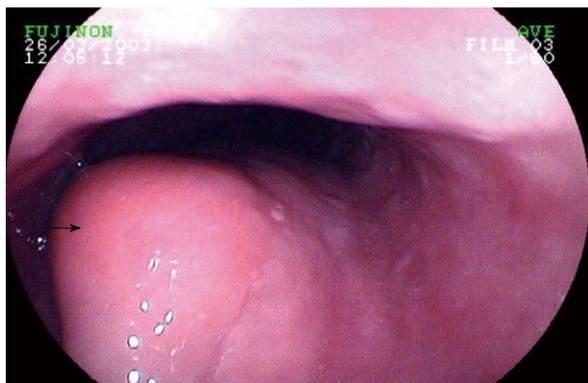


Figure 1 Gastroscopy showing a 4 cm fusiform polyp (black arrow) in mid-esophagus.

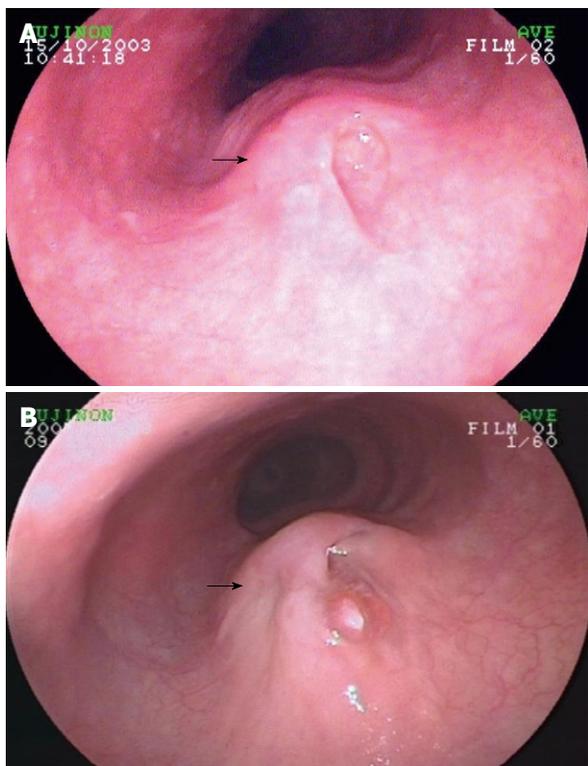


Figure 2 Gastroscopy showing an orifice-like lesion on the top surface of the polyp (black arrows). A: 1st; B: 2nd.

and tuberculous aortitis. Prompt diagnosis and emergent intervention are substantial to improve the survival of the patients. Its classical symptoms, named Chiari's triad, include dysphagia, mid-thoracic pain and sentinel minor hematemesis followed by exsanguination, as did our patient. Mid-thoracic pain may be caused by distension and dissection of the aortic wall, esophageal perforation, or tumor invasion. Spontaneous cessation of the sentinel minor hemorrhage may be caused by temporary occlusion of the fistula due to spasm of the arterial wall and/or periaortic hematoma, which is later digested by infection or gastrointestinal contents. Fifty-nine percent of the AEF patients had mid-thoracic pain, 45% experienced dysphagia, 65% had sentinel minor hematemesis, and 45% showed Chiari's triad<sup>[1]</sup>. Thus, intensive inquiry



Figure 3 Barium radiography reported a 2 cm filling defect (black arrow) in the mid-esophagus.



Figure 4 A subsequent enhanced computed tomography (CT) showing a descending aortic pseudoaneurysm (black arrow).



Figure 5 Aortography. The contrast extravasated from the descending aorta rupture with a diameter of about 0.5 cm (black arrow).

of patient's history and careful physical examination are necessary if AEF is suspected.

Early diagnosis is the key point to decrease the mortality from AEF. Endoscopy is always the first examination to be chosen. Endoscopy usually reveals esophageal compression in the mid-upper esophagus or a submucosal hematoma with bluish grey mucosa indicating the aorta wall. A pulsating mass covered with blood clots or a fistula opening is seldom seen. In our case, one of the causes for misdiagnosis is that gastroscopy showed a polyp with an orifice, without bluish grey mucosa or blood clot. However, whether gastroscopy is safe enough to AEF is still controversial. In our opinion, gastroscopy can be performed carefully for upper gastrointestinal

hemorrhage, except the definitive AEF is confirmed by CT or aortography<sup>[2,3]</sup>.

Subsequent CT or computed tomographic angiography, which is an accurate and non-invasive method for diagnosing AEF, can demonstrate the location of an aneurysm and its surrounding structures, especially esophagus. Barium radiography may display thoracic aortic aneurysm as an extrinsic compression<sup>[3]</sup>. Aortography is used for diagnosing the aneurysm, but rarely shows a fistula itself because of lacking blood. Moreover, aortography may provide more opportunities for endovascular aortic stenting in patients who are not actively bleeding<sup>[4,5]</sup>.

After definitive diagnosis, immediate repair is mandatory as surgical repairs offer the only chance to cure the patients with aneurysm and esophageal erosion. Surgery includes thoracic aorta replacement with a synthetic graft, use of cryopreserved arterial allografts and extra-anatomic bypass. Dacron graft is the most commonly used one. Primary repair and esophageal resection should also be done to avoid the complications of infection. In recent years, endovascular stent grafting has been used as an alternative to surgical treatment, but it has some limitations such as AEF remaining as it is, insufficient debridement or drain of mediastinum. Therefore, some authors suggested that an endovascular stent graft should only be used in patients with a high risk of open surgery<sup>[2,6]</sup>. Since 2005, Pirard *et al*<sup>[7,8]</sup> have attempted to combine endovascular with open surgical approach, which brings more hope to decrease the mortality of AEF.

In conclusion, AEF is an uncommon and life-threatening cause of upper gastrointestinal bleeding. The classical clinical triad, such as mid-thoracic pain or dysphagia,

a sentinel minor hematemesis followed by exsanguinations and gastroscopy will give a hint to suspected AEF, and thoracic CT scan or aortography can confirm this diagnosis. Surgery or endovascular stent grafting should be chosen individually. However, rapid diagnosis and immediate intervention are considered the best ways to save the patient's life.

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LETTERS TO THE EDITOR

## Frequency of alcohol and smoking cessation counseling in hepatitis C patients among internists and gastroenterologists

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### Abstract

Given the overwhelming evidence that both alcohol consumption and smoking accelerate the progression of hepatitis C virus (HCV)-induced liver disease, we evaluated the frequency of alcohol and smoking counseling of patients with HCV-induced liver disease by their primary care internists and gastroenterologists. One hundred and twenty-three medical records of consecutive patients with HCV-induced liver disease referred by an internist to a gastroenterologist for its management were reviewed. Patient gender, race, history of and counseling against alcohol and tobacco use by a physician and a gastroenterologist were obtained. A database was created using Microsoft Excel. There were 105 African-Americans, 12 Caucasians and six patients of other races/ethnicities. Forty-six (37%) patients were daily tobacco users and 34 (28%) patients were daily alcohol consumers. There was a statistically significant difference in the frequencies of alcohol ( $P = 0.0002$ ) and smoking cessation ( $P = 0.0022$ ) between gastroenterologists and internists. This study reveals that internists and gastroenterologists, alike, inadequately counsel patients with hepatitis C about tobacco and alcohol use.

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**Key words:** Alcohol; Hepatitis C virus; Counseling; Smoking; Hepatocellular carcinoma

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### TO THE EDITOR

We read with interest the article by Scognamiglio *et al*<sup>[1]</sup> "Impact of hepatitis C virus infection on lifestyle", *World J Gastroenterol* 2007; 13(19): 2722-2726, which reveals that a greater number of patients with hepatitis C virus (HCV) infection could modify their behavior and lifestyle habits with regard to alcohol consumption when compared to tobacco use after the diagnosis of HCV infection. The authors also emphasized the importance of counseling patients about the effects of both tobacco and alcohol use on the liver in patients with HCV infection. Tobacco use and alcohol consumption alone have been shown to accelerate the progression of hepatitis C toward chronic hepatitis<sup>[1-3]</sup>. Furthermore, multiple studies have shown a synergistic effect of alcohol and tobacco use on the progression of hepatitis C to hepatocellular carcinoma (HCC)<sup>[1,4]</sup>. Independently, tobacco use is also associated with a decreased response to interferon treatment<sup>[1,3]</sup>. Given the above evidence of lifestyle factors on progression of hepatitis C toward chronic hepatitis or HCC, it is imperative that patients with HCV infection not only receive counseling on alcohol consumption but also on tobacco use. We evaluated the frequency of alcohol and smoking counseling of patients with HCV infection by their primary care internists and gastroenterologists.

A retrospective medical record review of patients with HCV infection who were referred by internists to gastroenterologists for management of their liver disease was conducted. The records were evaluated for documentation of alcohol consumption, tobacco use and physician counseling during an evaluation by internists or during consultation by gastroenterologists. One hundred

and twenty three records (75 females, 48 males) were reviewed. There were 105 African-Americans, 12 Caucasians and six patients of other races. Of the 123 patients, 36 (29%) were admitted to smoking, 24 (20%) reported daily alcohol consumption, and 10 (8%) were using both tobacco and alcohol. Ten of the 36 patients who were admitted due to tobacco use were counseled by their primary care internist about the dangers of smoking and were offered assistance in cessation. None of the patients who used tobacco were counseled by their gastroenterologist on the effects of smoking. There was a statistically significant difference ( $P = 0.0022$ ) between the internists' and gastroenterologists' frequencies of consultation on the effects of smoking. Of the 24 patients who drank alcohol daily, 14 (17%) were counseled about the effects of alcohol on the liver by gastroenterologists. Only one of the daily alcohol consumers was counseled about alcohol use by their internists ( $P = 0.0002$ ). There was a statistically significant difference in the frequency of alcohol counseling between gastroenterologists and internists.

It is essential that physicians counsel patients on the effects of both tobacco and alcohol use in the setting of HCV infection<sup>[1]</sup>. Additionally, the discrepancy between the frequencies of addressing smoking and alcohol cessation in patients with HCV infection by internists and gastroenterologists is interesting. This study is important because it reveals that physicians inadequately counsel patients with HCV infection about tobacco and alcohol use despite the overwhelming evidence that these factors accelerate the progression of HCV-induced liver disease toward chronic hepatitis or HCC. The potential fragmentation of counseling may be due to a presumed transfer of responsibility of alcohol counseling by the internist to the gastroenterologist. This can result in decreased counseling by internists about alcohol cessation. Similarly,

gastroenterologists may presume that smoking cessation counseling is the internist's responsibility. It is crucial that efforts are made to ensure that all physicians counsel patients about the effects of alcohol and tobacco use. It is uncertain whether the lapse in counseling is a result of a lack of knowledge about the synergistic effect of tobacco and alcohol use on the progression of HCV-induced liver disease toward chronic hepatitis or HCC, or whether there is simply a failure of both specialties to document their counseling practice.

Although this study is small, it may offer a partial explanation on the findings in study by Scognamiglio *et al*<sup>[1]</sup> on the decreased incidence of smoking modification *vs* alcohol modification in hepatitis C patients. Since hepatitis C treatment is often managed by specialty physicians, alcohol cessation may be emphasized rather than tobacco cessation. Further studies investigating counseling barriers on lifestyle modifications in patients with HCV infection, in both primary care and gastroenterology offices, are necessary to prevent progression of hepatitis C toward an already increasing incidence of HCC.

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## Meetings

### Events Calendar 2009

January 12-15, 2009  
Hyatt Regency San Francisco, San Francisco, CA  
Mouse Models of Cancer

January 21-24, 2009  
Westin San Diego Hotel, San Diego, CA  
Advances in Prostate Cancer Research

February 3-6, 2009  
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)  
Second AACR Conference  
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009  
Hyatt Regency Boston, Boston, MA  
Translation of the Cancer Genome

February 8-11, 2009  
Westin New Orleans Canal Place, New Orleans, LA  
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009  
Hong Kong Convention and Exhibition Centre, Hong Kong, China  
19th Conference of the APASL  
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009  
Orlando, Florida  
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009  
Vienna, Austria  
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease  
[www.easl.ch/vienna2009](http://www.easl.ch/vienna2009)

March 13-14, 2009  
Phoenix, Arizona  
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009  
Marriott Wardman Park Hotel  
Washington, DC  
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009  
Glasgow, Scotland  
British Society of Gastroenterology (BSG) Annual Meeting  
Email: [bsg@mailbox.ulcc.ac.uk](mailto:bsg@mailbox.ulcc.ac.uk)

April 8-9, 2009  
Silver Spring, Maryland  
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009  
Colorado Convention Center, Denver, CO  
AACR 100th Annual Meeting 2009

April 22-26, 2009  
Copenhagen, Denmark  
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)  
<http://www.easl.ch/>

May 17-20, 2009  
Denver, Colorado, USA  
Digestive Disease Week 2009

May 29-June 2, 2009  
Orange County Convention Center  
Orlando, Florida  
45th ASCO Annual Meeting  
[www.asco.org/annualmeeting](http://www.asco.org/annualmeeting)

May 30, 2009  
Chicago, Illinois  
Endpoints Workshop: NASH

May 30-June 4, 2009  
McCormick Place, Chicago, IL  
DDW 2009  
<http://www.ddw.org>

June 17-19, 2009  
North Bethesda, MD  
Accelerating Anticancer Agent Development

June 20-26, 2009  
Flims, Switzerland  
Methods in Clinical Cancer Research (Europe)

June 24-27 2009  
Barcelona, Spain  
ESMO Conference: 11th World Congress on Gastrointestinal Cancer  
[www.worldgicancer.com](http://www.worldgicancer.com)

June 25-28, 2009  
Beijing International Convention Center (BICC), Beijing, China  
World Conference on Interventional Oncology  
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009  
Snowmass, CO, United States  
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009  
Aspen, CO, United States  
Molecular Biology in Clinical Oncology

August 1-7, 2009  
Vail Marriott Mountain Resort, Vail, CO, United States  
Methods in Clinical Cancer Research

August 14-16, 2009  
Bell Harbor Conference Center, Seattle, Washington, United States  
Practical Solutions for Successful Management  
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009  
Beijing International Convention Center (BICC), Beijing, China  
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists (IASGO)  
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009  
Taipei, China  
Asian Pacific Digestive Week  
<http://www.apdwcongress.org/2009/index.shtml>

October 7-11, 2009  
Boston Park Plaza Hotel and Towers, Boston, MA, United States  
Frontiers in Basic Cancer Research

October 13-16, 2009  
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States  
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009  
Versailles, France  
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009  
Boston, MA, United States  
The Liver Meeting

November 15-19, 2009  
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States  
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009  
London, UK  
Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

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*In press*

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 11 **Lam SK.** Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 15 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

### Statistical data

Write as mean  $\pm$  SD or mean  $\pm$  SE.

### Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as  $\chi^2$  (in Greek), related coefficient as *r* (in italics), degree of freedom as  $\nu$  (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

### Units

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose)  $6.4 \pm 2.1$  mmol/L; blood CEA concentration, *p* (CEA) =  $8.6 \pm 2.5$   $\mu$ g/L; CO<sub>2</sub> volume fraction, 50 mL/L CO<sub>2</sub>, not 5% CO<sub>2</sub>; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23243641.

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### Abbreviations

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### Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

Restriction enzymes: *EcoRI*, *HindI*, *BamHI*, *Kho I*, *Kpn I*, etc.

Biology: *H. pylori*, *E coli*, etc.

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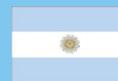
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## Pediatric non-alcoholic fatty liver disease: Preventive and therapeutic value of lifestyle intervention

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### Abstract

Nonalcoholic fatty liver disease (NAFLD), ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), and eventually cirrhosis and liver failure, is seen to be increasing amongst Western children. NAFLD rates are rising in parallel with the epidemic of childhood obesity, and in particular, fatty liver evolves more easily in NASH when poor dietary habits and sedentary lifestyle are combined. In fact, its general prevalence in the child population varies between 2.6% and 10%, but increases up to 80% in obese children. Since NASH is expected to become the most common cause of pediatric chronic liver disease in the near future, there is broad interest amongst clinical researchers to move forward, both in diagnosis and treatment. Unfortunately, to date, the expensive and invasive procedure of liver biopsy is seen as the gold standard for NASH diagnosis and few noninvasive diagnostic methods can be applied successfully. Moreover, there are still no approved pharmacological interventions for NAFLD/NASH. Therefore, current management paradigms are based upon the presence of associated risk factors and aims to improve an individual's quality of life, thus reducing NAFLD-associated morbidity and mortality. Today, lifestyle intervention (diet and exercise) is the treatment of choice for NAFLD/NASH. Thus far, no study has evaluated the potential preventive effect of lifestyle intervention on children at risk of NAFLD/NASH. Future studies will be required in this area with the perspective of developing a national program to promote nutrition education and increase physical activity as means of preventing the disease in individuals at risk. Here, we outline the clinical course,

pathogenesis and management of NAFLD in children, highlighting the preventive and therapeutic value of lifestyle intervention.

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**Key words:** Fatty liver; Children; Lifestyle; Diet; Exercise; Prevention

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### INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD), a worldwide health problem principally affecting millions of people in Western countries, ranges from simple steatosis to nonalcoholic steatohepatitis (NASH), and eventually cirrhosis and liver failure. A few population-based studies have reported that NAFLD/NASH prevalence has been increasing over the past three decades, both in children and adolescents, presenting a worldwide problem<sup>[1-3]</sup>. The rate of prevalence, ranging from 2.6% to 10%, increases with age and the number of risk factors associated with NAFLD. The most prominent risk factor for NAFLD/NASH is overweight/obesity and the disease is most common in male adolescents<sup>[4,5]</sup>. Studies of the prevalence of NAFLD in overweight/obese children have reported values which range from 8% to 80%<sup>[2,4]</sup>. Discrepancies in prevalence data reported in these studies depend on the methods used for diagnosis<sup>[6-11]</sup>. In fact, although definitive diagnosis of NASH and fibrosis staging and grading requires liver biopsy, most studies have been limited to the use of indirect measures, such as elevated serum alanine transaminase (ALT) and ultrasound to predict histological outcome<sup>[4,12]</sup>.

The clinical course of NAFLD, as well as the management of fatty liver and steatohepatitis, is complicated by limited knowledge of the natural history and pathogenesis of the disease, and the paucity of safe and effective treatment modalities<sup>[1,13]</sup>. Most NAFLD children are asymptomatic and the few signs and symptoms that are observed are often nonspecific. NAFLD is a multifactorial disease that clinically may or may not comprise elevated serum ALT levels, hyperlipidemia, hyperglycemia and insulin resistance, associated with increased body weight and echogenic liver that is suggestive of hepatic steatosis<sup>[13-15]</sup>. Over the initial period of disease, many patients progress to the more advanced form of NAFLD, which combines the mentioned dysmetabolic pattern with severe liver injury, including steatohepatitis, necroinflammation and fibrosis<sup>[16]</sup>.

In contrast to the past, there is today, a widespread and growing recognition of the disease, but some aspects of its pathogenesis and multi-causality still remain obscure<sup>[17,18]</sup>. As a consequence, currently, it is only the presence of associated risk factors that contributes to updating the management program of pediatric NAFLD, which is essentially focused on improving the individual's quality of life, thus reducing NAFLD-associated morbidity and mortality. In this management program, lifestyle intervention (diet and exercise) is the best choice of treatment for NAFLD/NASH in children<sup>[19,20]</sup>. However, the potential preventive effect of lifestyle intervention on children at risk of NAFLD/NASH has still not been evaluated.

Here, we outline the clinical course, pathogenesis and management of pediatric NAFLD in children, and highlight the preventive and therapeutic impact of lifestyle intervention. In addition, we suggest further studies in the area of NAFLD prevention in children, but we also presume that soon a national program will be undertaken to promote nutrition education and increase the physical activity for preventing the disease in individuals at risk.

## CLINICAL COURSE AND PATHOGENESIS OF PEDIATRIC NAFLD

Pediatric NAFLD, as in adults, is defined as fat accumulation in the liver that exceeds 5%-10% by wet weight, in the absence of excessive alcohol consumption<sup>[21]</sup>. Although, the natural history of NAFLD is poorly understood in children, it is a multifactorial liver disease that comprises a large spectrum of clinical features: simple fatty liver accumulation (hepatic steatosis); steatosis accompanied by inflammation and other evidence of cellular injury, including various degrees of fibrosis (NASH); and end-stage liver disease, such as rare cases of cirrhosis and hepatocellular carcinoma<sup>[12,22]</sup>.

All genetic and environmental factors responsible for fatty liver and its progression to NASH are still obscure. The most widely accepted model is a "multiple hits" process (Figure 1), during which a first hit induces accumulation of fat in the liver, which causes hepatic steatosis, and renders hepatocytes more susceptible to

additional cofactors (i.e. oxidative stress, mitochondrial dysfunction, overproduction and release of pro-inflammatory cytokines, adipocytokine imbalance, and stellate cell activation), which induce persistent liver injury that leads to NASH<sup>[17,23,24]</sup>.

### Causes of hepatic steatosis

Hepatic steatosis is caused by imbalance between the delivery of fat in the liver and its subsequent secretion or metabolism. Fat accumulates in the liver for different reasons, in particular because of: excessive intake of dietary free fatty acids (FFAs), *de novo* hepatic lipogenesis, and great liver FFA influx caused by insulin resistance<sup>[25,26]</sup>. Interestingly, Donnelly *et al*<sup>[27]</sup> have demonstrated that liver FFA accumulation in NAFLD subjects derives from non-esterified fatty acids for about 60%-80%; *de novo* lipogenesis for 26%, and originates from diet for about 15%. These findings reinforce the hypothesis that several intracellular pathways may contribute to the accumulation of hepatic fat in NAFLD. These pathways include deregulation of  $\beta$  oxidation, decreased hepatic lipid export *via* very low-density lipoproteins, increased lipogenesis due to insulin-resistance-dependent activation of sterol regulatory element-binding protein (SREBP-1c), and glucose-regulated activation of carbohydrate response element-binding protein (ChREBP)<sup>[28]</sup>.

### Steatohepatitis and fibrosis

Several factors play central roles in the second-hit progression from simple steatosis to NASH. Various mechanisms have been proposed, including increased oxidative stress, inflammation, hepatocellular apoptosis and fibrogenesis<sup>[17,29,30]</sup>. There is accumulating evidence that oxidative stress and mitochondrial dysfunction are relevant in the pathogenesis of steatohepatitis, whatever its initial cause<sup>[31,32]</sup>. Moreover, oxidative stress and mitochondrial dysfunction, with insulin resistance, form a complex network of interactions, which promotes progressive liver injury (fibrosis), which causes chronic accumulation of liver FFA, antioxidant depletion, enhanced cytokine-mediated hepatotoxicity, and promotion of stellate cell activation and proliferation<sup>[33-35]</sup>. This last event ultimately results in increased inflammation, apoptosis and liver fibrosis<sup>[36]</sup>.

## MANAGEMENT OF PEDIATRIC NAFLD/ NASH: FIRST-LINE TREATMENT AND PROMISING THERAPEUTIC AGENTS

Significantly high levels of triglycerides, glucose, insulin, serum ALT, increased body mass index (BMI) and waist circumference (central adiposity) are all possible clinical features of pediatric NAFLD, which suggests that interventions on these variables can help to cure fatty liver, as well as to prevent progression to NASH<sup>[37]</sup>. On the other hand, resolution of histological abnormalities revealed by liver biopsy, is, at this time, the main target of NASH treatment<sup>[20,38]</sup>.

Several recent studies have been carried out to

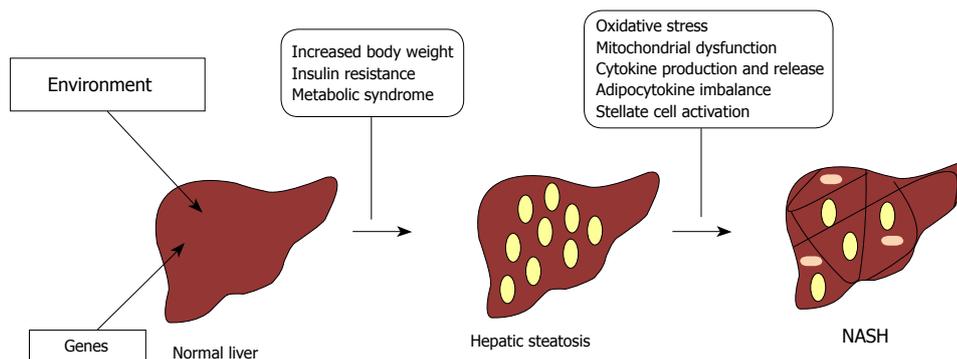


Figure 1 Nonalcoholic fatty liver disease (NAFLD)/nonalcoholic steatohepatitis (NASH) pathogenesis.

examine the effects of dietary composition on NAFLD/NASH in children<sup>[39-41]</sup>. However, some others have also looked at the effects of energy restriction combined with physical activity and pharmaceutical treatments (i.e. vitamin E)<sup>[42,43]</sup>.

#### Diet and exercise

As most NAFLD children are overweight/obese, weight loss may help to reduce pediatric NAFLD prevalence. Weight loss can be achieved through diet and proper exercise, it leads to significant improvement in serum ALT and liver histology in adults with NAFLD<sup>[44,45]</sup>. Studies on pediatric subjects have shown that moderate weight loss can improve serum BMI and levels of ALT, and reduce fatty liver infiltration and necro-inflammation, although no change has been demonstrated in degree of fibrosis<sup>[43,46]</sup>.

Based upon knowledge of NAFLD pathogenesis, a proper diet might be a low-glycemic index diet; in fact, a similar diet may lead to reduction in serum ALT and hepatic steatosis<sup>[47,48]</sup>. Nevertheless, a rapid and excessive caloric restriction and weight loss is not recommended because it might potentially increase dysmetabolism and liver injury<sup>[12]</sup>.

#### Vitamin E

As oxidative stress play a pivotal role in NASH pathogenesis, the use of natural antioxidants, such as vitamin E, is under investigation as a therapeutic approach for NASH patients. Vitamin E has been shown to improve ALT and liver histology in adults with NAFLD<sup>[49]</sup>. However, only one open-label study has demonstrated that 2-4 mo treatment with vitamin E normalizes serum ALT in obese children<sup>[50]</sup>. The efficacy of vitamin E is currently under investigation in a histology-based, double-blind, randomized, placebo-controlled study conducted by NASH Clinical Research Network, and its results will be available in 2010 (ClinicalTrials.gov Identifier: NCT00063635).

#### Insulin sensitizers

Most pediatric NAFLD patients present with insulin resistance, therefore, another approach to decreasing dysmetabolic and histological features associated with this liver disease is treatment with insulin-sensitizing

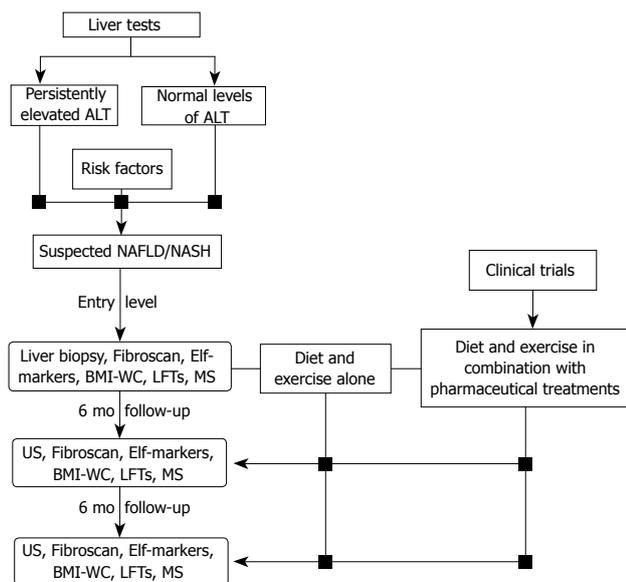
agents. Metformin, a biguanide, is the only insulin-sensitizing agent that has been evaluated for the treatment of pediatric NAFLD. Metformin seems safe and effective in treating type 2 diabetes in children<sup>[51,52]</sup>. In addition, metformin has been evaluated in several pilot studies, which have demonstrated a significant improvement in ALT and hepatic steatosis<sup>[3]</sup>. A pediatric, randomized controlled trial with metformin as a monotherapy in NAFLD is now underway (ClinicalTrials.gov Identifier: NCT00063635). Also, thiazolidinediones, such as pioglitazone and rosiglitazone, have been used successfully for improving insulin resistance and possibly liver histology in adults, but their use in children still requires an accurate control study before they can be considered for use in clinical practice<sup>[53-55]</sup>.

#### Ursodeoxycholic acid (UDCA)

UDCA may act as a cytoprotective and antioxidant agent. It is able to reduce ALT levels and improve liver histology in adults with NAFLD<sup>[56]</sup>. However, in a randomized control trial, Vajro *et al.*<sup>[57]</sup> have demonstrated by ultrasound that UDCA is ineffective in improving serum ALT or steatosis, both alone and in combination with diet. On the other hand, another randomized controlled trial has shown that UDCA in combination with vitamin E may improve serum ALT and liver histology, but also decrease hepatocellular apoptosis and restore serum levels of adiponectin<sup>[58,59]</sup>.

## PREVENTIVE AND THERAPEUTIC EFFECTS OF LIFESTYLE INTERVENTION ON SEVERITY AND OUTCOME OF NAFLD

Patients suffering early liver dysfunction, such as simple hepatic steatosis, or at risk of developing a severe disease, including NASH and cirrhosis, require early diagnosis and intensive treatment. As already discussed, treatment options are limited and dietary weight loss is recommended. Although diets are often difficult to adhere to, they also have an enormous preventive value. In fact, a management program (Figure 2) that incorporates and encourages an adequate diet and age-appropriate physical activities may not only promote a healthy lifestyle, but also prevent the development of NAFLD/NASH.



**Figure 2** Management flowchart for NAFLD/NASH treatment and prevention. EIf: Enhanced liver fibrosis; BMI-WC: Body mass index-waist circumference; LFTs: Liver function tests; MS: Metabolic syndrome; US: Ultrasonography.

### NAFLD-preventive effects of diet and exercise

Weight loss has been, until now, the only proven therapy for pediatric NAFLD. Thus, diet and exercise can be considered the first-line of defense for preventing the onset of NAFLD and progression to NASH in children at high risk. As demonstrated by several recent studies, the high-risk category comprises not only overweight/obese subjects, but also children with insulin resistance, metabolic syndrome, type 2 diabetes, and those with low birth weight<sup>[2,60,61]</sup>.

However, recommendations for lifestyle modifications should be chosen according to the patients' general health because very rapid weight loss might expose patients to severe metabolic consequences and increased mortality risk.

We recommend a hypocaloric diet of 25-30 cal/kg per day in case of overweight/obese subjects, and isocaloric diet (40-45 cal/kg per day) for normal-body-weight children. The amount of calories prescribed also takes into account physical activity and daily routines. Diet composition consists of low gastrointestinal carbohydrate (50%-60%), fat (23%-30%), and protein (15%-20%); a fat composition of two-thirds unsaturated and one-third saturated; and a  $\omega 6/\omega 3$  ratio of approximately 4:1, in accordance with the Italian Recommended Dietary Allowances. Diet is tailored to individual preferences to improve compliance, which may be particularly poor in children and adolescents. A moderate daily exercise program, consisting of 45 min/d of aerobic physical activity, is also recommended. At each visit, subjects or their parents fill out a 3-d dietary and physical activity recall to evaluate adherence to lifestyle recommendations. A multidisciplinary team including dietitians, hepatologists, endocrinologists, psychologists, and cardiologists evaluates and closely follows up the patients. Participants and their parents are instructed on how to exercise, and maintain adherence to the

**Table 1** Success and failure rates after therapeutic intervention in children with liver-biopsy-proven NAFLD *n* (%)

	Lifestyle intervention	Vitamin E	Metformin
<i>n</i>	29	25	28
Complete success (all endpoints)	11 (38)	9 (36)	2 (7)
Partial success			
Serum ALT normalization	23 (79)	13 (52)	23 (75)
HOMA-IR $\leq 1$ restoration	11 (38)	9 (36)	2 (7)
NAS amelioration	19 (65)	17 (68)	16 (57)
Complete failure	4 (14)	8 (32)	2 (7)

NAFLD: Nonalcoholic fatty liver disease; HOMA-IR: Homeostatic model assessment of insulin resistance; NAS: NAFLD activity score; ALT: Alanine transaminase.

exercise program, by a skilled exercise physiologist as part of our multidisciplinary program. Every 6 mo after treatment, children with NAFLD undergo ultrasonography, laboratory analyses, dietician evaluation and psychological tests. In Table 1, we report our success and failure rates after therapeutic intervention in children with biopsy-proven NAFLD.

Thus, recommendations for lifestyle intervention in children at high risk and in subjects with fatty liver should follow a program based on an integrated care model that encourages patients, as well as family members, to adopt diet and exercise goals to prevent NAFLD/NASH development.

### Integrated care model

NAFLD patients require a multidisciplinary approach that involves health professionals with different areas of expertise. This is even more crucial for pediatric patients, whose care also involves their families and other care providers, such as school personnel. In this respect, it is necessary to identify clearly a case manager with a strong leadership role, who can coordinate case management. Case management is defined as the process of planning, co-ordinating, managing and reviewing the care provided in order to ensure that it responds to the appraisal needs. The challenge of this model is for different professionals to work transversally and concurrently, placing the patient at the core of the system. An effective case management system should therefore be based on different steps<sup>[62]</sup>.

The first step is the strategic planning and preparation of services, which includes different activities, with the integration of skills in a team of professionals who identify and overcome individual and organizational barriers, to guarantee timely access to health care. Coordination, monitoring and evaluation of the results gained by the effort of these integrated teams should be conducted on a regular basis, because they are paramount to achieving proper implementation.

The second step is the management of information. In fact, in order to work in an integrated manner, it is essential to achieve good communication among all the professional specialties involved.

The third step of this model stems from the flow of information between the various professionals and

the patient. In this regard, there must be an educational function towards the patients and their relatives, in order that they may also become actively involved in the decisions and in the implementation of required treatments.

At the Bambino Gesù Hospital, this integrated care model is becoming the health-care model of choice. Beginning in 2003, the Liver Unit implemented the multidisciplinary outpatient clinic for the diagnosis and monitoring of patients affected by NAFLD/NASH. The hepatologist acts as case manager, establishes the individual patient's care program, and coordinates clinical activities with the goal of ensuring that all of them are conducted on the day of the outpatient visit.

In addition to the hepatologist, the multidisciplinary team includes: the endocrinologist, because these patients frequently present with metabolic syndrome, with a predisposition to hyperinsulinism, to glucose intolerance and to type 2 diabetes; the cardiologist, who takes care of cardiovascular issues, ranging from arterial hypertension to increased risk of cardiovascular diseases; the radiologist, for the monitoring of hepatic lesions; the dietician for following appropriate dietary changes and increased physical activity prescribed on an individual basis; and the psychologist to consider psychological attitudes which may have preceded, and perpetuate an unhealthy diet and sedentary life style.

As defined in the program, the results of health care and clinical outcomes are evaluated by the team, and are the object of collegial discussion in order to jointly identify obstacles and the most appropriate way to overcome them.

This organisational model, in which the case manager has a central role, guarantees the quality and efficiency of the multi-specialist care process, leading to a favorable cost/benefit ratio, both at the individual and societal level. In our hospital, this model has worked reasonably well. In fact, it has allowed us to improve considerably patient compliance and medication adherence, reaching values close to 80%<sup>[43]</sup>.

## CONCLUSION

NAFLD/NASH has become the leading cause of pediatric liver disease in westernized countries. Several trials are ongoing to establish pharmacological treatment of pediatric disease, but health and nutrition strategies, such as better exercise habits and comprehensive approach to weight management in the school and home surroundings, can reduce the public health impact of pediatric NAFLD. We believe that our integrated care model not only provides an efficient and effective model for the care management of NAFLD patients, who need the intervention of health-care providers with different background and expertise, but also offers a good starting point for a national program of nutritional education and exercise for preventing the disease in children at high risk (i.e. overweight/obese children).

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## Genetic polymorphisms in non-alcoholic fatty liver disease: Clues to pathogenesis and disease progression

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### Abstract

The spectrum of non-alcoholic fatty liver disease (NAFLD) ranges from simple steatosis through steatohepatitis to advanced fibrosis and cirrhosis. Although the reason why only a minority of patients develop progressive forms of disease still remains largely unclear, recent research has identified genetic factors as a possible basis for this variation in disease presentation. Most of the studies have been focused on finding associations between advanced disease forms and selected single nucleotide polymorphisms in genes encoding various proteins involved in disease pathogenesis. Although there are many limitations regarding the study design and interpretation of published data, further carefully planned studies together with implementation of new genetic technologies will likely bring new insights into disease pathogenesis and potential benefits to the management of patients with NAFLD.

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**Key words:** Genetics; Liver fibrosis; Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Single nucleotide polymorphisms

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### INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has emerged as the most common form of chronic liver disease. The spectrum of NAFLD ranges from simple steatosis through steatohepatitis (NASH) to advanced fibrosis and cirrhosis, and the minority of patients progress to end-stage liver disease requiring liver transplantation or develop hepatocellular carcinoma<sup>[1]</sup>. However, the vast majority of patients only have simple steatosis with a benign long-term prognosis. It has been observed that even when considering patients with similar environmental and metabolic NAFLD risk factors (diet, exercise, obesity and insulin resistance being the most important factors), they still differ largely in terms of disease phenotype and degree of progression<sup>[2]</sup>. This led to the research focus more recently being placed on genetic factors that may possibly have a role in NAFLD etiology, and genetic variability is now implied to be one of the most important determinants of disease phenotype and progression in individual patients.

### GENETIC INFLUENCES IN NAFLD

Possible genetic risk for advanced NAFLD was initially suggested in studies which showed coexistence of NASH and/or cryptogenic cirrhosis within several kindreds, and it was not invariably associated with similar major metabolic risk factors<sup>[3,4]</sup>. Further evidence comes from reports of ethnic differences in the prevalence of steatosis, NASH and cryptogenic cirrhosis. The prevalence of all forms of NAFLD was shown to be highest in Hispanic and lowest in African American populations, and this variability did not always correlate with differences in the prevalence of major risk factors<sup>[5-7]</sup>. Furthermore, it was reported that Asian patients with NAFLD had a significantly lower body mass index (BMI) than all other racial groups<sup>[8]</sup>.

As most of the common diseases today, NAFLD

is considered to be a genetically complex disorder. In complex diseases, several or many different genes interact with environmental factors in determining disease presence or its phenotype, and individual genes only have a small effect on disease risk and can therefore be very difficult to identify. Methods for detecting genes in complex disorders have included family-based linkage studies, hypothesis-based candidate gene allele association studies, genome-wide single nucleotide polymorphism (SNP) scanning and, recently, microarray and proteomic studies. Almost all of the data available on genes associated with NAFLD has so far come from the candidate gene association studies, where candidate genes are usually selected on the basis of their suggested role in disease pathogenesis, and the frequency of one or more known SNPs within or close to those genes is compared in cases and controls, in the search for a positive or negative association with the disease. Genes that are candidates for study in NAFLD have included genes influencing insulin resistance, fatty acid metabolism, oxidative stress, immune regulation and fibrosis development.

## GENETIC POLYMORPHISMS

### **Peroxisome proliferator-activated receptor $\gamma$ coactivator 1 $\alpha$ (PPARGC1A)**

*PPARGC1A* has been involved with different metabolic pathways, such as regulation of gene expression in glucose and lipid metabolism and transcriptional control of cellular metabolism, mainly through control of mitochondrial function and biogenesis<sup>[9,10]</sup>. Several studies have shown that *PPARGC1A* regulates several key hepatic gluconeogenic genes, is directly involved in the homeostatic control of systemic energy metabolism, and *PPARGC1A* Gly482Ser polymorphism has also been associated with the development of insulin resistance, obesity and diabetes<sup>[11-14]</sup>. *PPARGC1A* knockout mice are prone to develop hepatic steatosis due to a combination of reduced mitochondrial respiratory capacity and an increased expression of lipogenic genes<sup>[15]</sup>. Yoneda *et al*<sup>[16]</sup> therefore examined 15 SNPs in the *PPARGC1A* gene and found that the rs2290602 polymorphism was significantly associated with NAFLD (more closely with NASH than with simple steatosis), and the frequency of the T allele (allele with rs2290602 polymorphism) was significantly higher in the NASH patients than in the control subjects. They also found that intrahepatic *PPARGC1A* mRNA expression was significantly lower in the TT genotype group than in the GG or GT group. On the other hand, Hui *et al*<sup>[17]</sup> did not find any association between the Gly482Ser variant and NAFLD in Chinese Han people. However, they have reported a correlation between C161T PPAR- $\gamma$  gene SNP, consequent lower plasma levels of adiponectin and increased susceptibility to NAFLD.

### **Microsomal triglyceride transfer protein (MTTP)**

A higher incidence of -493G/T polymorphism in the MTTP gene promoter has been reported in patients with NAFLD; GG homozygosity was associated with

more severe liver histology and has been considered as a risk factor for NAFLD<sup>[18]</sup>. Gambino *et al*<sup>[19]</sup> suggested that NASH patients with GG homozygosity have more atherogenic postprandial lipoprotein profiles and lipoprotein metabolism, which leads to increased peroxidative liver injury.

### **Leptin**

Leptin is an adipocytokine whose main role is regulation of food intake. It probably has an important role in the pathogenesis of NAFLD; leptin-deficient ob/ob mice develop steatohepatitis when fed with a methionine-choline-deficient diet<sup>[20]</sup>. Common variants in the human leptin receptor (*LEPR*) gene have been associated with traits of metabolic syndrome such as obesity, insulin resistance, type 2 diabetes mellitus and altered lipid metabolism, and possibly with NAFLD<sup>[21-23]</sup>. The *LEPR* 3057 variant may link obesity to NAFLD in Chinese patients with type 2 diabetes mellitus through interference with leptin receptor signaling and regulation of lipid metabolism and insulin sensitivity<sup>[24]</sup>.

### **Adiponectin**

Adiponectin, an adipocyte-derived cytokine has an important role in mobilization, transport and muscle oxidation of free fatty acids leading to improvements in lipid profiles and insulin sensitivity<sup>[25,26]</sup>. High levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) mRNA in adipose tissue and high plasma TNF- $\alpha$  concentrations were detected in adiponectin-knockout mice, resulting in severe diet-induced insulin resistance<sup>[27]</sup>. Musso *et al*<sup>[28]</sup> reported that the adiponectin SNPs 45TT and 276GT/TT were more prevalent in Italian NAFLD patients than in the general population; these polymorphisms independently predicted the severity of liver disease in NASH and exhibited a blunted postprandial adiponectin response and higher postprandial triglyceride levels.

### **Hepatic lipase**

Zhan *et al*<sup>[29]</sup> investigated the prevalence of the hepatic lipase gene promoter polymorphism at position -514 in Chinese patients with NAFLD. They reported a higher frequency of the CC genotype and C allele in the NAFLD group and both the CC genotype and CT genotypes were associated with higher relative risk for development of NAFLD<sup>[29]</sup>.

### **Phosphatidylethanolamine N-methyltransferase (PEMT)**

Phosphatidylcholine is required for hepatic formation and secretion of very low density lipoproteins, and it has been shown that a choline-deficient diet leads to accumulation of fat droplets in hepatocyte cytosol and the development of fatty liver<sup>[30]</sup>. PEMT catalyzes *de novo* synthesis of phosphatidylcholine and is responsible for approximately 30% of phosphatidylcholine formed in liver, the rest of it being synthesized by another pathway from dietary choline. Song *et al*<sup>[31]</sup> showed that SNP (G to A substitution in exon 8) that leads to Val to Met substitution at residue 175 of PEMT is associated

Table 1 Studies of genetic polymorphisms in non-alcoholic fatty liver disease (NAFLD) included

Gene	Polymorphism	Ref.	No. of patients with NAFLD included in the study
Peroxisome proliferator-activated receptor $\gamma$ coactivator 1 $\alpha$ (PPARGC1A)	rs2290602	Yoneda <i>et al</i> <sup>[16]</sup> , 2008	115
Microsomal triglyceride transfer protein (MTTP)	Gly482Ser	Hui <i>et al</i> <sup>[17]</sup> , 2008	96
	-493G/T	Namikawa <i>et al</i> <sup>[18]</sup> , 2004 Gambino <i>et al</i> <sup>[19]</sup> , 2007	63 29
Human leptin receptor	G3057A	Lu <i>et al</i> <sup>[24]</sup> , 2009	104
Adiponectin	45G/T and 276G/T	Musso <i>et al</i> <sup>[28]</sup> , 2008	70
Hepatic lipase	-514C/T	Zhan <i>et al</i> <sup>[29]</sup> , 2008	106
Phosphatidylethanolamine N-methyltransferase (PEMT)	Val175Met	Song <i>et al</i> <sup>[31]</sup> , 2005	28
		Dong <i>et al</i> <sup>[32]</sup> , 2007	107
Methylenetetrahydrofolate reductase (MTHFR)	C677T and A1298C	Sazci <i>et al</i> <sup>[33]</sup> , 2008	57
Tumor necrosis factor- $\alpha$ (TNF- $\alpha$ )	-238 and -308	Valenti <i>et al</i> <sup>[38]</sup> , 2002	99
	-1031, -863, -857, -308 and -238	Tokushige <i>et al</i> <sup>[39]</sup> , 2007	102
Angiotensinogen	G-6A	Dixon <i>et al</i> <sup>[45]</sup> , 2003	105
Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1)	Pro25Arg		

with significantly diminished activity of the enzyme, and determined the frequency of this polymorphism in NAFLD patients and controls. The loss of function AA genotype (Met/Met) occurred significantly more frequently in NAFLD patients than in control subjects, which led to the conclusion that genetically inherited low PEMT activity is an important risk factor for developing NAFLD. This was further proven in a Japanese study published by Dong *et al*<sup>[32]</sup>. Although the polymorphism is much rarer in the Japanese population than in Caucasians, the frequency of A allele was significantly higher in NASH patients compared with controls. NASH patients who were carriers of the Val175Met variant had significantly lower BMI and were more frequently non-obese than NASH patients who were wild-type homozygotes, further proving the role of this polymorphism as an independent risk factor for NAFLD development.

#### **Methylenetetrahydrofolate reductase (MTHFR)**

Sazci *et al*<sup>[33]</sup> investigated whether the C677T and A1298C polymorphisms of the MTHFR gene which lead to hyperhomocysteinemia and development of liver steatosis were associated with NASH. They found that the MTHFR 1298C allele was associated with increased risk for NASH in patients of both genders, C1298C genotype and C677C/C1298C compound genotype in female and C677C/A1298C compound genotype in male NASH patients.

#### **TNF- $\alpha$**

TNF- $\alpha$  has long been proven to be one of the key cytokines in the development of all chronic liver diseases. In NAFLD, it has been shown that it may cause hepatocyte injury and apoptosis, neutrophil chemotaxis, and hepatic stellate cell activation, as well as contribute to systemic and hepatic insulin resistance<sup>[34-36]</sup>. Crespo *et al*<sup>[37]</sup> found that obese patients with NASH compared to those without NASH have significantly increased liver expression of TNF- $\alpha$  and its receptor p55, as well as increased expression of TNF- $\alpha$  in adipose tissue. Valenti *et al*<sup>[38]</sup> investigated the relationship between insulin resistance,

occurrence of NAFLD and -238 and -308 TNF- $\alpha$  promoter polymorphisms known to be associated with an increased release of this cytokine. The prevalence of the 238 TNF- $\alpha$  polymorphism was higher in subjects with NAFLD than controls, and patients with these polymorphisms had higher insulin resistance indices. Tokushige *et al*<sup>[39]</sup> determined the prevalence of several TNF- $\alpha$  promoter region polymorphisms (positions -1031, -863, -857, -308 and -238) in a group of Japanese NAFLD patients and control subjects. There were no significant differences in the allele frequencies of any of the six polymorphisms among the group of patients with NAFLD and the control group, including the -238 polymorphism which was previously reported to be associated with NAFLD in Italian patients, but this polymorphism was much less frequent in the Japanese population<sup>[38]</sup>. However, the frequency of the -1031C polymorphism was significantly higher in the NASH group compared to the simple steatosis group, as was the frequency of the -863A polymorphism. The frequency of other polymorphisms did not differ significantly between the two groups. These two polymorphisms were also associated with higher levels of insulin resistance measured by HOMA-IR.

#### **Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) and angiotensin II**

TGF- $\beta$ 1 and angiotensin II are two molecules that have been extensively studied in models of liver fibrogenesis. TGF- $\beta$ 1 has a major role in development of liver fibrosis by activation of hepatic stellate cells and stimulation of production of extracellular matrix proteins<sup>[40]</sup>. Besides its well-known effects in the cardiovascular and renal systems, angiotensin II also has an established role in liver fibrogenesis, and based on those observations, studies with angiotensin II receptor antagonists have been performed in patients with NASH<sup>[41,42]</sup>. There have been several suggestions that profibrotic effects of angiotensin II in heart and kidney are mediated by induction of transcription of TGF- $\beta$ 1<sup>[43,44]</sup>. Considering these data, and based on their previous study in hepatitis C patients, Dixon *et al*<sup>[45]</sup> investigated the relationship between the

presence of advanced fibrosis and angiotensinogen G-6A polymorphism or TGF- $\beta$ 1 Pro25Arg polymorphism in a group of severely obese patients. There was no correlation between either high angiotensin or TGF- $\beta$ 1 producing genotypes alone and hepatic fibrosis. However, patients who inherited both high angiotensin and TGF- $\beta$ 1 producing polymorphisms had a higher risk of advanced fibrosis. These data also support the hypothesis that angiotensin II stimulated TGF- $\beta$ 1 production promotes hepatic fibrosis.

A comprehensive list of the above-mentioned polymorphism studies is shown in Table 1.

## CONCLUSION

While all this and other evidence clearly indicates that genetic factors have a key role in determining susceptibility to advanced forms of NAFLD and its progression, the majority of studies mentioned here had small sample sizes and therefore limited statistical power, which makes it rather difficult to draw definitive conclusions. However, we believe that the development and wider availability of high throughput genetic technologies together with careful design and performance of large multicenter studies with adequate statistical power will soon provide new insights in this vast and very interesting area. Further study and new data on genetic effects have many potential benefits - advancement in understanding the pathogenesis of NAFLD, identification of new potential treatment targets, and, eventually, categorization of patients with respect to disease prognosis, leading to a change in management approach in specific subgroups of patients. Despite the currently limited data on genetic influences in NAFLD and all the difficulties in studying them, we believe that most of the variability in NAFLD presentation will eventually be attributed to and explained by variations in SNP frequencies and their effects on the function of factors involved in the pathogenesis of the disease.

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## Indian task force for celiac disease: Current status

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### Abstract

There are limited data on celiac disease (CD) from India. The limited knowledge about CD in India might be attributed to several factors. The first meeting of the Indian Task Force for Celiac Disease was held in the Asian Institute of Gastroenterology, Hyderabad, India in December 2008. The objectives of the meeting were to focus research on prevalence of CD in the

wheat-eating Northern vs the rice-eating Southern Indian population, low-budget serological assays to study the underprivileged population, to involve other medical subspecialties in CD, to suggest proper legislation regarding wheat food labeling, and to organize affordable food substitutes for patients with celiac disease.

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### INTRODUCTION

Celiac disease (CD) is an autoimmune disease that is caused by interaction of gluten in genetically predisposed individuals<sup>[1]</sup>. The diagnosis is based upon European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN)/United European Gastroenterology Foundation (UEGF) criteria<sup>[2]</sup>.

CD in India is submerged in an ocean of malnutrition. The limited research on CD in India can be attributed to several factors: (1) a common belief that CD is uncommon in India; (2) recognition of tropical sprue and gastrointestinal tuberculosis as major causes of chronic diarrhea and malabsorption syndromes; (3) non-realization that partial villous atrophy (PVA) may be a feature of CD; (4) more pressing problems of malnutrition; (5) lack of awareness regarding non-diarrheal manifestations of CD<sup>[3-5]</sup>.

The first meeting of Indian Task Force for Celiac Disease was held in the Asian Institute of

Gastroenterology, Hyderabad, India on December 6, 2008. The main objectives of this meeting were to evaluate the Indian data on CD and discuss future research. A panel of experts from different parts of India, who have special interest in CD, took part in this Task Force. Professor Mulder CJJ, VU University Medical Center, Amsterdam, also participated in the meeting as an international expert. All the participants addressed issues specific to CD and discussed the area of future research and the strategies to carry out these objectives. In addition, legal issues related to food labeling and availability of gluten-free food items in India were also discussed.

## EPIDEMIOLOGY OF CD IN INDIA

Based on epidemiological studies from Europe and the United States, 90% of CD remains undiagnosed. There are limited data on prevalence of CD from India<sup>[6-9]</sup>. The majority of data are from Northern India. The incidence of CD is increasing<sup>[10]</sup>. The prevalence of CD in India is probably not different from that in western Caucasian populations<sup>[11]</sup>. In a field study conducted among school children in Punjab, the estimated frequency of disease was 1 in 310 (0.3%)<sup>[12]</sup>. This prevalence is probably an underestimation. The siblings of CD patients have a high prevalence of CD (22%). In other studies, the prevalence of CD among the first-degree relatives has been reported to be 8%-25%<sup>[13-15]</sup>. There are regional variations in the prevalence of CD due to genetic and dietary factors, that is, the wheat-rice shift from the North to the South in India, which will be discussed in the next section.

## REGIONAL DIFFERENCES AND CHANGING EPIDEMIOLOGY IN INDIA

CD has a strong genetic predisposition. The main genetic factors are *HLA-DQ* genes, that is, the genes encoding DQ2 or DQ8 in the HLA complex. In the West, approximately 95% of CD patients have a DQ2 heterodimer comprised of DQB1\*02 and DQA1\*05, and most of the remaining 5% have a DQ8 heterodimer comprised of DQB1\*302 and DQA1\*03. Adequate data about DQ2 and DQ8 distribution in India are lacking<sup>[16-19]</sup>.

Regional differences in CD can be explained by genetic, dietary and immunological factors. High prevalence areas of CD such as Saharawis (North Africa, up to 5%) and Europe (1%) have a very high carrier rate of DQ2 and DQ8. On the other hand, Japan and Burkina Faso, which have very low prevalence of CD, have low or absent DQ2 and DQ8 carriage.

Dietary patterns also contribute to geographical differences in CD. Wheat consumption broadly parallels CD prevalence, being particularly low in the Far East and Sub-Saharan Africa. In India also, CD is reported frequently in high wheat-consuming states in Northern India.

Immune conditioning might also influence the development of CD. Dose of gluten in early childhood may be an important determinant of lifelong susceptibility.

Breast feeding during gluten induction probably reduces susceptibility<sup>[20]</sup>. Increased exposure to enteric infections in infancy confers modest increase (1.5 RR) in susceptibility to CD<sup>[21]</sup>.

## CASE FINDING IN HIGH-RISK GROUPS

A wide gap exists in India between the CD prevalence in the population (1%) and the prevalence based on diagnosis (0.02%-0.27%). Thus 90%-95% of CD remains undetected<sup>[22]</sup>. Several complications might occur among untreated CD subjects. Among non-diarrheal adult cases with gastrointestinal symptoms, diagnosis of CD and treatment with gluten-free diet results in a significant improvement in symptoms of abdominal pain, bloating and lactose intolerance. A twofold increase in standard mortality ratio has been reported in adult CD<sup>[23]</sup>. Excess mortality occurs in the first 5-10 years after diagnosis among subgroups of patients with malabsorption, delayed diagnosis and poor compliance. Enteropathy-associated T-cell lymphoma (EATL) is an important mortality risk in patients diagnosed above 50 years of age. There is a higher frequency of so-called associated disorders in CD in comparison to controls, such as endocrine disorders, type 1 diabetes mellitus and connective tissue disorders. Higher risk of malignancy in adult CD is known. Overpresentation of cancer occurs in the small bowel, esophagus and T-cell lymphoma. After diagnosis, despite dietary compliance, an increased risk was observed for EATL<sup>[24]</sup>. On the contrary, a protective role of gluten-free diet has been reported for these so-called associated malignancies<sup>[25]</sup>. The risk of breast cancer seems lower in CD. A higher frequency of CD occurs with autoimmune disorders. The incidence of autoimmune disorders in CD seems to be related to duration of gluten exposure<sup>[26]</sup>.

Targeted screening of CD might be important<sup>[27]</sup>. Among children, screening for CD in India is not indicated before age 1-3 years. Compliance with gluten-free diet and giving consent for small bowel biopsy are problems, because the subjects usually are not convinced about investigations and treatment in the absence of severe symptoms. Serological tests like tissue transglutaminase (tTGA) have a positive predictive value of 75%-80%, however, seronegative CD is well-recognized in milder degrees of villous atrophy<sup>[28]</sup>. In future, we have to define how to interpret serology positivity when biopsies are normal. There is no consensus regarding treating subjects with silent disease with positive serology.

## CD IN CHILDREN IN INDIA

CD was first reported in India in 1966<sup>[29]</sup>. The triad of symptoms of chronic diarrhea/malabsorption, failure to thrive and anemia were common until 2000 in India. However, the presentation of disease seems to have changed over the past few years. An upsurge has been observed by clinicians from North-West India. The so-called typical presentation is now below 50%<sup>[30-32]</sup>.

Symptomatic disease is just the tip of the iceberg but, because of the availability of new serological tests,

we are exploring the hidden CD groups in India. The demographic profile of CD in children in India is different from that in the West<sup>[33]</sup>. In one Indian study, the male/female ratio was 3:2. The sole atypical presentations were short stature in 20%, anemia in 14%, constipation in 5%, family history of CD in 5%, and rickets in 1.5% of patients. Common associations observed in children were IgA deficiency in 6%, asthma in 2%, type 1 diabetes in 1.5%, autoimmune hepatitis in 1.5%, seizures in 1.5%, juvenile rheumatoid arthritis in 0.7%, Down syndrome in 0.7%, and nephrotic syndrome in 0.7% of patients. The recent upsurge is due to factors like improved awareness among pediatricians, cost-effectiveness of serological tests, and increasing pediatric endoscopic facilities.

### CAPSULE ENDOSCOPY IN CD

Video capsule endoscopy (VCE) provides high resolution views of the small intestinal mucosa in a noninvasive manner. Characteristic mucosal abnormalities are seen on capsule endoscopy in CD, which include scalloping of mucosal folds, fissures or grooves, mosaic pattern, and absent or reduced mucosal folds<sup>[34]</sup>. Although esophagogastroduodenoscopy (EGD) and multiple duodenal biopsies continue to remain the gold standard, VCE may be used for initial diagnosis and follow-up of CD patients. VCE may be a reasonable alternative to upper gastrointestinal endoscopy in those patients who are strongly positive for tTGA or endomysial antibodies (EMAs), who are unwilling to undergo EGD. In patients with positive serology for CD and negative histology, VCE might be of help. VCE is useful in follow-up of patients of CD who remain symptomatic despite being on a gluten-free diet<sup>[35]</sup>.

### MAGNIFICATION ENDOSCOPY IN CD

The role of conventional endoscopy in the diagnosis of CD has been limited because of low and varying sensitivity and specificity. The small bowel mucosal damage associated with CD can be distributed unevenly and present as patchy villous atrophy, with some parts appearing normal and others severely diseased<sup>[36]</sup>. Endoscopic markers are not adequate to target biopsy sampling to sites of villous damage in the duodenum.

In the past few years, newly developed procedures and technologies have improved endoscopic recognition of the duodenum. These new technologies include the water immersion technique, chromoendoscopy, high-resolution magnification endoscopy, narrow band imaging, and optimal band imaging<sup>[37]</sup>. These new endoscopic techniques have increased the accuracy of CD diagnosis in patients with patchy villous atrophy, and achieve optimal accuracy for the recognition of severe villous atrophy<sup>[38]</sup>.

### HISTOLOGICAL FEATURES AND PROBLEMS IN INTERPRETATION

Diagnosis of CD is confirmed by biopsy, with a characteristic mucosal injury in association with a clinical

response to a gluten-free diet. Biopsy of the small bowel remains the gold standard for the diagnosis of CD<sup>[39]</sup>. Normal small intestinal mucosa contains long villi, varying in length depending on orientation and depth of biopsy. Histological features of CD comprise small intestinal mucosal injury, surface enterocyte damage, increased intraepithelial lymphocytes, crypt hyperplasia and villous blunting or flattening. A reliable histological diagnosis of CD requires lifelong adherence to a gluten-free diet, which is expensive, socially limiting and difficult on a contemporary diet with manufactured food stuffs. Pathologists should avoid overdiagnosis based on minimal nonspecific histological changes. The uniform classification according to Marsh and its modification as described by Rostami should be applied, which includes Marsh I lesion (lymphocytic enteritis); Marsh II (lymphocytic enteritis with crypt hyperplasia; Marsh IIIA in addition shows partial villous atrophy; Marsh IIIB, subtotal villous atrophy; and Marsh IIIC, total villous atrophy.

Jejunal biopsies are not necessary anymore if adequate duodenal biopsies are taken. Numerous intestinal disorders can present with a CD-like histology but are not responsive to a gluten-free diet, and therefore, are not CD cases. Villous atrophy is noted in various infections such as giardiasis, tropical sprue, HIV, Whipple's disease, and immune-mediated diseases. In the same way, increased intraepithelial lymphocytes (IELs) are seen in tropical sprue, after nonsteroidal anti-inflammatory use, Crohn's disease, and bacterial overgrowth. In cases of histological features suggestive of CD, the diagnosis should be based on ESPGHAN criteria.

Diagnosis of refractory CD, ulcerative jejunitis and EATL requires multiple biopsies. Identification of the two categories of refractory CD (RCD), Marsh type I without aberrant T cells and type II with aberrant T cells requires correlation with T-cell immunophenotyping by flow cytometric analysis and immunohistology. An increase in IELs in uncomplicated CD shows a phenotype of sCD3+, CD8+,  $\gamma^+$  population of T cells, which contrasts with RCD II, which shows an aberrant immunophenotype of sCD3- cCD3+, CD8-. Immunostaining methods using anti-CD3 and anti-CD8 antibodies distinguish active CD from RCD.

Pathologists should be attentive to recognize the less severe histopathological abnormalities of Marsh type I and II CD, and must be aware of the pitfalls in the assessment of mucosal biopsy specimens<sup>[40]</sup>. In general, we do not advise a gluten-free diet for Marsh type I lesions, unless serology (tTGA and EMA) is positive and the patients are symptomatic for CD.

### ATYPICAL CD

Atypical presentations of CD are on the rise in children and adults<sup>[41]</sup>. Patients may present with CD-related symptoms in other specialties, such as cardiology, hematology, ENT, endocrinology, dermatology and dental services. Clinicians should be aware of CD. Screening for CD should be considered in unexplained anemia, unexplained gastrointestinal symptoms, idiopathic

osteoporosis, unexplained infertility, first-degree relatives of CD patients, and autoimmune diseases.

## DIETARY COMPLIANCE IN CD

CD is well recognized in most parts of the world where wheat is the staple diet. Irrespective of the manifestations of CD, the mainstay of treatment is a gluten-free diet. Proper dietary compliance leads to alleviation of symptoms, improvement of anthropometry, improvement in quality of life, and prevention of EATL and osteoporosis. It is important to determine factors that affect dietary compliance. Non-compliance to any dietary modification is multifactorial and is determined by several socioeconomic and cultural factors. Dietary compliance can be assessed by questionnaires, serology or histology, or a combination of these methods.

In a study to determine factors to assess gluten-free dietary compliance, strict compliance was seen in 45%, 50% and 35% in pediatric, adolescent and adult populations, respectively. Temptation was the main reason for default in children. Ignorance combined with temptation were major problems in adolescents, whereas digression in adults was mainly due to sociocultural and economic factors. Overall compliance rates to GFD vary from 45% to 80%. tTGA normalizes in 75% of the compliant patients at 1 year and serves as a useful marker for medium-term compliance and beyond. Histological improvement lags behind serological response. Overall non-compliance was seen in 58% at 2 years<sup>[42]</sup>.

## MANAGEMENT PROBLEMS OF CD IN INDIA

The only treatment available for CD is strict adherence to a gluten-free diet for life. Data suggest that diagnosed but untreated patients with CD have significantly higher morbidity and mortality.

### Gluten-free diet

A gluten-free diet is defined as one that excludes wheat, rye and barley. Even small quantities of gluten may be harmful. The strict definition of a gluten-free diet remains controversial because of the lack of an accurate method to detect gluten in food, and the lack of evidence for what constitutes a safe amount of gluten ingestion. The patients and their relatives should be counseled by a trained dietician. Vitamin and mineral deficiencies, including iron, calcium, phosphorus, folate, B12, and fat-soluble vitamins should be looked for. It is important to have a team-based approach to management. In addition to treatment by a physician and participation in a local support group, consultation with a skilled dietician is essential (Table 1).

Dietary counseling of the patient and the family is the cornerstone of the treatment of CD. In India, it is common practice for families to purchase whole grain and have the flour processed at a small neighborhood flourmill, where other cereals like corn and rice are ground separately at a different time slot after cleaning

**Table 1** Key elements in the management of celiac disease (CD)

Consultation with a skilled dietician
Education about the disease
Lifelong adherence to a gluten-free diet
Identification and treatment of nutritional deficiencies
Access to a support group
Continuous long-term follow-up by a multidisciplinary team

**Table 2** Factors to improve compliance

Learning about CD
Identify gluten-containing products
Improved self-management
Trust in physicians and dietitians
Proactive follow-up measures
Understanding the risk factors and serious complications
Ability to reinforce positive changes internally
Positive coping skills
Participation in a support groups

the grinding machine. Despite cleaning of the flour-making machine, there may be mixing during grinding of cereals. The mixing occurs in the initial part of cereal grinding, therefore, initial flour should not be used by the patients with CD. These measures are inadequate, and some quantity of wheat becomes mixed with other cereals and may be a factor for non-response in a strictly compliant patient. It might make sense for patients to use solely home grinding for gluten-free flour. The major problem is faced by the patients and families on certain occasions: birthday cakes, chocolates, ice creams, biscuits, social functions, and traveling (Table 2).

CD has come to attention of physicians in the past two decades. The number of patients diagnosed with CD is also limited. Therefore, the market value of gluten-free products and food items has not been properly realized. With time, the exact number of patients with CD is going to rise and there will be a requirement for commercially available food items. Besides, there is no legislation for gluten labeling in India, therefore, a patient with CD will not be able to know if any of the food items is safe.

## MANAGEMENT OF RCD

A small subset of CD patients fails to respond to a gluten-free diet. This condition is referred to as RCD, which can be either primary or secondary. There is no standard definition of RCD. Currently, RCD is defined as persisting or recurrent villous atrophy with crypt hyperplasia and increased IELs, in spite of a strict gluten-free diet for more than 12 mo<sup>[43]</sup>.

Before making a diagnosis of RCD, the following causes must be ruled out: (1) dietary non-compliance; (2) ubiquitous gluten source (pill capsules); (3) wrong initial diagnosis; and (4) associated disease, such as collagenous colitis, lactose intolerance, or bacterial overgrowth syndrome.

There are no clear clinical or biological markers that

predict the development of RCD. This disorder usually manifests in patients diagnosed in adulthood, and all reported cases have been patients diagnosed over the age of 40-50 years. The exact incidence of RCD amongst CD is not known. However, in a small subgroup of patients, the clinical and histological abnormalities persist or recur while taking a gluten-free diet. This non-responsiveness leaves a poorly understood syndrome known as RCD<sup>[4,38,40]</sup>. RCD may appear in a subgroup of CD patients with persistent histological abnormalities. In all patients screened for RCD, DQ2 and DQ8 need to be checked. In non-DQ2/DQ8 patients, the diagnosis of CD has to be reconsidered and differentiated from diseases such as autoimmune enteropathy. Most of the patients referred for RCD are affected by other diseases. Probably, the commonest cause of non-responsiveness is continued gluten intake. Exocrine pancreas insufficiency, hyperthyroid disease and collagenous colitis are other common explanations. Immunosuppressive treatment might moderate this. We suggest azathioprine and steroids in RCD- I (without aberrant T lymphocytes). However, in RCD- II (with aberrant T lymphocytes), we suggest chemotherapy. As the prognosis of EATL is extremely poor, the early detection of CD is crucial<sup>[44]</sup>.

## CONCLUSION

The spectrum of CD in India is changing. There is a need to start studies to estimate prevalence of CD all over India. This task can be accomplished by establishing nodal centers in different parts of the country. In addition, competent authorities must be approached with specific recommendations to make food labeling regarding gluten content legally mandatory. Based on available data and discussion, the following recommendations are made. (1) Common questionnaire to collect data at different centers needs to be developed. (2) Studies to estimate community prevalence of CD must be started. (3) Prevalence of CD in high-risk groups should be studied. (4) Simple and low-budget serological assays should be developed for studies in underprivileged individuals. (5) Genetic studies to identify HLA typing of CD patients in India can be taken up in a small sub set of patients. (6) Subspecialties like endocrinology and neurology must be approached and involved in the Indian Task Force. (7) Rapid assays for CD serology need studies among populations suffering from parasitic infections to look for interference with CD. (8) Proper legislation about wheat food labeling should be framed.

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## Reduced normogastric electrical activity associated with emesis: A telemetric study in ferrets

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### Abstract

**AIM:** To characterize the gastric myoelectric activity (GMA) and intra-abdominal pressure changes induced by emetic stimuli (apomorphine and cisplatin) in the ferret.

**METHODS:** GMA and intra-abdominal pressure were recorded in conscious, unrestrained ferrets surgically implanted with radiotelemetry transmitters. Animals were challenged with apomorphine (0.25 mg/kg *sc*) and cisplatin (10 mg/kg *ip*), and the emetic response was quantified *via* direct observation and intra-abdominal pressure recording for 1 and 4 h, respectively. The GMA was analyzed by spectral analysis; the parameters used to characterize the GMA were the dominant frequency (DF) and the repartition of spectral power in the bradygastric, normogastric and tachygastric frequency ranges.

**RESULTS:** Retches were identified on the intra-abdominal pressure trace as peaks  $0.30 \pm 1.01$  s in duration and  $59.57 \pm 2.74$  mmHg in amplitude, vomit peaks were longer ( $0.82 \pm 0.06$  s,  $P < 0.01$ ) and reached a higher pressure ( $87.73 \pm 8.12$  mmHg,  $P < 0.001$ ). The number of retches and vomits quantified

*via* direct observation [apomorphine:  $65.5 \pm 11.8$  retches + vomits (R+V), cisplatin:  $202.6 \pm 64.1$  R+V] and intra-abdominal pressure (apomorphine:  $68.3 \pm 13.7$  R+V,  $n = 8$ ; cisplatin:  $219.0 \pm 69.2$  R+V,  $n = 8$ ) were correlated ( $r = 0.97$ ,  $P < 0.0001$ ) and the timing of emesis was consistent between the 2 methods. Apomorphine induced a decrease in normogastria from  $45.48\% \pm 4.35\%$  to  $36.70 \pm 4.34\%$  ( $n = 8$ ,  $P < 0.05$ ) but the DF of the slow waves was not changed [ $8.95 \pm 0.25$  counts/min (cpm) *vs*  $8.68 \pm 0.35$  cpm,  $n = 8$ ,  $P > 0.05$ ]. Cisplatin induced a decrease in normogastria from  $55.83\% \pm 4.30\%$  to  $29.22\% \pm 5.16\%$  and an increase in bradygastria from  $14.28\% \pm 2.32\%$  to  $31.19\% \pm 8.33\%$  ( $n = 8$ ,  $P < 0.001$ ) but the DF ( $9.14 \pm 0.13$  cpm) remained unchanged ( $P > 0.05$ ). The GMA changes induced by cisplatin preceded the emetic response as normogastria was reduced for 1 h before the onset of emesis ( $57.61\% \pm 5.66\%$  to  $39.91\% \pm 5.74\%$ ,  $n = 6$ ,  $P < 0.05$ ). Peri-emesis analysis revealed that the GMA was significantly disturbed during and immediately after, but not immediately before, the emetic episodes.

**CONCLUSION:** The induction of emesis is reliably associated with a disrupted GMA, but changes may also occur prior to and following the emetic response.

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**Key words:** Emesis; Nausea; Stomach; Ferret; Cisplatin; Apomorphine; Electromyography

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### INTRODUCTION

Nausea and vomiting are components of the body's

defense response against toxins either ingested (e.g. plant alkaloids: nicotine, veratridine, emetine), or released following bacterial (e.g. *Staphylococcus aureus*, *Salmonella enterica*, *Vibrio cholerae*) or viral infection (e.g. norovirus, rotavirus)<sup>[1]</sup>. However, in the clinic, the emetic response can also be triggered inappropriately, for example, as a sequelae of anesthesia with surgery (postoperative nausea and vomiting)<sup>[2]</sup>, or as a side effect of cancer chemotherapy (e.g. cisplatin)<sup>[3]</sup>. In addition, nausea and vomiting are also commonly encountered during the pre-clinical and early clinical development of novel chemical entities (NCE) for a variety of therapeutic indications. Indeed, nausea and vomiting has been ranked second, after abuse liability, in the side effects negatively impacting on the development of NCE<sup>[4]</sup>.

Pre-clinical studies using species capable of emesis such as the ferret (*Mustela putorius furo* L.) or the least shrew (*Cryptotis parva*) correctly identified an emetic liability for several NCE. For example, emetic side effects were observed for phosphodiesterase-IV inhibitors<sup>[5]</sup>, a nicotinic receptor agonist<sup>[6]</sup>, and a cannabinoid CB1 receptor antagonist<sup>[7]</sup>, considered for the treatment of asthma, pain and obesity, respectively. Studies in the ferret also identified the anti-emetic potential of 5-hydroxytryptamine-3 (5-HT<sub>3</sub>) and tachykinin NK<sub>1</sub> receptor antagonists against cancer chemotherapy agents (see<sup>[8]</sup> for review), however, their more limited efficacy against nausea<sup>[9]</sup> could not be predicted from pre-clinical studies. Emesis can be divided into 2 components: the pre-ejection (or prodromal) phase and the ejection phase. The prodromal phase is characterized by sympathetic outputs such as cold sweating, skin vasoconstriction and pupil dilation. Additionally, under vagal control, the proximal stomach relaxes, delaying gastric emptying, and the retrograde giant contraction carries to the stomach intestinal contents. This phase is usually temporally correlated with the sensation of nausea<sup>[10]</sup>. Retching and emesis (vomiting), which constitute the ejection phase, are therefore end-stages in activation of the emetic reflex and there would be considerable utility in the identification of biomarkers induced at sub-emetic doses of a compound, which could be used to better identify the separation between a therapeutic dose and one with emetic liability (therapeutic index). Such biomarkers may provide insights into the potential for induction or detection and reduction of nausea, which remains controversial in studies using laboratory animals<sup>[4]</sup>.

In humans, correlates of nausea include an increase in plasma levels of vasopressin and changes in the electrogastrogram (EGG) frequency, rhythm and power<sup>[11]</sup>. The EGG reflects gastric myoelectric activity (GMA), or gastric slow waves; it is usually recorded from cutaneous abdominal electrodes to investigate the frequency of pacemaker activity, which underlies the genesis of gastric contractile activity<sup>[12]</sup>. However, the precise relationship of the EGG to motility itself is unclear<sup>[13]</sup> but tachygastria has been linked to gastric motor quiescence<sup>[14]</sup>. Of the 2 markers, only the recording of the gastric slow waves

provides a method amenable to telemetry with an adequate temporal resolution to examine relationships to emesis in unrestrained animals.

This paper reports the use of radiotelemetry in the conscious unrestrained ferret to record gastric slow wave activity and investigate the effect of the emetic challenges, apomorphine and cisplatin. Data are also included on telemetric recordings of intra-abdominal pressure following administration of emetic agents in this species. The results show that both apomorphine and cisplatin are associated with a reduction of normal gastric rhythm, and demonstrate the potential applicability of telemetric recording techniques to the study of emetic mechanisms and the identification and understanding of emetic liability of NCE. This provides insights into the changes in gastric function occurring prior to the onset of emesis and which, in humans, have been associated with the occurrence of nausea.

## MATERIALS AND METHODS

### Animals

Castrated male fitch (pigmented) ferrets (*Mustela putorius furo* L.) (1.17-1.65 kg) were obtained from Southland Ferrets (Invercargill, New Zealand). Prior to the experiments, they were housed communally in a temperature-controlled room at 24 ± 1°C, under artificial lighting with lights on between 06:00 am and 18:00 pm. Water and pelleted cat food (Tri Pro Feline Formula Cat Food, American Nutrition®, Utah, USA) were available *ad libitum* until the start of the experiments. All animals were then housed individually from the day of surgery to the end of the experiment. The experiments were conducted under the authority of a license provided by the Government of the Hong Kong SAR and approval from the Animal Experimentation Ethics Committee, The Chinese University of Hong Kong.

### Surgical techniques

**Telemetry transmitter implantation for GMA and abdominal pressure recording:** Anesthesia was induced with ketamine (20 mg/kg im; Alfasan, Holland) and maintained with isoflurane (Halocarbon Products Corporation, USA) about 1.5%, in a 3:1 O<sub>2</sub> to N<sub>2</sub>O ratio using a custom-made face mask and an anesthetic machine (Narkomed 2C, Drager, USA). Animals were placed on a heating pad (UCI#390 Analog moist heating pad, Rebirth Medical & Design, Inc., Taiwan) and the level of anesthesia was assessed and monitored throughout the surgery by the pedal withdrawal reflex. Following a midline abdominal incision, the antrum was exposed and the biopotential wires of the telemetry transmitter (C50-PTX, DSI, USA) were inserted in the muscle and secured in place by suturing the serosa. The body of the transmitter (with the pressure catheter) was inserted in the peritoneal cavity and sutured to the muscle layer on the side. The abdominal cavity was treated with antibiotic (Nebacetin®, Altana Pharma, Germany), sutured closed

in layers and covered with a permeable spray dressing (Opsite<sup>®</sup>, Smith and Nephew, UK).

**Analgesia and post-operative recovery:** Buprenorphine (0.05 mg/kg *sc*; Temgesic<sup>®</sup>, Schering Plough, UK) was given as a preoperative analgesic 15 min before the induction of anesthesia, and 12 h post surgery. Recovery was unremarkable and the wound healed within a week.

### Experimental design

Following surgery, animals were housed individually in observation cages (W49 cm × L61 cm × H49.5 cm). They were allowed to recover for at least 7 d prior to further experimentation. Some ferrets were administered apomorphine (0.25 mg/kg *sc*) at least 7 d prior to the administration of cisplatin (10 mg/kg *ip*). These doses of apomorphine and cisplatin have been shown to induce a reliable emetic response in the ferret<sup>[15,16]</sup>. At the end of the observation period, animals were killed with an overdose (> 100 mg/kg *ip*) of pentobarbital sodium (Dorminal<sup>®</sup>, Alfasan, Woerden, Holland).

Baseline telemetry recordings (GMA and abdominal pressure) were made for a least 1 h prior to presenting the animals with food, or an emetic challenge. Recordings then continued for 1 h in studies assessing the effect of food alone or apomorphine (0.25 mg/kg *sc*), or for 4 h in experiments assessing the action of cisplatin (10 mg/kg *ip*). Emesis was characterized by rhythmic abdominal contractions that were either associated with the oral expulsion of solid or liquid material from the gastrointestinal tract (i.e. vomiting), or not associated with the passage of material (i.e. retching movements). An episode of retching and/or vomiting was considered separate when the animal changed its location in the observation cage, or when the interval between retches and/or vomits exceeded 5 s<sup>[17]</sup>. The latency was defined as the time between the administration of the drug and the first emetic episode.

**Effect of feeding on GMA:** Food was withdrawn for 12-14 h before the start of the studies. The ferrets were then presented with 20 g of pelleted cat food, and all uneaten food was withdrawn 10 min later. This design was chosen to mimic human studies describing the effect of a meal on GMA in humans<sup>[12]</sup>. GMA data were analyzed during the 5 min period prior to presentation of food, and during a 5 min post-prandial period, starting 5 min after the uneaten food was withdrawn.

**Effect of apomorphine (0.25 mg/kg *sc*) and cisplatin (10 mg/kg *ip*) on GMA and abdominal pressure:** On the day of the experiment water was freely available but food was withdrawn for approximate 2 h before the start of the studies. Ferrets were presented with pelleted cat food and 30 min later animals were injected subcutaneously with apomorphine (0.25 mg/kg) or saline (0.5 mL/kg NaCl 154 mmol/L); or injected intraperitoneally with cisplatin (10 mg/kg) or saline (10 mL/kg NaCl 154 mmol/L).

**Drugs:** Cisplatin [cis-diamminedichloroplatinum(II), David Bull Laboratories, Victoria, Australia] was purchased as a sterile saline solution at an active concentration of 1 mg/mL. Apomorphine hydrochloride (Sigma-Aldrich, St. Louis, USA) was dissolved in sodium metabisulphite (526 μmol/L, Riedel-de Haën, Germany) and injected at a concentration of 0.5 mg/mL and a volume of 0.5 mL/kg *sc*. Doses are expressed as the free base.

### Telemetry system and analysis of the data

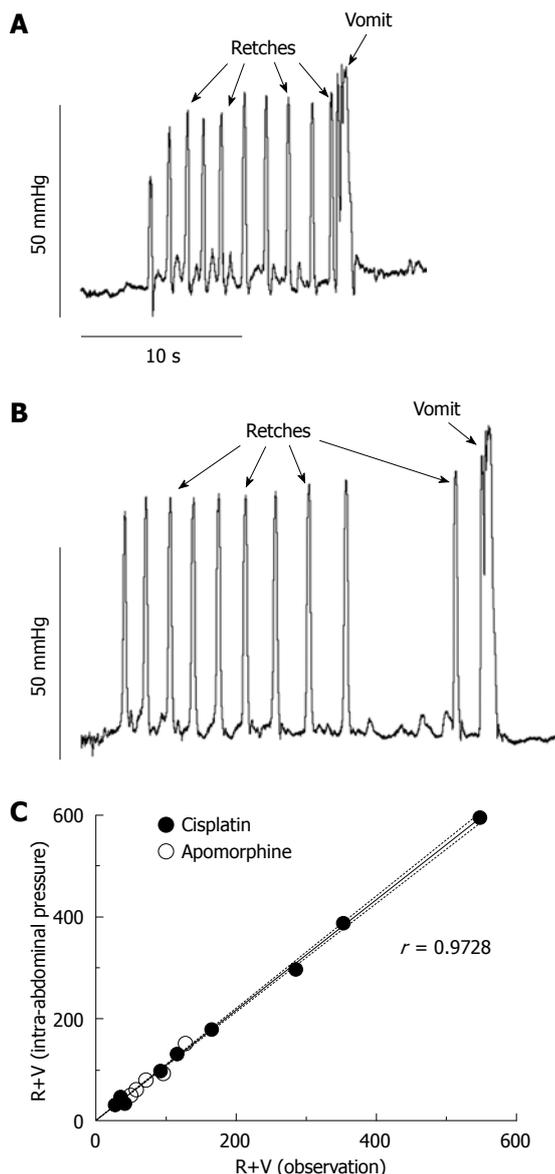
A DSI Dataquest<sup>®</sup> A.R.T. telemetry system (Data Science International, Minnesota, USA) was used. The GMA and intra-abdominal pressure were recorded using PhysioTel<sup>®</sup> C50-PXT Small Animal Transmitters. Telemetric signals were recorded *via* 2 receiver plates (PhysioTel<sup>®</sup> RPC-1) placed under the cages. The receivers were connected to a PC desktop computer *via* a matrix (Dataquest ART Data Exchange Matrix). An ambient pressure reference monitor (APR-1) was connected to the exchange matrix. Data was recorded with the Dataquest Acquisition software (DQ ART 4.0). Analysis of telemetry recordings was carried out using Spike2<sup>®</sup> (version 6.06, Cambridge Electronic Design, UK).

**Quantification of the retches and vomits *via* intra-abdominal pressure:** The abdominal pressure signal was recorded with a sampling frequency of 500 Hz. Retches and vomits were quantified from the intra-abdominal pressure recordings in a semi-automated manner. Thus, the traces of each ferret were inspected visually and then a detection threshold was set and pressure profiles corresponding to retches and vomits were isolated manually. To test the validity of this method, the number of retches + vomits (R+V) detected was compared to the number obtained *via* direct observation using a Spearman test for non-parametric correlation.

**GMA recordings:** The GMA signal was recorded with a sampling frequency of 1000 Hz; selected steps of the analysis procedure are presented in Figure 1. Briefly, a low pass finite impulse response (FIR) filter with a cut-off frequency of 2.5 Hz (transition gap: 10 Hz) was used to remove any signal with a frequency higher than 150 counts/min (cpm). The traces were then interpolated to a sampling frequency of 10.24 Hz and a second low pass FIR filter with a cut-off frequency of 0.3 Hz (18 cpm, transition gap: 0.1 Hz) was applied. This cut-off was chosen to filter out signals of probable cardiac (about 200 cpm in the ferret) and respiratory (33-36 cpm) origins<sup>[18]</sup>.

The following parameters were used to characterize the GMA (Figure 1): (1) the dominant power (DP, the highest power in the 0 to 15 cpm range); (2) the dominant frequency (DF, frequency bin with the highest power in the 0 to 15 cpm range); (3) the repartition of power in the bradygastric, normal and tachygastric ranges (i.e. bradygastria, normogastria and tachygastria). The DF





**Figure 2** Intra-abdominal pressure recordings. Intra-abdominal pressure was recorded via a telemetry transmitter in the abdominal cavity (C50-PTX, DSI®), showing emetic episodes—each composed of several retches and one vomit—induced by apomorphine (0.25 mg/kg sc) (A) and cisplatin (10 mg/kg ip) (B). Both recordings were obtained from the same animal. C: Correlation between the number of retches + vomits (R+V) induced by cisplatin (10 mg/kg ip) and apomorphine (0.25 mg/kg sc), quantified by direct observation and from the intra-abdominal pressure traces. Results plotted as individual values,  $n = 16$  (data obtained from 11 animals). Spearman correlation coefficient ( $r$ ), the linear regression (plain line) and 95% confidence interval (dotted line) are indicated on the graph.

increased by a ratio of  $6.80 \pm 3.61$ , to  $9.45 \times 10^{-4} \pm 2.84 \times 10^{-4}$  mV<sup>2</sup>. These changes were not statistically significant,  $P = 0.38$  and  $P = 0.13$  for the DF and DP respectively (paired  $t$ -tests,  $n = 10$ ).

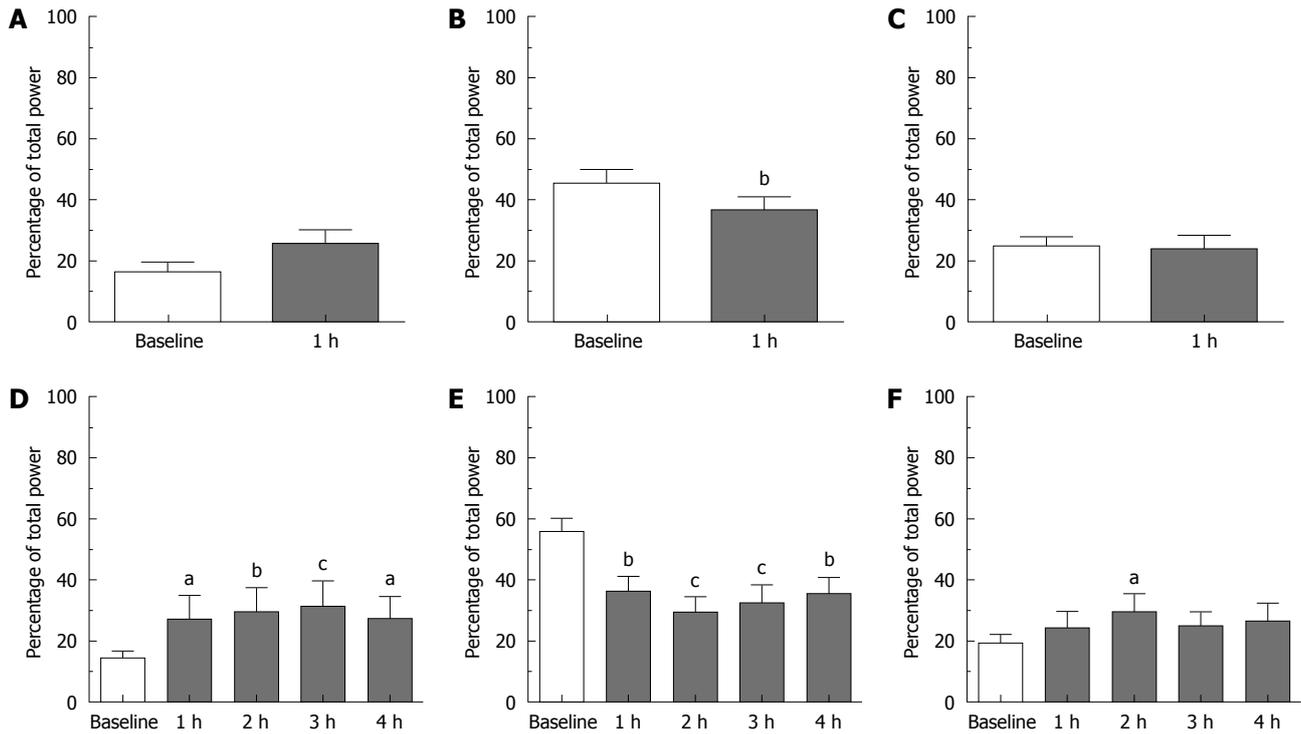
#### Effect of apomorphine and cisplatin on the GMA

**Effect of apomorphine:** During the 1 h that preceded the injection of saline (0.5 mL/kg sc), baseline bradycardia, normogastria and tachygastria were  $13.57\% \pm 3.53\%$ ,  $63.31\% \pm 9.99\%$  and  $16.26\% \pm 5.82\%$  of the total power, respectively. Baseline DF was  $9.54 \pm 0.26$  cpm

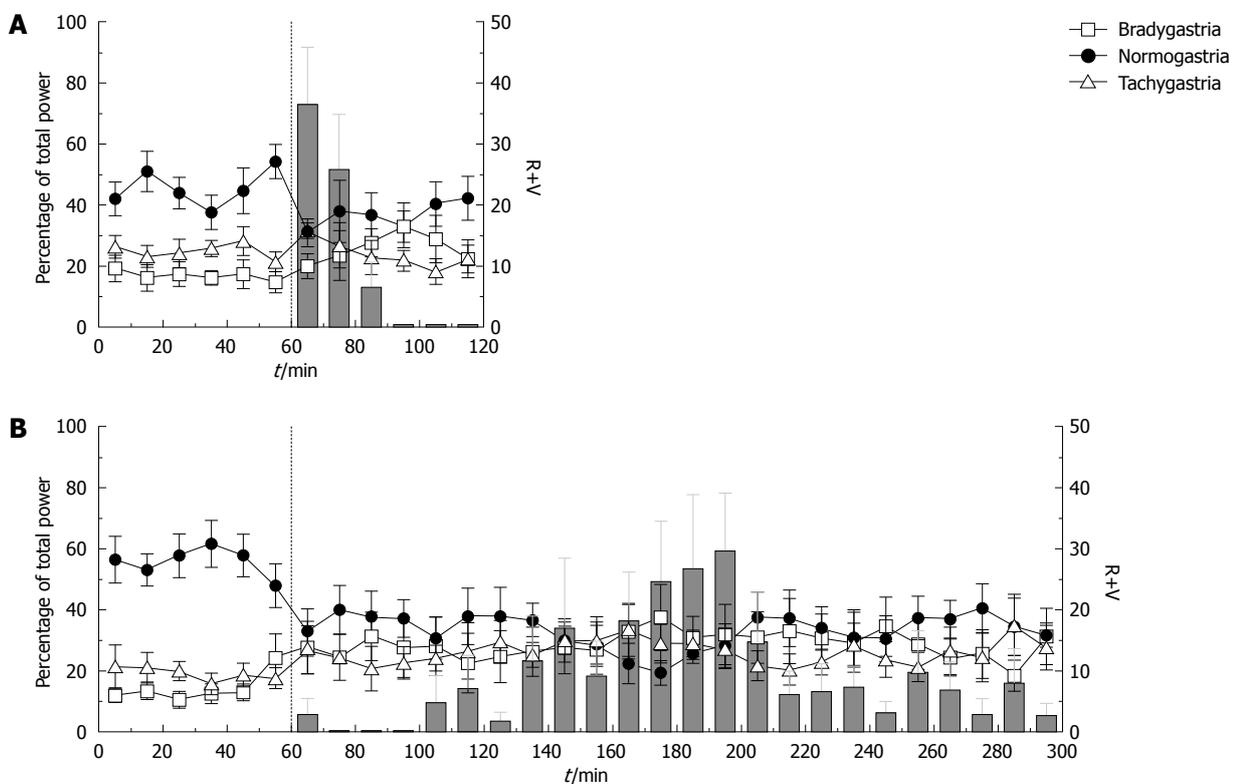
( $n = 4$ ). Following saline injection, the GMA repartition was  $15.14\% \pm 1.28\%$ ,  $57.40\% \pm 8.03\%$  and  $18.46\% \pm 6.13\%$  in the bradycardia, normal and tachygastria ranges respectively; the DF was  $9.73 \pm 0.24$  cpm. None of these values were significantly different from the baseline and no differences were detected between the saline and apomorphine groups during baseline ( $P > 0.05$ ). During the 1 h that preceded the injection of apomorphine, the GMA was repartitioned as follows: bradycardia:  $16.57\% \pm 3.17\%$ , normogastria:  $45.48\% \pm 4.35\%$  and tachygastria:  $25.66\% \pm 2.72\%$ ; DF:  $8.95 \pm 0.25$  cpm ( $n = 8$ ). As shown in Figures 3 and 4, following the administration of apomorphine, the percentage of power in the normal range decreased to  $36.70\% \pm 4.34\%$  ( $P < 0.01$ ), whereas the percentage of power in the bradycardia and the tachygastria ranges was not significantly altered:  $25.76\% \pm 4.65\%$  and  $22.77\% \pm 4.67\%$ , respectively ( $P > 0.05$ ). The DF did not change, and was  $8.68 \pm 0.35$  cpm following apomorphine [ $P > 0.05$ , two-way ANOVAs followed by Bonferroni post-tests,  $n = 8$  (apomorphine) and  $n = 4$  (saline)].

**Effect of cisplatin:** During the 1 h baseline recordings prior to the injection of saline (10 mL/kg ip), baseline bradycardia, normogastria and tachygastria were  $17.11\% \pm 5.35\%$ ,  $43.32\% \pm 6.37\%$  and  $25.85\% \pm 3.83\%$ , respectively; baseline DF was  $8.44 \pm 0.61$  cpm ( $n = 4$ ). The saline treatment had no significant effect on the GMA up to 3 h following intraperitoneal injection, however 4 h post injection the percentage of power in the tachygastria range was significantly increased compared to baseline to  $40.40\% \pm 8.31\%$  ( $P < 0.05$ ). During the 1 h baseline prior to the injection of cisplatin, the percentages in the bradycardia, normogastric and tachygastria ranges were  $14.28\% \pm 2.32\%$ ,  $55.83\% \pm 4.30\%$  and  $19.17\% \pm 3.08\%$ , respectively. The DF was  $9.14 \pm 0.13$  cpm ( $n = 8$ ). As shown in Figures 3 and 4, following the administration of cisplatin the percentage of power in the normogastric range decreased and reached a nadir in the second hour post-injection ( $29.22\% \pm 5.16\%$ ), whereas the percentage of bradycardia and tachygastria increased. Bradycardia reached a peak during the third hour after the injection ( $31.19\% \pm 8.33\%$ ) and tachygastria reached a peak during the second hour post-injection ( $29.56\% \pm 6.01\%$ ); the effects on normogastria and bradycardia were statistically significant during the entire observation period whereas the increase in tachygastria were only statistically significant 2 h post-injection ( $P < 0.05$ , Bonferroni post-tests compared to the 1 h baseline). The DF was not significantly altered after cisplatin administration ( $P < 0.05$ ). No differences could be detected at any time points between saline and cisplatin [ $P > 0.05$ , repeated measures two-way ANOVA followed by Bonferroni post-tests,  $n = 8$  (cisplatin),  $n = 4$  (saline)].

A secondary analysis was carried out only on the animals with a latency to the onset of emesis greater than 1 h ( $n = 6$ ). In these animals, the percentage of power in the normogastric range was significantly reduced in



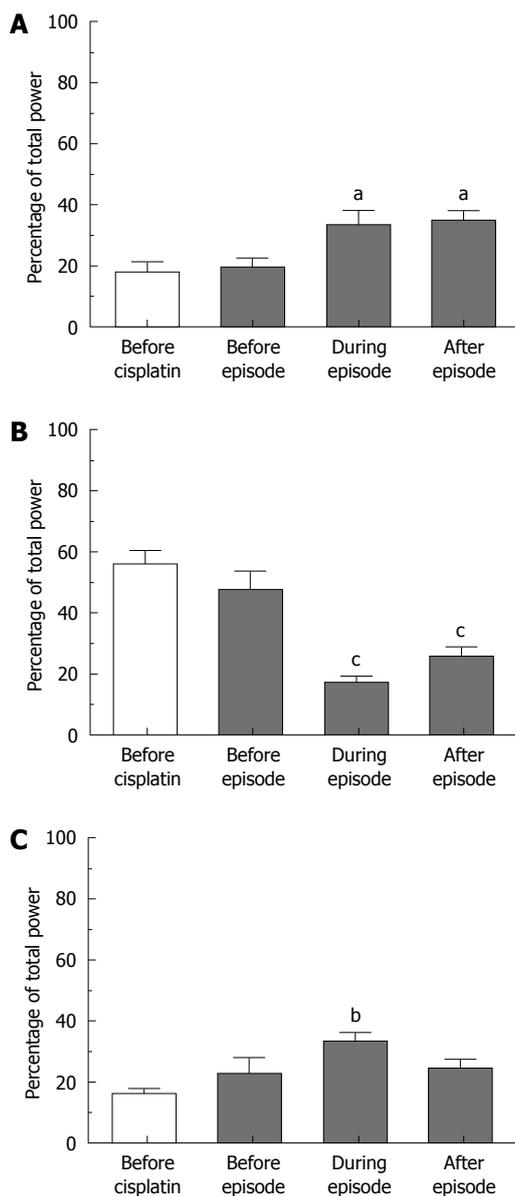
**Figure 3** Effect of apomorphine (0.25 mg/kg sc, A-C) and cisplatin (10 mg/kg ip, D-F) on the gastric myoelectric activity (GMA) in ferrets. The graphs represent the percentage of total (0-18 cpm) power in the bradygastric range [0 - (DF - 1 cpm), A and D], in the normogastric range [(DF - 1 cpm) - (DF + 1 cpm), B and E] and in the tachygastric range [(DF + 1 cpm) - 15 cpm, C and F]. Results are plotted as mean ± SE (n = 8). Differences compared to the effect of a dose of saline (sc or ip as appropriate, data not shown on this graph) were computed using two-way ANOVA (factors: treatment and time) and Bonferroni post-tests. Differences with baseline are indicated as <sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01, <sup>c</sup>P < 0.001.



**Figure 4** Profile of emesis and GMA repartition in the bradygastric, normogastric and tachygastric ranges following the administration of apomorphine (0.25 mg/kg sc) (A) and cisplatin (10 mg/kg ip) (B) in the ferret. Data plotted as mean ± SE per 10 min, n = 8.

the first hour post-cisplatin injection from 57.61% ± 5.66% to 39.91% ± 5.74% (*P* < 0.05) even though no

emesis was observed during that period. Percentages of power in the bradygastric and tachygastric ranges



**Figure 5** Repartition of the GMA in the bradygastric (A), normogastric (B) and tachygastric (C) ranges before the injection of cisplatin, immediately before emetic episodes, during emetic episodes and immediately after emetic episodes induced by cisplatin (10 mg/kg ip) in the ferret. Results are plotted as mean  $\pm$  SE,  $n = 8$ . Differences compared to baseline are calculated using repetitive measures one-way ANOVA followed by Bonferroni post tests. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>c</sup> $P < 0.001$ .

were unchanged [ $P > 0.05$ , repeated measures two-way ANOVA followed by Bonferroni post-tests,  $n = 6$  (cisplatin),  $n = 4$  (saline)].

We investigated the GMA repartition surrounding and during emetic episodes; altogether, 40, 41, 62 and 41 determinations were used to characterize the GMA repartition before cisplatin and before, during and after emetic episodes, respectively. Bradygastric, normogastric and tachygastric values during the 10 min preceding the injection of cisplatin were  $17.67\% \pm 3.50\%$ ,  $56.00\% \pm 4.41\%$  and  $16.15\% \pm 1.66\%$  (mean  $\pm$  SE for 8 animals), respectively. Peri-emesis, the percentage repartition in all 3 ranges was significantly altered ( $P = 0.0085$ ,  $P < 0.0001$  and  $P = 0.0261$  for bradygastric, normogastric

and tachygastric respectively, repeated measures one-way ANOVAs, Figure 5). Bonferroni post-tests revealed that these values did not change significantly immediately before an episode (bradygastric:  $19.36\% \pm 3.02\%$ , normogastric:  $47.56\% \pm 6.18\%$  and tachygastric:  $22.87\% \pm 5.20\%$ ,  $P > 0.05$ ), but were altered during (bradygastric:  $33.08\% \pm 4.90\%$ , normogastric:  $17.23\% \pm 2.06\%$  and tachygastric:  $33.27\% \pm 3.00\%$ ,  $P < 0.05$ ) and immediately after emetic episodes (bradygastric:  $34.68\% \pm 3.33\%$ , normogastric:  $25.57\% \pm 3.27\%$  and tachygastric:  $24.38\% \pm 3.12\%$ ,  $P < 0.05$ ).

## DISCUSSION

### Recording and analysis of GMA in the ferret

The present study is the first report of ambulatory gastric myoelectric recordings in conscious unrestrained ferrets. The frequency of the gastric slow waves was  $9.12 \pm 0.23$  cpm (mean  $\pm$  SE of 12 animals), which is in accordance with post-prandial frequency of antral contractions ( $8.8 \pm 0.5$  cpm)<sup>[19]</sup>, and the frequency of the antral slow waves (9.5 cpm) reported by Diamant *et al.*<sup>[20]</sup> in abstract form; both were measured in conscious but restrained ferrets. In contrast to common protocols used to investigate the GMA in humans<sup>[21-23]</sup> and other animals (dog)<sup>[24,25]</sup>, which used a fixed normogastric range (typically 2.5-3 to 3.7-4 cpm in humans and 4-6 cpm in dogs)<sup>[12,26,27]</sup>, normogastric was defined according to each animal's intrinsic slow waves frequency during baseline. This was done for 2 reasons: (1) because of a relative paucity of literature, not enough data on the ferret's GMA was available to determine with confidence what the normogastric range should be (DF range 7.8-9.2 cpm in 12 animals in the present study) or how wide it should be; (2) the purpose of the present study was to focus on the changes induced by an experimental stimulus; setting the normogastric range relative to each individual animal reduced the influence of inter-animal variability and rendered the analysis more powerful to detect the effect of an emetic stimulus on the GMA in a limited number of animals.

Regarding the GMA analysis parameters, we chose to report the DF and compute the repartition of power in the normogastric, bradygastric and tachygastric ranges. An alternative analysis, which has been used in most preclinical studies<sup>[27-29]</sup> and some studies in humans<sup>[30]</sup>, consists of calculating the percentage of time during which the DF falls within each frequency range. The analysis parameters used in the present study were chosen as the power repartition encompass all the data present in the GMA signal and does not solely focus on the time at which the DF is included within a range of interest<sup>[12]</sup>.

### GMA changes induced by apomorphine and cisplatin

Similar GMA changes were observed following apomorphine and cisplatin; both stimuli induced a decrease in the percentage of power in the normogastric range, which was accounted for in the case of cisplatin by a clear increase in power in the bradygastric range,

without changing the DF. Our findings on the effects of apomorphine are partially supported by a number of studies in conscious, restrained dogs, which also reported a transient disruption of the gastric antral rhythm following the administration of apomorphine<sup>[31-33]</sup>. To the best of our knowledge, the present study represents the first clear evidence that apomorphine is associated with a reduction of normogastria for up to 1 h, which outlasts the emetic response as the last emetic episode was observed  $21.12 \pm 1.76$  min ( $n = 8$ ) post-apomorphine.

Regarding the effect of cisplatin, GMA changes were temporally correlated with the occurrence of emesis and maximal changes in the GMA repartition were observed when the emetic response was the most intense (2-3 h post cisplatin). However, GMA changes preceded the emetic response as evidenced by a decrease in normogastria during the first hour post-cisplatin in animals, which had not yet developed emesis during that period. Consistent with our findings, a recent study in the dog showed that cisplatin reduced the percentage of normal gastric slow waves in the hours preceding the onset of emesis and during the emetic response<sup>[34]</sup>. In the present study, a peri-emesis analysis revealed that normogastria was decreased and bradygastria and tachygastria were increased during and immediately after-but not immediately before-an emetic episode, which is in accordance with the short periods of dysrhythmia associated with emetic episodes have been reported in human patients during the administration of cisplatin<sup>[35]</sup>. Our findings are partly supported by human studies, which reported dysrhythmias in a few patients treated with anti-cancer chemotherapy. However, such events appeared to be transient rather than an overall change in slow wave activity<sup>[30,35]</sup>. The apparent differences in the effect of chemotherapy in human patients and in the ferret model could have four explanations:

(1) In the present study, the ferret model of acute cisplatin-induced emesis was used. This model uses a high bolus dose of cisplatin (10 mg/kg ip)-a highly emetic chemotherapeutic agent-to provoke an intense, reliable emetic response in all the animals, typically quantifiable over 4 h<sup>[36]</sup>. In human patients however, chemotherapy (platinum-based or not) is infused over hours and the emetic response is less reliable. Correspondingly, the GMA disturbance may be more severe in the ferret model than it is in human patients.

(2) Additionally, cancer patients treated with chemotherapy are not healthy subjects and their GMA may already be altered before they receive chemotherapy, rendering it less likely to detect an additional effect of the anti-cancer treatment. It is interesting that following a chemotherapy session, Riezzo *et al.*<sup>[30]</sup> reported a higher percentage of tachygastria in cancer patients compared to healthy volunteers. However, they did not compare the EGG repartition in the cancer patients prior to chemotherapy, the measurements were collected 7 d post-chemotherapy and the chemotherapy regimens were not reported, precluding any direct comparison with the present study.

(3) The use of anti-emetic prophylaxis may also have an influence on GMA, and 5-HT<sub>3</sub> receptor antagonists-commonly administered with chemotherapy and used in the 2 above-mentioned human studies-have been reported to reduce vection-induced dysrhythmias<sup>[37]</sup>.

(4) The analysis of the data is an important factor to consider; as discussed above, in the present study, the DF and the percentage repartition of the power in the bradygastric, tachygastria and normogastria ranges were computed. In contrast, Samsom *et al.*<sup>[35]</sup> identified bradygastria and tachygastria as intervals of at least 2 min, during which the DF was  $< 2.4$  cpm or  $> 3.6$  cpm and no overall assessment of normogastria was made, apart from the mean DF. Also, Riezzo *et al.*<sup>[30]</sup> calculated the percentage of successive spectra in which the DF falls within one of the 3 ranges, or used visual inspection of the waveform traces or the regular spiking activity (RSA).

### ***Intra-abdominal pressure recordings***

On the intra-abdominal pressure traces, retches were identified as brief peaks whereas vomits were more prolonged. These findings in the freely moving, conscious ferret are consistent with what McCarthy *et al.*<sup>[38]</sup> described in the decerebrate cat. Our technique represents a great advantage in that quantifying the retches and vomits from the intra-abdominal pressure traces is more accurate than from a video or even direct observation; the distinction between retches and vomits is unequivocal and abdominal pressure recordings enable precise analysis regarding the timing and the frequency of the retches. Further information regarding the central neuronal circuitry involved in the mechanism of various emetics and anti-emetics can be gained from, for example, the interval between retches, the pattern of retching preceding a vomit or the characteristics of episodes including a vomit and those of episodes of retches. The major disadvantage is that this is an invasive technique, which requires abdominal surgery.

## **CONCLUSION**

The use of telemetry enables the recording of gastric slow waves in freely moving, conscious animals; additionally, analysis of the emetic response *via* intra-abdominal pressure permits a more rigorous data collection and the investigation of the precise temporal correlation between gastric changes and emesis. Using an EGG-like analysis of GMA recordings, disruption of the gastric rhythm was detected in ferrets challenged with apomorphine and cisplatin. Both emetic stimuli were associated with a reduction of normogastria, and cisplatin increased bradygastria. The GMA changes were subtle and not detectable by a simple DF analysis but comparison of the power repartition of the gastric signal, before and after the emetic challenges, indicated a clear change, which was temporally correlated with the development of the emetic response but not restricted to the immediate peri-emetic period. In the ferret, recording and analysis of the GMA, which is a physiological correlate of nausea in

humans, will improve the understanding of the changes in gastric function associated with emesis.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Nausea is a subjective sensation, therefore impossible to study directly in animals. However physiological correlates of nausea have been identified in humans and include disruption of the gastric electrical rhythm. As nausea usually precedes the onset of emesis, recording the gastric electrical rhythm in animals potentially enables the detection of emetic pathway activation before the emetic threshold is reached.

### Research frontiers

The application of human biomarkers of nausea to an animal model enables the refinement of this model as it improves its translational potential. The research hotspot is the correlation of physiological events occurring around the time of vomiting, which may give insight into nausea.

### Innovations and breakthroughs

Recent *in vivo* research studies investigating the relationship between emesis and gastric myoelectric activity (GMA) have used either restrained or anesthetized animals (mainly dogs) with wires connected to the serosa and externalized through the skin. The use of telemetry represents a novel approach and the present study is the first report of GMA recording in freely moving animals, which represents an indisputable advantage in terms of animal welfare.

### Applications

The ferret is a species commonly used in emesis research and our approach could be integrated to standard study designs, therefore refining this animal model by enabling the detection of emetic pathway(s) activation before the emetic threshold is reached.

### Terminology

The GMA consists of electrical pacemaker signals, which trigger contractions of the stomach. Gastric dysrhythmia refers to a departure from the normal gastric electrical rhythm, the term encompasses bradygastria, tachygastria and gastric arrhythmia.

### Peer review

This is a well written manuscript from an established group expert in this field using a novel relevant approach to assess GMA and intra-abdominal pressure in the conscious ferret implanted with radiotelemetry transmitters.

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## MRI versus 64-row MDCT for diagnosis of hepatocellular carcinoma

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### Abstract

**AIM:** To compare the diagnostic capability of multi-detector computed tomography (MDCT) and magnetic resonance imaging (MRI) for the detection of hepatocellular carcinoma (HCC) tumour nodules and their effect on patient management.

**METHODS:** A total of 28 patients (25 male, 3 female, mean age  $67 \pm 10.8$  years) with biopsy-proven HCC were investigated with 64-row MDCT (slice 3 mm native, arterial and portal-venous phase, 120 mL Iomeprol, 4 mL/s, delay by bolus trigger) and MRI (T1fs f12d TE/TR 2.72/129 ms, T2tse TE/TR 102/4000 ms, 5-phase dynamic contrast-enhanced T1fs f13d TE/TR 1.56/4.6, Gadolinium-DTPA, slice 4 mm). Consensus reading of both modalities was used as reference. Tumour nodules were analyzed with respect to number, size, and location.

**RESULTS:** In total, 162 tumour nodules were detected by consensus reading. MRI detected significantly more tumour nodules (159 vs 123,  $P < 0.001$ ) compared to MDCT, with the best sensitivity for early arterial phase MRI. False-negative CT findings included nodules  $\leq 5$  mm ( $n = 5$ ),  $\leq 10$  mm ( $n = 17$ ),  $\leq 15$  mm ( $n = 12$ ),  $\leq 20$  mm ( $n = 4$ ), and 1 nodule  $> 20$  mm.

MRI missed 2 nodules  $\leq 10$  mm and 1 nodule  $\leq 15$  mm. On MRI, nodule diameters were greater than on CT ( $29.2 \pm 25.1$  mm, range 5-140 mm vs  $24.1 \pm 22.7$  mm, range 4-129 mm,  $P < 0.005$ ). In 2 patients, MDCT showed only unilobar tumour spread, whereas MRI revealed additional nodules in the contralateral lobe. Detection of these nodules could have changed the therapeutic strategy.

**CONCLUSION:** Contrast-enhanced MRI is superior to 64-row MDCT for the detection of HCC nodules. Patients should be allocated to interventional or operative treatment according to a dedicated MRI-protocol.

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**Key words:** American Association for the Study of Liver Diseases; European Association for the Study of the Liver; Hepatocellular carcinoma; Multidetector computed tomography; Magnetic resonance imaging

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### INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and the third most common cause of cancer death, with 600 000 to 1 million new cases being diagnosed each year<sup>[1,2]</sup>. In North America and Europe, the most common risk factors are alcoholic cirrhosis and chronic hepatitis C and hepatitis B infection<sup>[3-6]</sup>. Patient survival has not significantly improved in the last 30 years because most cases are still not diagnosed until the disease is already in an advanced stage, which limits the most effective therapeutic options<sup>[7]</sup>. Therefore, early tumour detection is one of the most important issues in HCC therapy<sup>[8]</sup>. Currently, magnetic

resonance imaging (MRI) and multidetector computed tomography (MDCT) are both equally used for HCC diagnosis, although in the past MRI has been reported to produce significantly higher detection rates<sup>[9-11]</sup>. State-of-the-art MRI provides fast imaging techniques with dynamic contrast-enhanced sequences to detect the mostly hypervascularized HCC tumour nodules with a high sensitivity and specificity. However, current developments in MDCT techniques provide better spatial resolution than MRI and quick scan times potentially cause fewer motion artifacts and improve the accuracy of MDCT. The exact number and the distribution of tumour nodules is crucial for allocating these patients to adequate treatment regimens; however, it is well known that particularly small nodules often remain undetected using radiological methods<sup>[8,12,13]</sup>. The purpose of this study was to compare the diagnostic capability of 64-row MDCT and MRI for the detection of hypervascularized tumour nodules in the cirrhotic liver to allow for adequate treatment.

## MATERIALS AND METHODS

Between July 2006 and July 2007, 28 patients with a suspected diagnosis of HCC on ultrasound or CT (25 male, 3 female, mean age  $67.0 \pm 10.8$  years, range 46-89 years) were included in the study protocol. Diagnosis was confirmed by liver biopsy of at least one of the tumour nodules in 25 cases (Table 1). According to the guidelines of the European Association for the Study of the Liver (EASL), the other 3 patients were diagnosed with two different imaging techniques by arterial hypervascularization of nodules  $> 2$  cm and/or a corresponding increase in serum levels of alpha-feto-protein<sup>[14]</sup>. For final diagnosis, all patients were included in this comparative imaging protocol that comprised contrast-enhanced MDCT and MRI in order to evaluate number, size, and location of HCC tumours for subsequent treatment allocation. The study protocol was approved by the local ethical review board. All patients gave their informed consent before entering the study.

The CT diagnosis was based on a triphasic contrast-enhanced protocol using a 64-row MDCT scanner (Brilliance 64<sup>®</sup>, Philips Medical Systems, Eindhoven, Netherlands, 120 kV, 200 mAs, collimation 64 mm  $\times$  0.6 mm, pitch 0.625, reconstruction interval 1.172 mm, slice thickness 5 mm native and 3 mm in contrast-enhanced phases). 1 mm slices were reconstructed for CT-angiography of liver arteries if the patients were considered for surgery. The contrast bolus consisted of 120 mL Iomeprol (Imeron 300<sup>®</sup>, Altana Pharma, Konstanz, Germany) administered at a flow rate of 4 mL/s using a bolus trigger technique (positioning of the respective region of interest (ROI) in the abdominal aorta just above the celiac trunk, threshold 150 Hounsfield Units (HU), start delay 10 s). The portal phase started with a delay of 50 s after reaching the threshold.

MRI was performed using a 1.5-Tesla MR scanner (Magnetom Vision<sup>®</sup>, Siemens Medical Solutions, Erlangen, Germany; software: syngo MR 2004A 4VA25A) with two body coils (CP Body Array Flex<sup>®</sup>). The study

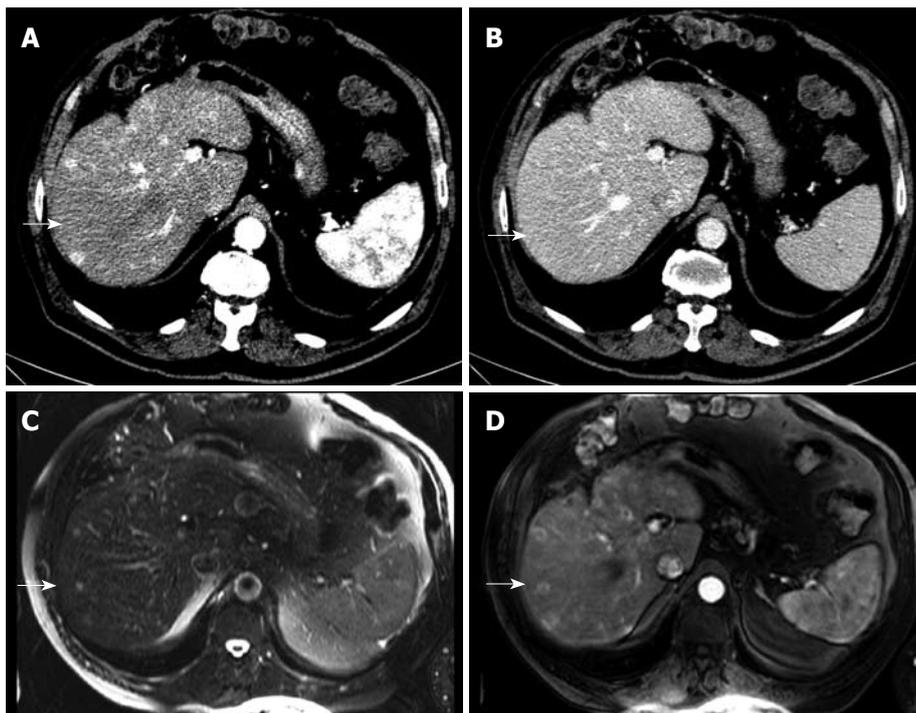
**Table 1** Demographics, aetiology of liver cirrhosis and clinical condition of the patients ( $n = 28$ )

	<i>n</i>
Gender	
Male	25
Female	3
Mean age (yr)	$67.0 \pm 10.8$ (range 46-89)
Aetiology of liver cirrhosis	
Ethanol	13
Hepatitis B	2
Hepatitis C	7
Cryptogenetic	6
Clinical stage	
BCLC stage	
A	6
B	22
C	0
D	0
Child Pugh	
A	24
B	4
C	0
Okuda	
I	25
II	3
III	0
ECOG	
0	26
I	2
II	0
III	0
IV	0
Histological tumour grading	
Well	16
Moderate	4
Poor	2
Unknown	3
No biopsy	3

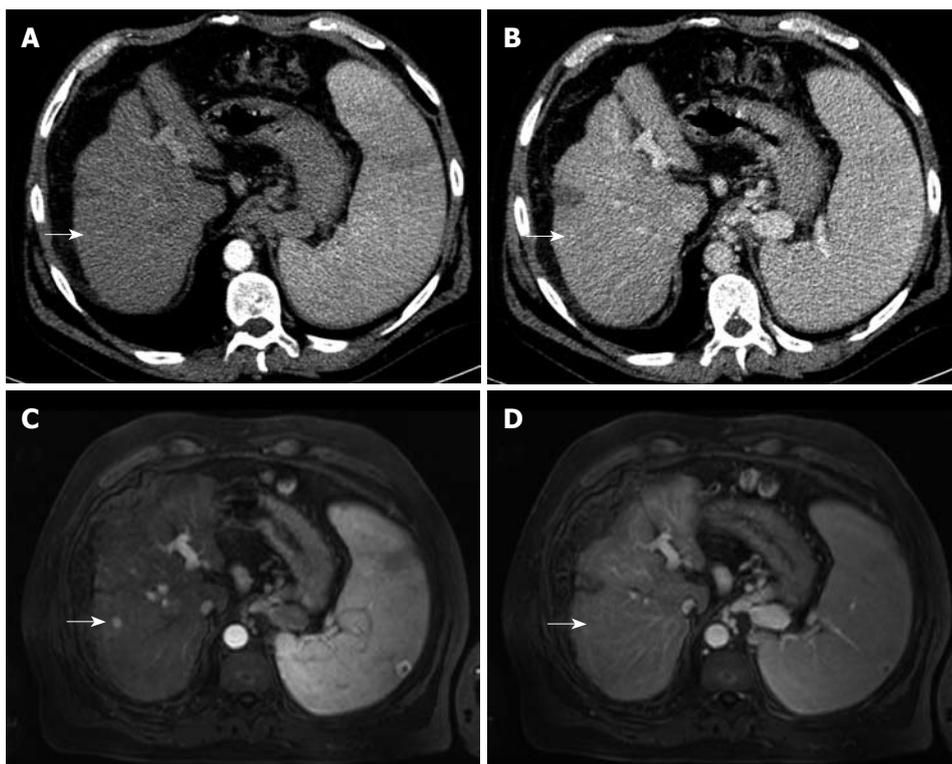
BCLC: Barcelona Clinic Liver Cancer; ECOG: Eastern Cooperative Oncology Group.

protocol covered (1) T1w-2D-Flash fatsat (TE/TR 2.72/129 ms, flip 70°, slice 6 mm, matrix 256\*), (2) T2w TSE (TE/TR 102/4000 ms, flip 150° slice thickness 6 mm, matrix 256\*), (3) in phase and out of phase (TE/TR 2.36/4.76/108 ms, flip 70°, slice 6 mm, matrix 256\*), and (4) five dynamic contrast-enhanced T1w-3D-Flash fat sat sequences (TE/TR 1.56/4.6 ms, flip 15°, slice 4 mm, matrix 256\*) with 0, 20, 45, 90, and 300 s start delay after contrast material injection (0.1 mmol/kg Gadolinium-DTPA (Magnevist<sup>®</sup>, Bayer Schering Pharma AG, Berlin, Germany), 2 mL/s by power injector (Spectris<sup>®</sup>, Medrad, Dusseldorf, Germany).

All phases of the MDCT and MRI scans were independently analyzed by two independent investigators with respect to the number, size, and location of the tumours. Both investigators had at least 10 years experience in evaluating HCC in daily practice. In order to gain the highest diagnostic sensitivity, each nodule was rated positive whenever CT or MRI or both modalities were equivocally positive by both investigators in consensus. Positive diagnosis was based on the EASL and American Association for the Study of Liver Diseases (AASLD) guidelines which require hypervascularization in



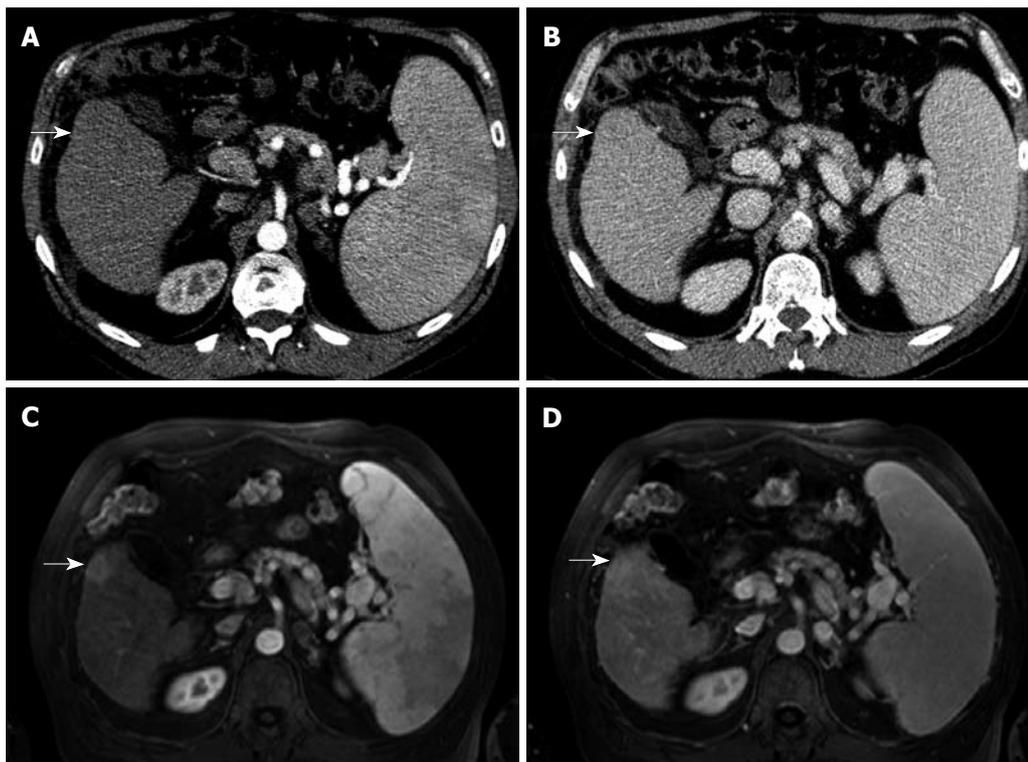
**Figure 1** 71-year-old man with biopsy-proven hepatocellular carcinoma (HCC). Detection of an additional tumor nodule by magnetic resonance imaging (MRI), size 12 mm (size category  $\leq 15$  mm). Multi-detector computed tomography (MDCT) demonstrates two hypervascularized tumor nodules in the contrast-enhanced arterial phase (A, arrow) but not in the portal venous phase (B, arrow). MRI arterial phase depicts one more tumor nodule (arrows) in the T2w (C) and the T1w contrast-enhanced early arterial phase (D).



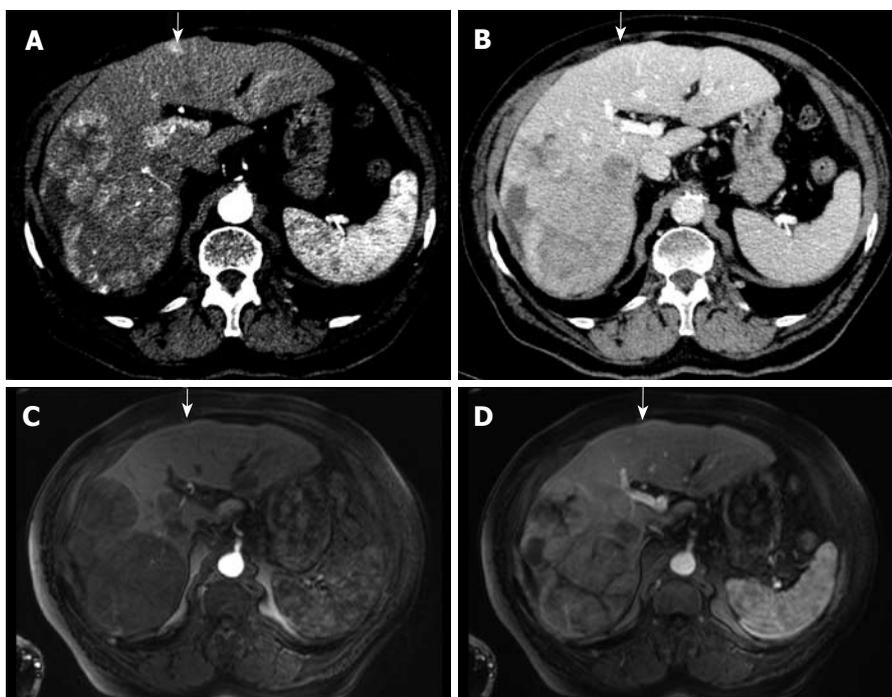
**Figure 2** 70-year-old man with biopsy-proven HCC. Detection of an additional tumour nodule by MRI, size 10 mm (size category  $\leq 10$  mm). MDCT does not show any contrast enhancement in the arterial (A, arrow) or portal venous phase (B, arrow). MRI arterial phase depicts one more tumour nodule (C, arrow) which is hypo- to isointense on the portal venous phase (D, arrow).

arterial phase and contrast washout in the early or delayed venous phase<sup>[15]</sup>. All but three cases were histologically proven by biopsy from one representative nodule. Biopsy proof from each nodule, however, is not feasible *in vivo* due to ethical reasons. The other three cases were not histologically proven because of poor coagulation status and inappropriate subcapsular location for biopsy in order to avoid tumor cell spreading. However, these cases fulfilled the diagnosis criteria according to EASL (hypervascularized nodules  $> 2$  cm in two imaging modalities).

All images were analyzed on a separate workstation with magnification. Tumour diameters were sized with a measuring tool integrated in the workstation software. All nodules visible in both modalities were compared in size. Additionally, the influence of HCC aetiology on tumour detection was analysed. Explanted liver specimens from patients who underwent liver transplantation (3  $\times$ ) or hemihepatectomy (1  $\times$ ) were analyzed pathologically. The specimens were cut in 4 mm slices in the same orientation as in CT and MRI in order to compare the findings. All nodules found by the



**Figure 3** 70-year-old man with biopsy-proven HCC. Detection of an additional tumour nodule by MRI, size 19 mm (size category  $\leq 20$  mm). MDCT demonstrates no hypervascular enhancement in the contrast-enhanced arterial phase (A, arrow) or the portal venous phase (B, arrow). MRI arterial phase depicts a hypervascularized area in the T1w phase (C, arrow) which became isointense in the portal venous phase (D, arrow).



**Figure 4** 82-year-old man with biopsy-proven HCC. Detection of an additional tumour nodule by MDCT. The contrast-enhanced arterial phase MDCT demonstrates large tumours in the right liver lobe and one additional hypervascularized nodule in segment 4 (A, arrow) but not in the portal venous phase (B, arrow). Contrast-enhanced MRI depicts the large tumours in the right liver lobe but not in segment 4 (arrows) in early arterial phase (C) and portal venous phase (D).

pathologist were correlated to the CT and MRI data and investigated histologically.

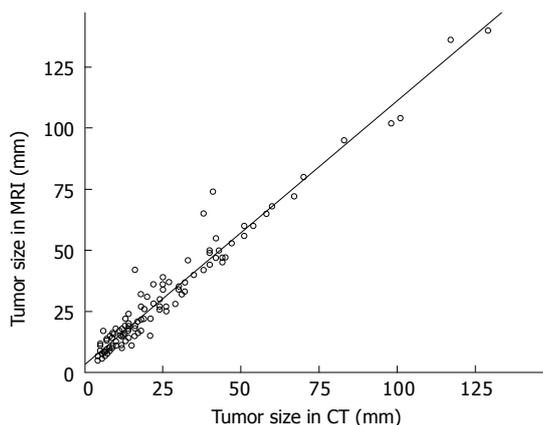
**Statistical analysis**

Sensitivity for tumour detection was calculated with “R” (R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Version 2.5.0, Vienna, Austria, 2007), including Geepack (Generalized

Estimating Equations). *P* values less than 0.05 were considered statistically significant. Statistical testing was performed by an independent statistician to avoid review bias.

**RESULTS**

Consensus reading of MRI and MDCT depicted a total of 162 nodules. On a per nodule basis, MRI detected



**Figure 5** Correlation of tumor sizes measured with MDCT and MRI using a scatterplot. There is a tendency towards greater diameters on MRI compared to MDCT ( $y = 1.08x + 3.2$ ).

**Table 2** Diagnostic impact of imaging protocols on tumour detection  $n$  (%)

Imaging protocols of MDCT and MRI	Tumour nodules detected
MRI, T1w 3D-Flash, arterial phase (20 s start delay)	158 (97.5)
MRI, T1w 3D-Flash, portal-venous phase (45 s start delay)	145 (89.5)
MRI, T1w 3D-Flash, equilibrium phase (90 s start delay)	127 (78.4)
MDCT, arterial phase (bolus trigger for start delay)	119 (73.5)
MRI, T1w 3D-Flash, delayed phase (300 s start delay)	115 (71.0)
MRI, T1w 3D-Flash, dynamic phase Phase (T1 native)	109 (67.3)
MRI, T1w 2D Flash native	104 (64.2)
MRI, Dual-GRE in-phase	98 (60.5)
MRI, Dual-GRE out-phase	96 (59.3)
MDCT, portal-venous phase (55 s start delay)	84 (51.9)
MRI, T2w TSE	72 (44.4)
MDCT, native phase	56 (34.6)

MDCT: Multidetector computed tomography; MRI: Magnetic resonance imaging.

significantly more nodules than MDCT (159 *vs* 123,  $P \leq 0.001$ , Figures 1-3), resulting in an overall sensitivity of 0.98 for MRI and 0.76 for MDCT. The best diagnostic sensitivity was ascertained for the early arterial phase MRI, followed by portal venous phase MRI, equilibrium phase MRI, arterial phase MDCT, and late phase contrast enhanced MRI (Table 2). For native MRI phases (T1w, T2w, Dual-GRE in/out phase) and native and portal venous MDCT phases, sensitivities were low. Negative MDCT findings included 5 nodules  $\leq 5$  mm, 17 nodules  $\leq 10$  mm, 12 nodules  $\leq 15$  mm, 4 nodules  $\leq 20$  mm, and 1 nodule greater than 20 mm. In contrast, MRI missed two nodules  $\leq 10$  mm and one nodule  $\leq 15$  mm. With respect to unilobular and bilobular tumour dissemination, MRI detected 7 MDCT-negative nodules in 2 patients which were located in the contralateral liver lobe and could have changed the therapeutic strategy if not detected. In contrast, the three nodules missed in MRI (Figure 4) had no influence on the treatment regimen because the patient had multinodular disease in both lobes.

Figure 5 shows the sizes of the nodules positive in

both, CT and MRI. Compared to MDCT, the diameters of the tumour nodules were slightly greater in MRI ( $29.2 \pm 25.1$  mm *vs*  $24.1 \pm 22.7$  mm,  $P < 0.005$ , Figure 5). The median lesion diameter was 15.5 mm (range 5 mm to 140 mm) in MRI compared to 12 mm (range 4 mm to 129 mm). Irrespective of the false-negative MDCT findings, tumour diameters were underestimated with MDCT in 43 nodules compared to MRI. In contrast, MRI underrated tumour diameter in only one case compared to MDCT (Table 3).

During the study period, three patients underwent liver transplantation and one was allocated to hemihepatectomy. The explanted specimens (three complete organs and one right liver lobe) were transected in 4 mm slices in transverse orientation for a comparative correlation with the respective MDCT and MRI slices (Figure 6). These four specimens revealed a total number of 20 tumour nodules, 16 of which were depicted by MRI (80%) and only 13 by MDCT (65%).

## DISCUSSION

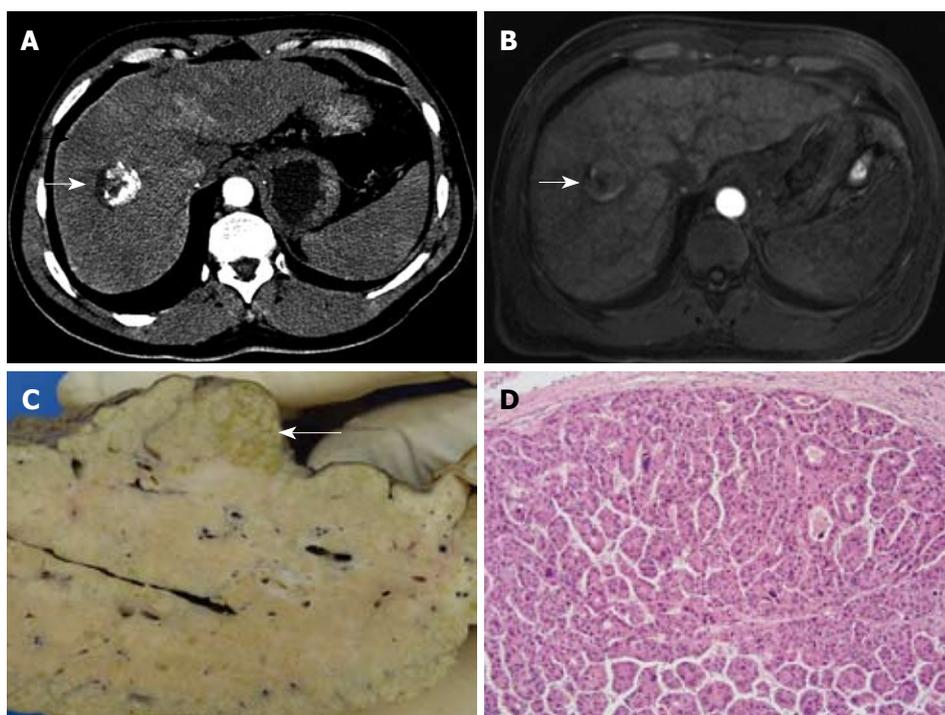
The main reason for the poor survival of HCC patients is the fact that most cases are not diagnosed until disease has reached an advanced stage, which limits the most effective therapeutic options<sup>[7]</sup>. HCC cases that fulfil the Milan criteria (one nodule  $< 5$  cm or three nodules  $< 3$  cm) might be indicated for liver transplantation with curative intention because it not only completely removes the tumour but also the critical precancerous liver cirrhosis<sup>[8]</sup>. However, a significant number of additional intrahepatic tumours have been missed in comparative radiological studies, particularly small nodules  $< 20$  mm<sup>[8,13,16]</sup>, calling the decision-making process into question. Moreover, resection and local tumour ablation with percutaneous ethanol injection (PEI) or radiofrequency ablation (RFA) might have curative potential if the tumour nodules are not multilobular and do not exceed defined nodule diameters<sup>[17]</sup>. Thus, for optimal treatment allocation, current efforts in diagnostic work-up focus on increasing the correctness of preoperative diagnosis with respect to number, size, and location of tumour nodules.

The purpose of the present study was to evaluate the diagnostic potential of 64-row MDCT and MRI for the detection of hypervascularized tumour nodules in the cirrhotic liver for adequate treatment allocation of HCC patients. MDCT has advantages compared to MRI, such as fewer motion artifacts due to much shorter scanning time (3 to 5 s *vs* 18-25 s) and higher spatial in-plane resolution (512\* *vs* 256\* Matrix). However, overall sensitivity of state-of-the-art 64-row MDCT has been demonstrated to be significantly inferior compared to contrast-enhanced MRI and thereby confirms respective findings from older studies with less sophisticated CT technology<sup>[9-11,18,19]</sup>. Recent data have reported slightly higher detection rates for MDCT compared to MRI<sup>[20]</sup>. In our study, however, the outstanding contrast resolution of MRI scored much better with greater sensitivity particularly in small lesions of  $\leq 10$  mm compared to MDCT (0.95 *vs* 0.48,  $P < 0.001$ ) which is in concordance

**Table 3** Results of consensus reading of MDCT and MRI: No. of detected tumour nodules by MDCT and MRI depending on tumour size scaling

	Negative	MRI					Total
		≤ 5 mm	≤ 10 mm	≤ 15 mm	≤ 20 mm	> 20 mm	
Negative		5	17	12	4	1	39
MDCT ≤ 5 mm		2	2 <sup>1</sup>	2 <sup>1</sup>			6
MDCT ≤ 10 mm	2		13	11 <sup>1</sup>	4 <sup>1</sup>		30
MDCT ≤ 15 mm	1		1 <sup>2</sup>	12	11 <sup>1</sup>	4 <sup>1</sup>	29
MDCT ≤ 20 mm					3	9 <sup>1</sup>	12
MDCT > 20 mm						46	46
Total	3	7	33	37	22	60	162

<sup>1</sup>Nodules which appeared greater in MRI compared to CT; <sup>2</sup>Only the single nodule was bigger in CT compared to MRI.



**Figure 6** 54-year-old man with biopsy-proven HCC. False-negative finding in the two modalities. Contrast-enhanced early arterial and portal venous phase MDCT (A) and arterial and portal venous phase MRI (B) detected a 3 cm tumour in the right liver lobe (A, B, arrows) but failed to detect another tumour nodule at the posterior surface of the left liver lobe. The explanted liver specimen clearly depicts this additional 2 cm tumour nodule on gross-sectional pathology (C, arrow) and histology (D, 10 × magnification, HE staining).

with recent reports from the literature<sup>[18,21]</sup>.

Our study has some limitations. First, there was an obvious bias in patient recruitment since prior imaging results had suggested HCC in all patients before they entered the study protocol. Only 4 patients were diagnosed at an early stage with tumour nodules of small diameters so that they could be allocated to either local-ablative treatment with radiofrequency ablation or liver transplantation if they complied with the Milan criteria<sup>[8]</sup>. The majority, however, was diagnosed in an intermediate stage with multilobar disease and sometimes large tumour nodules which might have biased sensitivity. Second, the start delay for the arterial phase CT was 10 s after reaching the trigger threshold. This might have been slightly too short in light of recent reports<sup>[22]</sup> and could possibly explain the great difference between the sensitivity rates of MDCT and MRI. Third, for histological confirmation of HCC diagnosis, in most cases only one representative lesion was examined pathologically,

meaning that the diagnosis of additional tumour nodules relied on imaging only. Fourth, the reference diagnosis might not actually represent the final pathological diagnosis in all cases. For example, the explanted specimen of four cases (3 × liver transplantation, 1 × hemihepatectomy) revealed a total of 20 tumour nodules, whereas MRI and MDCT had depicted only 16 and 13, respectively. So far, we have to concede that even the dedicated MRI protocol used, underrated the intrahepatic tumour spread compared to histological examination. This is consistent with the findings of previous studies which demonstrated an even worse overall tumour detection rate of around 50%-70%<sup>[8,13,16]</sup>. Fifth, the consensus reading of MDCT and MRI could have potentially overestimated nodules in MDCT and MRI by mistaking a benign hypervascularized lesion for a malignant nodule due to the lack of an absolute standard of reference. However, in light of the specimens mentioned above, the underrating of the nodule numbers

and tumour spread is still a major issue for HCC diagnosis with both modalities.

Despite these limitations, the data demonstrate that diagnostic results depend considerably on the multiphasic imaging protocols. Although the early arterial phase in MRI depicts the greatest numbers of tumour nodules<sup>[23]</sup>, it potentially underestimates the real tumour spread in particular cases and might result in incorrect treatment allocation with respect to the Barcelona Clinic Liver Cancer (BCLC) classification<sup>[8,12,24-28]</sup>. Substantial efforts are required to improve the diagnostic correctness, and MRI seems to have better pre-requisites and a greater potential for future developments, either by improving MRI sequences or by employing more specific contrast materials<sup>[9,29-31]</sup>, the double contrast technique<sup>[32,33]</sup> or special imaging techniques<sup>[34]</sup>. Since MDCT failed to demonstrate equivalence with MRI, triphasic contrast-enhanced MDCT protocols might only be used in the first instance. However, if CT suggests local-ablative treatment, resection, or allocation to liver transplantation, dynamic multiphasic contrast-enhanced MRI should be used in order to exclude additional tumour nodules which would probably change the initial strategy.

In conclusion, dynamic contrast-enhanced MRI is superior to triphasic 64-row MDCT for detecting numbers, sizes, and distribution of HCC tumour nodules. HCC patients should be assigned to operative or interventional treatment according to a dedicated MRI protocol.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and the third most common cause of cancer death with 600 000 to 1 million new cases being diagnosed each year. Due to the lack of specific early symptoms, most cases are not diagnosed until the disease is already in an advanced stage, which limits the most effective therapeutic options. That is why, although new treatments are available, patient survival has not significantly improved in the last 30 years. Therefore, early tumor detection is one of the most important issues in HCC therapy. The study was to compare the diagnostic capability of two imaging systems, computed tomography (CT) and magnetic resonance imaging (MRI), for tumor detection.

### Research frontiers

In recent years, an enormous improvement has been taking place in the field of imaging systems. With new generations of CT and MRI scanners available, the question arises, which one is the better technique to depict this tumor.

### Innovations and breakthroughs

Previous studies used older equipment, e.g. single slice CT. The advance in this study is the evaluation of a new scanner generation (64-row CT) which allows faster acquisition and therefore fewer motion artifacts with a lower slice thickness.

### Applications

This article should help radiologists, gastroenterologists and other physicians dealing with HCC patients in daily practice to use the correct method of imaging in the right patient.

### Peer review

The study aimed at determination of the nodule detection sensitivity of MDCT compared with MRI. The presentation of the data should be more concise.

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BRIEF ARTICLE

## Different faces of gastroparesis

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### Abstract

**AIM:** To test the hypothesis that pain and affect rather than impaired emptying determine symptom severity in patients with gastroparesis.

**METHODS:** Adult patients with documented gastroparesis were enrolled prospectively in a single center and asked to complete the Gastroparesis Cardinal Symptom Index (GCSI), Hospital Anxiety and Depression Scale (HADS), the Short Form 12 (SF-12) as quality of life index, rate pain severity and answer 10 open-ended questions.

**RESULTS:** A total of 55 patients (44 women) participated. Idiopathic ( $n = 29$ ) or diabetic ( $n = 11$ ) gastroparesis and connective tissue disease ( $n = 8$ ) were the most common underlying causes. Antiemetics ( $n = 30$ ) and prokinetics ( $n = 32$ ) were most often prescribed. Seventeen patients used opioids on a daily basis. Nausea and/or vomiting ( $n = 28$ ), pain ( $n = 24$ ) and bloating ( $n = 14$ ) were most commonly listed as dominant symptoms. Patients subjectively attributed symptom improvement to nutritional and dietary therapy ( $n = 11$ ), prokinetics ( $n = 11$ ), antiemetics ( $n = 10$ ) or analgesic agents ( $n = 3$ ). In univariate analyses, the physical subscore of the SF-12 and HADS, but not gastric emptying delay or symptom duration significantly correlated with disease severity as measured by the GCSI. In multivariate analyses, the

combination of vomiting, bloating and depression best predicted the overall impact on quality of life.

**CONCLUSION:** The study confirms the importance of pain and affect in gastroparesis, which requires novel approaches to improve more effectively the quality of life in patients with this disorder.

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**Key words:** Pain; Depression; Gastroparesis; Quality of life

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### INTRODUCTION

Gastroparesis is an increasingly diagnosed functional disorder, characterized by delayed emptying of gastric contents. Considering the defining abnormality, prokinetics continue to play a primary role in managing this disorder<sup>[1-3]</sup>. However, cross-sectional and longitudinal studies have demonstrated a poor correlation between the documented delay in gastric emptying and symptom severity<sup>[4-6]</sup>. Moreover, motilin agonists do not improve symptoms even though they significantly accelerate gastric emptying<sup>[5,7-10]</sup>. Thus, other mechanisms must contribute to the clinical manifestation of gastroparesis, such as impaired gastric accommodation, altered distribution of ingested material within the stomach, and peripheral and/or central hyperalgesia<sup>[1-4]</sup>. The relative importance of any of these potential mechanisms remains unclear. More importantly, there is no evidence that medication that targets these abnormalities is indeed beneficial when used clinically. As we typically cannot change the underlying processes that lead to gastroparesis, treatment remains largely symptomatic with the primary goal of improving the overall quality of life of patients suffering from this, at times, disabling disorder.

We performed a cross-sectional study in patients with confirmed gastroparesis to define better the relative importance of key symptoms of gastroparesis, their

impact on the patients' functional status, and the potential differences between patient subgroups defined by the underlying etiology. The main hypotheses were: that pain significantly contributes to the impaired quality of life in patients with gastroparesis, and that affect plays an important role in determining symptom severity in patients with gastroparesis.

## MATERIALS AND METHODS

### Patient recruitment

Adult patients with confirmed gastroparesis seen by one investigator (Bielefeldt K) in the Digestive Disorders Clinic of the University of Pittsburgh Medical Center were invited to participate. Patients with gastroparesis had to be symptomatic for at least 6 wk and have objective evidence of impaired gastric function with delayed gastric emptying by scintigraphy, or documentation of retained food within the stomach after a 12-h fasting period obtained by endoscopy or contrast study. Structural abnormalities, such as gastric outlet obstruction or mechanical obstruction in the more distal gastrointestinal tract had to be excluded. The protocol was approved by the Institutional Review Board of the University of Pittsburgh (PRO8020059).

### Surveys

After obtaining informed consent, all patients were asked to provide basic demographic information, complete the Gastroparesis Cardinal Symptom Index (GCSI), the Hospital Anxiety and Depression Scale (HADS), and the Medical Outcomes Study Short Form 12 (SF-12).

The GCSI is a self-administered assessment tool developed for patients with gastroparesis. The original survey comprises three subscales: postprandial fullness/early satiation (4 items); nausea/vomiting (3 items), and bloating (2 items). Considering the potential importance of pain, pain rating was added to the scale and scored similarly to the other items. Internal consistency and test-retest reliability were 0.84 and 0.76, respectively<sup>[11]</sup>.

The HADS is a 14-item self-report questionnaire that asks patients to rate symptoms using statements that are converted into a numeric score between 0 and 3. The total score of can range between 0 and 21 for anxiety and depression. Extensive studies support an internal consistency exceeding 0.8. When used in different populations, scores of > 8 have a sensitivity around 80% for depressive or anxiety disorders<sup>[12]</sup>.

The SF-12 is a self-report measure of perceived health, which consists of 12 questions. Two summary scores are generated: a physical health factor score (PHS) and a mental health factor score (MHS). The scores have been constructed with the population norm for each score being 50. Compared with the more extensive Short Form 36 (SF-36), the measure has been shown to be valid with correlations of 0.905 for the SF-36 Physical Component Summary and 0.938 for the SF-36 Mental Component Summary<sup>[13]</sup>.

### Open ended questions

Patients were also asked a series of open-ended questions

(Table 1) that focused on the impact of gastroparesis on professional and private spheres of their lives. These questions included verbal assessments of the most significant symptoms, perceived treatment effects, and concerns related to the disease.

### Data abstraction

Clinical data about nature and duration of symptoms, severity, character, location of pain, weight changes, documented delay in gastric emptying, prior and ongoing treatment were abstracted after the clinical encounter, de-identified and linked to the survey data.

### Statistical analysis

Responses to open-ended questions were analyzed by two independent coders who were aware of the underlying diagnosis of gastroparesis, but were blinded to patient identity, survey data as well as duration and etiology of the disorder. The qualitative analysis focused on frequency rather than expressions of severity of factors. A codebook was generated to record responses. Statements supporting the code were highlighted and added to a master file that contained all qualitative data.

Dichotomous variables were analyzed using  $\chi^2$  statistics. Continuous and categorical variables are given as mean  $\pm$  SE. Group comparisons were performed using analysis of variance. The GCSI summary score and quality of life as measured by the SF-12 were defined as the main endpoints. In an initial univariate analysis, correlations between the main endpoints and between different measures and the main endpoints were analyzed using Spearman correlation. Only variables with  $P < 0.1$  were entered into the multilinear regression model.

## RESULTS

### Patients

Between June 2008 and February 2009, a total of 55 patients (mean age:  $42.4 \pm 1.9$  years; 81% women) agreed to participate (Table 2). Gastric emptying studies had documented delayed emptying in all but five patients, who could not tolerate or complete studies because of emesis. In these individuals, retained food ( $n = 4$ ) or a bezoar ( $n = 1$ ) had been demonstrated endoscopically prior to enrollment. Thus, all patients met the entry criterion of objectively documented impairment in gastric emptying. However, studies were not performed in a single center with differences in test meals, test duration and data reporting. In 26 patients, the half-emptying time for solid-phase gastric emptying had been calculated and given. One additional patient only underwent a gastric emptying study for liquids that showed a significant delay. In five patients, only gastric retention at 90 or 120 min was quantified and compared with institutional norms. In the remaining patients, only a qualitative comment described delayed gastric emptying without further quantification by radiologists in outside institutions. The mean half-emptying time for the available studies was  $319 \pm 50$  min.

The most common causes of gastroparesis were diabetes mellitus ( $n = 11$ ), connective tissue disease ( $n$

Table 1 Open-ended questions

How does the stomach problem affect your life?  
 How has gastroparesis affected your relationships with family and friends?  
 How has gastroparesis affected your ability to work and your professional life?  
 What do you worry about when you think about your stomach problem?  
 What symptom or problem related to your gastroparesis disturbs you the most?  
 What helped you the most in the treatment of your stomach problem?  
 What do you know and understand about your stomach problem?  
 Patients are often left with questions about their condition. What do you want to know about your disease?  
 What were your experiences as a patient with gastroparesis when you met physicians, nurses and other healthcare providers?  
 What can we do to better serve patients with your condition?

Table 2 Baseline patient characteristics *n* (%)

Variable	All patients	Idiopathic gastroparesis	Diabetic gastroparesis	Connective tissue disease
Women	55 (80)	29 (90)	11 (55)	8 (100)
Age (yr)	42.4 ± 1.9	38.1 ± 2.8 <sup>b</sup>	46.0 ± 2.9	57 ± 2.2
Education				
High school	38	20	8	5
Bachelor	9	5	1	1
Graduate	8	4	2	2
Employed	15 (27)	11 (38)	3 (27)	0
Annual household income (\$)				
< 20000	20	10	7	0
20000-39000	13	8	2	2
40000-59000	8	5	0	2
60000-79000	7	3	0	2
> 80000	7	3	2	2
Symptom duration (mo)	32 ± 4	24.2 ± 4.2	44 ± 15	25.8 ± 6.7
Weight loss (pounds)	3.7 ± 1.6	4.5 ± 2.6	2.9 ± 2.7	7.1 ± 3.6

<sup>b</sup>*P* < 0.01 vs patients with gastroparesis due to connective tissue disease.

= 8), abdominal surgery or trauma (*n* = 4), osteogenesis imperfecta (*n* = 1), mitochondrial myopathy (*n* = 1) and Marfan syndrome (*n* = 1). In the remaining 29 patients, no underlying cause could be identified. Four of the patients with idiopathic gastroparesis recalled an acute illness prior to the onset of the disease, which suggested a post-infectious form of gastroparesis. Patients with idiopathic gastroparesis were significantly younger than those in the other two major groups (Table 2). Gastroparesis caused by connective tissue disease showed an expected female predominance, considering the preferential manifestation of systemic sclerosis in women<sup>[14]</sup>. Similarly, 90% of patients with idiopathic gastroparesis were women, while nearly half of the patients with diabetic gastroparesis were men (*P* < 0.05).

Symptoms of gastroparesis had been present for 32 ± 4 mo. Ten patients reported a significant weight loss that exceeded 5% of their body weight within the preceding 6 mo. The average weight loss for the entire group was 1.7 ± 0.7 kg. All but four patients (93%) complained about nausea. A sense of bloating, fullness and/or early satiation was present in 47 (87%) patients. At least mild pain was mentioned by 44 (81%) patients. The pain was primarily postprandial in 11 patients, with the remaining 34 participants complaining about constant discomfort. The pain was typically located in the epigastrium and described

Table 3 Treatment of gastroparesis

Variable	All patients	Idiopathic gastroparesis	Diabetic gastroparesis	Connective tissue disease
Prokinetics				
Metoclopramide	25	11	7	4
Erythromycin	7	4	2	1
Other	4	1		3
Antiemetics				
Phenothiazine	21	10	5	2
Ondansetron	20	12	4	1
Scopolamine	6	3	2	
Meclizine	2	1	1	
Dronabinol	5	3		
Antidepressives				
TCA	3	1	1	
SSRI	15	4	5	4
Benzodiazepines	16	10	3	1
Opioids (daily)	17	7	5	1
Nutritional support				
Jejunostomy	8	1	4	
TPN	3	2		1

TCA: Tricyclic antidepressant; SSRI: Selective serotonin reuptake inhibitor; TPN: Total parenteral nutrition.

as pressure (*n* = 18), sharp (*n* = 12) or burning sensation (*n* = 7). Three patients mentioned generalized abdominal pain. In one patient, the pain radiated to the right upper quadrant and in another to the chest.

Seven patients (6 idiopathic gastroparesis; 1 diabetic gastroparesis) reported undergoing a cholecystectomy for biliary dyskinesia after symptom onset, which led to transient improvement in five patients. However, symptoms recurred within 3 mo in all but one of these patients, who continued to do well for 4 years before experiencing recurrent problems. Within the month prior to enrollment, 32 (60%) patients were using prokinetic agents, most commonly metoclopramide (Table 3). Ten of these patients experienced at least moderate side effects with extrapyramidal motor disorders (*n* = 3) worsening fatigue (*n* = 5) or depression (*n* = 1), which led to discontinuation of the agent. One patient was switched to domperidone. Three patients with systemic sclerosis and gastroparesis complicated by chronic intestinal pseudo-obstruction were using octreotide (*n* = 2) or pyridostigmine (*n* = 1). A total of 30 (55%) patients required daily antiemetic medication, most commonly phenothiazines and/or ondansetron. Eighteen patients (33%) received chronic antidepressant medication; mostly serotonin reuptake inhibitors. In four

Variable	All patients	Idiopathic gastroparesis	Diabetic gastroparesis	Connective tissue disease
GCSI	25.7 ± 1.4	24.6 ± 2.0	30.7 ± 2.3	20.8 ± 2.5
HADS				
Anxiety	8.3 ± 0.6	8.6 ± 0.9	9.3 ± 1.1	6.6 ± 1.7
Depression	7.7 ± 0.7	7.1 ± 1.1	9.9 ± 1.2	6.3 ± 1.5
SF-12				
PHS	31.5 ± 1.4	33.3 ± 2.0	28.7 ± 2.8	31.7 ± 3.6
MHS	41.7 ± 1.6	41.4 ± 2.1	37.7 ± 3.5	47.7 ± 4.1

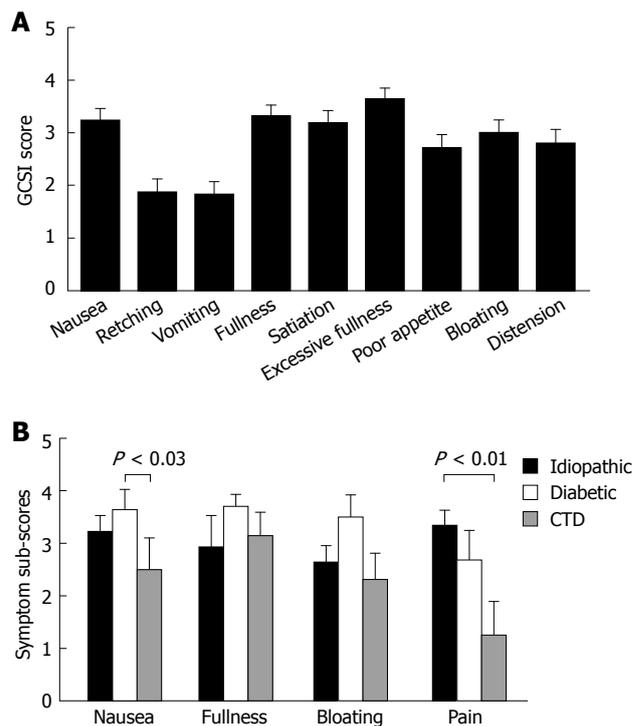
GCSI: Gastroparesis Cardinal Symptom Index; HADS: Hospital Anxiety and Depression Scale; SF-12: Short Form 12; PHS: Physical health factor score; MHS: Mental health factor score.

Variable	Spearman coefficient	P
GCSI		
SF-12 PHS	-0.56	0.0001
SF-12 MHS	-0.212	0.12
HADS-anxiety	0.17	0.22
HADS-depression	0.33	0.02
T-1/2	0.05	0.82
Symptom duration	-0.08	0.56
Age	-0.08	0.55
PHS		
GCSI-sub score nausea	-0.52	0.0001
GCSI-sub score fullness	-0.4	0.003
GCSI-sub score bloating	-0.4	0.003
GCSI-sub score pain	-0.35	0.01
HADS-depression	-0.44	0.001

patients, tricyclic antidepressants or duloxetine were given to improve pain control. Regular use of benzodiazepines was reported by 16 (29%) patients. A total of 17 patients (31%) received chronic opioids for pain management. In two patients, narcotics were given for painful diabetic neuropathy. One patient had sickle cell anemia with severe bone pain. An additional patient with severe joint and muscle pain caused by systemic sclerosis and polymyositis used a fentanyl patch, which left 13 (24%) patients who were taking opioids daily for control of their abdominal pain. As opioids can impair gastric emptying, assessment of gastric function was performed after transient discontinuation of narcotics in all but the two patients who suffered from painful diabetic neuropathy. A total of 11 (20%) patients received nutritional support *via* enteral (*n* = 8) or parenteral (*n* = 3) nutrition.

**Survey data**

As shown in Table 4, the GCSI summary score demonstrated a moderate symptom severity for the entire group. Group comparisons only showed a trend for a lower overall score in patients with connective tissue disorders as the underlying etiology (*P* = 0.075). Figure 1A summarizes the individual symptom scores for the entire group. When asked to rate abdominal pain using the GCSI coding scale, the mean pain severity was 2.97 ± 0.24, which was similar to the subjectively rated severity of other symptoms. The pain rating correlated with the



**Figure 1 Symptom severity scores in patients with gastroparesis.** The individual GCSI scores are shown for the entire group (A). Component sub-scores for the GCSI and pain ratings are summarized for the three major patient groups, to allow comparison between the different etiologies of gastroparesis (B). Significant differences were seen for nausea (diabetes vs connective tissue disease) and pain (idiopathic vs connective tissue disease). CTD: Connective tissue disease; GCSI: Gastroparesis Cardinal Symptom Index.

pain scale of the SF-12 that assessed the impact of pain over the preceding month (*R*<sup>2</sup> = 0.22, *P* < 0.01), and the routinely assessed patient pain rating that addressed pain presence on the day of the clinic visit (*R*<sup>2</sup> = 0.36, *P* < 0.001). A group comparison of component scores revealed more significant nausea for patients with diabetic gastroparesis and higher pain scores for patients with idiopathic gastroparesis (Figure 1B).

Patients in all groups had moderately elevated scores for both anxiety and depression, without significant differences between the groups. Using the proposed cutoff score of > 8<sup>[12]</sup>, 40 participants (74%) met screening criteria for anxiety or depression. In 16 patients (29%), anxiety and depression were both above the proposed threshold for clinically relevant affective spectrum disorders.

Compared to a population norm of 50, the mental and the physical component of the SF-12 showed a significant impairment of health-related functional status. There were no significant differences between the groups.

The GCSI summary score showed a significant inverse correlation with the physical but not mental score of the SF-12. It was correlated positively with depression scores, but not HADS anxiety scores, age, symptom duration or degree of gastric emptying delay (Table 5). In order to identify the relative contribution of symptoms, we performed univariate analyses, correlating the physical health scale of the SF-12 with GCSI subscores and pain ratings. Based on these results, we performed

multiple linear regressions to identify the most important predictors of poor health function. A combination of GCSI subscores for nausea and bloating and the HADS score for depression best predicted the PHS subscore with an adjusted  $R^2$  of 0.44.

### Qualitative data

When asked to describe the impact of gastroparesis on their lives, most patients focused on the effects of interactions with family, friends or colleagues ( $n = 37$ ). Three main topics emerged in the more detailed analyses of responses. First, eating out, dinners or other social functions were seen as causing difficulties because of the limited ability to tolerate food ( $n = 26$ ). One patient explained these problems: “[With] every- and anything that goes on socially there is food. When you can’t eat, it is really hard to cope with. People feel funny asking you out to dinner or invite you to something where there will be food.” The second problem was related to fatigue, which made it difficult for patients to meet expectations ( $n = 14$ ) as exemplified by statements such as “I push myself to get out of bed every morning” or “I can barely make it through the day”. The third major topic revolved around the frustration and emotional impact of the disease as patients experienced difficulties interacting with others ( $n = 16$ ). One patient summarized her experience: “...enjoying life has become difficult because my mind is always on my stomach”.

Participants cited a significant strain on their relationships with partners and/or family members ( $n = 13$ ). Such problems could be a simple consequence of limitations imposed by their disease, as this woman explained: “...having to sleep sitting up doesn’t contribute to closeness with your partner”. A young mother with idiopathic gastroparesis reported that her disease “...has prevented me from caring for my children and participating in their activities”. In other cases, it was more anxieties and concerns in response to the physical evidence of illness, such as frequent vomiting: “...children - always frightened I will never get well or I will die”. Some patients reported a gradual withdrawal of others in the face of ongoing illness: “My friends don’t really call anymore because they know I am always feeling sick. ... I also broke off a wedding engagement with someone I was with for 9 years because he couldn’t stand how sick I was and felt I was holding him back”.

When asked about the impact of gastroparesis on their professional activity, three patients described themselves as retired and seven responded that they were on disability (in 2 cases, for reasons other than gastroparesis). An additional eight patients mentioned that they felt forced to quit jobs or school because of their disease. One patient who had successfully completed college education explained his difficulties: “...nausea is consuming... [making it] difficult to concentrate on anything else...” Another participant described the impact as being “...unable to work... therefore, I feel worthless. I feel like my stomach problems wasted everything I achieved”.

Patients were also asked to explain their most

bothersome symptoms and their concerns related to gastroparesis. Nausea or vomiting were listed by 28 participants, followed by pain ( $n = 24$ ), bloating ( $n = 14$ ) and emotional difficulties ( $n = 6$ ). Nearly half ( $n = 26$ ) of the patients mentioned the fear of an unrelenting chronic disease as their main concern, as shown by the following quote: “I worry that I’ll never feel like a normal person”. These concerns expressed themselves in some instances through anxiety that a more serious, perhaps even lethal disease caused the problems. Despite prior and extensive investigations and explanations, five patients were afraid that they had cancer.

When describing the perceived impact of treatment they had received for gastroparesis, patients most commonly listed the benefits of dietary and nutritional therapy ( $n = 11$ ), prokinetics ( $n = 11$ ) and antiemetics ( $n = 10$ ). However, 10 patients mentioned that no treatment had led to any improvement. Others reported benefits of acid suppression ( $n = 5$ ), relaxation techniques ( $n = 3$ ) and analgesics ( $n = 3$ ).

Patients also described their experiences with healthcare providers, with 15 (27%) expressing frustration or dissatisfaction. They primarily focused on a perceived lack of knowledge or understanding, which may have contributed to a perceived diagnostic delay and limited symptomatic improvement. One patient summarized her experiences: “I’ve had horrifying experiences from the medical community. Most physicians just say what you can [eat] or drink [like] Ensure”.

## DISCUSSION

Our results provide a cross-sectional picture of gastroparesis and its impact on patients seen in a tertiary referral center. Consistent with prior studies, most patients were women in their thirties and early forties, with the majority suffering from idiopathic forms of gastroparesis<sup>[11,15-18]</sup>. Overall disease severity as judged by the GCSI score, other symptom severity scores or the physical and mental component of the SF-12 were comparable to those reported in previously published studies<sup>[6,11,15,16]</sup>. Similarly, the frequency of different symptoms, such as nausea, vomiting, early satiation and pain, were consistent with prior reports<sup>[6,16,18-21]</sup>. About 10% of patients with idiopathic gastroparesis recalled an acute symptom onset after a flu-like illness, which suggested a post-infectious form of the disease. These results are slightly lower than previously reported<sup>[15,22]</sup>. We also did not see differences in the apparent clinical course based on patient recall, as all individuals with post-infectious forms of gastroparesis had symptoms for more than 1 year and complained about ongoing and at least moderately severe symptoms. However, the cross-sectional design and relatively small sample size do not allow definitive conclusions to be drawn. While the symptom pattern of patients with diabetic and idiopathic gastroparesis did not differ, patients with systemic sclerosis complained less about pain compared to the other groups, which may be important in management decisions<sup>[18]</sup>.

### **Impact of gastroparesis**

When asked to rate their overall health, more than two thirds used descriptors of fair or poor, which demonstrated the significant impact of gastroparesis, which is also shown by the low score in the physical functioning domain of the SF-12. We included a qualitative analysis of open-ended questions to capture important details that led to impairment of quality of life. Most of the comments focused on social interactions with family, friends or colleagues rather than symptoms. A striking finding was the high number of patients who reported significant problems in their professional activities that may have contributed to unemployment or disability. Despite a mean age below 45 years, only about one quarter of study participants was fully employed. The number of individuals not working and/or on disability benefit was higher than reported for inflammatory bowel disease<sup>[23,24]</sup>. Consistent with the impact of the disease on professional lives, more than half of the patients reported household incomes well below the national average, which also has been observed in other disorders associated with chronic pain<sup>[25]</sup>. Chronic illness certainly increases work absenteeism and may even result in permanent disability<sup>[26-29]</sup>. Our findings suggest a disproportionate impact in patients with gastroparesis, which corresponded with patient statements about their own disease experience and functional health status. Additional studies with larger patients groups and longitudinal design are needed to better define the effect of impaired gastric function on professional productivity.

Not surprisingly, our results also highlighted the importance of food intake. Dietary management and nutritional therapy primarily and appropriately target the caloric and nutritional value of ingested materials, which relies on changes in meal volume and frequency, food consistency, and in more severe cases, even enteral or parenteral alimentation<sup>[2,30]</sup>. Although successful in preventing the development of nutritional deficiencies, these measures do not address the hedonic and social aspects related to eating. Aversive reactions to the previously pleasant experience of food intake, the inability to fully participate in daily routines, such as dinners, fully meet professional obligations associated with eating or drinking, or enjoy dining out with friends reminded patients on a daily basis how the illness had changed their lives. The answers often hinted at resentments, when healthcare providers did not appreciate this fact and briefly told them that simple dietary adjustments would solve their problems, without acknowledging the tremendous impact on quality of life.

### **Gastroparesis and affect**

The high scores for anxiety and depression and patient statements certainly demonstrated the association between mood and impaired gastric function. Our design did not allow us to determine whether and how these factors are causally related. Interestingly, in the multivariate model, depression but not anxiety significantly contributed to the overall impairment in health status. Only one study has described the prevalence of depression in a large

group of patients with gastroparesis, with nearly one quarter carrying the diagnosis of depressive illness<sup>[15]</sup>. This figure corresponds with the reported chronic use of antidepressant medications in our patient group. Our findings also fall in line with previous results obtained in patients with functional dyspepsia, in whom depression significantly correlated with the physical domain of quality of life<sup>[31,32]</sup>. Recent evidence suggests that depression is the key determinant of symptom severity in functional dyspepsia, which is primarily mediated through somatization<sup>[33]</sup>. Considering the overlap between functional dyspepsia and gastroparesis, additional studies will be needed to better define the role of psychological mechanisms in disease severity and manifestations.

### **Pain and gastroparesis**

The recent consensus statement of the American Gastroenterological Association comments on pain in gastroparesis as being relatively common, but typically not the primary symptom or concern<sup>[3]</sup>. Our findings, especially the patient comments, argue against this statement, when one considers the high prevalence of pain and subjective pain ratings, which has been emphasized previously by Hoogerwerf *et al*<sup>[18]</sup>. The relevance of pain was also reflected in the treatments received by patients. Despite concerns about the use of opioids in patients with gastroparesis, about 30% of the patients regularly used narcotics. Only one study has mentioned specifically narcotic use in patients with gastroparesis, and has reported an even higher number of close to 50%, which may have been caused partly by selection bias, because all patients underwent implantation of a gastric electrical stimulator<sup>[16]</sup>. The interaction between narcotic use and impaired gastric emptying is admittedly complex, as opioids affect gastrointestinal motility. However, virtually all assessments of gastric function were performed when patients did not receive narcotics. It is thus unlikely that the documented delay in gastric emptying seen in our patients was solely a consequence of medication.

Beyond its prevalence, pain remains a significant challenge in managing gastroparesis. The conventional approaches with dietary and/or prokinetic therapy do not provide significant benefit<sup>[18]</sup>. Tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs) or serotonin/norepinephrine reuptake inhibitors have been used in functional dyspepsia and other visceral pain syndromes<sup>[34-36]</sup>. Although they may acutely lower pain thresholds in healthy volunteers<sup>[37-39]</sup>, efficacy in patients has not been demonstrated consistently<sup>[32,40,41]</sup>. Gabapentin or pregabalin similarly may decrease sensory thresholds acutely<sup>[42]</sup>, but have not been shown to possess true analgesic properties in patients<sup>[43]</sup>. Considering the negative impact of opioids on gastric emptying and their addictive potential, kappa opioids agonists have been tried in gastroparesis or functional dyspepsia, but were not superior to placebo<sup>[44,45]</sup>. In view of recent advances in our understanding of visceral sensory mechanisms, we will have to see whether other potential targets for pharmacological interventions, such as purinergic receptors, TRPV1 or TRPA1, will provide greater benefit for these patients<sup>[46,47]</sup>.

### **Gastroparesis and gastric emptying**

Although gastroparesis is defined by delayed emptying, our study did not show a correlation between symptom severity and half-emptying time as an objective and quantitative measure of this impairment. Only minor differences emerged in comparisons between symptomatic individuals with (gastroparesis) and without (functional dyspepsia) delayed gastric emptying, which did not allow differentiation between the groups based on symptoms only<sup>[6,48]</sup>. In addition, potent prokinetics have a limited or no impact on symptoms, which further argues against a primary role of transit delay as a determinant of disease severity<sup>[8,9]</sup>. Consistent with this conclusion, only a small fraction of patients reported subjectively perceived benefit when taking prokinetics. This relatively low response rate contrasts starkly with the relatively frequent adverse effects. More than one third of the patients who received metoclopramide had to discontinue the medication because of side effects; a rate that is in line with previous reports<sup>[1,2]</sup>. These results are likely caused by the complex pathophysiology of gastroparesis, which includes impaired accommodation, delayed emptying, hypersensitivity and (preexisting or secondarily evolving) affective spectrum disorders, all of which contribute to the clinical manifestation of the disorder<sup>[4,30]</sup>.

### **Study limitations**

As is true for most clinical studies, our investigation was conducted in a tertiary referral center, which biases and skews findings as a result of the likely higher proportion of difficult-to-treat patients. However, patient age, sex distribution, the high proportion of patients with idiopathic gastroparesis and self-reported symptom severity were comparable with prior studies that used similar scoring systems<sup>[4,15,16,49]</sup>. The relatively high number of patients with systemic sclerosis and the slightly smaller group of diabetic patients likely reflects institutional idiosyncrasies. A cross-sectional study design certainly comes with limitations. It enabled us to identify association between symptom severity and its potential determinants, from underlying etiology to treatment. Although we noticed an important impact of depressive symptoms, longitudinal studies are needed to better define this relationship and correlate time- and/or treatment-dependent changes in the various indices. Moreover, our data cannot identify psychological or physiological mechanisms that mediate the interactions between affect and symptoms, which will require more detailed investigations. Similarly, our study was not designed to measure systematically the effects of different treatments in patients with gastroparesis. More than half of the patients were enrolled during their initial encounter, with data thus reflecting the approaches of many referring physicians. We also used previously obtained data on gastric emptying, which provided less standardized but still objective evidence of impaired gastric function in all patients. Although appropriate for reducing increasing healthcare costs and typical for clinical practice<sup>[11]</sup>, it limited our ability to correlate fully this disease-defining

variable with symptom severity.

Taken together, our data raise questions about some of the key premises that we as clinicians use when approaching patients with gastroparesis. Delayed gastric emptying still remains the defining and only routinely available diagnostic tool to identify this disorder. However, it may detract from the much more complex pathophysiology and may even misguide our treatment. As we cannot cure the illness, our treatment has to focus on improving the overall quality of life. A paradigm shift may be needed to take into account the important influence of under-recognized and under-treated problems, such as pain or affect. Pain assessment should be included in the GCSI. Gastric electrical stimulation was initially thought to alter gastric motility, with investigators now increasingly speculating on its effect on visceral sensation and affect<sup>[20,50,51]</sup>. Some data are promising, especially in diabetic patients<sup>[16,20,50,52]</sup>. However, the high cost, frequent need of reoperation, and the limited benefit in patients with significant pain and/or idiopathic gastroparesis clearly force us to look for alternatives. Our results may be seen as initial, yet circumstantial evidence that supports psychologically based interventions, which have been quite successful in functional bowel disease and have shown promise in a small case series<sup>[53]</sup>.

## **COMMENTS**

### **Background**

Gastroparesis is a chronic disorder that is characterized by significant dyspeptic symptoms and delayed gastric emptying. Despite the defining abnormality in gastric motor function, prokinetics typically have limited efficacy, which leaves patients with persistent symptoms and poor quality of life.

### **Research frontiers**

Anxiety and depression play an important role in the clinical manifestation of many functional disorders, which range from irritable bowel syndrome to fibromyalgia. We prospectively enrolled patients with gastroparesis to better understand the relative importance of different gastrointestinal symptoms and associated or coexisting emotional factors on their quality of life.

### **Innovations and breakthroughs**

Our data highlight that depression plays a significant role in the overall impact of gastroparesis on quality of life. The findings also emphasize the importance of pain as a symptom of gastroparesis, which is under-appreciated and under-treated.

### **Applications**

The results stress the need to shift treatment strategies for gastroparesis away from approaches that simply try to accelerate gastric emptying. In our clinical assessment, we need to assess the different symptoms including pain, and design a therapy that takes into account the primary problems the individual patients experience. Because affect, especially depression, significantly impairs quality of life, diagnostic approaches and treatment should address emotional factors. In future research, we will have to define better the interrelationship between altered gastric function and emotion to understand underlying mechanisms. Interactions will likely be reciprocal, which raises the question of how much psychologically or psychiatrically oriented treatment may be beneficial in patients with gastroparesis.

### **Terminology**

Gastroparesis is a chronic disorder that is characterized by symptoms of nausea, vomiting, fullness and bloating after meals, and a delay in gastric emptying, which is typically determined using standardized scintigraphic tests.

### **Peer review**

The article fits into the increasing evidence that structural and especially functional disorders of the gastrointestinal tract should be seen as biopsychosocial phenomena that require more holistic diagnostic and therapeutic approaches.

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## Association of the *GNAS1* T393C polymorphism with tumor stage and survival in gastric cancer

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### Abstract

**AIM:** To analyze the impact of the *GNAS1* T393C polymorphism on prognosis and histopathology of gastric cancer.

**METHODS:** Genomic DNA was extracted from paraffin-embedded tissues of 122 patients with primary gastric carcinoma and from the blood of 820 healthy white

individuals. Allelic discrimination was performed by quantitative real-time polymerase chain reaction. Genotyping was correlated with histopathologic parameters and with overall survival according to the Kaplan-Meier approach and with multivariate analysis by multiple stepwise regression.

**RESULTS:** Thirty-nine (32%) patients displayed a CC genotype, 57 (46.7%) a CT genotype and 26 (21.3%) a TT genotype. The frequency of the C allele (fC) in the patient group was 0.55, which was not significantly different from that of healthy blood donors. The distribution was compatible with the Hardy-Weinberg equilibrium. Analysis of clinicopathological parameters did not show any significant correlation of the T393C genotype with gender ( $P = 0.50$ ), differentiation ( $P = 0.29$ ), pT-category ( $P = 0.19$ ), pN-category ( $P = 0.30$ ), pM-category ( $P = 0.25$ ), R-category ( $P = 0.95$ ), the classifications according to WHO ( $P = 0.34$ ), Lauren ( $P = 0.16$ ), Goseki ( $P = 1.00$ ) and Ming ( $P = 0.74$ ). Dichotomization between C+ (CC+CT) and C-genotypes (TT), however, revealed significantly more advanced tumor stages ( $P = 0.023$ ) and lower survival rates ( $P = 0.043$ ) for C allele carriers.

**CONCLUSION:** The present study provides strong evidence to suggest that the *GNAS1* T393C allele carrier status influences tumor progression and survival in gastric cancer with higher tumor stages and a worse outcome for C allele carriers.

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**Key words:** Gastric cancer; G Protein; Polymorphism; Prognosis; Tumor stage

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## INTRODUCTION

Gastric cancer has substantially decreased in incidence over the past decades, but it still remains one of the most common cancers in the world and the second most frequent cause of cancer-related death after lung cancer<sup>[1]</sup>. Most patients are diagnosed with advanced gastric cancer, and overall survival remains poor<sup>[2,3]</sup>. The 5-year survival rate for gastric cancer is still only at 40%<sup>[4,5]</sup>.

Of particular interest are prognostic factors, as they give the basis to identify gastric cancer patients with high-risk and poor prognosis. The identification of patients with poor outcome can help to set up novel treatment strategies at the beginning of treatment and may lead to better and more individualized therapy strategies with better survival<sup>[2]</sup>. Current efforts in research are therefore focused on the detection and validation of biomarkers and genetic markers that give additional information about prognosis to classical prognostic factors such as the TNM classification. The majority of new detected markers are related to properties of the tumor itself, e.g. somatic mutations or differential expression of genes or proteins. However, difficulties in standardization of such markers often prevent their routine application in clinical practice<sup>[6]</sup>.

In recent years, studies have focused on the detection of single nucleotide polymorphisms (SNPs) that have a prognostic impact in cancer. One major advantage of SNPs as prognostic markers is that they can be determined independently from the availability and quality of tumor material as they can be easily evaluated from a blood sample from individual patients.

The T393C polymorphism of the gene *GNAS1* is one such polymorphism. This SNP is located in exon 5 of the gene *GNAS1*, which encodes the ubiquitously expressed G $\alpha$ s subunit of heterotrimeric G proteins. Previous studies indicate that increased expression of G $\alpha$ s enhances apoptosis<sup>[7,8]</sup> and that G $\alpha$ s mRNA expression is different between T393C genotypes<sup>[9]</sup>. For various solid tumors, previous studies demonstrated that patient survival and tumor progression depended on T393C genotype<sup>[10-17]</sup>.

Until now, nothing has been published about the impact of the *GNAS1* T393C polymorphism on gastric cancer. Thus, the aim of the present study was to determine the influence of this polymorphism on prognosis in gastric cancer. Furthermore, we looked for possible correlations between the *GNAS1* T393C polymorphism and clinicopathological parameters.

## MATERIALS AND METHODS

### Patients

Of 159 patients, who were treated surgically between May 1996 and January 2005 for primary gastric carcinoma at the Department of General, Visceral and Cancer Surgery of the University of Cologne, 13 (8.2%) patients with a second tumor, a previous operation of the upper digestive tract or missing paraffin-embedded tissue from normal cells, and 24 (15.1%) patients with neoadjuvant

treatment received before surgery were excluded. Excluded patients did not differ in age and gender from the remaining patients.

All of the included 122 patients [median age 67.6 years, range 33-87 years; 78 (63.9%) male, 44 (36.1%) female] were initially treated by operation with curative intention. Gastroscopic examination, endoscopic ultrasound and computed tomography (CT) of the chest and abdomen were performed before surgery on all patients for clinical staging.

One hundred and six (86.9%) of the 122 patients underwent a gastrectomy with D2-lymphadenectomy (compartment I and II) and in 16 (13.1%) cases, a subtotal gastrectomy with D2-lymphadenectomy was performed. The median number of resected lymph nodes was 36.0 (range 15-80).

The present study was performed according to the guidelines of the local Research Ethics Commission.

### Histopathology

The specimens were removed *en bloc* and the lymph nodes of the specimens were dissected with the cooperation of surgeons and pathologists according to a standardized protocol. The resected specimens were routinely fixed in 5% phosphate-buffered formalin and embedded in paraffin. Histopathologic examination of all resected specimens consisted of a thorough and standardized evaluation of the tumor stage, residual tumour (R) category, grading and the number of resected and infiltrated lymph nodes. The gastric lymph nodes were documented according to the classification of the Japanese Research Society of Gastric Cancer (JRS GC) with lymph node groups 1 to 13<sup>[18]</sup>. The tumor localization was defined according to the International Classification of Diseases for Oncology. The lesions were further classified and graded in accordance with WHO recommendations, the Laurén-classification and tumor differentiation. Postoperative staging was performed according to the 6th edition of the TNM-classification of malignant tumors<sup>[19]</sup>.

### Genotyping

DNA was extracted from paraffin-embedded tissues from resection boundaries containing exclusively normal cells using a DNA extraction kit (QIAamp, Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genotyping was performed in 96-well plates by 5' nuclease assay (TaqMan) using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Darmstadt, Germany).

The pre-developed TaqMan assay ID C\_9901536\_10 (Applied Biosystems, Darmstadt, Germany) was used for genotyping of *GNAS1* T393C polymorphism (dbSNP rs7121). Polymerase chain reaction (PCR) reactions contained 10 ng DNA, 200  $\mu$ mol/L dNTPs and 900 nmol/L primers (Figure 1).

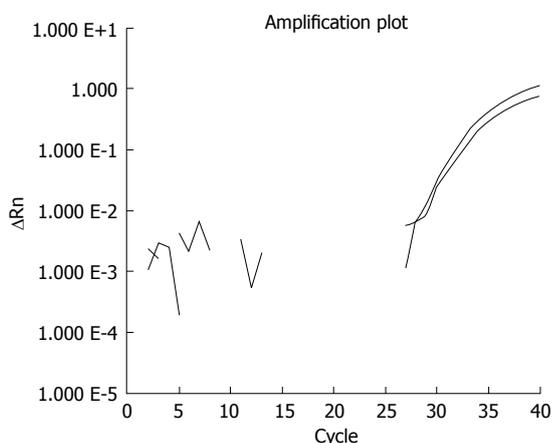
PCR conditions were: 95°C for 10 min followed by 40 cycles of 15 s at 92°C and 60 s at 60°C.

### Reference group

The Caucasian control sample consisted of 820 healthy

**Table 1** Allele frequencies and genotype distribution of *GNAS1* T393C polymorphism in 122 gastric cancer patients and in the reference group ( $n = 820$ )  $n$  (%)

T393C	Patients ( $n = 122$ )			Reference group ( $n = 820$ )			$\chi^2$	$P$	Odds ratio	95% CI
Allele	C 135 (55.3)		T 109 (44.7)	C 873 (53.2)		T 767 (46.7)	0.38	0.54	0.92	0.70-1.20
Genotype	CC 39 (32.0)	CT 57 (46.7)	TT 26 (21.3)	CC 235 (28.7)	CT 403 (49.1)	TT 182 (22.2)	0.37	0.54	0.92	

**Figure 1** Amplification plot of one heterozygous *GNAS1* T393C (CT) by two allele specific TaqMan probes.

white individuals who were recruited at the local Department for Transfusion Medicine, University Hospital, Essen. All samples were collected at random from subjects donating blood. The details of this sample have been published previously<sup>[12]</sup>.

### Statistical analysis

Associations between T393C genotype and clinicopathological parameters were evaluated using the  $\chi^2$  test. Pearson's  $\chi^2$  was used for Hardy-Weinberg analysis and to examine differences in allele frequencies between our patient group and the reference group. Relations to overall survival were evaluated with univariate analysis according to the Kaplan-Meier approach using the log-rank test to assess statistical differences between groups. Prognostic factors were determined by multiple stepwise regression analysis using the Cox model. Only potential prognostic factors were included in the multivariate analysis. The level of significance was set at  $P < 0.05$  and  $P$  values were for 2-sided testing. All statistical tests were performed using the Software Package SPSS for Windows, Version 17.0 (Chicago, IL, USA).

## RESULTS

### Genotype distribution and reference group

Thirty-nine (32.0%) patients displayed a CC genotype, 57 (46.7%) a CT genotype and 26 (21.3%) a TT genotype. The frequency of the C allele (fC) in the entire patient group was 0.55, which is not significantly different from that of healthy blood donors (Table 1). The distribution was compatible with the Hardy-Weinberg equilibrium.

### Clinicopathological characteristics

Clinicopathological characteristics of the whole patient

group with genotype distribution are displayed in Table 2. Thirty (24.6%) patients showed an early gastric carcinoma (pT1). In 73 (59.8%) cases, lymph node metastasis (pN+) was detected. An M1 category was found in 23 (18.9%) patients with localized peritoneal carcinosis, distant lymph node metastasis (M1 lymph) or single liver metastasis (M1 Hep). Patients with diffuse peritoneal or multiple liver metastasis had been treated non-surgically and were excluded from the study.

Analysis of clinicopathological parameters did not show any significant correlation of the T393C genotype with gender ( $P = 0.50$ ), differentiation ( $P = 0.29$ ), pT-category ( $P = 0.19$ ), pN-category ( $P = 0.30$ ), pM-category ( $P = 0.25$ ), R-category ( $P = 0.95$ ), or the classifications according to WHO ( $P = 0.34$ ), Laurén ( $P = 0.16$ ), Goseki ( $P = 1.0$ ), Ming ( $P = 0.74$ ) and UICC ( $P = 0.15$ ).

When genotypes were dichotomized in C+ (CC+CT) and C-genotypes (TT), a significantly higher rate of advanced tumor stages (stage III and IV), according to the UICC classification, was seen for C allele carriers ( $P = 0.023$ ). Only 6 (23.1%) of 26 patients with TT genotype were diagnosed with a tumor stage of III or IV. In contrast, an advanced tumor stage was detected in 50 (52.1%) of 96 C allele carriers.

### Univariate survival analysis

Overall survival dependent on T393C genotypes is displayed in Figure 2. The 5-year survival rate for patients with a TT genotype was 56.9% (SE  $\pm$  10.4%), followed by patients with CC genotype with a 5-year survival rate of 42.6% (SE  $\pm$  8.3%). Heterozygous CT patients showed a 5-year survival rate of 32.7% (SE  $\pm$  6.3%). Survival was not significantly associated with the T393C genotype when the three genotypes were compared ( $P = 0.082$ ). However, dichotomization between C+ (CC+CT) and TT demonstrated a significantly ( $P = 0.043$ ) lower survival rate for C allele carriers (Figure 3) with a 5-year survival rate for the C+ group of only 36.7% (SE  $\pm$  5.1%) *vs* 56.9% (SE  $\pm$  10.4%) for the TT group.

### Multivariate survival analysis

In the multivariate Cox regression analysis, known prognostic factors for gastric cancer (pT, pN, pM and R-category) and T393C genotype with dichotomization between C+ (CC+CT) and TT were included. pT-category ( $P < 0.001$ ), R-category ( $P = 0.022$ ) and pM-category ( $P = 0.027$ ) maintained their prognostic independence (Table 3). pN-category ( $P = 0.55$ ), and the T393C genotype ( $P = 0.33$ ) lost their prognostic independence.

## DISCUSSION

Gastric cancer is the fourth most common cancer with

Table 2 Clinicopathological characteristics of 122 patients with gastric cancer *n* (%)

	All	T393C genotypes			<i>P</i>
		CC	CT	TT	
<i>n</i> (%)	122 (100)	39 (32)	57 (46.7)	26 (21.3)	
Gender					
Male	78 (63.9)	25 (32.1)	34 (43.6)	19 (24.4)	0.274
Female	44 (36.1)	14 (31.8)	23 (52.3)	7 (15.9)	
WHO					
Papillary/Tubular/Mucinous	76 (62.3)	23 (30.3)	34 (44.7)	19 (25)	0.340
Signet-ring cancer	38 (31.1)	12 (31.6)	19 (50)	7 (18.4)	
Other	8 (6.6)	4 (50)	4 (50)	0	
Differentiation					
Well/Moderate (G1-G2)	42 (34.4)	12 (28.6)	22 (52.4)	8 (19)	0.805
Poor (G3-G4)	80 (65.6)	27 (33.8)	35 (43.8)	18 (22.5)	
Laurén					
Intestinal	52 (42.6)	16 (30.8)	25 (48.1)	11 (21.2)	0.171
Diffuse	55 (45.1)	17 (30.9)	29 (52.7)	9 (16.4)	
Mixed	15 (12.3)	6 (40)	3 (20)	6 (40)	
Ming					
Expanding	47 (38.5)	14 (29.8)	24 (51.1)	9 (19.1)	0.620
Infiltrative	75 (61.5)	25 (33.3)	33 (44)	17 (22.7)	
pT-category					
T1	30 (24.6)	7 (23.3)	13 (43.3)	10 (33.3)	0.110
T2	44 (36.1)	12 (27.3)	22 (50)	10 (22.7)	
T3	38 (31.1)	14 (36.8)	18 (47.4)	6 (15.8)	
T4	10 (8.2)	6 (60)	4 (40)	0	
pN-category					
N0	49 (40.2)	11 (22.4)	24 (49)	14 (28.6)	0.196
N1	34 (27.9)	13 (38.2)	13 (38.2)	8 (23.5)	
N2	14 (11.5)	6 (42.9)	6 (42.9)	2 (14.3)	
N3	25 (20.5)	9 (36)	14 (56)	2 (8)	
pM-category					
M0	99 (81.1)	30 (30.3)	45 (45.5)	24 (24.2)	0.101
M1	23 (18.9)	9 (39.1)	12 (52.2)	2 (8.7)	
R-category					
R0	118 (96.7)	38 (32.5)	54 (46.2)	25 (21.4)	0.950
R1/R2	4 (3.3)	1 (25)	2 (50)	1 (25)	
UICC stage					
I a	26 (21.3)	5 (19.2)	11 (42.3)	10 (38.5)	0.023
I b	22 (18)	7 (31.8)	12 (54.5)	3 (13.6)	
II	18 (14.8)	4 (22.2)	7 (38.9)	7 (38.9)	
III a	11 (9)	4 (36.4)	5 (45.5)	2 (18.2)	
III b	4 (3.3)	2 (50)	2 (50)	0	
IV	41 (33.6)	17 (41.5)	20 (48.8)	4 (9.8)	

*P* values are given for dichotomization between C+ (CC+CT) and C- (TT) genotypes.

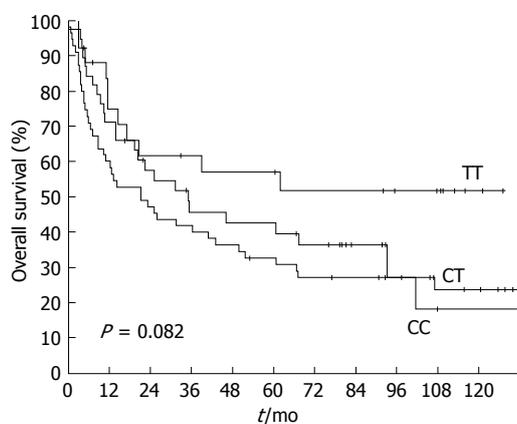


Figure 2 Overall survival of 122 resected gastric cancer patients based on *GNAS1* T393C genotype (Kaplan-Meier analysis), *P* = 0.082 (Mantel-Cox log-rank test).

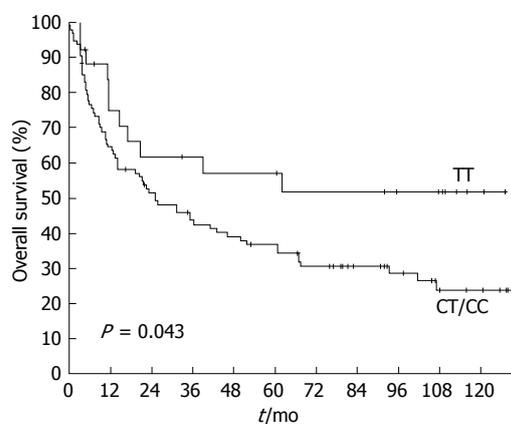


Figure 3 Overall survival of 122 resected gastric cancer patients based on *GNAS1* T393C genotype with dichotomization between C+ and C- genotypes, *P* = 0.043.

Table 3 Univariate and multivariate survival analysis of 122 gastric cancer patients

Covariate	n	Univariate analysis			Multivariate analysis		
		P value	5-yr-SR (%)	SE ( $\pm$ %)	P value	HR	95% CI
pT-category		< 0.001			< 0.001		
pT1	30		85.4	6.8		1	
pT2	44		44.5	7.8	< 0.001	6.212	2.31-16.70
pT3	38		5.4	3.7	< 0.001	13.026	4.44-38.23
pT4	10		33.3	15.7	0.001	7.838	2.24-27.46
pN-category		< 0.001			0.549		
pN0	49		61.6	7.4		1	
pN1	34		47.1	8.6	0.226	0.663	0.34-1.29
pN2	14		16.9	10.9	0.986	0.993	0.43-2.27
pN3	25		8.0	5.4	0.814	0.905	0.40-2.07
T393C SNP		0.043			0.333		
CC/CT	96		36.7	5.1		1	
TT	26		56.9	10.4		0.712	0.36-1.42
pM-category		< 0.001			0.027		
M0	99		48.1	5.2		1	
M1	23		9.2	6.2		2.087	1.09-4.01
R-category		< 0.001			0.022		
R0	118		42.3	4.7		1	
R+	4		0	0		3.128	1.18-8.27

SNP: Single nucleotide polymorphism; 5-yr-SR: 5-yr-survival; HR: Hazard ratio.

Table 4 Summary of the effect of the *GNAS1* T393C polymorphism on various carcinomas

Cancer type	Yr	n	Effect	Benefit (survival)
Gastric cancer	2009	122	The present study demonstrates a significant survival benefit for the TT genotype with a 5-yr-survival rate of 56.9% vs the CC/CT group with a 5-yr-survival rate of only 36.7% ( $P = 0.043$ )	TT-genotype
Squamous cell cancer of larynx <sup>[15]</sup>	2008	157	Survival was significantly dependent on the T393C genotype in advanced American Joint Committee on Cancer (AJCC) stages (III-IV) with higher 5-yr survival rates for TT, followed by TC and CC ( $P = 0.0437$ )	TT-genotype
Oro- and hypopharyngeal squamous cell carcinoma <sup>[16]</sup>	2008	202	C homozygous patients displayed a higher risk for disease progression than T homozygous patients ( $P = 0.019$ ) and a higher risk for death ( $P = 0.015$ ). In multivariate analysis, besides cancer stage and tumor localization, the T393C polymorphism was an independent prognostic factor for disease progression and death	TT-genotype
Clear cell renal cell carcinoma <sup>[11]</sup>	2006	150	Tumor progression, development of metastasis and tumor-related death was significantly associated with the T393C polymorphism. In multivariate analysis CC patients were at highest risk for progression or tumor-related death compared with T-allele carriers ( $P = 0.018$ )	TT-genotype
Chronic lymphocytic leukemia <sup>[17]</sup>	2006	144	Median progression-free survival was significantly higher for T-allele carriers ( $P = 0.007$ ). In multivariate analysis, the T393C polymorphism kept its prognostic independence ( $P = 0.01$ ) besides of ZAP-70 ( $P = 0.005$ ) and Binet stage ( $P < 0.001$ ). Regarding overall survival, CC genotypes were significantly at highest risk for death compared to T-alleles both in univariate ( $P < 0.001$ ) and multivariate analysis ( $P = 0.002$ )	TT-genotype
Bladder cancer <sup>[10]</sup>	2005	254	Progression-free survival ( $P = 0.011$ ), metastasis-free survival ( $P = 0.001$ ) and cancer-specific survival ( $P = 0.014$ ) were significantly increased in TT genotypes compared with CC genotypes. In multivariate analysis, the T393C polymorphism kept its prognostic independence	TT-genotype
Sporadic colorectal cancer <sup>[12]</sup>	2005	151	In UICC stages I to II, the 5-yr survival rate was significantly ( $P = 0.009$ ) higher in TT genotypes (88%) compared with TC (71%) and CC genotypes (50%). In multivariate analysis, the T393C polymorphism was also an independent prognostic factor. No significant effect could be seen for UICC stages III to IV	TT-genotype
Cholangiocarcinoma <sup>[14]</sup>	2007	87	Disease-specific overall survival was significantly dependent on the T393C genotype ( $P = 0.02$ ), with TT genotypes showing reduced survival compared to patients carrying at least one C allele. In multivariate analysis (TT/C+) the T393C genotype kept its prognostic independence ( $P = 0.04$ )	CC-genotype
Breast carcinoma <sup>[13]</sup>	2007	279	Overall survival was significantly ( $P = 0.033$ ) associated with the T393C polymorphism with lowest survival rates for the TT-genotype and highest survival rate for the CC-genotype. In multivariate analysis, the TT-genotype still had a significant survival benefit compared to the CC genotype ( $P = 0.045$ )	CC-genotype
Esophageal cancer <sup>[28]</sup>	2009	51	T393C polymorphism was significantly associated with tumor response to Cisplatin/5-FU-based radiochemotherapy. 63% of the T allele carriers had a minor histopathologic response (MiHR) with more than 10% residual vital tumor cells in resection specimens. For the CC genotype MiHR was seen only in 20%. In binary logistic regression analysis, the T393C genotype kept its independence ( $P < 0.05$ )	CC-genotype

approximately 800000 new cases per year and the second leading cause of cancer-related death worldwide<sup>[20]</sup>. Many patients have advanced disease at the time of diagnosis, resulting in poor prognosis and high mortality<sup>[2,21,22]</sup>. Pre-treatment staging of the disease is of high importance as it provides the basis for selecting the most appropriate therapeutic strategy<sup>[23]</sup>. Based on the preoperative staging, patients with early stage tumors are treated by endoscopic mucosal resection, while patients with advanced tumors are treated by partial or total gastrectomy<sup>[5]</sup>. Accurate staging is also the basis for selecting patients for neoadjuvant, adjuvant or palliative treatment<sup>[24]</sup>. By identifying patients with poor outcome, novel treatment strategies could be set up at the beginning of treatment which can lead to better and more individualized therapy strategies with superior survival<sup>[2]</sup>.

The present study demonstrated that besides the known prognostic factors pT, pM, pN and R-category, the T393C polymorphism was also a significant prognostic factor in the univariate analysis with a survival benefit for homozygous TT patients. In addition, it demonstrated that compared to C allele carriers, homozygous TT patients were diagnosed with significantly less advanced tumor stages according to UICC, which is possibly the main reason why the T393C genotype lost its independence in the multivariate analysis.

The gene *GNAS1* is mapped to chromosome 20q13 and consists of 13 exons. Somatic activating mutations of *GNAS1* have been implicated in the etiology of McCune Albright Syndrome<sup>[25]</sup> and sporadic, isolated endocrine tumors<sup>[26,27]</sup> which supports a role of *GNAS1* in tumor initiation and progression.

Recent studies have shown that genotypes of the T393C polymorphism are significantly associated with survival of patients suffering from colorectal cancer, bladder cancer, clear cell renal carcinoma, intrahepatic cholangiocarcinoma, invasive breast carcinoma and squamous cell carcinoma of the larynx, oropharynx and hypopharynx (Table 4)<sup>[10-14]</sup>.

Comparable to previous results in bladder cancer, clear cell renal carcinoma and colorectal cancer, the present study also demonstrated significantly higher survival rates for TT genotypes in gastric cancer (Figure 3). Patients with the TT genotype showed a 5-year survival rate of 57%, whereas the 5-year survival rate for C allele carriers was only at 37%.

In contrast to our findings in gastric cancer and previous findings in the above-mentioned tumor types, an unfavourable clinical course for T allele carriers has been described in studies of invasive breast cancer and intrahepatic cholangiocarcinoma, suggesting that the biological effect of the T393C polymorphism may be different in different tumor types. In a recent study, we demonstrated that this polymorphism is a predictive molecular marker for tumor response to cisplatin/5-FU-based radiochemotherapy in esophageal cancer, with CC genotypes mostly showing a major response<sup>[28]</sup>.

*In vitro* experiments suggest that expression of G $\alpha$ s is associated with enhanced apoptosis<sup>[7,8]</sup>. The second messenger, cyclic AMP, which is generated by activated G $\alpha$ s,

seems to play a major role in this proapoptotic process. An increased concentration of the intracellular second messenger, cyclic AMP promotes apoptosis in several cell types including leukemic cells<sup>[29]</sup>, ovarian cancer cells<sup>[30]</sup>, and lymphoma cells<sup>[25]</sup>. Increased G $\alpha$ s expression in tissues of patients with TT genotypes may therefore confer enhanced apoptosis in 393T allele carriers. Hypothetically, this mechanism may contribute to the described more favorable clinical course and the less advanced tumor stages of homozygous TT patients. This hypothesis remains to be supported by additional functional studies which were beyond the scope of the present study. The T393C polymorphism as a risk factor for gastric cancer could not be established in the present study.

In conclusion, this study demonstrated for the first time that in primary gastric cancer, homozygous *GNAS1* 393T patients have less advanced tumor stages and higher survival rates than C allele carriers. These findings further support the concept of a general role for the *GNAS1* T393C polymorphism in tumor progression.

## COMMENTS

### Background

Identification of gastric cancer patients with poor outcome can help to set up novel treatment strategies at the beginning of treatment and may lead to better and more individualized therapy strategies with better survival. In recent years, studies have focused on the detection of single nucleotide polymorphisms (SNPs) as prognostic molecular markers in cancer.

### Research frontiers

The *GNAS1* T393C polymorphism is located in exon 5 of the gene *GNAS1*. In this study the authors describe, for the first time, the impact of this SNP in gastric cancer. The study demonstrates that the *GNAS1* T393C polymorphism affects tumor stage and prognosis in gastric cancer.

### Innovations and breakthroughs

For various solid tumors, previous studies have demonstrated that patient survival and tumor progression depend on the *GNAS1* T393C genotype. In the present study, the authors have described for the first time that the *GNAS1* T393C polymorphism affects tumor stage and prognosis in gastric cancer.

### Applications

The *GNAS1* T393C polymorphism will contribute to identifying high-risk patients with gastric cancer and might help to establish a more individualized treatment strategy for gastric cancer.

### Terminology

A single-nucleotide polymorphism (SNP) is a DNA sequence variation occurring when a single nucleotide - A, T, C, or G - in the genome differs between members of a species. The *GNAS1* T393C is located in exon 5 of the gene *GNAS1*. For several cancer types, studies have demonstrated that patient survival is affected by this SNP.

### Peer review

Overall, this paper provides information on *GNAS1* T393C allele carrier status which influences tumor progression and survival in gastric cancer, with higher tumor stages and worse outcome for C allele carriers.

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BRIEF ARTICLE

## Elevated pro-inflammatory and lipotoxic mucosal lipids characterise irritable bowel syndrome

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tients with IBS. The most significant upregulation was seen for pro-inflammatory lysophosphatidylcholines. Other lipid groups that were significantly upregulated in IBS patients were lipotoxic ceramides, glycosphingolipids, and di- and triacylglycerols. Among the metabolites, the cyclic ester 2(3H)-furanone was almost 14-fold upregulated in IBS patients compared to healthy subjects ( $P = 0.03$ ).

**CONCLUSION:** IBS mucosa is characterised by a distinct pro-inflammatory and lipotoxic metabolic profile. Especially, there was an increase in several lipid species such as lysophospholipids and ceramides.

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**Key words:** Functional gastrointestinal diseases; Irritable bowel syndrome; Histopathology

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### Abstract

**AIM:** To investigate the pathophysiology of irritable bowel syndrome (IBS) by comparing the global mucosal metabolic profiles of IBS patients with those of healthy controls.

**METHODS:** Fifteen IBS patients fulfilling the Rome II criteria, and nine healthy volunteers were included in the study. A combined lipidomics (UPLC/MS) and metabolomics (GC × GC-TOF) approach was used to achieve global metabolic profiles of mucosal biopsies from the ascending colon.

**RESULTS:** Overall, lipid levels were elevated in pa-

### INTRODUCTION

Irritable bowel syndrome (IBS) is a functional bowel disorder characterised by abdominal pain or discomfort and an irregular bowel habit<sup>[1]</sup>. The prevalence is up to 20% in Western adults, which makes IBS the most common diagnosis in gastroenterology. The precise aetiology and pathophysiology of IBS are incompletely understood, despite extensive interest and investigation. The current knowledge does, however, suggest that altered gut motility, visceral hyperalgesia, and dysregulation of the brain-gut axis are central to IBS.

IBS is diagnosed by the presence of symptoms according to the Rome criteria<sup>[2]</sup>, with concomitant exclusion of organic diseases; hence, there is no specific biological,

radiological, endoscopic, or physiological marker for IBS. Medical treatment options for IBS are limited, possibly due to the lack of biomarkers and data about the pathophysiology of the condition. Different immune markers are among the most studied putative biomarkers in IBS. Increased mast cell counts, mast cells in close proximity to nerves, and mast cell mediators that are able to stimulate murine visceral sensory nerves appear to be characteristic of IBS<sup>[5-6]</sup>. Elevated plasma levels of pro-inflammatory interleukin (IL)-6 and IL-8 have been observed in IBS<sup>[7]</sup>, and peripheral blood mononuclear cells obtained from IBS patients produce higher amounts of tumour necrosis factor (TNF)- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-12 than cells from healthy controls<sup>[8,9]</sup>. Furthermore, an increased number of immunocytes have frequently been observed in mucosa from IBS patients<sup>[10,11]</sup>. In addition to immune markers, other markers, such as 5-hydroxytryptamine (5-HT, serotonin) and gut hormones, have also been associated with IBS. Decreased expression of 5-HT has been associated with constipation-predominant IBS<sup>[12]</sup>. Elevated plasma 5-HT concentrations have been observed in a mixed IBS population<sup>[13]</sup> and in post-infectious IBS<sup>[14]</sup>, while the opposite was demonstrated in constipation IBS<sup>[14]</sup>. Moreover, IBS patients have also been demonstrated to have decreased 5-HT levels and turnover, and lower 5-HT transporter mRNA concentrations<sup>[14,15]</sup>.

The recent technological development of analytical instruments combined with rapid progress in bioinformatics has opened novel opportunities to quickly and simultaneously measure and model huge numbers of molecular metabolites in biological samples<sup>[16,17]</sup>. This metabolomic approach is considered a powerful tool for characterising complex phenotypes and developing biological markers for specific physiological states. Thus, metabolomics provides an interesting platform to investigate the pathophysiology of a complex syndrome like IBS at the molecular level. Studies on the molecular abnormalities in IBS are needed to understand the mechanisms behind the emergence of symptoms, and to enable the development of novel therapies.

The aim of the current study was to compare the global metabolic profiles of mucosal biopsies from IBS patients with those from healthy subjects using a high-throughput approach comprising lipidomics and metabolomics.

## MATERIALS AND METHODS

### Subjects

Sixteen adult IBS patients fulfilling the Rome II criteria<sup>[18]</sup> and without organic intestinal diseases were recruited to participate in the study. One statistical outlier IBS patient was left out of the analyses after an initial quality check of the results, and thus a total of 15 patients were analysed (Table 1). Nine healthy subjects (mean age 49 years, SD 14; 4 male) without organic intestinal diseases or gastrointestinal symptoms consistent with IBS and undergoing colonoscopy for clinical reasons were recruited as controls. The healthy subjects were either polyp control patients having a minimum of 3 years since the previous

**Table 1** Sociodemographic and clinical characteristics of IBS patients ( $n = 15$ ) (mean  $\pm$  SD)

Age (yr)	42 $\pm$ 16
Sex (n): F/M	11/4
BMI (kg/m <sup>2</sup> )	23.3 $\pm$ 5.0
Predominant bowel habit <sup>1</sup> : (n)	
Diarrhoea	2
Constipation	3
Alternating	10

<sup>1</sup>According to the Rome II criteria; BMI: Body mass index.

polyp finding or anaemic patients. Inclusion criteria for all subjects were: an age between 20 and 65 years; normal blood count (erythrocytes, haemoglobin, haematocrit, MCV, MCH, MCHC, platelets, leukocytes); serum creatinine, alanine aminotransferase (ALT) and alkaline phosphatase (ALP) within reference values; and normal gut histology as evaluated by an experienced pathologist (PS). Subjects were excluded if they had a history of major or complicated gastrointestinal surgery, severe endometriosis, complicated abdominal adhesions, malignant tumours, were pregnant or lactating, or had received antimicrobials during the previous month. Patients with lactose intolerance were allowed to participate if they followed a continuous low-lactose or lactose-free diet.

The Human Ethics Committee of the Hospital District of Pirkanmaa, Finland, approved the study protocol. All subjects provided written informed consent.

### Sample collection and preparation

Mucosal biopsies (mean weight 5.2 mg/sample; SD 1.5) from the ascending colon were obtained from each subject during colonoscopy after bowel cleansing. The samples were immediately frozen at -20°C, and stored at -70°C until required for analysis. The samples for lipidomic analysis were weighed into Eppendorf tubes, and 10  $\mu$ L of 0.9% sodium chloride and 10  $\mu$ L of an internal standard mixture (10 lipid compounds, 0.1  $\mu$ g each) were added. The samples were extracted with 100  $\mu$ L of chloroform:methanol (2:1; two min vortexing, one h extraction time) and centrifuged (7800 *g*, 3 min). Of the lower organic phase, 60  $\mu$ L aliquots were transferred into vial inserts and 10  $\mu$ L of a standard mixture containing three labelled lipid compounds was added. The internal standard mixture contained the following lipid compounds with heptadecanoic acid (C17:0) as the esterified fatty acid: D-erythro-Sphingosine-1-Phosphate (C17 Base, Avanti Polar Lipids, Alabaster, AL, USA), LysoPC (Avanti Polar Lipids), MG (17:0)[rac] (Larodan Fine Chemicals, Malmö, Sweden), PG (17:0/17:0)[rac] (Avanti Polar Lipids), Cer (d18:1/17:0) (Avanti Polar Lipids), PS (17:0/17:0) (Avanti Polar Lipids), PC (17:0/17:0) (Avanti Polar Lipids), PA (17:0/17:0) (Avanti Polar Lipids), PE (17:0/17:0) (Avanti Polar Lipids), DG (17:0/17:0)[rac] (Larodan Fine Chemicals), and TG (17:0/17:0/17:0) (Larodan Fine Chemicals). The labeled standard mixture consisted of LysoPC (16:0-D3) (Larodan Fine Chemicals), PC (16:0/16:0-D6) (Larodan Fine Chemicals), and TG (16:0/16:0/16:0-13C3) (Larodan Fine Chemicals).

For water-soluble compounds, the samples were weighed into Eppendorf tubes and 10°C of 1000 ppm (mg/mL) labelled palmitic acid (16:0-16, 16, 16D3) was added as an internal standard. The samples were extracted with 500 µL methanol (two min vortexing, 0.5 h extraction time) and centrifuged (7800 g, 3 min). The separated supernatants were evaporated to dryness under nitrogen, and the residues were derivatised with 2% methoxyamine HCl in pyridine (MOX; 25 µL, 90 min at 30°C) and N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA; 50 µL, 30 min at 37°C). All samples were run in duplicate.

### **Analysis of lipids by UPLC/MS**

Characterisation of lipid molecular species in colonic mucosa was performed by a lipidomics strategy using ultra performance liquid chromatography coupled to mass spectrometry (UPLC/MS, Waters Micromass Q-ToF Premier). The column (50°C) was a Waters Acquity UPLC™ BEH C18 (Waters Inc., Milford, MA, USA) 1 mm × 50 mm with 1.7 µm particles. The solvent system included: (A) ultra pure water (1% 1 mol/L NH<sub>4</sub>Ac, 0.1% HCOOH) and (B) LC/MS grade acetonitrile:isopropanol (5:2, 1% 1 mol/L NH<sub>4</sub>Ac, 0.1% HCOOH). The gradient started from 65% A and 35% B, reached 100% B in 6 min and remained there for the next 7 min. There was a 5 min re-equilibration step before the next run. The flow rate was 0.200 mL/min and the injected amount 1.0 µL. Lipid profiling was carried out using ESI+ mode, and the data were collected at a mass range of m/z 300-2000 with a scan duration of 0.08 s.

Lipids were identified using an internal spectral library or with tandem mass spectrometry. The normalisation of lipidomics data was performed as follows: all monoacyl lipids, except cholesterol esters (such as monoacylglycerols and monoacylglycerophospholipids), were calibrated with lysophosphatidylcholine lysoPC (17:0) internal standard; all diacyl lipids, except ethanolamine phospholipids, were normalised with phosphatidylcholine PC (17:0); the diacyl ethanolamine phospholipids were calibrated with phosphatidylethanolamine PE (17:0); and the triacylglycerols and cholesterol esters were calibrated with triacylglycerol TG (17:0). Other molecular species were normalised by lysoPC (17:0) for retention time < 310 s, PC (17:0) for retention time between 310 and 450 s, and TG (17:0) for higher retention times. Data was processed using the MZmine software, version 0.60<sup>[19]</sup>, and metabolites were identified using an internal spectral library or with tandem mass spectrometry (UPLC/MS/MS).

### **Analysis of water-soluble metabolites by GC × GC-TOF**

A broad screening of water-soluble metabolites was conducted by a comprehensive two-dimensional gas chromatography coupled to a high speed time-of-flight mass spectrometry (GC × GC-TOF)<sup>[20]</sup>. The instrument used was a Leco Pegasus 4D GC × GC-TOF with Agilent 6890N GC from Agilent Technologies, USA and CombiPAL autosampler from CTC Analytics AG, Switzerland. Modulator, secondary oven, and time-of flight mass spectrometer were from Leco Inc., USA. The GC

was operated in split mode (1:20) using helium as carrier gas at 1.5 mL/min constant flow. The first GC column was a relatively non-polar RTX-5 column, 10 m × 0.18 mm × 0.20 µm, and the second was a polar BPX-50, 1.10 m × 0.10 mm × 0.10 µm. The temperature programme was as follows: initial 50°C, 1 min → 280°C, 7°C/min, one min. The secondary oven was set to +30°C above the primary oven temperature. The second dimension separation time was set to 3 s. The mass range used was 40 to 600 amu and the data collection speed was 100 spectra/s. A commercial mass spectral library, Palisade Complete 600K, was used for identifying metabolites.

### **Statistical analysis**

Partial least squares discriminant analysis (PLS/DA) was utilized as a supervised modelling method using the SIMPLS algorithm to calculate the model. The contiguous-blocks cross-validation method and  $Q^2$  scores were used to develop the models. Top loadings for latent variables associated with drug-specific effects were reported. The VIP (variable importance in the projection) values were calculated to identify the most important molecular species for clustering of specific groups. Multivariate analyses were performed using Matlab, version 7.2 (Mathworks Inc., Natick, MA, USA), and the PLS Toolbox, version 4.0, for the Matlab package (Eigenvector Research Inc., Wenatchee, WA, USA). One statistical outlier IBS patient was left out of the analyses after an initial quality check of the results. Univariate comparisons for individual metabolites between the groups were performed using the Wilcoxon rank-sum test. A *P* value < 0.05 was considered statistically significant. To account for multiple comparisons, the False Discovery Rate (FDR)  $Q$ -value is also reported<sup>[21]</sup>.

## **RESULTS**

### **Lipidomic analysis**

By applying UPLC/MS, a total of 651 lipid peaks were found, and 75 of them were identified using the internal spectral library, as described by Yetukuri *et al.*<sup>[22]</sup>, or with tandem mass spectrometry using UPLC/MS/MS. PLS/DA analysis of lipidomic data revealed significant differences in the mucosal lipid profiles of IBS patients and healthy controls. Overall, lipid species were upregulated in biopsies from IBS patients compared to those from healthy subjects. The 20 lipids with the largest differences between the groups by fold change appear in Table 2. A significant upregulation in the concentrations of typical cell membrane metabolites, lysophospholipids, in IBS patients was the most obvious finding (Figure 1A). Other lipid groups with a significant contribution to the distinction between IBS patients and healthy controls were ceramides (Figure 1B), glycosphingolipids, and di- and triacylglycerols. All of these showed upregulation in the IBS group.

### **Metabolomic analysis**

Broad metabolite screening by GC × GC-TOF resulted in several hundred mucosal metabolites, of which 107 were

**Table 2** The 15 lipids with the largest and most significant differences between patients and controls

Lipid name	Fold (IBS/healthy control)	P value <sup>1</sup>	FDR Q value
Cer (d18:1/24:1)	1.3	0.001	0.022
Cer (d18:1/24:2)	1.4	0.00004	0.0018
DG (36:2)	1.9	0.000001	0.0003
GlycoSL (m/z = 1199.805)	1.9	0.001	0.018
GlycoSL (m/z = 1195.851)	2.0	0.0003	0.0069
lysoPC (16:0)	2.1	0.00006	0.0020
lysoPC (18:0)	1.9	0.0002	0.0049
lysoPC (18:1)	2.8	0.00002	0.0013
lysoPE (18:1e)	2.4	0.00002	0.0013
7TG (46:5)	1.4	0.04	0.19
TG (48:5)	1.6	0.03	0.17
TG (48:6)	1.7	0.03	0.18
TG (49:3)	2.1	0.009	0.089
TG (51:4)	1.6	0.04	0.19
TG (51:5)	1.8	0.02	0.12

<sup>1</sup>Wilcoxon rank sum test. Cer: Ceramide; DG: Diacylglycerol; GlycoSL: Glycosphingolipid; lysoPC: Lysophosphatidylcholine; lysoPE: Lysophosphatidylethanolamine; TG: Triacylglycerol.

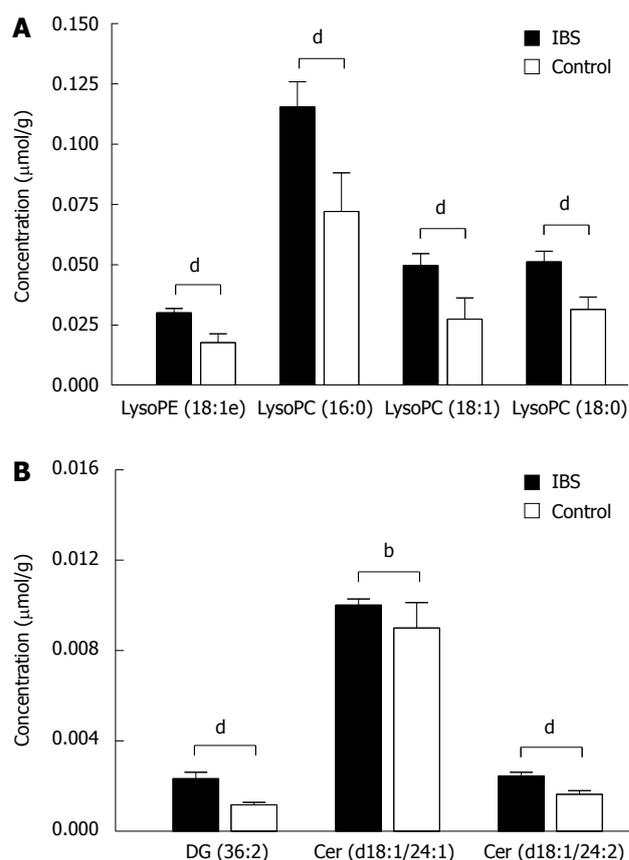
**Table 3** Major water soluble metabolites contributing to differentiation between patients and controls<sup>1</sup>

Metabolite	Fold (IBS/healthy control)	P value <sup>2</sup>	FDR Q value
2(3H)-furanone	13.7	0.03	0.25
Ribitol	3.6	NS	0.25
Heptan	2.9	0.02	0.25
L-Mannose	2.8	NS	0.25
Creatinine	1.7	0.04	0.25
Dodecane	1.5	NS	0.29
Decanoic acid	1.3	NS	0.52
Dodecanoic acid	-1.5	NS	0.25
<i>n</i> -Butylamine	-1.5	0.01	0.25
D-ribose	-1.5	NS	0.25
Glucopyranose	-1.6	NS	0.29
Azelaic acid	-1.8	0.02	0.25
Adipic acid	-2.7	0.0008	0.05

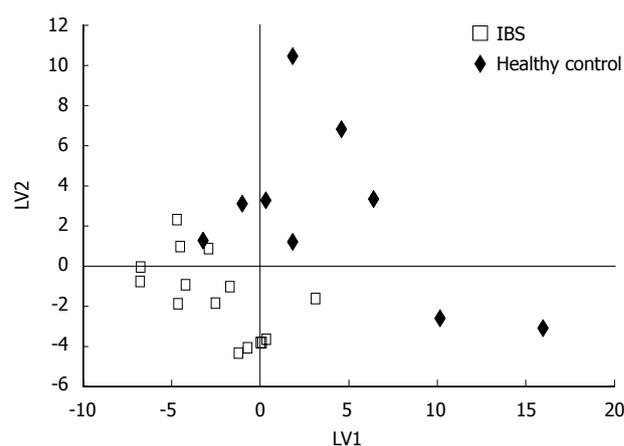
<sup>1</sup>Separation is based on a variable importance projection (VIP) analysis with a cut-off value of 2; <sup>2</sup>Wilcoxon rank sum test. NS: Not significant.

identified and kept in analyses. Based on PLS/DA analysis, a clear distinction between IBS cases and controls was obtained (Figure 2). Both upregulation and downregulation of metabolites were observed in IBS patients *vs* controls. The top ranked metabolites contributing to the distinction between the groups appear in Table 3.

The metabolite contributing most to the distinction was 2(3H)-furanone, a cyclic ester commonly produced in biochemical pathways, which was almost 14-fold upregulated in IBS patients compared to healthy subjects ( $P = 0.03$ ). The fold changes for other top ranked metabolites were clearly lower (a 3.7 to -2.7 fold change). Other basic metabolites frequently found in biochemical pathways, such as the second messenger, D-ribose, were also among the major factors contributing to the distinction between cases and controls. Organic, carboxylic acids were found to be both slightly downregulated (dodecanoic, azelaic,



**Figure 1** The concentrations (mean  $\pm$  SE) of selected lysophospholipids in mucosal biopsies from irritable bowel syndrome (IBS) patients ( $n = 15$ ) and healthy controls ( $n = 9$ ) as measured by UPLC/MS. (A) Patients and controls differ significantly from each other for all presented lysophospholipids, as well as for (B) diacylglycerol and ceramides. LysoPE: Lysophosphatidylethanolamine; LysoPC: Lysophosphatidylcholine.  $P$  values are based on Wilcoxon rank sum test with <sup>b</sup> $P < 0.01$  and <sup>d</sup> $P < 0.001$ .



**Figure 2** Partial least squares discriminant analysis (PLS/DA) of GC  $\times$  GC-ToF-based metabolic profiles for IBS patients ( $n = 15$ ) and healthy controls ( $n = 9$ ). Two latent variables (LVs) were used ( $Q^2 = 61\%$ ).

and adipic acid) as well as slightly upregulated (decanoic acid) in IBS patients compared to healthy controls.

## DISCUSSION

Data on the molecular abnormalities in IBS are urgently

required, as the pathophysiology of the condition is largely unknown and current therapies are therefore also limited. In this study, the differences between colonic mucosa from IBS patients and healthy controls were investigated by employing two high-throughput metabolomic platforms, UPLC/MS based lipidomics and GC × GC-TOF based metabolomics. Data indicated multiple differences between IBS mucosa and healthy mucosa, including an increase in the IBS group of several lipid species, such as lysophospholipids and ceramides.

The present study is the first to utilise a metabolomic approach to investigate molecular differences between IBS patients and healthy controls. Metabolomics can be seen as an optimal tool for studying diseases with unknown or complex pathophysiology, as a global study of the metabolome is a non-targeted approach that requires no preselection of markers, in contrast to the traditional way of limiting the analysis to a predefined set of known compounds<sup>[23]</sup>. Recently, metabolomics has been utilised in the investigation of multiple diseases, e.g. inflammatory bowel disease<sup>[24]</sup>, obesity<sup>[25]</sup>, and cancer<sup>[26]</sup>.

In the present study, lipids - particularly lysophosphatidylcholines (lysoPCs) and ceramides - were the most upregulated molecules in IBS patients. Mounting data suggest that certain lipids, including phospholipid derivatives and ceramides, play a role in modulating and enhancing pain sensitivity<sup>[27,28]</sup>, which could be one explanation for their involvement in IBS pathophysiology. LysoPCs have not previously been associated with IBS, but studies indicate elevated levels of lysoPCs or phospholipase A2, an enzyme involved in lysoPC formation, in inflammatory bowel disease (IBD)<sup>[29,30]</sup>. A high lysoPC concentration has been suggested to impair mucosal barrier function and increase gastrointestinal permeability *in vivo* and *in vitro*<sup>[31-34]</sup>. The role of permeability defects in IBS is not fully elucidated, but a recent review by Camilleri and Gorman concludes that there appears to be at least one IBS subgroup with increased gut permeability<sup>[35]</sup>. Interestingly, lysoPCs have also been associated with vascular inflammation, endothelial dysfunction, and coronary atherosclerosis<sup>[36]</sup>, implying that lysoPCs might also play a role in the subtle type of mucosal inflammation present in IBS.

Lipids of the ceramide/sphingomyelin pathway are another lipid type that we observed to be altered in IBS. Previous studies indicate that ceramides might be involved in the pathology of IBD<sup>[37,38]</sup>, whereas no reports on ceramides in IBS are available. Ceramides have, however, been shown to be toxic in several cell types. Current data reveal a possible role for ceramides in the damage of cells and tissues, and ultimately in the development of chronic metabolic diseases, such as diabetes and cardiovascular disease<sup>[39]</sup>. The toxic effects of ceramides might be partly mediated *via* the production of reactive oxygen species in cells<sup>[40]</sup>. It has been proposed that, similar to lysophosphatidylcholines, epithelial oxidative stress might also contribute to gut barrier dysfunction<sup>[41]</sup>. Our results thus suggest that the molecular mechanism behind increased permeability could, to some extent, be similar in IBS and IBD.

Based on a global analysis of water-soluble metabolites, IBS cases and healthy controls were rather well separated into two distinct groups. The physiological relevance of the main molecules contributing to the separation is, on the other hand, less evident than in the case of lipid species. Differences were seen in basic metabolites, such as 2(3H)-furanone (also known as lactone) and D-ribose, both of which are produced in common biochemical pathways in cells. A recent study investigating a *Trichinella spiralis* infected mouse model of post-infectious IBS and utilizing metabolomics, demonstrated an increase in molecules involved in energy metabolism (lactate, citrate, and alanine) in the IBS group<sup>[42]</sup>. The authors suggest that this might reflect a muscular hypercontractility possibly present in IBS, though it should be underlined that this was an experimental model. Concerning organic acids, our results are in line with the study by Martin *et al*<sup>[42]</sup>, in that we found that organic acids contributed to the separation between cases and controls. Specifically, we did observe increased concentrations of alanine in the IBS group, but the difference between IBS and control groups was not significant (fold = 1.3,  $P = 0.14$ ). Organic acids are known to be produced by the intestinal microbiota, and a disruption in the acid profile could reflect a possible deviance in microbiota previously reported in IBS<sup>[43,44]</sup>. In further support of findings by Martin *et al*<sup>[42]</sup>, we detected elevated levels of creatinine in the IBS group. Creatine and creatinine are tightly interlinked with the energy metabolism in smooth muscle, and a raised creatinine concentration might be a sign of increased energy consumption and muscle contractility<sup>[45]</sup>.

The field of metabolomics is evolving rapidly, and it is already considered a sensitive analytical tool for investigating the health-disease continuum<sup>[17]</sup>. Like any method, however, it has its own limitations. As large numbers of metabolites are included in the studies, caution is necessary in the interpretation of results<sup>[16]</sup>. The relevance of a single identified biomarker might not be high, but it could be that systematic up- or down-regulation in specific groups of molecules (such as certain lipids in the current study) indicates a biologically relevant metabolite type. Another drawback of metabolomics is that a large proportion of spectral peaks are still unknown, and consequently more effort has to be placed on the compilation of standardised metabolite libraries<sup>[16,23]</sup>. Considering the current study setting, one obvious weakness is the small number of subjects. On the other hand, it is highly encouraging to see that IBS patients and healthy controls were fairly well differentiated, even with this limited sample size. Moreover, it would have been interesting to investigate whether differences between IBS and healthy subjects could also be observed in metabolic profiles from non-invasive tissues, such as faecal material or blood, as these are more easily obtained in clinical settings.

Taken together, our results suggest significant differences in the global mucosal metabolic profile between IBS patients and healthy controls. The current study is the first to attempt to identify colonic mucosal metabolites typical for IBS using a high-throughput

metabolomic approach. In this study, IBS was particularly characterised by an upregulation of specific lipid groups, such as lysophosphatidylcholines and ceramides. These lipid species have been associated with the modulation of pain sensitivity and gut permeability, and our data thus indicate that these molecules might be involved in the pathophysiology of visceral pain and gut barrier dysfunction associated with IBS.

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## COMMENTS

### Background

Irritable bowel syndrome (IBS) is the most common diagnosis in gastroenterology. The syndrome is classified as functional, and no biological marker exists for IBS. The precise aetiology and pathophysiology is not fully known, and this might partly explain why pharmacotherapy is considered rather ineffective.

### Research frontiers

More data on the molecular abnormalities in IBS are required to better understand the mechanism behind the emergence of symptoms, and to be able to treat the patients in a safe and efficient way.

### Innovations and breakthroughs

This study characterises the differences between colonic mucosa from IBS patients and healthy controls using two high-throughput metabolomic platforms, UPLC/MS based lipidomics and GC × GC-TOF based metabolomics. Metabolomics is a useful tool for investigating diseases with complex or unknown backgrounds, because it is possible to simultaneously measure and model a huge number of metabolites. Data indicated multiple differences between IBS mucosa and healthy mucosa, thus providing novel information about the pathophysiology of IBS. An increase in the IBS group of several lipid species, such as lysophospholipids and ceramides, was the major difference observed.

### Applications

By better understanding the mucosal abnormalities behind IBS, it might be possible to improve the diagnosis and therapy of patients.

### Peer review

This article is surely innovative, not only in the hypothesis, but also in the methodology. This could be a hot article if could be popularized appropriately. A review of the current literature indicates that the present article is a pioneer in its field.

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## Wireless capsule endoscopy in detecting small-intestinal polyps in familial adenomatous polyposis

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### Abstract

**AIM:** To detect the prevalence of small bowel polyps by wireless capsule endoscopy (WCE) in patients with familial adenomatous polyposis (FAP).

**METHODS:** We examined prospectively 14 patients with FAP to assess the location, size and number of small-intestinal polyps. Patients' age, sex, years of observation after surgery, type of surgery, duodenal polyps and colorectal cancer at surgery were analyzed.

**RESULTS:** During WCE, polyps were detected in 9/14 (64.3%) patients. Duodenal adenomatous polyps were found in nine (64.3%) patients, and jejunal and ileal polyps in seven (50%) and eight (57.1%), respectively. The Spigelman stage of duodenal polyposis was associated with the presence of jejunal and ileal polyps. Identification of the ampulla of Vater was not achieved with WCE. Importantly, the findings of WCE had no immediate impact on the further clinical management of FAP patients. No procedure-related complications were observed in the patients.

**CONCLUSION:** WCE is a promising noninvasive new method for the detection of small-intestinal polyps. Further investigation is required to determine which phenotype of FAP is needed for surveillance with WCE.

### INTRODUCTION

Familial adenomatous polyposis (FAP) is a dominant inherited syndrome with an incidence of 1 in 11 000. It is caused by an alteration of the FAP (APC) gene that is located on chromosome 5q21, which causes multiple disorders of the development of the ecto-, endo- and mesoderm. The syndrome is characterized by the presence of adenomatous polyps in the gastrointestinal tract, mainly in the colon, rectum and duodenum, with a demonstrated adenoma-carcinoma sequence<sup>[1-3]</sup>. The duodenum is characterized by the presence of adenomas in 80% of patients with FAP and the development of periampullary cancer in 4%<sup>[4,5]</sup>. In patients who have undergone colectomy, periampullary cancer is the main cause of death. Between five and 10% of FAP patients die from upper gastrointestinal cancer, which is frequently periampullary in origin. In an attempt to prevent malignancy, a screening program appears to be mandatory to detect particularly those patients most at risk of developing the disease. Therefore, endoscopic surveillance of the second part of the duodenum with side-viewing endoscopy is advised. Since it was introduced in 2000, wireless capsule endoscopy (WCE) has opened the way for the noninvasive and painless test of the entire small intestine, thereby becoming the gold standard for endoscopic evaluation of the small bowel

in several clinical situations, including surveillance of polyposis syndromes. There have been only a few studies that have evaluated the utility of WCE in detecting small-intestinal polyps in patients with FAP<sup>[6-12]</sup>.

The aim of our prospective study was to investigate the diagnostic yield of WCE in being able to detect adenomatous polyps in a Greek FAP cohort, and to establish potential risk factors for small-bowel polyp development for a more targeted surveillance with WCE.

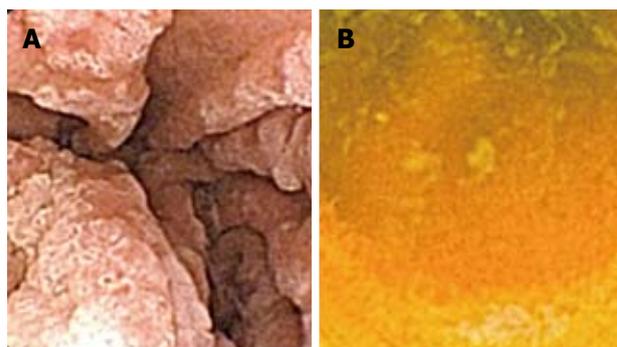
## MATERIALS AND METHODS

We performed an open prospective, non-randomized clinical trial from September 2007 to September 2008, which evaluated the use of WCE in FAP patients. The study was conducted in accordance with good clinical practice, as set forth by the Helsinki agreements and their later amendments. The study was approved by our hospital Ethics Committee and informed consent was obtained from all patients.

We included male and female patients, aged 18-70 years, who were referred to our clinic. Patients excluded were those with severe swallowing disorders, implanted cardiac pacemaker or other electronic devices, pregnant women, patients with a clinical suspicion of small-bowel obstruction/pseudo-obstruction, strictures or fistulas, and children under 10 years old<sup>[13,14]</sup>.

The following information was gathered from patients' records: age, sex, diagnostic [endoscopy, small-bowel radiography, computed tomography (CT)] and surgical procedures (type of colectomy and time of surgery) before WCE. All the procedures were performed on an outpatient basis, in the morning, after an overnight fast. Bowel preparation was performed with 4 L polyethylene glycol solution given 15 h before the procedure. Patients were allowed to drink clear fluids 2 h after capsule ingestion. Furthermore, the patients were able to maintain their normal activities while the capsule was passing through the digestive tract. Patients returned to the hospital 8 h after capsule ingestion. The registration device and the antennae were disconnected from the patient and a questionnaire about symptom occurrence and overall satisfaction with the procedure was completed. On each of the 2 d following the procedure, a telephone call was made to inquire about any symptoms and to confirm that the capsule had been expelled. In view that the major risk from WCE is capsule retention or impaction, all patients were instructed to contact the study staff should they develop any gastrointestinal symptoms during or after WCE.

Capsule videorecordings were reviewed by a single experienced endoscopist (Katsinelos P) who previously had performed more than 200 WCE procedures. A polyp was defined as a discrete mass of tissue that protruded into the bowel lumen. The location of small-bowel polyps was approximately estimated as duodenal (Figure 1A), jejunal, or ileal (Figure 1B), according to the timing of polyp appearance after entrance of the capsule to the duodenum, the total small-bowel passage time, and the endoscopic appearance of the small-



**Figure 1** Wireless capsule endoscopy (WCE) view. A: Large and small mushroom-shaped adenomas in the distal duodenum; B: Small ileal polyp.

intestinal mucosa. Entrance to the duodenum is easy to detect because it begins just after the pylorus, which can be identified easily. The location of small-bowel polyps was estimated by analyzing the WCE transit time between pylorus passage and ileocecal valve or pouch ileostoma. The duodenum was designated to be the small bowel that was visualized during the first 15 min after the capsule exited the pylorus, while the jejunum and ileum were designated to be the small bowel that was visualized after < 50% and > 50% of small-bowel transit time, respectively. Moreover, the prominent folds and high narrow villi characterized the jejunum, while fewer folds and shorter villi were observed in the ileum. WCE allows only an approximate estimation of the size of polyps, therefore, based on previous experience<sup>[11]</sup>, we classified polyps as small or large, using an open pylorus orifice (diameter 10 mm) as a reference for polyp size estimation. Small and large polyps were classified with a diameter < 10 mm and > 10 mm, respectively.

Following WCE, conventional endoscopy was performed within 2 wk in all patients. Standard duodenoscopy up to the second part of the duodenum was performed with a forward-looking gastroscope and a side-viewing duodenoscope, on an outpatient basis. To reduce motility artifacts, 20 mg butylscopolamine were administered intravenously. Biopsies and polypectomies were performed for staging of duodenal disease according to the Spigelman classification (Table 1)<sup>[15]</sup>.

The primary end point of the study was to identify the number and size of small-bowel polyps in each patient, and the secondary end point was the impact of WCE findings on the management of the patients.

## RESULTS

Fourteen patients (9 men, median age 34 years, range: 22-56 years) with FAP were recruited. Eight patients had undergone total proctocolectomy with ileal-pouch-anal anastomosis, four had undergone ileorectal anastomosis, and two were examined before colectomy (Table 2).

Endoscopic investigation of the entire length of the small bowel was achieved in all patients. The quality was considered as good except for one case in which food debris in the duodenum, jejunum and ileum made reading the film very difficult; in the last case, the procedure

**Table 1** Spigelman classification of duodenal polyposis (adenomas in FAP)<sup>[15]</sup>

	Number of points		
	1P	2P	3P
Number of polyps	1-4	5-20	> 20
Polyp size (mm)	1-4	5-20	> 10
Histology	Tubulous	Tubulovillous	Villous
Dysplasia	Mild	Moderate	Severe
Stage	Spigelman score		
0	0		
I	1-4		
II	5-6		
III	7-8		
IV	9-12		

**Table 2** Clinical characteristics of the 14 patients with FAP studied with WCE

Patient No.	Sex	Age (yr)	Time of surgery before WCE (yr)	Type of surgery	No. of colon polyps	Colon cancer
1	M	54	4	IPAA	> 1000	No
2	F	23	BS	BS	> 100	No
3	M	53	5	IPAA	> 100	No
4	F	27	5	IPAA	> 100	No
5	M	28	4	IPAA	> 100	No
6	M	22	BS	BS	> 100	No
7	M	53	2	IRA	> 1000	No
8	F	26	3	IPAA	> 100	No
9	M	56	18	IRA	> 1000	No
10	F	29	10	IPAA	> 100	No
11	M	54	4	IPAA	> 1000	Yes
12	M	36	15	IRA	> 100	No
13	F	41	17	IRA	> 100	No
14	M	32	7	IPAA	> 100	No

FAP: Familial adenomatous polyposis; WCE: Wireless capsule endoscopy; IPAA: Ileal pouch-anal anastomosis; IRA: Ileorectal anastomosis; BS: Before surgery.

was repeated and the patient was asked to avoid food intake prior to the examination. Mean gastric and small-bowel transit time were 36 (range: 12-58) and 256 (range: 128-360) min, respectively. No abnormal additional findings were identified. Overall, 81 polyps, mainly small (96.3%), were detected by WCE. The presence, size and location of duodenal, jejunal and ileal polyps were related to the Spigelman stage of duodenal polyposis and age of the patient, but not sex (Table 3). None of the five young FAP subjects with Spigelman stage 0 had small-bowel polyps detected. Three large sessile polyps were found in the duodenum in one patient with Spigelman stage IV disease. All other polyps detected were small (Table 3). WCE was inferior diagnostically to standard duodenoscopy and gastroscopy regarding the second part of the duodenum and especially the ampullary region. The capsule technique could not identify the papilla of Vater in any of our patients and four small ampullary adenomas were missed by WCE as compared with duodenoscopy. Endoscopic polypectomy of duodenal adenomas was performed in five patients, and biopsies were taken from the rest of the patients. Histological

**Table 3** Distribution and size of polyps according to Spigelman stage as assessed by WCE

Patient No.	Sex	Spigelman stage	Duodenal	Jejunal	Ileal	Rectal stump
1	M	IV	13	3	5	No
2	M	0				
3	F	II	3	2	4	No
4	M	I	3		2	No
5	F	I	2	1	3	No
6	M	0				No
7	M	III	4	4	3	Yes
8	F	0				Yes
9	M	III	7	3	2	Yes
10	M	0				No
11	M	II	4	1	2	Yes
12	M	I	3			No
13	F	I	4	1	2	No
14	M	0				No

examination of the specimens confirmed the diagnosis of tubular or tubulovillous adenomas with low-grade dysplasia in one case with large polyps. We detected small ileal white-colored polyps with a normally appearing mucosal surface in two young patients (both Spigelman stage 0), and we classified these lesions as lymphoid hyperplasia, which occurs commonly in the terminal ileum and rectum associated with FAP, especially in young patients<sup>[16]</sup>.

All the patients described the procedure as comfortable and were willing to repeat it had it been deemed necessary. Difficulty/inability to swallow the capsule or clinically significant complications, including symptomatic capsule retention and aspiration, did not occur during the procedure. Five patients previously had undergone enteroclysis, and although a comparison of the two methods was not within the scope of this study, as there was a significant time lapse between them, all the patients preferred WCE when they were asked to compare it with enteroclysis. All patients reported no pain or discomfort when contacted 1 wk after the WCE examination.

## DISCUSSION

Small-intestinal adenomas can occur in FAP patients, but their prevalence varies, depending of the modality used for their detection<sup>[17]</sup>. The advent of WCE in 2000 has changed noticeably the diagnosis and management of numerous diseases of the small intestine, including polyps associated with FAP<sup>[14]</sup>. Our study shows that WCE is able to detect even small polyps in the entire small intestine in subjects with FAP. We found jejunal and ileal polyps to be common. The frequency and number of polyps and the length of small bowel involvement was found to increase with Spigelman classification (Table 3). All polyps were small except for three in the duodenum. These findings were similar to those previously reported by other studies<sup>[6-11]</sup>, although Iaquino *et al.*<sup>[12]</sup> have found the presence of duodenal adenomas to be the only clinical feature predictive of small-intestinal adenoma, but not associated with Spigelman

stage. We cannot define the true sensitivity of WCE for detection of small-bowel adenomas because the lack of visualization of the entire small-bowel mucosa by WCE leads to underestimation of polyp burden. To achieve this, we would have to compare the performance of WCE with that of the criterion standard of surgical enteroscopy. However, given the invasiveness and the high morbidity rate of the latter procedure, such a study would be extremely difficult to perform. The advent of double or single balloon enteroscopy of the small bowel may have opened a new avenue to gain less invasive access, even to polyps located in the distal small bowel. Double balloon enteroscopy appears to be equivalent to an intraoperative enteroscopy for scrutiny of small-intestinal polyps in FAP<sup>[17]</sup>. The region around the papilla of Vater was not visualized in any of our patients, which calls for the mandatory use of side-view duodenoscopy for staging duodenal disease.

Magnetic resonance enteroclysis combines the advantages of cross-sectional resonance with those of the volume challenge of conventional enteroclysis in the recognition and characterization of small-bowel-wall abnormalities, including initial tumors. There are few promising reports about the role of magnetic resonance enteroclysis and CT enteroclysis in the diagnostic algorithm of small-bowel neoplasms<sup>[18]</sup>. Whether the use of WCE in combination with these new diagnostic techniques will lead to earlier diagnosis of small-intestinal polyps in FAP patients remains to be elucidated in the future.

We observed no complications from WCE in our study. Other reports of WCE performed in individuals with FAP also have failed to detect any complications<sup>[6-12]</sup>.

Forward and side-viewing endoscopic surveillance for gastric and duodenal/periapillary neoplasia is recommended for all individuals with FAP<sup>[19,20]</sup>. The frequency of surveillance should be based on the Spigelman classification of duodenal polyposis<sup>[19,20]</sup>. However, the implication of jejunal and ileal adenomas in FAP is unknown. The risk of cancer distal to the duodenum in FAP has been reported much more rarely than that of duodenal and periampillary carcinoma<sup>[21]</sup>. The lack of data may rely on the fact that patients with FAP usually are not studied because of the low incidence of non-duodenal small-bowel cancer<sup>[21]</sup>. Therefore, should a search for small bowel adenomas with WCE be performed in all patients with FAP? Keeping in mind the high cost of WCE, identification of a subset of FAP patients who might be at the highest risk for developing small bowel tumor is desired. The analysis of germline APC gene mutation was not available in our patients, to compare with WCE findings. However, as reported by other investigators, the incidence of small-intestinal adenomas is correlated with mutations found in exon 15<sup>[22]</sup>. Mutations in this exon traditionally have been associated with a more aggressive phenotype<sup>[22,23]</sup>. The identification of genotypic factors that predict the phenotype of small-bowel adenomas is important. It has been suggested that WCE should be performed only in patients with exon 15 mutations<sup>[12]</sup>, thereby requiring

relative WCE surveillance. This approach may allow for a more cost-effective evaluation of FAP patients. Obviously, the current genotype-phenotype correlation must be confirmed in a larger cohort of FAP patients.

The frequency of WCE surveillance of jejunal and ileal adenomatous polyps in patients with FAP remains unknown. The detection of these small polyps in our study and previous studies had no immediate impact on the clinical management, other than establishing further surveillance intervals in these patients<sup>[6-12]</sup>. The tendency is for WCE to become the standard imaging modality for small-bowel surveillance, since Spigelman stage III and IV patients have a high burden of small-intestinal adenomas on WCE (Table 3). With the potential exception of the mentioned high risk of FAP patients developing small-bowel cancer, we recommend surveillance every 3-5 years in these patients; despite more data on the prevalence of small-bowel polyps in patients with advanced stage (III or IV) duodenal polyposis being needed to understand the utility of WCE in these groups. The small number of polyps observed in our FAP patients with Spigelman stage 0-II disease (Table 3) is in accordance with other studies<sup>[6-8]</sup>. We agree with other investigators<sup>[6-10]</sup> recommendations that WCE is not useful for routine small-bowel surveillance in these patients. Although management of jejunal and ileal polyps has not as yet been well defined considering the adenomatous nature of polyps in FAP, it seems reasonable to remove these polyps that are easily accessible by endoscopy. Whenever endoscopic polypectomy cannot be performed, although there is not enough evidence to propose surgical resection, surveillance with WCE seems advisable.

In conclusion, WCE is noninvasive, safe and comfortable, and can be performed on an ambulatory basis in FAP patients. It is effective for the detection of small-bowel polyps, but larger studies are needed to define better the impact of WCE on the clinical outcome of FAP patients with small-intestinal polyps, to elaborate which mutant gene carries the highest prevalence of small-intestinal adenomas, and to decide the timing of surveillance and polypectomy treatment by double or single balloon enteroscopy.

## COMMENTS

### Background

Endoscopic surveillance of the duodenum and periampillary area is recommended in patients with familial adenomatous polyposis (FAP), because 4% of patients develop cancer. However, the significance of the presence of jejunal and ileal polyps in patients with FAP is unknown.

### Research frontiers

FAP is a dominant inherited syndrome characterized by the presence of adenomatous polyps in the gastrointestinal tract, with a demonstrated adenoma-carcinoma sequence. In the present study, the authors investigated the diagnostic utility of wireless capsule endoscopy (WCE) in detecting small intestine polyps in a Greek FAP cohort.

### Innovations and breakthroughs

Few studies have evaluated the utility of WCE in detecting small-intestinal polyps and their clinical significance in patients with FAP. The rate of detection of small polyps in our patients was high but had no immediate impact on clinical management, other than establishing further surveillance intervals in these patients.

### Applications

This study represents a new role for WCE in the examination of the small intestine in FAP patients and emphasizes the need for a highly targeted surveillance based on Spigleman classification.

### Terminology

WCE is a technology that uses a swallowed video capsule to take photographs of the inside of the esophagus, stomach, and small intestine. Since it was introduced in 2000, it has become the gold standard for endoscopic evaluation of the small bowel in several clinical situations, including surveillance of polyposis syndromes.

### Peer review

This is an interesting observational study of the role of WCE in screening for small-intestinal polyps in a small cohort of patients with established FAP.

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BRIEF ARTICLE

## Sphincter of Oddi dysfunction: Psychosocial distress correlates with manometric dyskinesia but not stenosis

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Psychosocial distress may help to explain the finding of SO dyskinesia in some postcholecystectomy patients.

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**Key words:** Gender; Functional gastrointestinal disorders; Psychosocial distress; Sphincter of Oddi dyskinesia

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### Abstract

**AIM:** To compare postcholecystectomy patients with Sphincter of Oddi (SO) dyskinesia and those with normal SO motility to determine the psychosocial distress, gender and objective clinical correlates of dyskinesia, and contrast these findings with comparisons between SO stenosis and normal SO motility.

**METHODS:** Within a cohort of seventy-two consecutive postcholecystectomy patients with suspected SO dysfunction, manometric assessment identified subgroups with SO dyskinesia ( $n = 33$ ), SO stenosis ( $n = 18$ ) and normal SO motility ( $n = 21$ ). Each patient was categorized in terms of Milwaukee Type, sociodemographic status and the severity of stress-coping experiences.

**RESULTS:** Logistic regression revealed that *in combination* certain psychological, sociodemographic and clinical variables significantly differentiated SO dyskinesia, but not SO stenosis, from normal SO function. Levels of psychosocial stress and of coping with this stress (i.e. anger suppressed more frequently and the use of significantly more psychological coping strategies) were highest among patients with SO dyskinesia, especially women. Higher levels of neuroticism (the tendency to stress-proneness) further increased the likelihood of SO dyskinesia.

**CONCLUSION:** A motility disturbance related to psy-

### INTRODUCTION

Sphincter of Oddi (SO) dysfunction (SOD), characterized by recurring episodes of severe biliary-like abdominal pain following cholecystectomy, is one of a number of functional disorders of the biliary tract<sup>[1]</sup>. However, based on SO manometry, which is the “gold standard” for the diagnosis of SOD, the condition is a heterogeneous one, currently considered within the context of the functional disorders, but known to include both stenotic and non-stenotic (functional) groups. Thus, SO dyskinesia can be differentiated from SO stenosis, a structural defect of the sphincter orifice.

Unlike other functional gastrointestinal disorders (FGIDs)<sup>[2]</sup>, the psychosocial and gender concomitants of SOD have received little attention. In particular, psychosocial issues specifically relating to SO dyskinesia have not been addressed. Although two previous studies have implicated psychosocial factors in the genesis of symptoms in SOD, patients were not differentiated manometrically<sup>[3,4]</sup>. A more recent study found that health-related quality of life is impaired, and abuse histories are common, in SOD patients, but again patients were not differentiated manometrically<sup>[5]</sup>.

Given the strength of the relations between psychosocial disturbance and alterations in gastrointestinal transit, motor activity and sensitivity previously documented

in irritable bowel syndrome (IBS)<sup>[6-12]</sup>, our central hypothesis was that a similar association may exist with respect to SO dyskinesia. While the term stress or chronic stress can be viewed as a generic or umbrella term to include all features of a social stressor situation including psychophysiological effects, we have applied the term psychosocial distress to represent a person's experience of and transactions with a stressor situation including on-going attempts to gain or regain emotional and psychological equilibrium/control.

The aims of the present study, therefore, were to determine, among subgroups of postcholecystectomy patients exhibiting SO dyskinesia, SO stenosis ( $\pm$  dyskinesia) or normal SO motor activity: (1) whether levels of psychosocial distress are higher in patients with manometrically proven SO dyskinesia than in those with normal SO motor activity, and (2) whether higher levels of psychosocial distress are specific to SO dyskinesia (that is, relative to patients with SO stenosis), and (3) whether the presence of objective clinical criteria, according to the Milwaukee classification<sup>[13]</sup>, influences these associations. We chose specific psychological assessments of emotional state, personality and coping behaviour based on previously documented associations in other FGIDs<sup>[6-12]</sup>. Thus, during an individual's exposure to high levels of threat, powerful emotions such as fear, anger, anxiety and frustration are aroused and coping strategies, strongly influenced by personality and past experiences, are employed in an attempt to reduce the impact of both the emotions and the situation. A high level on any cluster of these psychological dimensions suggests high levels of psychosocial distress.

## MATERIALS AND METHODS

### Patients

The study population comprised seventy-two consecutive postcholecystectomy patients with biliary-like pain consistent with SOD [64 women, mean age 45 (SD  $\pm$  12) years], referred to the Gastrointestinal Investigation Unit for SO manometry. The presence of recurrent biliary-like pain fulfilling the criteria for SOD was confirmed from responses on the Bowel Disease Questionnaire (BDQ)<sup>[14]</sup> and defined according to the Rome criteria<sup>[1]</sup>, namely - episodes of pain in the right upper quadrant or epigastrium, rated by the patients as severe or very severe, steady and lasting from 30 min to 6 h. In all patients, organic disease had been excluded on the basis of normal physical examination, negative screening blood tests, negative gastroduodenoscopy and upper abdominal ultrasound or computed tomography scan, and the absence of calculi and strictures as demonstrated by endoscopic retrograde cholangiopancreatography (ERCP). Approval for the procedures was given by the Medical Research Ethics Committee of the Royal North Shore Hospital, and all subjects gave written informed consent.

### Clinical assessment

SO Milwaukee Type was determined according to Hogan *et al.*<sup>[13]</sup>, with patients sub-grouped into patients with objective clinical criteria (Types I & II), including

abnormalities of the biliary tree at cholangiography or abnormalities in hepatic biochemistry associated with episodes of pain, and patients without objective criteria (Type III) but with recurrent episodes of biliary-like pain. Thus, biliary-type I patients exhibited elevated liver biochemistries documented on two or more occasions, delayed contrast drainage, and a dilated common bile duct with a corrected diameter equal to or greater than 12 mm at ERCP; biliary-type II patients exhibited only one or two of the above criteria; while biliary-type III patients exhibited none of the above criteria<sup>[1]</sup>.

### SO manometry

Manometry of the SO was performed in standard fashion according to the technique of Toouli *et al.*<sup>[15]</sup>, using an Olympus JFIT10 duodenoscope and a triple-lumen catheter with inner lumen diameters each of 0.5 mm, an outer diameter of 1.7 mm and side holes radially orientated 2 mm apart (Wilson-Cook Medical, Winston-Salem, NC, USA). SO manometric tracings were analyzed by two experienced observers, blinded to the results of the symptom questionnaire. The following parameters<sup>[15,16]</sup> were determined: basal sphincter pressure, peak sphincter pressure, and phasic wave contractile frequency and propagation. Abnormalities of these parameters were defined as values outside normal ranges established previously using an identical recording technique<sup>[15]</sup>: basal pressure < 40 mmHg, contractile frequency < 8/min, and proportion of retrograde contractions < 50%. Complete inhibition of phasic contractions following cholecystokinin (CCK) was considered a normal CCK response. Failure of such a response, including a "paradoxical response" of the sphincter to CCK (i.e. increase in either the basal pressure and/or phasic contractions)<sup>[15]</sup>, was considered an abnormal response. SO manometric recordings were classified<sup>[15,17,18]</sup> as either: (1) sphincter dyskinesia, defined as an abnormally high basal pressure resolving after CCK, or an abnormally high phasic contractile frequency, and/or an elevated proportion of retrograde contractions, and/or an abnormal response to CCK in the absence of sphincter stenosis or (2) sphincter stenosis, defined as an abnormally high basal pressure persisting after CCK irrespective of the presence of some features of dyskinesia or (3) normal sphincter motor function. The presence of SO stenosis had hierarchical importance over any other feature of dyskinesia in stratifying patients.

### Psychosocial assessments

Psychometric measures assessed sociodemographic and psychological factors. The following data were collected prior to the SO manometry.

**Sociodemographic data:** Sex, age, marital status, highest education level, current employment status (i.e. whether working full-time, part-time or unemployed) and highest occupation level of self and father.

**Emotional distress/mood state:** Depression - in particular the affective component of a depressed mood state - was assessed using The Centre of Epidemiological Studies

Depression Scale<sup>[19]</sup> and state anxiety using the Spielberger State and Trait Anxiety Inventory (STAI)<sup>[20]</sup>. Responses on these scales reflect complementary dimensions of psychosocial distress arising from stressful life situations.

**Personality traits:** Trait anxiety (the tendency to anxious states) was also assessed from responses on the STAI<sup>[20]</sup>, and neuroticism (high scores reflect a tendency to stress-proneness and to excessive worry) and extroversion (orientation to things external or internal) were assessed using the Eysenck Personality Inventory<sup>[21]</sup>.

**Coping style:** The Defense Style Questionnaire (DSQ 40) measured the tendency to use emotion-focused coping defenses or strategies to reduce emotional distress classified as mature (e.g. using humor, suppression, anticipation, sublimation), immature (e.g. using denial, acting out, being passive aggressive) and/or neurotic [e.g. using pretence (pseudo-altruism), idealization, undoing, reaction formation]<sup>[22]</sup>. Normative and reliability data from patient and non-patient groups show these factors to have the internal consistency and the temporal stability of a trait measure - the mature dimension proving to be the primary discriminating factor<sup>[23]</sup>.

**Emotional suppression/expression:** The Courtauld Emotional Control Scale<sup>[24]</sup> assessed the tendency to suppress unwanted emotions such as anger and anxiety and the Anger Expression Scale<sup>[25]</sup> assessed the tendency to hold in anger (anger-in) to express anger (anger-out) and to control and/or resist becoming angry (anger-control). Anger-in and anger-out are empirically independent, factorially orthogonal dimensions<sup>[26]</sup>. Differences in the physiological effects of suppression *vs* expression of powerful emotions on autonomic, neuroendocrine and digestive functioning underlie the inclusion of these scales.

**Locus of control of behavior:** This scale<sup>[27]</sup> assessed the extent to which a person believes that personal efforts more than external factors can achieve a positive outcome.

Each of the above measures has established reliability and validity and relevance with respect to the investigation of the hypothesized links between psychosocial distress and the development of SO dyskinesia.

### Statistical analysis

Univariate and multivariate analyses were performed to compare the SO dyskinesia subgroup, and for comparison purposes the SO stenosis subgroup, with the normal SO motility subgroup, on a range of clinical, sociodemographic and psychological factors<sup>[28]</sup>. The relation of individual continuous variables such as age, sociodemographic and psychosocial factors was assessed by logistic regression, while  $\chi^2$  analyses were performed to determine any sex or clinical differences with respect to SO subgroups. Using a Stepwise regression - a model-building procedure by which sample data (not the investigator) determines order of entry into the model - an optimal subset of clinical, sociodemographic and psychological factors that had independent, statistically significant effects in relation to SO subgroups was then identified.

**Table 1** Clinical features of the post-cholecystectomy patient subgroups of SO dyskinesia, SO stenosis with or without dyskinesia and normal SO motility (mean  $\pm$  SD) *n* (%)

	SO dyskinesia ( <i>n</i> = 33)	SO stenosis ( <i>n</i> = 18)	Normal SO motility ( <i>n</i> = 21)
Age (yr)	45 $\pm$ 10	44 $\pm$ 13	48 $\pm$ 16
Gender: % female	91	89	86
SOD			
Type I	2 (6)	2 (11)	0 (0)
Type II	19 (58)	12 (67)	14 (67)
Type III	12 (36)	4 (22)	7 (33)

*P* > 0.05 for all comparisons. SO: Sphincter of Oddi; SOD: SO dysfunction.

**Table 2** Clinical, psychosocial and demographic variables which significantly differentiated between SO dyskinesia and normal SO motility in postcholecystectomy patients: logistic regression model of best fit

	Effect size and significance		
	B	SE	<i>P</i> -value
Female sex	5.023	1.981	0.01
Frequent suppression of anger <sup>1</sup>	2.399	1.239	0.05
Frequent use of mature coping strategies <sup>1</sup>	0.232	0.085	0.006
Frequent use of immature coping strategies <sup>1</sup>	0.03	0.057	0.026
Infrequent use of neurotic coping strategies <sup>1</sup>	0.004	0.266	0.091
Higher occupational status of father	1.475	0.588	0.01
Neuroticism (stress-proneness)	4.901	4.901	0.02

<sup>1</sup>See text for further details; Model variance explained = 36.3%; B: Regression coefficient.

## RESULTS

SO manometry revealed evidence of SO dyskinesia (with no stenosis) (*n* = 33), SO stenosis [*n* = 18, some with concurrent SO dyskinesia features (*n* = 8)], or normal SO motility (i.e. no SO dyskinesia or SO stenosis (*n* = 21)). The three groups did not differ with respect to age, gender, or any of the independent clinical variables (Table 1), sociodemographic or psychological variables assessed.

When logistic regression was used to determine the effect of combinations of independent variables in relation to manometric outcome, however, a particular subset of clinical demographic and psychological variables significantly differentiated SO dyskinesia from normal SO motility (Table 2). In this model, variables positively associated with SO dyskinesia were being female, and the psychological variables of frequently suppressing anger, frequently using stress coping strategies, and neuroticism (the propensity to an overly anxious response to stressors). The negative association with abnormal motility (or positive association with normal motility) was related to the sociodemographic background, namely a lower occupational status of the patient's father. The variables of anxiety and depression were not associated with SO dyskinesia, nor were there positive associations with objective clinical criteria.

In contrast, duplicate analyses revealed no significant differences between patients with SO stenosis and those with normal SO motor activity, on psychosocial distress and gender and also on objective clinical criteria (data

not shown). This was the case whether the SO stenosis group included patients with ( $n = 18$ ) or without SO dyskinesia features ( $n = 10$ ).

## DISCUSSION

The novel finding in this study was the identification of a cluster of psychosocial and gender factors which together differentiate postcholecystectomy patients with manometric SO dyskinesia - but not those with SO stenosis - from those with normal SO motor activity. In comparison with the normal motor activity group, patients with SO dyskinesia, especially women, used significantly more stress-management strategies that were problem-focused (i.e. they suppressed anger more frequently) and emotion-focused (i.e. they frequently used mature and immature but not neurotic coping strategies), findings which implicitly represent on-going attempts to reduce psychosocial stress. While the prominence of the emotion anger (but not depression or anxiety) in the stress-coping profile reveals the potent nature of the psychosocial distress, for each individual the effectiveness of the particular range of coping strategies used to reduce emotional distress (and the associated physiological responses) is unclear. Thus, although patients with SO dyskinesia displayed a preference for strategies such as anticipation, humor, suppression, and sublimation (especially adaptive in the short term), other less effective (immature) strategies such as passive aggression and denial were also employed from time to time. Neurotic coping was rare in this group.

Our other major finding was that the biopsychosocial model of SO dyskinesia described above was, in essence, independent of objective clinical non-manometric criteria. This was despite the fact that the distribution of our patients with manometric evidence of SOD according to the Milwaukee classification was generally in keeping with that of other published reports: half of our patients with Type I exhibited stenosis, 61% of Type II exhibited sphincter dyskinesia, and 75% of Type III exhibited sphincter dyskinesia. The fact that our patients with Type III SOD exhibited a higher overall proportion of manometric dysfunction than published reports may reflect the fact that we employed CCK provocation, which is not now routinely undertaken in Units performing SO manometry. Moreover, our use of CCK considerably strengthens the distinction between SO hypertension due to sphincter hypertonicity and that due to a true fixed stenosis.

The significant association between psychosocial distress and sphincter dyskinesia is a new finding with respect to the sphincter of Oddi. It is, however, conceptually consistent with reports of similar links between stress and alterations in gut motility and sensation in patients with FGID categorized as IBS<sup>[29,30]</sup>. Studies using measures of stress-coping behavior similar to those used in the present study, suggest that higher levels of stress correspond with an increasing degree of dysmotility (and heightened perceptual sensitivity to mechanical distension) in the jejunum of women with

IBS<sup>[11]</sup>, and with the severity<sup>[9,12]</sup> and the extent (number of regions) of gut stasis<sup>[12]</sup>, especially gastric stasis, in mixed gender groups. Similar relations have been reported with respect to functional gut symptomatology. For example, higher levels of distress, assessed as outlined above, and also an objective measure of life stress, namely chronic stressor threat, are associated with a larger number of FGID syndromes<sup>[31]</sup>, with the overall intensity of FGID symptoms, and with the direction and extent of change in symptom intensity over time<sup>[7]</sup>. Also for patients with FGID, anger provoked in real life situations is the emotion which most strongly contributes to the net severity and extent of symptoms<sup>[31]</sup> and sensorimotor dysfunction<sup>[9,11]</sup>, while anger provoked in the laboratory inhibits antral motor activity in patients with these disorders in contrast to its enhancing effects on antral motor activity in healthy control subjects<sup>[32]</sup>. Consistent with all of these findings, the psychosocial distress model which described postcholecystectomy patients with SO dyskinesia in this study also included anger and female gender; these findings suggest both the potency of the stressful input on the one hand, and perhaps the more subtle and discriminating influence of sex hormones in SO motility on the other.

There is only very limited data available relating to psychosocial associations with SOD. Psychological disturbance has been implicated in one study<sup>[3]</sup> in the recurrence of biliary-type pain in some patients following cholecystectomy. In comparison to healthy controls, psychological factors assessed indirectly in terms of the number of concurrent multisystem gastrointestinal and non-gastrointestinal symptoms, was higher in patients with a diagnosis of SOD. However, patients were not differentiated manometrically in this study. In another report, psychological disturbance (anxiety, somatization, depression and obsessive-compulsive behavior) was found to be higher in patients with SOD Type III<sup>[4]</sup> than in other types. Interestingly, our findings did not confirm significantly higher levels of anxiety and depression in patients evaluated according to their manometric findings and not their Milwaukee criteria alone. In a longitudinal study, Jørgensen *et al.*<sup>[33]</sup> reported that psychological vulnerability (assessment prior to cholecystectomy in terms of the severity of multisystem somatic and neurotic symptoms) predicted failure to achieve a full recovery post-operatively, after controlling for age, sex, pre-operative pain characteristics, history of disease, type of surgery, histology and complications. The contribution of the present study is the notion that a stress-related sensorimotor dysfunction may help to explain the presence and the persistence of the syndrome for some patients with a diagnosis of SO dyskinesia, while high rates of recovery following endoscopic sphincterotomy in patients with manometric features of SO stenosis<sup>[16,34]</sup> suggest that fixed structural or anatomical defects may explain the syndrome in others. Indeed, an important feature of the stress-related sensorimotor dysfunction in this study is that it was determined in the absence of confounding influences arising from the presence of SO stenosis.

We are aware of the potential limitations of our

findings, especially the potential selection bias because patients with certain personality or mood-state characteristics may be more likely to seek medical attention than other patients, and also that recurrent pain may have influenced some of their responses to the various questionnaires. However, we sought to limit any such bias by also including patients with the same symptoms who had presented for medical care but were found to have sphincter stenosis. Moreover, as all patients reported intermittent episodes of pain as severe or very severe it was not feasible to relate the psychosocial measures to pain scores. Further studies will be required to confirm and extend these findings, as they are of potential clinical importance given that the psychological distress levels including clinical levels of anxiety and depression may be eminently treatable (e.g. with medication and/or psychotherapy). Although individual psychotherapies (e.g. biofeedback, cognitive-behavioral, psychodynamic, hypnotherapy), achieve reductions in emotional distress and symptom severity in patients with IBS, an integrated psychophysiological approach to the management of these disorders that is sensitive to the unique nature of each stressor situation would seem most likely to be helpful long term.

In summary, the close association found, for the first time, in this study between psychosocial distress and SO dyskinesia, but not between psychosocial distress and SO stenosis or normal SO motor activity, suggests that, for some patients with a diagnosis of SO dyskinesia, a stress-related motor dysfunction may help to explain the recurrence of their biliary-like symptoms following cholecystectomy. This is consistent with pathophysiological models of the FGIDs in general<sup>[35-37]</sup>.

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## COMMENTS

### Background

Sphincter of Oddi (SO) dysfunction (SOD) is one of a number of functional disorders of the biliary tract. It is known to include both stenotic and non-stenotic (functional) groups. Thus, SO dyskinesia can be differentiated from SO stenosis, a structural defect of the sphincter orifice. Unlike other functional gastrointestinal disorders, the psychosocial and gender concomitants of SOD have received little attention. In particular, psychosocial issues specifically relating to SO dyskinesia have not been addressed.

### Research frontiers

Because of the strong relationships between psychosocial disturbance and alterations in gastrointestinal transit, motor activity and sensitivity previously documented in irritable bowel syndrome, we hypothesized that a similar association may exist with respect to SO dyskinesia.

### Innovations and breakthroughs

This is the first study to identify a cluster of psychosocial and gender factors which together differentiate postcholecystectomy patients with manometric SO dyskinesia - but not those with SO stenosis - from those with normal SO motor activity. The findings suggest that a stress-related motor dysfunction may help to explain the recurrence of biliary-like symptoms following cholecystectomy. This is consistent with pathophysiological models of the functional gastrointestinal disorders in general.

## Applications

These findings are of potential clinical importance given that psychological distress levels including clinical levels of anxiety and depression may be eminently treatable (e.g. with medication and/or psychotherapy).

## Terminology

Sphincter of Oddi dysfunction: a disorder characterized by recurring episodes of severe biliary-like abdominal pain following cholecystectomy. Sphincter dyskinesia: an abnormally high basal pressure resolving after cholecystokinin (CCK), or an abnormally high phasic sphincter contractile frequency, and/or an elevated proportion of retrograde contractions, and/or an abnormal response to CCK in the absence of sphincter stenosis. Sphincter stenosis: an abnormally high basal pressure persisting after CCK irrespective of the presence of some features of dyskinesia.

## Peer review

It is a solid research, well-written paper with reasonable conclusion. Although there are limitations to the study, these are nicely outlined and discussed.

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BRIEF ARTICLE

## Balloon overtube-guided colorectal endoscopic submucosal dissection

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### Abstract

**AIM:** To evaluate the usefulness of a balloon overtube to assist colorectal endoscopic submucosal dissection (ESD) using a gastroscope.

**METHODS:** The results of 45 consecutive patients who underwent colorectal ESD were analyzed in a single tertiary endoscopy center. In preoperative evaluation of access to the lesion, difficulties were experienced in the positioning and stabilization of a gastroscope in 15 patients who were thus assigned to the balloon-guided ESD group. A balloon overtube was placed with a gastroscope to provide an endoscopic channel to the lesion in cases with preoperatively identified difficulties related to accessibility. Colorectal ESD was performed following standard procedures. A submucosal fluid bleb was created with hyaluronic acid solution. A

circumferential mucosal incision was made to marginate the lesion. The isolated lesion was finally excised from the deeper layers with repetitive electrosurgical dissections with needle knives. The success of colorectal ESD, procedural feasibility, and procedure-related complications were the main outcomes and measurements.

**RESULTS:** The overall *en bloc* excision rate of colorectal ESD during this study at our institution was 95.6%. *En bloc* excision of the lesion was successfully achieved in 13 of the 15 patients (86.7%) in the balloon overtube-guided colorectal ESD group, which was comparable to the results of the standard ESD group with better accessibility to the lesion (30/30, 100%, not statistically significant).

**CONCLUSION:** Use of a balloon overtube can improve access to the lesion and facilitate scope manipulation for colorectal ESD.

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**Key words:** Balloon overtube; Colorectal neoplasm; Early colorectal cancer; *En bloc* tumor excision; Endoscopic submucosal dissection; Laterally spreading tumor

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### INTRODUCTION

Endoscopic submucosal dissection (ESD) has evolved to become one of the therapeutic options for the treatment of early stage gastric cancers in Asian countries<sup>[1,2]</sup>. In Japan, ESD has been increasingly applied to various levels of the gastrointestinal tract and results of initial experiences in a few high volume endoscopy centers have demonstrated the technical feasibility of this unique and aggressive approach, even in the colon. However, the

number of institutions allowed to perform this procedure is still restricted because colorectal ESD is technically more challenging, and may carry a higher risk of perforation and the most common sequela, bacterial peritonitis<sup>[3-6]</sup>. Inherited anatomic variability in the colon such as a long tubal structure, folds, or looping in mobile segments of the colon attached to the mesentery may hinder any endoscopic intervention in the colon. A further challenge arises from the need for a longer colonoscope; this can increase procedural workloads due to the need for careful, intuitive manipulation of the tip of the scope during needle knife dissections of the paper thin submucosal tissue plane<sup>[7,8]</sup>. At our institution, the gastroscope is therefore often preferentially used even for colorectal ESD, despite the shorter scope length, for deep scope intubation.

Use of a balloon overtube in enteroscopy provides optimal traction on the intestinal wall, thereby facilitating scope intubation. By inflation and withdrawal of a balloon attached to the tip of the overtube, the intestinal wall can be pleated on the overtube. This balloon overtube-guided technique has enabled a standardized total enteroscopy and has also provided a shorter direct access to the innermost locations in the gastrointestinal tract<sup>[9-11]</sup>. In addition, this approach has facilitated various interventions in the small intestine including biopsy, hemostasis and most recently polypectomy and endoscopic mucosal resection (EMR). Based on these results in the small intestine, we postulated that the a balloon overtube could form an ideal platform for colorectal ESD<sup>[12,13]</sup>.

We used a balloon overtube as an endoscopic channel and platform for colorectal ESD in cases in which access to the lesion with a gastroscope was difficult. Here we review the results of colorectal ESD in our institution to evaluate whether the balloon overtube-guided technique could improve access and scope manipulation during colorectal ESD.

## MATERIALS AND METHODS

### Patients

From October 2008 to March 2009 we performed colorectal ESD in 45 patients. The mean age of the patients was 70.7 years (range, 58 to 83 years). Indications for colorectal ESD were: 1. laterally spreading tumors (LST) over 20 mm in size, 2. lesions evaluated as being difficult to remove *en bloc* regardless of lesion size, e.g. local residual recurrent tumors after endoscopic removal and flat or depressed mucosal lesions. Histopathology of the lesions was preoperatively confirmed as adenoma or cancer with magnifying endoscopy or biopsy.

In all cases, access to the lesion was preoperatively evaluated using a therapeutic gastroscope (GIF Q260-J, Olympus, Tokyo, Japan). When circumferential access to the lesion with the tip of the endoscope was difficult, the access was considered difficult with a standard gastroscopic approach and the lesion was indicated for balloon overtube-guided ESD.

### Instrumentation and ESD procedure

**ESD procedure with a gastroscope:** A transparent cap (D201-11802; 2 mm Olympus, Tokyo, Japan) fitted thera-

peutic gastroscope (GIF-Q260-J, Olympus, Tokyo, Japan) was used for the conventional ESD group. The gastroscope is equipped with a water jet system, which supplies a continuous jet of high-pressure water to wash out blood and mucous during the endoscopic dissection. Two types of needle knives specially designed for ESD with minor modifications to the diathermy wire tip (Flex knife, KD-630L, Olympus, Tokyo Japan or Dual knife, KD-650Q, Olympus, Tokyo Japan) were used for the standard ESD procedure with a VIO300D high-frequency generator (ERBE, Elektromedizin, Tübingen, Germany). Ten percent sodium hyaluronate solution mixed with a small amount of indigo carmine and epinephrine hydrochloride was used as the injection solution to create a submucosal safety bleb<sup>[8]</sup>. A circumferential mucosal incision was made using one of the needle knives on endocut I (effect 2, interval 2, duration 2) mode. After the horizontal margination of the lesion from the surrounding normal mucosa, the electrosurgical dissection of the submucosal tissue plane was continuously performed with repetitive electrosurgical needle knife dissections. When bleeding and vascular structures were encountered, hemostasis were performed with point cauteries with the diathermy tip of the needle knife with Swift Coag mode 45W (effect 3) or a coagulation forceps (Coagrasper FD411-QR, Olympus, Tokyo, Japan) with Swift Coag mode 45W (effect 3). Patient posture rotations were carried out as needed to improve access to the lesion and deflect the overlying mucosa away from the dissection plane by gravity.

**Single balloon overtube-guided ESD:** Balloon overtube-guided ESD was performed with a standard diagnostic gastroscope (GIF-Q260, Olympus, Tokyo, Japan) with a 9.2-mm outer diameter. A balloon overtube with a 13.2-mm outer diameter, 11-mm inner diameter over-tube (ST-SB1, total length 1400 mm Olympus, Tokyo, Japan) designed for enteroscopy was shortened to 70 cm in length from the distal end leaving the balloon inflation tube intact. A gastroscope preloaded into the length-adjusted overtube was then inserted into the colon and the lesion was accessed using techniques similar to balloon enteroscopy (Figure 1). A transparent hood (D201-10704; 4 mm, Olympus, Tokyo, Japan) was attached to the tip of the endoscope. The procedural processes for ESD and the tool set used in the balloon overtube-guided procedure were the same for the ESD procedure performed without the overtube.

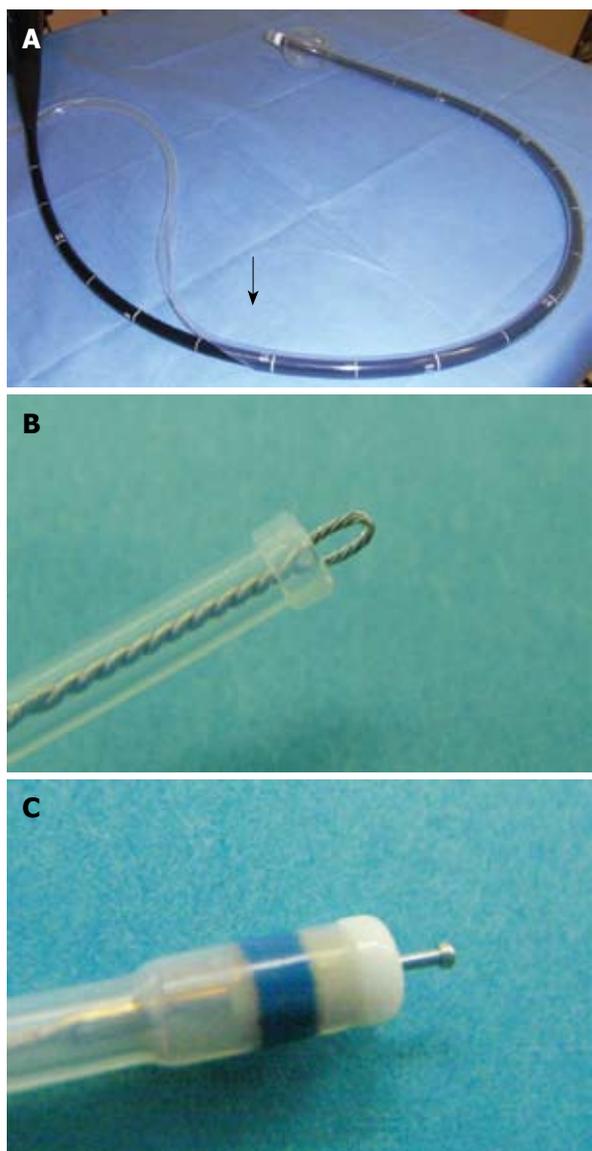
The study protocol was approved by the Institutional Ethical Committee of Kanto Medical Center NTT. Written informed consent was obtained from each patient before the ESD procedure.

### Statistical analysis

The significance of differences between patient characteristics and clinicopathological features was determined using  $\chi^2$  test, the Mann-Whitney *U* test, or Student *t* test as appropriate. *P* values < 0.05 were considered statistically significant.

## RESULTS

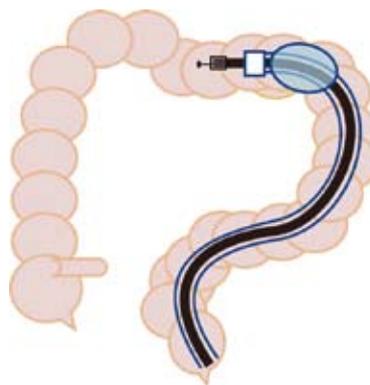
In the preoperative evaluation of accessibility with a



**Figure 1** Balloon over-tube and endoscopic submucosal dissection (ESD) devices. A: Single balloon over-tube shortened to 70 cm in length (arrow) from the distal end leaving the balloon inflation tube intact. A standard diagnostic gastroscope (GIF Q260, Olympus, Tokyo, Japan) is preloaded into the shortened over-tube; B: Flex knife (KD-630L, Olympus, Tokyo, Japan) used for ESD procedure; C: Dual knife (KD-650Q, Olympus, Tokyo, Japan) used for ESD procedure.

gastroscope, fifteen patients were identified as difficult, and were enrolled in the balloon overtube-guided ESD group. Thirty patients, who met the circumferential access criteria, were treated with the standard ESD method using a gastroscope without the overtube.

The overall *en bloc* excision rate of colorectal ESD was 95.6%. In the patients treated with the standard ESD method with a gastroscope, *en bloc* excision of the lesion was performed successfully in all 30 patients (100%). The lesions were located in the cecum in 2 patients, in the ascending colon in 10 patients, in the transverse colon in 2 patients, in the descending colon in 2 patients, in the sigmoid colon in 6 patients, and in the rectum in 8 patients. The median procedure time was 60 min (12-200 min). The median size of the lesion was 35 mm (SD: 13-98), and the median resected specimen size was 43 mm (17-112 mm).



**Figure 2** Scheme shows anchoring of the transverse colon with the single balloon over-tube.

There were no perforations, however, post-ESD bleeding occurred in one case, which required repeated endoscopic hemostasis.

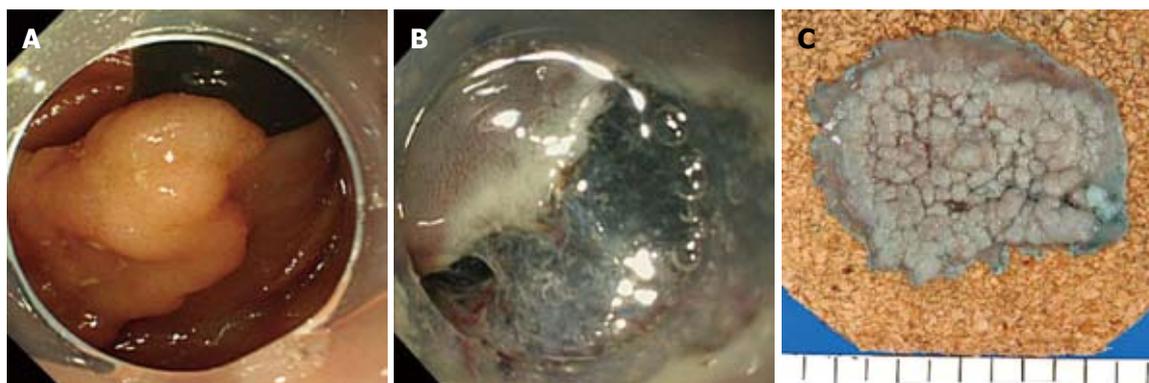
*En bloc* excision of the lesion was successfully achieved in 13 of the 15 patients (86.7%) in the balloon overtube-guided colorectal ESD group. In 2 cases of failure in the single balloon overtube-guided group, the endoscope did not reach the lesion due to elongation of the sigmoid colon. These two patients were eventually treated by piecemeal snare EMR using a colonoscope (CF-Q240L, Olympus, Japan). One lesion was located in the ascending colon and the other was in the transverse colon. Lesions in the balloon overtube-guided group were located in the transverse colon in 10 patients, the descending colon in 3 patients, the ascending colon in 1 patient and the sigmoid colon in 1 patient. The median procedure time was 80 min (30-160 min). The median size of the tumors was 27 mm (10-46 mm), and the median resected specimen size was 38 mm (18-57 mm). There were no severe complications such as perforation or bleeding.

There were no significant differences in the age ( $P = 0.352$ ), sex ( $P = 0.292$ ), lesion size ( $P = 0.472$ ), or resected specimen size ( $P = 0.597$ ) between the two groups. Lesions were more frequently located in the transverse colon in the balloon overtube-guided ESD group (10 vs 2,  $P < 0.001$ ). Operation time was longer in the balloon overtube-guided group ( $P = 0.050$ ).

On pathology, twenty lesions were diagnosed as tubular adenomas [44.4%, 15 in the ESD without overtube group, 5 in the ESD with overtube group;  $P = \text{NS}$  (not statistically significant)], and 25 were diagnosed as adenocarcinomas (55.6%, 15 in the ESD without overtube group, 10 in the ESD with overtube group;  $P = \text{NS}$ ). Four patients had submucosal invasion (3 in the ESD without overtube group, 1 in the ESD with overtube group,  $P = \text{NS}$ ) and one patient also had venous involvement. None of the patients had lymphatic involvement.

## DISCUSSION

The development of endoscopic snare polypectomy represents one of the most important achievements in the history of flexible endoscopy. This approach benefits patients enormously by reducing the physical burden associated with colonic polyp removal compared to traditional



**Figure 3** A colorectal laterally spreading tumor (LST) in the transverse colon that was difficult to approach with a standard endoscope. A: Retroflex view of the sessile granular-type lesion in the hepatic flexure of the transverse colon. The sharp angle of the colon made it difficult for a stabilized scope maneuver; B: Submucosal dissection plane with good elevation using hyaluronic acid injection; C: Gross specimen, showing the sessile, raised lesion resected *en bloc*.

surgical colectomy. Due to the limited size of the snare, removal of large polyps by snare polypectomy requires a piecemeal resection, which may lead to incomplete tumor removal. Local residual recurrence may occur after piecemeal colonic polyp excision in between 3% to 27% of cases<sup>[4]</sup>. In general, the majority of recurrent colorectal lesions are not clinically significant and can be managed with repeated endoscopic interventions. However, since some endoscopically removed lesions require additional surgical resection due to invasion into the deeper layers and vasculature, *en bloc* tumor excision is of interest not only for minimization of local tumor recurrence, but also for ensuring the precise histopathological evaluation of the sampled specimen<sup>[14]</sup>. In this study, three patients had shallow submucosal invasions and one patient had a solid submucosal invasion with vascular involvement that required additional surgical treatment. If it were possible to overcome the major technical difficulties associated with colorectal ESD, we believe this treatment could be an appropriate therapeutic option for colonic lesions that are difficult to remove *en bloc*, and this approach may be better accepted in western countries with a higher incidence of colonic polyps and cancers<sup>[15]</sup>.

In this study, preoperative evaluation showed that the majority of lesions in the transverse colon were in a difficult location and were assigned to the balloon overtube-guided group (10 *vs* 2). The transverse colon is the mobile segment most distant from the anus, and hence could be embarrassing due to the situation of the distal side of the colon. Mid-transverse colon may present as a sharp bend in patients with a redundant and drooping transverse colon. Reformation of sigmoid looping may generate friction for scope passage interfering with scope manipulation. Use of the balloon overtube provided an anchor on the colon wall giving optimal traction to maintain a shorter, straighter and more stabilized access to the lesion during ESD (Figure 2). Lesions that were preoperatively identified as being in a difficult location in the transverse colon could be accessed repeatedly using a diagnostic gastroscope by guidance of the balloon overtube, with the exception of one case with a surprisingly elongated sigmoid and severe adhesion. In addition, stabilized access *via* the overtube allowed direct and intuitive scope ma-

nipulation and *en bloc* tumor excision could be completed with the standard ESD technique in all attempts (Figure 3). Furthermore, a thin diagnostic gastroscope used in the balloon overtube-guided group could be more smoothly inserted into the submucosal tissue plane following a minimal mucosal isolation of the surgical margin. Once the cap fitted tip of the endoscope was inserted into the submucosal space thus created, electrosurgical dissection of the submucosa, the most error prone procedural process in ESD, could be safely performed with a clear visualization of the working field by deflecting the overlying isolated mucosa from the dissection plane. Although the single balloon-guided technique seems to be a promising approach to reduce the technical challenges of colorectal ESD, some important questions still remain unanswered. Two lesions, one located in the ascending colon and the other in the transverse colon were still difficult to access even with use of the balloon overtube. Both lesions were eventually treated with a colonoscope in a piecemeal fashion. In order to conclusively demonstrate that use of the balloon overtube can reduce technical difficulties in colorectal ESD, this novel approach should be directly compared with the standard ESD techniques using a colonoscope. Additionally, development of an overtube specially designed for the colorectal ESD of larger diameter to enable passage of a colonoscope could potentially reduce procedural difficulties and operation time further.

In conclusion, our preliminary experience suggests that the combined use of the balloon overtube and a thin diagnostic gastroscope is an effective and useful platform for colorectal ESD, especially in cases with difficult to access target lesions. Further studies are needed in which this novel technique is compared to the existing ESD techniques.

## COMMENTS

### Background

Endoscopic submucosal dissection (ESD) is technically more challenging for colorectal lesions than other locations in the gastrointestinal tract due to the anatomic characteristics of the colon and difficulties establishing stabilized manipulation of a long colonoscope.

### Research frontiers

Use of a balloon overtube in enteroscopy provides optimal traction on the intestinal wall thereby facilitating scope intubation. This balloon overtube-guided

approach has facilitated various interventions in the small intestine including biopsy, hemostasis and most recently polypectomy and endoscopic mucosal resection (EMR). Based on these results in the small intestine, the authors postulated that the balloon overtube could form an ideal platform for colorectal ESD. In this study, the authors reviewed the results of the balloon overtube-guided colorectal ESD technique.

### Innovations and breakthroughs

Colorectal ESD for the treatment of large superficial colorectal tumors is technically feasible, can improve *en bloc* resection rates, and is also less invasive compared to surgical treatment. However, colorectal ESD is technically more difficult and carries a higher risk of perforation than ESD at other levels of the gastrointestinal tract. Use of a balloon overtube improved access to the lesion and scope manipulation during colorectal ESD by shortening and straightening the access.

### Applications

If it were possible to overcome the major technical difficulties associated with colorectal ESD, the authors believe colorectal ESD could be an appropriate therapeutic option for colonic lesions that are difficult to remove *en bloc*, and this approach may be better accepted in Western countries with a higher incidence of colonic polyps and cancers.

### Terminology

ESD has evolved to become one of the therapeutic options for treatment of early stage gastric cancers in Asian countries. In Japan, ESD has been increasingly applied to various levels of the gastrointestinal tract and results of initial experiences in a few high volume endoscopy centers have demonstrated the technical feasibility of this unique and aggressive approach, even in the colon.

### Peer review

ESD for colorectal tumors is not generally recommended because of the technical difficulties and complications, including perforation. The authors performed ESD in 45 cases using gastroscope with a low perforation rate.

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## Stereotactic body radiotherapy for isolated paraaortic lymph node recurrence from colorectal cancer

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### Abstract

**AIM:** To evaluate the efficacy and complications of stereotactic body radiotherapy in localized paraaortic lymph node recurrence from colorectal cancer.

**METHODS:** From 2003 to 2009, 7 patients with paraaortic lymph node recurrence (1-3 lesions) from colorectal cancer were treated with stereotactic body radiotherapy. Total gross tumor volumes ranged from 4 to 40 mL. The doses were escalated from 36 Gy/patient to 51 Gy/patient and were delivered in 3 fractions.

**RESULTS:** One and 3 year overall survival rates were 100% and 71.4%, respectively, and median survival was 37 mo. Grade IV intestinal obstruction was reported in 1 of 7 patients. This patient received 48 Gy in 3 fractions with a maximum point dose to the intestine of 53 Gy and  $V_{45Gy} = 3.6$  mL. However, 6 patients received an intestinal maximum point dose of < 51 Gy and  $V_{45Gy}$  of < 1 mL, and did not develop any severe complications.

**CONCLUSION:** This pilot study suggests selected paraaortic lymph node recurrence (1-3 closed lesions) that failed to respond to chemotherapy can be potentially salvaged by stereotactic body radiotherapy.

**Key words:** Stereotactic body radiotherapy; Paraaortic lymph node recurrence; Colorectal cancer

**Peer reviewer:** Luis Bujanda, PhD, Professor, Department of Gastroenterology, CIBEREHD, University of Country Basque, Donostia Hospital, Paseo Dr. Beguiristain s/n, 20014 San Sebastián, Spain

Kim MS, Cho CK, Yang KM, Lee DH, Moon SM, Shin YJ. Stereotactic body radiotherapy for isolated paraaortic lymph node recurrence from colorectal cancer. *World J Gastroenterol* 2009; 15(48): 6091-6095 Available from: URL: <http://www.wjgnet.com/1007-9327/15/6091.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.6091>

### INTRODUCTION

Metastatic hepatic and pulmonary lesions from colorectal cancer (CRC) are commonly resected, and if tumorectomy for liver and lung recurrence from CRC is performed successfully, about 20% of patients are expected to achieve a long-term cure. On the other hand, isolated paraaortic lymph node (PALN) recurrences are rarely encountered from CRC. Moreover, surgical treatment in these cases is not widely accepted even when lesions are localized, due to their relative rarity, high postoperative morbidity, and poor prognosis. However, if such patients are left untreated, the survival rates are 31% at 1 year, 7.9% at 2 years, and 0.9% at 4 years<sup>[1,2]</sup>. Therefore, this patient subset is considered for various chemotherapy regimens. However, despite optimal treatment and initial response, overall survival approaches only 20 mo<sup>[3]</sup>. Furthermore, radiotherapy usually provides only temporary symptom relief in most cases.

On the other hand, stereotactic body radiotherapy (SBRT) is an emerging technology in the radiation oncology field. This technique utilizes stereotactic principles for localization and delivers multiple beams to well defined targets in a few fractions, and therefore, SBRT can deliver higher doses to tumors due to reduced mechanical error margins, and thus, causes less normal tissue damage. To our knowledge, no previous report has described the role of radiation, including conventional RT, intensity-modulated RT, and SBRT, for PALN recurrence from CRC, although several reports have been issued on the treatment of cervical cancer with isolated PALN recurrence, which responds well to both chemo- and radiation therapy<sup>[4-6]</sup>. Therefore, the aim of this study was to evaluate the feasibility and efficacy and

the complications associated with SBRT in patients with isolated PALN recurrence from CRC.

## MATERIALS AND METHODS

### Patients

From May 2003 to March 2009, we reviewed retrospectively 7 patients with isolated PALN recurrence from rectal cancer after curative resection who were treated with SBRT using a CyberKnife (Accuray Inc., Sunnyvale, CA). Isolated PALN recurrence was initially detected by computed tomography (CT) or by an elevated serum carcinoembryonic antigen (CEA) level during a routine check-up, and was confirmed by elevated standardized uptake values of paraaortic lesions by positron emission tomography (PET) or PET/CT. According to our hospital's protocol, patient eligibility criteria for curative SBRT for paraaortic lymph node recurrence from CRC were as follows: (1) resection of CRC after diagnosis; (2) PALN recurrence after primary cancer resection; (3) progression after chemotherapy for recurrence; (4) a single conglomerate recurrent node or 2-3 recurrent nodes in close proximity (< 1 cm); (5) a greatest tumor diameter of < 8 cm; and (6) an Eastern Cooperative Oncology Group (ECOG) performance score of 1 or 2. Exclusion criteria were as follows: (1) a tumor attached to the stomach or intestine by CT; (2) extra-lymphatic active lesion by CT or PET/CT; (3) more than three separate lymph nodes affected (4) time from primary operation to recurrence of < 6 mo; or (5) previous radiation therapy applied to the treatment site. Patients' characteristics are summarized in Table 1. Ages ranged from 47 to 73 years (median 59 years) and the male:female ratio was 5:2. All patients underwent primary tumor resection. Liver resection was also performed in 2 patients with liver metastasis. Adjuvant chemotherapy was performed in all patients. Initial pathologic stages were stage II in 2, stage III in 3, and stage IV in 2. Pathologic diagnoses were adenocarcinoma in all 7. Times between operation and first relapse ranged from 7 to 44 mo (median 21 mo). Three patients had a conglomerated LN and the other four had 2 or 3 separate enlarged lymph nodes on a paraaortic lesion. Greatest tumor diameters and heights were calculated using measurements taken from CT scans during planning, and are itemized in Table 1. Total gross tumor volumes (GTV) ranged from 4 to 40 mL (median 22 mL). After recurrence had been detected, all patients received chemotherapy based on 5-FU before SBRT. Chemotherapy regimens were variable because of the different initial adjuvant chemotherapy regimens used. All 7 patients demonstrated disease progression despite chemotherapy, and thus, were defined as non-responders. After performing SBRT, patients were followed up every 2 or 3 mo. When recurrence was detected after SBRT, salvage or palliative treatment was performed according to the status of recurrence. All patients provided written informed consent for SBRT. This study was approved by Institutional Review Board-approved protocol for SBRT at the Korea Institute of Radiological and Medical Sciences (KIRAMS).

### SBRT technique

In accord with our hospital protocol, gold fiducials

(4 mm long and 0.8 mm in diameter) were used as markers for tumor localization. Six fiducials were placed percutaneously on transverse processes of the spine located nearest tumors using an 18 gauge spinal needle under fluoroscopic guidance. Patients were immobilized using an Alpha Cradle (Smithers Medical products, North Canton, OH) 5-7 d after fiducial placement. Panning CT scans were performed with patients in the planned treatment position, and these images were then processed for the CyberKnife planning system. GTV were determined based on CT tumor visualizations. To better delineate tumor volumes, PET/CT images were used as a reference. Clinical target volumes (CTV) were considered to be identical to GTV. Planning target volumes (PTV) were CTV plus a 2-3 mm margin. Radiation doses were prescribed to the 76%-83% isodose line of the maximum dose covering the PTV (Table 1). Critical structures, such as the esophagus and spinal cord, were contoured. Treatment plans involved the use of hundreds of pencil beams shaped using a single 20, 25, or 30 mm diameter circular collimator. The method used to increase SBRT dose is described in detail in our previous report<sup>[7]</sup>. Briefly, the protocol adopted was follows; if at least 5 patients who received SBRT due to PALN from variable primary tumors (cervical cancer, CRC, or gastric cancer) did not develop Grade IV or V complications for 3-4 mo after radiation was administered, escalations of 1 Gy/fraction (to a total increase of 3 Gy/fraction) were administered for the next cohort. According to this protocol, total SBRT doses ranged from 36 to 51 Gy (median 48 Gy), and were delivered in 3 fractions. Maximum point dose limits were applied for critical organs, i.e. 18 Gy for the spinal cord and 24 Gy for the esophagus. No constraints were applied to limit intestinal or colon exposure. Table 1 summarizes SBRT dosage details.

### Survival and complications

Overall survival was calculated from the commencement of SBRT using the Kaplan-Meier method. Disease progression free survival was also measured from the commencement of SBRT to the date of local progression, distant metastasis, or both. All statistical calculations were performed using SPSS, version 13.0 (SPSS, Inc., Chicago, IL).

Tumor response during follow up was assessed using Response Evaluation and Criteria for Solid Tumors (RECIST)<sup>[8]</sup>. Local progression was defined as an increase in tumor size vs the previous CT image or the development of a new lesion in the radiation field. Regional failure was defined as the development of a new lesion in the PALN region.

Acute and late toxicities were defined as symptoms that developed within or after 3 mo of treatment completion, respectively. Toxicities were graded using the National Cancer Institute Common Toxicity Criteria version 3.0<sup>[9]</sup>. To identify factors related to complications, total CTV, intestinal maximum point dose ( $D_{max}$ ) and intestinal volume administered  $\geq 45$  Gy ( $V_{45}$ ) were calculated retrospectively (Table 1). Total CTV was defined as sum of the CTV of affected lymph node as determined by the CyberKnife planning system.  $V_{pre}$  was defined as the total volume administered the prescribed dose or more.

Table 1 Demographic data of 7 patients

Patient No.	Age (yr)/ Sex	Latent time (mo)	GTV (mL)	Dose (Gy)	Prescribed isodose (%)	Dmax of intestine (Gy)	V45 (mL)	Failure pattern	F/U (mo)	Final status
1	63/M	9	20	36	83	33	0	Lt SCLN (23) Lung (32)	70	AWD
2	64/F	44	4	41	82	38	0	-	37	Died due to lymphoma
3	52/F	29	24	45	78	48	0.2	Spine (26)	41	DOD
4	59/M	21	22	48	80	51	0.7	Lung (7)	22	DOD
5	56/M	10	9	48	80	40	0	PALN(9)	21	AWD
6	47/M	18	40	48	76	53	3.6	Rectum (7) Lt SCLN (7) Peritoneal seeding (25) Local recur (13)	25	DOD
7	73/M	7	29	51	78	50	0.9	-	26	CDF

GTV: Gross tumor volume; AWD: Alive with disease; CDF: Continuously disease-free; DOD: Died of disease; Latent time: Disease free interval from operation to first relapse; F/U: Follow-up from commencement of SBRT to last follow-up or death; Lt SCLN: Left supraclavicular lymph node; Dmax: Maximum point dose of intestine; V45: Intestinal volume receiving 45 Gy or more; Vpre: Total volume receiving the prescribed dose or more; Parenthesis (mo) means the period from SBRT to detection of complication.

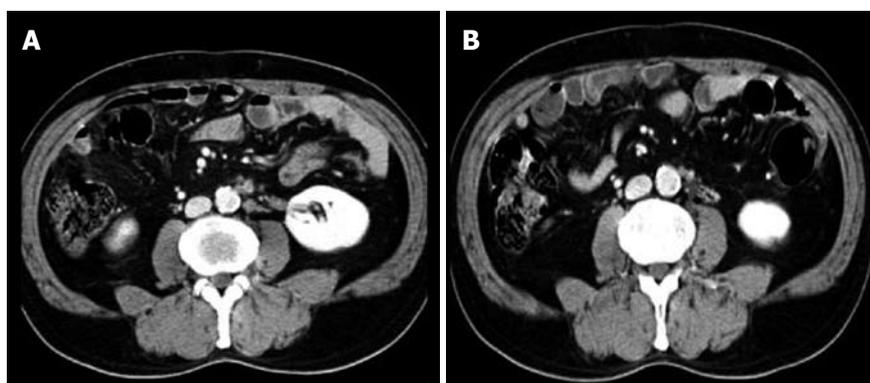


Figure 1 Computed tomography (CT) images obtained in a patient with paraaortic lymph node recurrence before (A) and 2 mo after (B) stereotactic body radiotherapy (SBRT).

## RESULTS

Follow up durations ranged from 15 to 70 mo (median; 26 mo). Final outcomes were as follows: 1 patient remained alive without evidence of disease; 2 patients remained alive with disease; 3 patients died of disease; and 1 patient died of an unrelated disease without recurrence (Table 1). During follow-up, 3 patients achieved a complete response and 4 patients achieved a partial response (Figure 1). One- and 3-year overall survival rates were 100% and 71.4%, respectively, and median survival was 37 mo. Local recurrence was observed at 13 mo after SBRT in patient No 6 (Table 1). Regional recurrence in the PALN region was observed in patient No. 5. However, 4 patients experienced distant failure with/without primary rectal recurrence (Table 1).

Grade I acute toxicity (nausea and vomiting) occurred in 2 patients at first date of SBRT, and these resolved spontaneously without medication. Grade 4 toxicity occurred in 1 patient (patient No. 6; Table 1). This patient had one conglomerated lymph node and another lymph node and had received 48 Gy in 3 fractions. Abdominal pain developed at 4 mo post-SBRT, and at 5 mo an obstruction was observed during a colon study. He underwent bypass surgery and recovered completely. A photograph of the excised obstructive lesion is shown in Figure 2. This patient had a larger CTV than CTV of the other patients. In this patient, the maximum point intestine dose was 53 Gy and V<sub>45Gy</sub> was 3.6 mL. No late complications occurred in any patients.

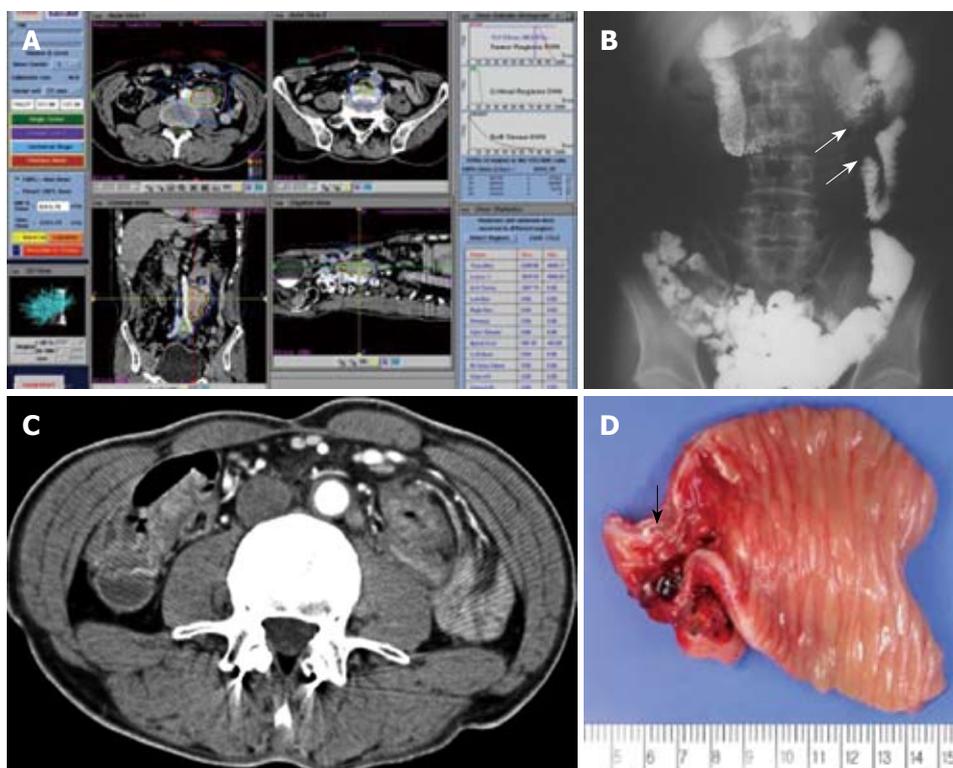
## DISCUSSION

The management of patients with locally recurrent rectal cancer is challenging. For technically resectable recurrent tumors, complete resection can be achieved by limited surgery and outcomes are relatively favorable. Vassilopoulos *et al*<sup>[10]</sup> and Pihl *et al*<sup>[11]</sup> reported 5-year survival rates of 49% and 42%, respectively, after resection for anastomotic recurrence. However, the treatment of isolated PALN recurrence from CRC is not well established. Recently, Min *et al*<sup>[12]</sup> categorized PALN recurrence as a retroperitoneal recurrence, which is a type of locoregional recurrence. Furthermore, several studies<sup>[13-16]</sup> have investigated the therapeutic efficacies of surgery for retroperitoneal, intraabdominal, and PALN recurrences, and several reported outstanding survival rates, which appear to have resulted from the selection of patients with a resectable mass at time of recurrence. In these studies, reported 5-year survival rates approached a maximum of 56% after complete resection, whereas they ranged from 0% to 7% after incomplete resection (Table 2). Because radical surgery is rarely feasible for PALN recurrence, traditionally, those affected have been considered for chemotherapy. However, despite optimal treatment and the achievement of initial response, patients invariably become non-responsive and achieve overall survivals approaching 20 mo. On the other hand, conventional radiotherapy has played a limited palliative role in the treatment of recurrent CRC involving locoregional recurrence, especially PALN

Table 2 Comparison of survival for recurrence from CRC treated by surgery or other treatment method

Study	Failure site	Treatment	n	Median survival (mo)	Survival rate (yr)
Gwin <i>et al</i> <sup>[15]</sup> , 1993	Non-hepatic intra-abdominal	R0 <sup>1</sup>	15	25.5	60 (2), 0 (3)
		R1 <sup>2</sup>	6	8	34 (2), 34 (3)
		R2 <sup>3</sup>	7	3.5	15 (2)
Shibata <i>et al</i> <sup>[13]</sup> , 2002	Retroperitoneum	R0	15	81	56 (5)
		R1	5	29	0 (5)
		R2	4	3	0 (5)
Bowne <i>et al</i> <sup>[14]</sup> , 2005	Retroperitoneum	R0	8	44	NA
		R1	8		
Min <i>et al</i> <sup>[12]</sup> , 2008	PALN	R0	6	34	80 (3), 0 (5)
		Chemotherapy <sup>4</sup>	33	12	18 (3), 7 (5)
Present study	PALN	SBRT	7	41	71.4 (3)

<sup>1</sup>Curative resection; <sup>2</sup>Marginal resection; <sup>3</sup>Incomplete, bypass or colostomy; <sup>4</sup>Resection is not planned. CRC: Colorectal cancer; PALN: Paraaortic lymph node; SBRT: Stereotactic body radiotherapy; NA: Not assessed.



**Figure 2** A case with complications. A: Isodose distribution and target coverage with SBRT to a dose of 48 Gy in 3 fractions prescribed to planning target volume; B: Small bowel series 5 mo after SBRT. Near total obstruction (arrows) in left side of abdomen; C: Computed tomography at 4 mo after SBRT. Wall thickening of proximal jejunal loop was observed; D: 2.5 cm × 1.5 cm ulceration & stricture lesion (arrow) in operation specimen. Eventually, he underwent bypass surgery and recovered completely.

recurrence. The proximities of involved lymph nodes and critical organs, such as the spinal cord, intestine, and colon, often prevent the delivery of sufficient radiation to achieve local control when conventional radiation modalities are used. However, SBRT can deliver higher doses to tumor and cause less tissue damage. Furthermore, it can have three times the biological effect of fractionated radiation therapy. However, the SBRT field is usually directed at the tumor burden, and thus, the prophylactic effect of SBRT in peritumoral regions is limited which in turn means the incidence of regional failure after SBRT seems higher than that after conventional radiation therapy. Fortunately, in the present study, only 1 regional failure pattern was observed.

At our institute, SBRT has been utilized for isolated PALN from gastric or cervical cancer, and 3-year survival

rates of 43%<sup>[17]</sup> and 63%<sup>[18]</sup> have been achieved, respectively. In addition, an excellent 3-year overall survival rate of 71.4% was achieved in the present study. Accordingly, the findings of studies on PALN recurrence from variable primary cancers treated by SBRT appear to support our hypothesis that a subset of isolated PALN recurrence cases exist that are likely to pursue an indolent disease course and be salvaged by adjustable treatments.

Theoretically, rectal cancer is classified as a slow-growing tumor that is likely to respond better to hypofractionation. However, no recommended optimal doses, fraction numbers, or planning constraints for SBRT of PALN recurrence are available in the literature. SBRT doses and fractions for PALN recurrence from variable tumors were started at our hospital from 33 Gy in 3 fractions. In the

present study, converted 58 Gy in normalized total doses of 2 Gy was used, and escalated step by step<sup>[18]</sup>. Although, 1 of our patients developed grade 4 toxicity due to an intestinal obstruction and required surgery, this patient recovered after surgery. Specifically, this patient received 48 Gy in 3 fractions, and had a larger GTV than the other 6 patients. Based on our experience, we consider the factors that most contribute to severe complications are maximum point dose delivered to normal tissue and the volume of normal tissue administered a high dose. Therefore, we tentatively consider maximum intestinal point dose of 51 Gy or V<sub>45Gy</sub> < 1 mL as constraints of the intestine for SBRT.

Summarizing, the findings of this preliminary study suggest that selected isolated PALN recurrence patients that fail to respond to chemotherapy with 1-3 closely located attached lymph nodes may be salvaged by SBRT. However, a further larger-scale study is required to define optimal dose, intestinal constraints, and adequate indications for SBRT in recurrent CRC.

## COMMENTS

### Background

Isolated paraaortic lymph node (PALN) recurrences are rarely encountered from colorectal cancer (CRC). Moreover, surgical treatment in these cases is not widely accepted even when lesions are localized, due to their relative rarity, high postoperative morbidity, and poor prognosis. This patient subset is considered for various chemotherapy regimens. However, despite optimal treatment and initial response, overall survival approaches only 20 mo.

### Research frontiers

Stereotactic body radiotherapy (SBRT) is an emerging technology in the radiation oncology field. This technique utilizes stereotactic principles for localization and delivers multiple beams to well defined targets in a few fractions. SBRT can deliver higher doses to tumors due to reduced mechanical error margins, and thus, causes less normal tissue damage.

### Innovations and breakthroughs

Delivery of a therapeutic dose of radiation to the PALNs is limited by the sensitivity of the surrounding normal tissues, such as those in the gastrointestinal tract, liver, spinal cord, and kidneys. However recent technologies such as intensity-modulated RT, image-guided RT, and SBRT have allowed higher doses to be delivered to tumor and caused less normal tissue damage. SBRT could lead to better local control through delivery of a higher radiation dose to the tumor and this could ultimately translate into survival gain. Furthermore, SBRT requires complex planning and relatively long treatment times (30-45 min) but generally is completed in 3-5 treatments. SBRT is associated with few side effects because the treatment field is generally very small and treatment is precisely delivered.

### Applications

This pilot study suggests selected paraaortic lymph node recurrence (1-3 closed lesions) that failed to respond to chemotherapy can be potentially salvaged by stereotactic body radiotherapy.

### Terminology

Isolated PALN metastasis is defined as metastasis only to the PALNs. SBRT is an image-guided radiation method. SBRT is directed to extremely well-defined targets within the body. SBRT has evolved from the intracranial experience of stereotactic radiosurgery (single fraction treatment) or stereotactic radiotherapy (multiple fractions of treatment).

### Peer review

The authors evaluate efficacy and complications of stereotactic body radiotherapy in localized paraaortic lymph node recurrence from colorectal cancer. It is well written.

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BRIEF ARTICLE

## First endoscopic procedure for diagnosis and staging of mediastinal lymphadenopathy

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Khoo KL, Ho KY, Khor CJL, Nilsson B, Lim TK. First endoscopic procedure for diagnosis and staging of mediastinal lymphadenopathy. *World J Gastroenterol* 2009; 15(48): 6096-6101 Available from: URL: <http://www.wjgnet.com/1007-9327/15/6096.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.6096>

### Abstract

**AIM:** To compare a first diagnostic procedure of trans-bronchial needle aspiration (TBNA) with selection of endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) or TBNA for mediastinal lymphadenopathy.

**METHODS:** Sixty-eight consecutive patients with mediastinal lymphadenopathy on computed tomography (CT), who required cytopathological diagnosis, were recruited. The first 34 underwent a sequential approach in which TBNA was performed first, followed by EUS-FNA if TBNA was unrevealing. The next 34 underwent a selective approach where either TBNA or EUS-FNA was selected as the first procedure based on the CT findings.

**RESULTS:** The diagnostic yield of TBNA as the first diagnostic procedure in the sequential approach was 62%. In the selective approach, the diagnostic yield of the first procedure was 71%. There was no significant difference in the overall diagnostic yield, but there were significantly fewer combined procedures with the selective approach.

**CONCLUSION:** Selecting either EUS-FNA or TBNA as the first diagnostic procedure achieved a comparable diagnostic yield with significantly fewer procedures than performing TBNA first in all patients.

### INTRODUCTION

Lung cancer is the commonest cause of mediastinal lymphadenopathy. For non-small cell lung cancer (NSCLC), which accounts for about 80% of lung cancers, mediastinal lymph node enlargement occurs in up to 38% of cases at diagnosis<sup>[1]</sup>. As surgical resection of NSCLC offers the best chance of cure in patients without distant metastases, the pathological confirmation of cancer spread to enlarged mediastinal lymph nodes is crucial to staging because this excludes curative surgical resection.

In the approach to suspected lung cancer without distant metastases, the lung mass is the initial target for cytopathological diagnosis. Following a diagnosis of NSCLC, mediastinal staging is the next step. In patients with mediastinal lymphadenopathy however, the mediastinum may be targeted first, even when a lung mass is present. This might achieve simultaneous diagnosis and mediastinal staging of lung cancer with a single procedure.

The esophagus and tracheobronchial tree offer endoluminal access to mediastinal lymph nodes, therefore endoscopic techniques such as endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) and trans-bronchial needle aspiration (TBNA) offer minimally invasive approaches for diagnosis of mediastinal lymphadenopathy.

Although EUS-FNA has a higher accuracy than TBNA, the transbronchial approach is preferred for anterior and right paratracheal lymph nodes. Real-time endobronchial ultrasound-guided TBNA (EBUS-TBNA) is now available but requires expensive specialized equipment and operator training. TBNA does not require specialized equipment and can be performed during the initial diagnostic bronchoscopy<sup>[2-4]</sup>. When we evaluated patients with mediastinal lymphadenopathy with bronchoscopy and TBNA, the diagnostic yield for mediastinal lymphadenopathy was 65%<sup>[3]</sup>.

We have also used EUS-FNA for cases in which TBNA was unrevealing or non-diagnostic, given its higher accuracy<sup>[4]</sup>. However, this resulted in subjecting these patients to two diagnostic procedures even though both procedures could be performed in the same outpatient session<sup>[4,5]</sup>.

We then hypothesized that bronchoscopy with TBNA need not be performed as the first procedure in all cases of mediastinal lymphadenopathy, and that by selecting the appropriate endoscopic procedure based on anatomical access, a higher diagnostic yield could be obtained after the first procedure. This could also result in subjecting the patient to fewer diagnostic procedures. Therefore, in this study, we compared an approach utilizing TBNA as the first diagnostic procedure with one utilizing selection of either EUS-FNA or TBNA.

## MATERIALS AND METHODS

Between December 2003 and June 2006, consecutive patients with mediastinal lymphadenopathy on thoracic computed tomography (CT) who presented to, or were referred to our respiratory division for cytopathological diagnosis were recruited for the study. Mediastinal lymphadenopathy was defined as a node larger than 1 cm in its short axis. The institutional review board of our hospital approved the study and informed consent was obtained for all the procedures.

### Sequential approach

During the first 16 mo of the study period, we employed a sequential approach for which bronchoscopy with TBNA was performed as the first diagnostic procedure, with or without other conventional bronchoscopic techniques. If TBNA was unrevealing on rapid on-site cytopathological evaluation (ROSE), EUS-FNA was performed immediately after TBNA, during the same session. Details of this approach and the results of the first 20 patients have been described when we explored the one-stop approach to mediastinal lymphadenopathy<sup>[4,5]</sup>.

### Selective approach

From April 2005, we employed a selective approach for which either EUS-FNA or TBNA was performed as the first diagnostic procedure. This was selected based on the predominant location of the lymphadenopathy on CT. If either the esophageal or transbronchial approach could access the nodes, the pulmonologist was left to

decide which procedure he deemed most appropriate. In general, TBNA was selected mainly for patients with right paratracheal lymphadenopathy, whereas EUS-FNA was the preferred option for left paratracheal lymphadenopathy. Subcarinal lymph nodes could be approached by either procedure. If TBNA was selected as the first diagnostic procedure, EUS-FNA remained a subsequent option.

### TBNA, EUS-FNA and ROSE

Bronchoscopy was performed by experienced pulmonologists using standard flexible videobronchoscopes (Olympus Optical Co. Ltd., Tokyo, Japan). Premedication with pethidine and atropine and sedation with midazolam were optional, while all patients received topical anesthesia with xylocaine. TBNA was performed blind with a Wang 22-gauge (MW 222) cytology needle (Bard Endoscopic Technologies, Billerica, MA, USA) at sites of mediastinal lymph node enlargement based on review of the CT scan. TBNA was performed before other conventional bronchoscopic procedures to avoid contamination.

EUS-FNA was performed as previously described using the curved linear array echoendoscope (GF-UC30P; Olympus) by experienced gastroenterologists<sup>[6]</sup>. Patients received topical anesthesia with xylocaine and sedation with a combination of midazolam and pethidine.

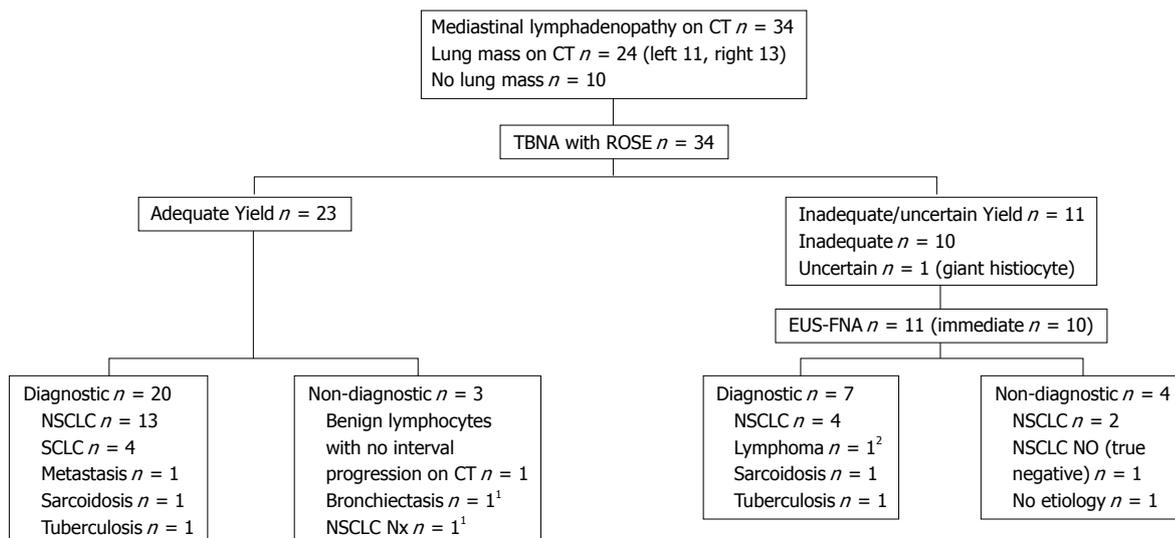
ROSE was employed to determine the adequacy of the needle aspirates. The aspirated material was blown onto a slide using the direct smear technique<sup>[7]</sup>. The smears were either air-dried and stained with Diff-Quik (American Scientific Products, McGraw Park, IL, USA) or fixed immediately in 95% ethanol and stained with Papanicolaou stain. Solid particles were fixed in formalin, routinely processed, and made into cell blocks for histological examination. The air-dried smears for Diff-Quik staining were reviewed immediately by an experienced cytotechnician. Endoscopists were then advised as to the need for additional needle aspirates (up to a maximum of six passes).

### Diagnostic yield

The final cytopathological diagnoses were made based upon analysis of the aspirated material by experienced cytopathologists. The diagnostic yield of TBNA was the number of patients in whom a definite diagnosis was made by TBNA over the total number of patients subjected to TBNA. The diagnostic yield after the first procedure was the number of patients in whom a definite diagnosis was made after the first procedure over the total number of patients. The overall diagnostic yield for each approach was the number of patients in whom a definitive diagnosis was made by needle aspiration over the total number of patients. When a diagnosis could not be made by either procedure, the final diagnostic categories were determined by review of further tests and clinical assessments.

### Statistical analysis

Descriptive statistics are presented as mean  $\pm$  SD. Discrete



**Figure 1 The sequential approach.** <sup>1</sup>False positive TBNA with ROSE; <sup>2</sup>False negative TBNA with ROSE. CT: Computed tomography; TBNA: Transbronchial needle aspiration; ROSE: Rapid on-site cytopathological evaluation; NSCLC: Non-small cell lung cancer; EUS-FNA: Endoscopic ultrasound-guided fine-needle aspiration.

variables were analyzed with  $\chi^2$  test and  $P < 0.05$  was defined as statistically significant.

## RESULTS

Sixty-eight consecutive patients with mediastinal lymphadenopathy on CT were recruited during the study period. The main indication for CT was suspected malignancy ( $n = 58$ ). Other indications included suspected pulmonary embolism ( $n = 4$ ), pyrexia of unknown origin ( $n = 2$ ), suspected aortic dissection ( $n = 1$ ), investigation of weight loss ( $n = 1$ ), suspected sarcoidosis ( $n = 1$ ), and follow-up of non-Hodgkin's lymphoma ( $n = 1$ ).

The baseline characteristics and diagnostic categories of the sequential group ( $n = 34$ ) and the selective group ( $n = 34$ ) were similar (Table 1).

Results of the sequential approach are shown in Figure 1. TBNA was performed at the following mediastinal sites according to regional lymph node map definitions as described by Mountain *et al*<sup>[8]</sup>: 4R in 10 patients, 7 in 24 patients, and 4L in 7 patients. The TBNA obtained adequate specimens in 23 of the 34 patients. In the remaining 11 patients, TBNA with ROSE showed the specimens to be inadequate or unrevealing, thus, EUS-FNA was performed immediately after bronchoscopy, at lymph node stations 7 (seven patients), 4L (10 patients) and 4R (one patient). Some patients had TBNA or EUS-FNA performed at more than one lymph node station. When the final cytopathological results were analyzed, TBNA with ROSE was falsely negative in one patient. In another patient, TBNA with ROSE showed a giant histiocyte and a decision was made to proceed with EUS-FNA. The final cytopathological diagnosis for both specimens returned as granulomatous inflammation. Results of the first 20 patients with this approach have been described previously<sup>[4]</sup>.

Results of the selective approach are shown in Figure 2. TBNA was performed in 22 patients in the following mediastinal sites: 4R (six patients) and 7 (19 patients).

**Table 1 Baseline characteristics and diagnostic categories of study population  $n$  (%)**

Variables	Sequential approach	Selective approach	$P$ value
No. of patients	34	34	
Male/female	24/10	25/9	
Age (mean $\pm$ SD, yr)	64.7 $\pm$ 11.2	65.1 $\pm$ 12.7	
Mass on CT	24	23	
Right-sided/left-sided,	13/11	12/11	
No. of patients undergoing			
TBNA	34 (100)	22 (65)	< 0.001
TBNA and EUS-FNA	11 (32)	2 (6)	< 0.05
Diagnostic yield (%)			
First procedure	62	71	0.6
TBNA	62	73	0.6
Overall	79	73	0.8
Diagnostic categories			
Malignancy	26	28	
NSCLC/SCLC	22/3	21/7	
Benign tumor	8	6	
Sarcoid/tuberculosis	2/2	1/1	

CT: Computed tomography; TBNA: Transbronchial needle aspiration; NSCLC: Non-small cell lung cancer; EUS-FNA: Endoscopic ultrasound-guided fine-needle aspiration.

EUS-FNA was performed as a first diagnostic procedure in 12 patients at lymph node stations 7 (12 patients), 4L (five patients) and 2R (one patient, Figure 3). In contrast to the sequential approach for which all 34 patients had TBNA performed first, 35% (12/34) of patients in the selective approach had EUS-FNA performed first, while the remaining 65% (22/34) had TBNA performed first. In the selective approach, TBNA was performed only for right paratracheal and subcarinal stations, whereas EUS was performed predominantly in the left paratracheal and subcarinal stations.

The diagnostic yield of TBNA as the first diagnostic test was 62% in the sequential approach, while the diagnostic yield of the first diagnostic procedure in the selective approach was 71%. The diagnostic yield of

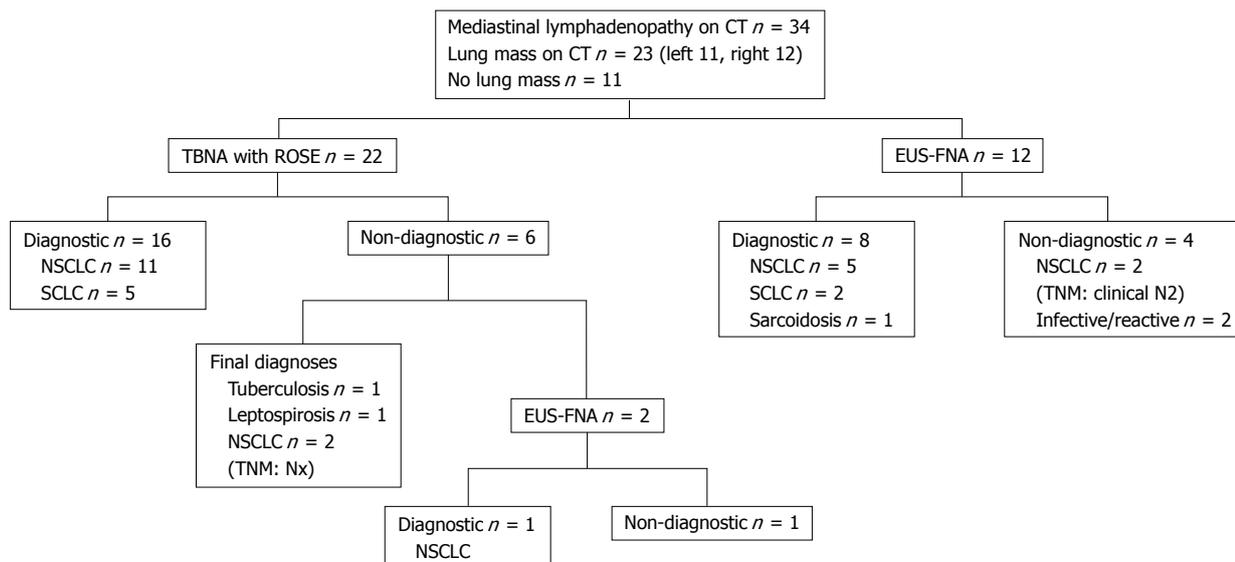


Figure 2 The selective approach.



Figure 3 CT showing right paratracheal lymphadenopathy that was sampled by EUS-FNA.

EUS-FNA was 67% (8/12). The overall diagnostic yield of the sequential approach was 79% (27/34) and that of the selective approach was 74% (25/34). There was no significant difference in the overall diagnostic yields. Significantly fewer combined diagnostic procedures (6% *vs* 32%,  $P < 0.05$ ) were required with the selective approach. The yield of TBNA was higher with the selective approach (73%) as compared to the sequential approach (62%). There were no complications with either TBNA or EUS-FNA, or damage to the bronchoscopes or endoscopes.

## DISCUSSION

The present study compared a diagnostic approach utilizing TBNA as the first diagnostic procedure with one in which EUS-FNA or TBNA was selected as the first procedure. The selection was based on whether the optimal anatomical approach was transesophageal or transbronchial. We found a higher diagnostic yield after the first diagnostic procedure with the selective approach, and this translated to a significant reduction in the number of diagnostic procedures performed.

The transesophageal and transbronchial routes to the mediastinum are complementary. The transesophageal approach has limited access to the right paratracheal nodes, therefore, the endobronchial route offers better access, as shown by Herth *et al*<sup>9</sup>. Therefore, the procedure of choice for right paratracheal lymphadenopathy with the selective approach was TBNA, unless CT showed a peri-esophageal location of these nodes that was easily accessed by EUS-FNA (Figure 3). Harrow *et al*<sup>10</sup> also have shown that the right paratracheal and subcarinal locations are predictors of a positive aspirate with TBNA. With the selective approach, TBNA was limited to these two locations, and the yield of TBNA improved from 62% to 73%.

Although mediastinoscopy remains the diagnostic standard for the mediastinal staging of lung cancer, with a sensitivity of 80%-85%, this invasive surgical procedure requires general anesthesia and has a morbidity and mortality rate of 2% and 0.08%, respectively<sup>[11]</sup>. In contrast, both TBNA and EUS-FNA are minimally invasive and can be performed in the outpatient setting under local anesthesia and sedation. EUS-FNA permits real-time visualization of needle sampling and has been shown to be highly accurate in the mediastinal staging of lung cancer, as well as in the diagnosis of mediastinal lymphadenopathy of unknown etiology<sup>[6,12-18]</sup>.

The development of EBUS-TBNA for mediastinal lymph nodes has lagged behind EUS-FNA by more than a decade<sup>[19,20]</sup>. As such, the new convex-probe EBUS is still not as widely available as EUS. Wallace *et al*<sup>[21]</sup> have suggested that the use of ultrasound-guided needle sampling of mediastinal lymph nodes in patients with suspected lung cancer, whether by EUS or EBUS, is superior to conventional TBNA. By combining EUS-FNA and EBUS-TBNA, they have achieved a near-complete medical mediastinoscopy, thus reinforcing the complementary nature these procedures<sup>[22]</sup>.

Our aim was not to achieve comprehensive staging of the mediastinum in the setting of lung cancer, but

rather, to demonstrate that, with appropriate selection of the first endoscopic procedure, a higher diagnostic yield could be obtained. This would mean that EUS-FNA could be selected as the first procedure, rather than routinely subjecting all patients to bronchoscopy. Indeed, a recent meta-analysis has suggested that EUS-FNA is the diagnostic test of choice for mediastinal lymphadenopathy<sup>[23]</sup>. In addition, the transesophageal route may be better tolerated as compared to the transbronchial route, with less coughing and the absence of obstruction of the needle by cartilaginous rings.

Most studies with EUS-FNA for mediastinal evaluation have been performed in patients only after confirmation of the diagnosis of NSCLC. Singh *et al*<sup>[24]</sup>, however, have demonstrated that EUS-FNA may be performed as the first diagnostic procedure for suspected lung cancer. In the setting of mediastinal lymphadenopathy in NSCLC, this diagnostic procedure also has enabled simultaneous mediastinal staging. Thus, besides showing that bronchoscopy need not be the first diagnostic procedure in patients with suspected lung cancer, they also have demonstrated that diagnosis and staging of lung cancer need not be performed sequentially or require multiple procedures. This highlights a paradigm shift where mediastinal staging is no longer performed only after confirming the diagnosis of NSCLC.

We believe that, in the diagnostic approach to the mediastinum, the transesophageal and transbronchial routes are complementary rather than competing. Instead of pitting TBNA against EUS-FNA, this study emphasizes that the complementary value of these endoscopic approaches is best exploited by appropriate procedure selection. Thus, when either EUS-FNA or TBNA was selected as the first procedure, the diagnostic yield increased from 62% to 71%, thereby significantly reducing the need for additional procedures. Targeting the mediastinum first to enable simultaneous diagnosis and staging, and optimizing the yield of the first diagnostic procedure may lead to fewer delays in the treatment of lung cancer patients. Devbhandari *et al*<sup>[25]</sup> have reported that a negative initial bronchoscopy in suspected lung cancer resulted in significant delays in diagnosis and treatment. In that study, initial bronchoscopy was diagnostic in less than 50% of cases.

The present study had several limitations. Firstly, this was not a randomized trial and the patient population was small. However, they were consecutive patients with similar baseline characteristics and diagnostic categories (Table 1). Secondly, a definitive diagnosis could not be made in all cases because some patients and their referring physicians declined further invasive surgical sampling. However, the aim of this study was to determine the diagnostic yield of the sequential and selective approaches rather than the accuracy of either endoscopic procedure. Thirdly, conventional TBNA was employed rather than EBUS-TBNA. This was because at the time of the study, EBUS-TBNA was not available at our center.

Three practical clinical points are highlighted here. Firstly, the cytopathological diagnosis of mediastinal lymphadenopathy may be achieved in the majority of

patients utilizing widely available endoscopic techniques. Secondly, targeting the mediastinum first may establish simultaneously diagnosis as well as mediastinal staging for patients with NSCLC. Finally, appropriate selection of the first diagnostic procedure may optimize the yield and minimize the number of procedures required for the diagnosis and/or staging of mediastinal lymphadenopathy. Thus, with the availability of EUS-FNA, bronchoscopy may no longer be required in selected patients with suspected lung cancer.

Endoscopic techniques are becoming essential high-utility tools in the investigative approach to the mediastinum. With the rapid evolution of newer endoscopic techniques, the physician's diagnostic armamentarium is likely to expand. The question of which is the most appropriate initial diagnostic procedure for mediastinal lymphadenopathy, given what is available, will become even more important. While awaiting further studies comparing the different emerging endoscopic techniques and combination of techniques, we suggest that the optimal diagnostic approach for mediastinal lymphadenopathy depends on selection of the most appropriate initial diagnostic procedure.

## COMMENTS

### Background

In the absence of distant metastasis, mediastinal staging remains crucial for determining prognosis and therapy of non-small cell lung cancer. An approach to patients with mediastinal lymphadenopathy regardless of whether a lung mass is present, is to target the mediastinum first. This may achieve simultaneous diagnosis and mediastinal staging with a single procedure, in the event of diagnosis of lung cancer, which is the commonest cause of mediastinal lymphadenopathy.

### Research frontiers

The transbronchial and transesophageal routes allow minimally invasive endoscopic needle sampling of mediastinal lymph nodes. These endoscopic procedures may be done under sedation in contrast to the gold standard mediastinoscopy, which requires general anesthesia.

### Innovations and breakthroughs

Minimally invasive mediastinal staging with endoscopic ultrasound-guided fine-needle aspiration and blind or endobronchial ultrasound guided transbronchial-needle aspiration may be a substitute for mediastinoscopy.

### Applications

Not all patients require all three procedures, therefore, appropriate initial procedure selection may be important in the diagnostic approach to mediastinal lymphadenopathy, because only a single procedure may be diagnostic in the majority of cases.

### Peer review

This is a very interesting topic for the readers of *World Journal of Gastroenterology*.

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BRIEF ARTICLE

## Azithromycin-containing *versus* standard triple therapy for *Helicobacter pylori* eradication: A meta-analysis

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Dong J, Yu XF, Zou J. Azithromycin-containing *versus* standard triple therapy for *Helicobacter pylori* eradication: A meta-analysis. *World J Gastroenterol* 2009; 15(48): 6102-6110 Available from: URL: <http://www.wjgnet.com/1007-9327/15/6102.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.6102>

### Abstract

**AIM:** To evaluate whether adding azithromycin to first-line *Helicobacter pylori* (*H pylori*) eradication improved eradication and reduced side effects.

**METHODS:** Eligible articles were identified by searches of electronic databases. We included all randomized trials that compared azithromycin-containing with standard triple-therapy regimens for first-line treatment of *H pylori* infection. Statistical analysis was performed with Review Manager 5.0.10. Sub-analyses were also performed.

**RESULTS:** We identified 14 randomized trials (1431 patients). Pooled *H pylori* eradication rates were 72.01% (95% CI: 58.09%-85.93%) and 69.78% (95% CI: 66.47%-73.09%) for patients with or without azithromycin by intention-to-treat analysis, and the odds ratio (OR) was 1.17 (95% CI: 0.64-2.14). The occurrence of side effects differed significantly and was 15.81% (95% CI: 12.50%-19.12%) and 25.20% (95% CI: 21.44%-28.96%) for treatment with or without azithromycin, respectively, and the summary OR was 0.58 (95% CI: 0.41-0.82). Furthermore, the azithromycin-containing group had a lower occurrence of diarrhea, nausea and taste disturbance.

**CONCLUSION:** Our review suggests that azithromycin-containing triple-therapy regimens could be equally effective in eradication of *H pylori* compared with standard first-line triple-therapy regimens.

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### INTRODUCTION

Infection caused by *Helicobacter pylori* (*H pylori*), one of the most common pathogens worldwide, causes chronic gastritis and increases the risk of peptic ulcer and gastric cancer. Although some *H pylori*-positive individuals are asymptomatic, many experience symptoms such as dyspepsia. It is increasingly common to screen patients, even those with mild symptoms, for *H pylori* infection, and to treat them actively. The first-line treatment for *H pylori* infection, as recommended by the Maastricht III Consensus Report, is 7-d triple therapy that includes clarithromycin, amoxicillin and a proton-pump inhibitor (PPI)<sup>[1]</sup>. Even though this triple therapy is effective and its short duration helps maintain patient compliance, a considerable number of patients experience undesirable side effects.

In first-line therapy, eradication rates using combinations of PPI-based triple therapies range from 75% to 98%, with most of them near 80%<sup>[2]</sup>. This signifies that up to 20% of patients are expected to be treatment failures, a value which could be even higher in areas with a high prevalence of resistant *H pylori* strains. The recommended second-line therapy is a quadruple regimen composed of tetracycline, metronidazole, bismuth salts and a PPI; however, the efficacy of this regimen is limited by poor compliance, treatment duration, number and dose of the prescribed drugs, and bacterial antibiotic resistance.

Gastroenterologists and microbiologists continue the search for new therapies because of the increasing number of target subjects for *H pylori* and the physiological and pharmacoeconomic burden of a second course of therapy.

Among the new options against *H pylori* brought to light recently, azithromycin has attracted substantial interest. Azithromycin is a macrolide antibiotic that has been shown to reach high concentrations in gastric tissue after oral administration; furthermore, these high concentrations are maintained for several days, which make it potentially useful in the eradication of *H pylori*<sup>3]</sup>. Clinical trials with triple therapy regimens that contain azithromycin have reported eradication rates of approximately 60%-80%, depending on the regimen and azithromycin dose used<sup>14,5]</sup>. However, results from some other available trials utilizing azithromycin have yielded conflicting results. The primary aim of the present meta-analysis was to evaluate whether adding azithromycin to *H pylori* eradication regimens could improve eradication and reduce side effects.

## MATERIALS AND METHODS

### Selection of studies

Studies evaluating azithromycin-containing triple therapy for the eradication of *H pylori* were considered. For the meta-analysis, the selection criteria were as follows: (1) articles that reported comparative randomized controlled trials (RCTs); (2) studies had to include at least two branches of treatment that consisted of (a) triple first-line therapy (one PPI and two antibiotics) and (b) azithromycin-containing triple regimen; (3) study population consisted of subjects who had never been treated for *H pylori* infection previously; and (4) data for successful eradication and/or side effects were available.

### Search strategy for identification of studies

Trials were identified by searching the Cochrane Controlled Trials Register (Issue 2, 2009), PubMed (1966 to May 2009), Embase (1980 to May 2009), Science Citation Index (1945 to May 2009) and the Chinese Biomedical Database (1981 to May 2009). A search strategy was constructed by using a combination of the following words: (*Helicobacter pylori* OR *H pylori*) AND (azithromycin). Articles published in any language were included. Reference lists from the trials selected by electronic searching were hand-searched to identify further relevant trials. We also conducted a manual search of abstracts from 1995 to May 2009 from the following congresses: International Workshop of the European Helicobacter Study Group, American Digestive Disease Week (DDW), and United European Gastroenterology Week (UEGW). Abstracts of the articles selected in each of these multiple searches were reviewed and those meeting the inclusion criteria were recorded. References of reviews on *H pylori* treatment with azithromycin, and from the articles selected for the study, were also examined for articles that met the inclusion criteria. Authors of some identified trials were asked whether they knew of additional studies, including unpublished randomized ones. In case of duplicate reports, or studies obviously reporting results from the same study population, only the latest published results were used.

### Data extraction

Standardized data abstraction sheets were prepared.

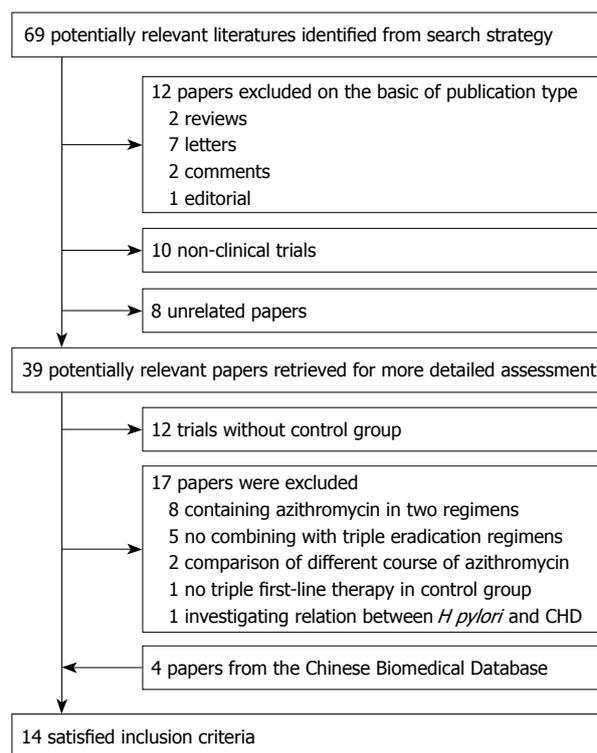


Figure 1 Flowchart of study selection. *H pylori*: *Helicobacter pylori*.

Data were exacted for study quality, dose and duration of azithromycin treatment, anti-*H pylori* regimens, and the number, sex and age of enrolled subjects, diagnostic methods of testing *H pylori* infection before enrolling and after completing the study, and scoring systems for assessing side effects. Key outcome data, such as eradication rates, occurrence of diarrhea, nausea, taste disturbance and abdominal pain were abstracted from all included studies. All articles were examined independently for eligibility by two reviewers. Disagreements were resolved by consulting a third reviewer. Quality was assessed using the Jadad score system based on three items, randomization, double blinding and description of withdrawals/dropouts. We considered that they were low quality when scores were < 3.

### Data synthesis

Data were entered into the Cochrane Collaboration review manager programme RevMan 5.0.10 (released on May 16, 2008). The outcome measure examined was the OR of improving *H pylori* eradication rates and reducing side effects with azithromycin compared to without azithromycin-containing triple regimens. Categorical variables were compared with the  $\chi^2$  test, and  $P < 0.05$  was considered statistically significant. Eradication rates and side effects were analyzed based on a fixed-effects model using the methods of Mantel-Haenszel<sup>6]</sup>, both by intention-to-treat and per-protocol. Heterogeneity between the studies was assessed by  $\chi^2$  test. Statistical significance of heterogeneity was set at 0.10. If significant heterogeneity existed, it would have been inappropriate to combine the data for further analysis using a fixed-effects model, while the random model was used for calculations.

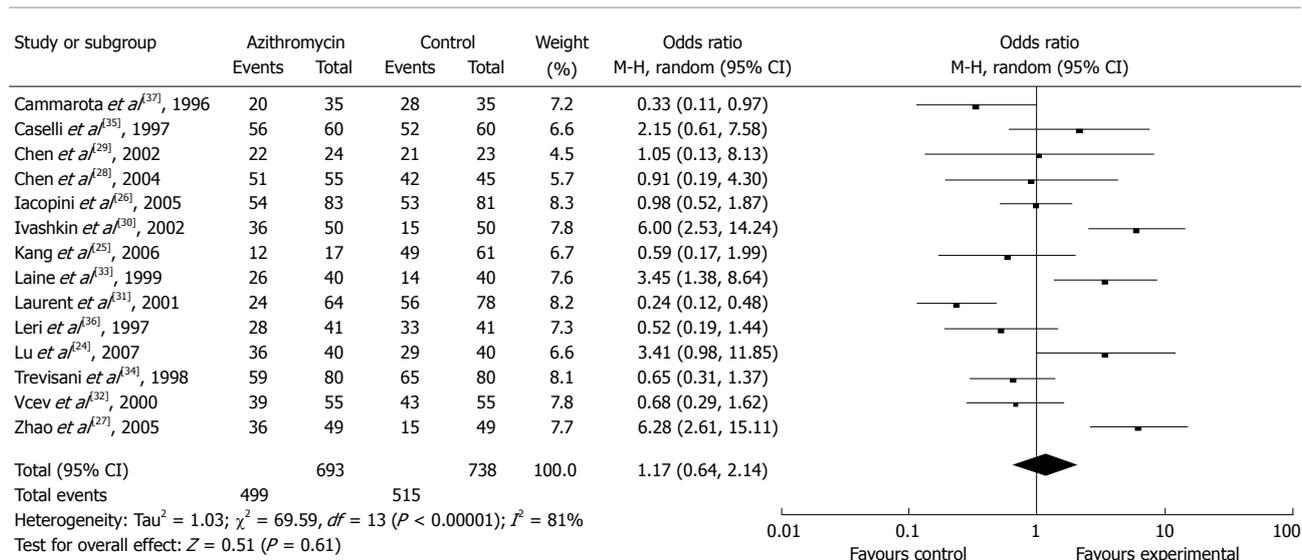


Figure 2 Effect of azithromycin-containing triple therapy versus standard triple therapy on eradication rates by intention-to-treat analysis.

### Sub-analyses

In the meta-analysis, sub-analyses of *H pylori* eradication efficacy were planned, depending on: (1) the type of drugs co-prescribed with azithromycin (combination with amoxicillin and a PPI was the most widely prescribed); (2) the duration and dose of azithromycin therapy; (3) age of the subjects involved; and (4) quality of the studies (based on quality score proposed by Jadad, see appropriate section). Finally, we used funnel plot asymmetry to detect any publication bias in the meta-analysis, and Egger's regression test to measure funnel plot asymmetry.

## RESULTS

### Description of the studies

The bibliographical search yielded a total of 69 studies. Of these, 12 articles were excluded owing to publication type, i.e. two reviews, seven letters, two comments, and one editorial. We excluded 18 articles (10 non-clinical trials and eight unrelated articles) after examining the title and abstract, which left 39 potentially relevant articles for more detailed assessment. Of these potential eligible articles, 12 trials without a control group were excluded, and then we excluded another 17 articles, because of no combining with triple eradication regimens<sup>[7-11]</sup>, containing azithromycin in two regimens<sup>[12-19]</sup>, comparison of different treatment course of azithromycin<sup>[20,21]</sup>, no triple first-line therapy in control group<sup>[22]</sup>, and investigating relation between *H pylori* eradication and coronary heart disease<sup>[23]</sup>. Furthermore, we identified four additional articles from the Chinese Biomedical Database (1981 to May 2009). Finally, 14 RCTs met the inclusion criteria<sup>[24-37]</sup>. The flowchart of reviews showed the detailed process of selection (Figure 1). The characteristics of 14 trials included in the meta-analysis are summarized in Table 1, including quality score.

### Eradication rates

Fourteen studies that described *H pylori* eradication rates were selected for the meta-analysis. Four of these

reported significantly improved eradication rates, and the remaining 10 had similar efficacy for *H pylori* eradication. Pooled eradication rates were achieved in 499 of 693 patients with azithromycin supplementation (72.01%, 95% CI: 58.09%-85.93%) and in 515 of 738 patients with azithromycin without regimen (69.78%, 95% CI: 66.47%-73.09%) by intention-to-treat analysis, the OR was 1.17 (95% CI: 0.64-2.14) (Figure 2). Overall, per-protocol eradication rates were 75.81% (95% CI: 72.44%-79.18%) and 72.44% (95% CI: 69.05%-75.83%) for azithromycin supplementation and azithromycin without regimen, respectively (OR 1.22, 95% CI: 0.61-2.43).

### Side effects

Total side effects were initially performed for meta-analysis. Data for the occurrence of side effects were obtained from 10 RCTs. Five of these studies reported a significant decrease in the occurrence of gastrointestinal side effects. The total number of side effects with azithromycin supplementation differed significantly from azithromycin without regimen: 15.81% (95% CI: 12.50%-19.12%) and 25.20% (95% CI: 21.44%-28.96%), and the summary OR was 0.58 (95% CI: 0.41-0.82) (Figure 3A). Individual symptoms during eradication therapy, such as nausea, diarrhea, abdominal pain, and taste disturbance were also analyzed. Incidence of diarrhea (2.13% *vs* 6.98%) (Figure 3B), nausea (3.85% *vs* 10.14%) (Figure 3C) and taste disturbance (3.17% *vs* 11.05%) (Figure 3D) were lower in the azithromycin supplementation group (OR: 0.33 *vs* 0.37 *vs* 0.28, 95% CI: 0.12-0.96 *vs* 0.14-0.96 *vs* 0.11-0.70).

### Sub-analyses

Sub-analyses for the meta-analysis were planned depending on subject age, symptoms before enrollment, course of azithromycin, and choice of antibiotics. We divided all eligible trials into long- and short-course subgroups, Az+A subgroup, Az+Lev subgroup and Az+M/T subgroup. There was no significant difference between the long-course and short-course subgroups; the summary ORs were 0.89 (95% CI: 0.43-1.85) and 1.56 (95% CI:

**Table 1** Characteristics of included studies comparing *Helicobacter pylori* (*H pylori*) eradication efficacy of azithromycin-containing triple therapy versus standard triple therapy

Authors	Country	Form	Trial design	Case No. (Az/con)	Patients	Diagnostic methods	Azithromycin regimen	% Eradication (n)	% Adverse effects (n)	Triple therapy	Days of antibiotics	% Eradication (n)	% Adverse effects (n)
Lu <i>et al</i> <sup>[24]</sup> , 2007	China	JA	Single centre RCT	85 (43/42)	<i>H pylori</i> positive	RUT/	O (20 mg <i>bid</i> )	ITT 84 (36/43)	26 (11/43)	O (20 mg <i>bid</i> )	7	ITT 69 (29/42)	19 (8/42)
Kang <i>et al</i> <sup>[25]</sup> , 2006	Korea	JA	Single centre RCT	78 (17/61)	<i>H pylori</i> positive	RUT (30 d later)	Lev (200 mg <i>bid</i> )	PP 90 (36/40)		A (1 g <i>bid</i> )		PP 72.5 (29/40)	
							Az (500 mg <i>o.d.</i> )			C (500 mg <i>bid</i> )			
Iacopini <i>et al</i> <sup>[26]</sup> , 2005	Italy	JA	Single centre RCT	164 (83/81)	<i>H pylori</i> positive	Histology + RUT or UBT/	Lev (500 mg <i>o.d.</i> )	PP 70.6 (12/17)	11.8 (2/17)	A (1 g <i>bid</i> )	7	ITT 80.3 (49/61)	41.0 (25/61)
							Az (500 mg <i>o.d.</i> )			C (500 mg <i>bid</i> )			
Zhao <i>et al</i> <sup>[27]</sup> , 2005	China	JA	Single centre RCT	98 (49/49)	<i>H pylori</i> positive	Histology + RUT/	E (20 mg <i>o.d.</i> )	ITT 65 (54/83)	12 (9/77)	E (20 mg <i>bid</i> )	7	ITT 65 (53/81)	30 (22/70)
							Lev (500 mg <i>o.d.</i> )	PP 70 (54/77)		A (1 g <i>bid</i> )			
Chen <i>et al</i> <sup>[28]</sup> , 2004	China	JA	Single centre RCT	100 (55/45)	<i>H pylori</i> positive	UBT + HpSA (8 wk later)	Az (500 mg <i>o.d.</i> )	ITT 73.5 (36/49)	/	C (500 mg <i>bid</i> )	7	ITT 30.6 (15/49)	/
							O (20 mg <i>bid</i> )	PP 76.6 (36/47)		O (20 mg <i>bid</i> )			
Chen <i>et al</i> <sup>[29]</sup> , 2002	China	JA	Single centre RCT	47 (24/23)	<i>H pylori</i> positive	Histology + RUT/	A (1 g <i>bid</i> )	PP 92.7 (51/55)	5 (3/55)	A (1 g <i>bid</i> )	7	ITT 93.3 (42/45)	17.8 (8/45)
							Az (500 mg <i>o.d.</i> ) 3 d	ITT 92 (22/24)	4 (1/24)	M (500 mg <i>bid</i> )			
Ivashkin <i>et al</i> <sup>[30]</sup> , 2002	Russia	JA	Multicenter RCT	100 (50/50)	Chronic gastritis adults	UBT (6 wk later)	L (30 mg <i>o.d.</i> ) 3 d	PP 92.7 (51/55)		A (1 g <i>bid</i> )	7	ITT 91 (21/23)	9 (2/23)
							O (20 mg <i>bid</i> )	PP 92 (22/24)		O (20 mg <i>bid</i> )			
Laurent <i>et al</i> <sup>[31]</sup> , 2001	France	JA	Multicenter RCT	247 (64/78/70)	<i>H pylori</i> positive	Histology + RUT/	M (400 mg <i>bid</i> ) 3 d	PP 92 (22/24)		M (400 mg <i>bid</i> )	7	ITT 71.8/61.4 (56/78)	57.7/66.1 (41/71)
							Az (500 mg <i>o.d.</i> ) 3 d	PP 92 (22/24)		C (500 mg <i>bid</i> )			
Veev <i>et al</i> <sup>[32]</sup> , 2000	Croatia	JA	Single centre RCT	110 (55/55)	<i>H pylori</i> positive	Histology + RUT/	O (20 mg <i>bid</i> )	PP 75 (36/48)	56.9 (33/58)	O (20 mg <i>bid</i> )	7	ITT 78.9/69.4 (56/71)	41/62
							A (1 g <i>bid</i> )	PP 41.4 (24/58)		C (500 mg <i>bid</i> )			
Laine <i>et al</i> <sup>[33]</sup> , 1999	USA	JA	Single centre RCT	120 (40/40/40)	<i>H pylori</i> positive	UBT (4-6 wk later)	Az (500 mg <i>o.d.</i> ) day 1 + (250 mg <i>o.d.</i> ) days 2-5	ITT 71 (39/55)	14 (7/50)	A (1 g <i>bid</i> )	7	ITT 78 (43/55)	17 (9/53)
							P (40 mg <i>bid</i> )	PP 78 (39/50)		A (1 g <i>bid</i> )			
Laine <i>et al</i> <sup>[33]</sup> , 1999	USA	JA	Single centre RCT	120 (40/40/40)	<i>H pylori</i> positive	Histology + RUT (8 wk later)	Az (500 mg <i>o.d.</i> ) 6 d	ITT 65 (26/40)	3 (1/38)	A (1 g <i>bid</i> )	10	ITT 35/78 (14/40)	8/15 (3/37)
							O (80 mg <i>o.d.</i> )	PP 66 (25/38)		O (80 mg <i>o.d.</i> )			
Laine <i>et al</i> <sup>[33]</sup> , 1999	USA	JA	Single centre RCT	120 (40/40/40)	Symptomatic and	UBT (6 wk later)	M (750 mg <i>o.d.</i> )	PP 66 (25/38)		M (750 mg <i>o.d.</i> )	7	PP 35/79 (13/37)	(5/33)

Author(s)	Year	Country	Study Design	Sample Size	Population	Intervention	Control	ITT	PP	OR	95% CI
Trevisani et al <sup>[54]</sup> , 1998	1998	Italy	JA Single centre RCT	160 (80/80)	Asymptomatic adults <i>H pylori</i> positive	Az (500 mg o.d.) 7 d L (30 mg bid) days 1-4	A (1.5 g o.d.) / C (1 g o.d.) O (20 mg o.d.)	ITT 73.3 (59/80)	ITT 81.2 (65/80)	1.3 (1/73)	2.6 (2/76)
Caselli et al <sup>[55]</sup> , 1997	1997	Italy	JA Multicenter RCT	120 (60/60)	Symptomatic adults <i>H pylori</i> positive	T (2000 mg o.d.) day 3 Az (500 mg o.d.) days 2-4 L (30 mg o.d.)	C (250 mg bid) T (500 mg bid) O (20 mg o.d.)	PP 80.8 (59/73)	ITT 86.7 (52/60)	/	/
Leri et al <sup>[56]</sup> , 1997	1997	Italy	Ab Single centre RCT	123 (41/41/41)	Gastritis with or without peptic ulcer <i>H pylori</i> positive	M (250 mg bid) 3 d Az (500 mg o.d.) 3 d O (20 mg bid)	C (250 mg bid) T (500 mg bid) O (20 mg bid)	PP 93.3 (56/60)	ITT 80/97 (33/41) (40/41)	/	/
Cammarota et al <sup>[57]</sup> , 1996	1996	Italy	JA Single centre RCT	70 (35/35)	Symptomatic adults <i>H pylori</i> positive	M (500 mg bid) 10 d Az (500 mg o.d.) 6 d L (30 mg o.d.)	A (1 g bid) / C (500 mg t.d.) L (30 mg o.d.)	ITT 57 (20/35)	ITT 80 (28/35)	18 (6/33)	26 (9/34)

Ab: Abstract; JA: Journal article; C: Clarithromycin; A: Amoxicillin; Az: Azithromycin; M: Metronidazole; T: Tinidazole; Lev: Levofloxacin; E: Esomeprazole; P: Pantoprazole; L: Lansoprazole; O: Omeprazole; UBT: <sup>13</sup>C-urea breath test; RUT: Rapid urease test; HpSA: *H pylori* stool antigen; Q: quality score; RCT: Randomized controlled trial; ITT: Intent-to-treat analysis; PP: Per-protocol analysis.

0.60-4.08), respectively (Figure 4A). For antibiotics sub-analysis, Az+A subgroup, Az+Lev subgroup and Az+M/T subgroup all had no significant difference; the summary ORs were 1.11 (95% CI: 0.32-3.89), 1.19 (95% CI: 0.51-2.81) and 1.20 (95% CI: 0.53-2.69), respectively (Figure 4B).

**Publication bias**

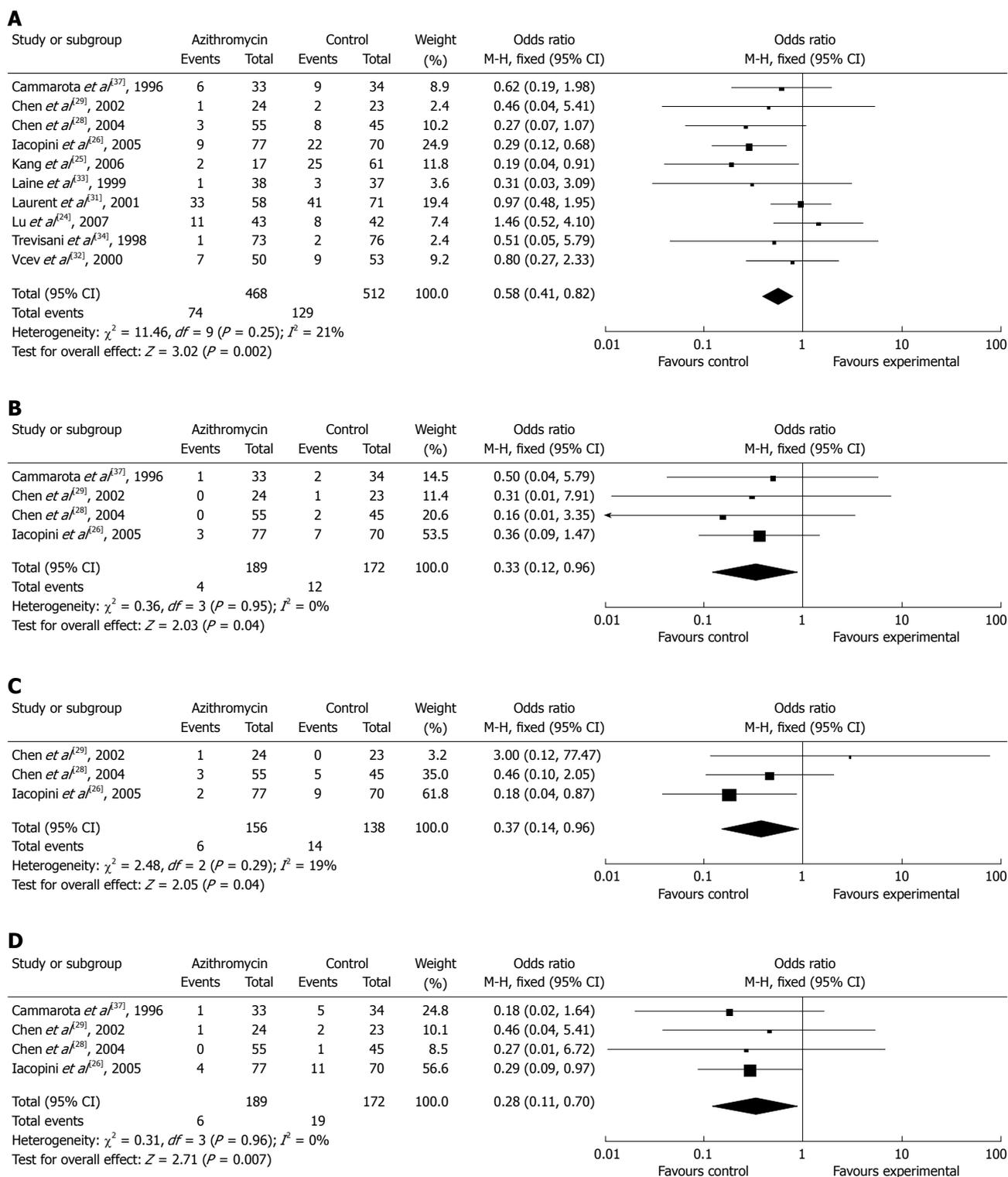
We found that the funnel plot had a slightly asymmetrical distribution, but Egger’s regression test<sup>[38]</sup> suggested no significant asymmetry of the funnel plot (P = 0.84), which indicated no evidence of substantial publication bias.

**DISCUSSION**

For *H pylori* eradication therapy, clinical trails are undertaken to search for simpler but equally or more effective regimens. The modern macrolides are a focus of attention from that point of view. Azithromycin, a new-generation macrolide, has some special attributes that make it a promising compound in regimens for *H pylori* eradication. Following the administration of a single oral dose, azithromycin readily accumulates in the human gastric mucosa, subsequently redistributes from mucosal tissue to the mucus layer, and from the mucus to gastric juice. There, it reaches gastric tissue concentrations that persist above the minimal concentration for 90% inhibition (MIC<sub>90</sub>) for *H pylori* (0.25 µg/mL) over a 5-d period, thus leading to exposure of the microorganism to consistent amounts of this drug. The high tissue affinity and the absorption of the drug after oral administration are reduced when given during or after a meal. The pharmacological properties of azithromycin make it possible to use shorter courses, therefore, the problem was to define an optimal dose and duration of azithromycin in triple therapy.

Azithromycin is able to reach high gastric concentrations that persist for several days, and therefore, it can be administered at a dose of 500 mg once daily for only 3 d during a 7-d triple eradication regimen. The published trials that have used this antibiotic have yielded conflicting results, and have reported a wide range of eradication rates. Administration with meals markedly reduces azithromycin absorption, therefore, this might account for the low eradication rates observed in some studies<sup>[21]</sup>. In treatment regimens in which azithromycin was given to fasting patients, the cure rate was in the range 86%-93%<sup>[13,37]</sup>. Recently, short-term treatments of only 3 d, using a PPI plus azithromycin 500 mg and tinidazole 1000-2000 mg daily, have been found to promote eradication in 81%-88% of cases<sup>[23,39]</sup>. In contrast with the results reported in early studies that have used azithromycin for 2 wk and in repeated daily doses<sup>[40]</sup>, side effects are scarce if the drug is administered once daily for a few days. In subanalyses, we also found that *H pylori* eradication rate had no significant difference between the long-course and short course subgroups.

*H pylori* eradication depends on a number of factors, including patient compliance, side effects, bacterial resistance, poor drug distribution or concentration, geographic differences, and socio-economic conditions. Optimization of *H pylori* eradication therapy remains an



**Figure 3** Effect of azithromycin-containing triple therapy versus standard triple therapy on the incidence of total side effects (A), diarrhea (B), nausea (C), and taste disturbance (D).

ongoing challenge worldwide. Although a great deal of research has focused on treatment of *H pylori* since the discovery of its crucial role in gastrointestinal disease, currently up to 25% of patients enrolled in clinical trials are treatment failures, even using the widely accepted and efficacious regimens that have gained inclusion in consensus guidelines<sup>[41]</sup>. A disappointing cure rate of < 80% after 7-d triple therapy was confirmed in

the present study. Guidelines often suggest that an acceptable success rate for a particular therapy against *H pylori* infection should be > 80% on an intention-to-treat basis. However, clinical trials with azithromycin have displayed considerable variation with respect to the regimens used and the results obtained. Eradication rates varying between 93% and 22% have been reported<sup>[20,30,42]</sup>. The results of our meta-analysis demonstrated pooled

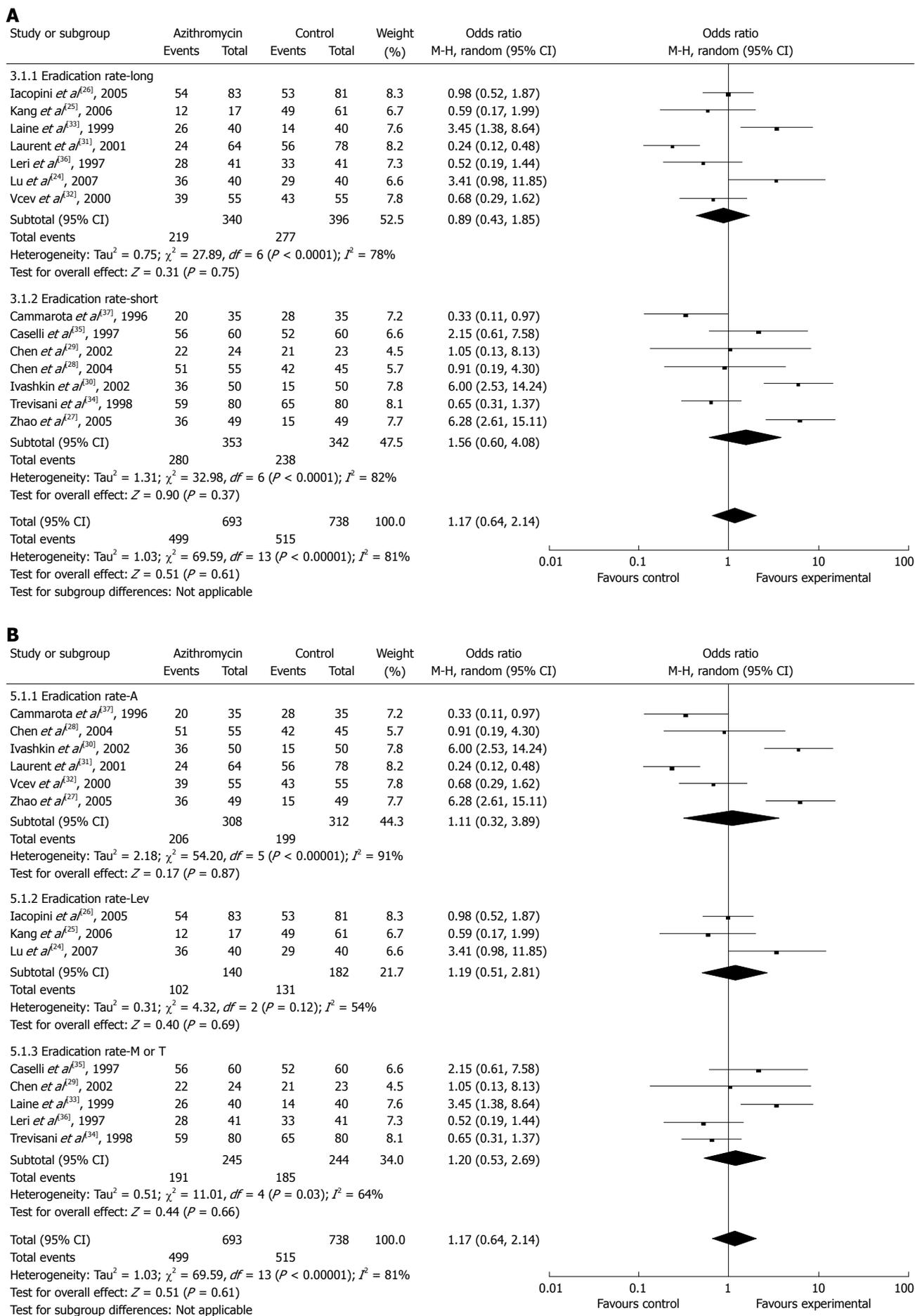


Figure 4 Meta-analysis of eradication rates by treatment course (A) and different antibiotics (B).

*H pylori* eradication rates were 72.01% and 69.78% for patients with or without azithromycin by intention-to-treat analysis, respectively, and no significant difference was observed between the two regimens.

*H pylori* has cross resistance to macrolides; e.g. a strain that is resistant to clarithromycin is resistant to every other macrolide. The level of clarithromycin resistance is unfortunately showing a tendency to increase. The effect of drug synergism is of great value in combination treatment to heal *H pylori* infection. Lepper *et al*<sup>[43]</sup> have demonstrated an *in vitro* synergistic effect of azithromycin and the PPI lansoprazole. They have speculated that this effect might enhance eradication rates even with macrolide-resistant *H pylori* strains, because of the unique pharmacological properties of the combination. Azithromycin could provide a potent anti-*H pylori* effect and could simplify the bulky triple therapy.

Antibiotic-associated gastrointestinal side effects such as diarrhea, nausea, vomiting, bloating and abdominal pain represent a serious drawback of anti-*H pylori* therapy, although they are mild in most cases, but usually result in non-compliance. The quadruple regimen is associated with a relatively high incidence of side effects. In contrast, azithromycin is generally well tolerated, and most side effects associated with its use are mild to moderate in severity and transient. In our systematic review, we found that the total number of side effects with azithromycin supplementation was significantly lower than with azithromycin without regimen: 15.81% *vs* 25.20%; the summary OR was 0.58 (95% CI: 0.41-0.82). Moreover, the incidence of diarrhea (2.13% *vs* 6.98%), nausea (3.85% *vs* 10.14%) and taste disturbance (3.17% *vs* 11.05%) were lower in the azithromycin supplementation group. Our results showed that azithromycin had a positive impact on some *H pylori* therapy-related side effects. Several methodological weaknesses may limit the validity and generalizability of our meta-analysis. For example, there were no studies involving patients from Africa and South America.

In summary, the conclusion of this systematic review and meta-analysis is that, for first-time treatment, azithromycin-containing triple therapy has equal efficacy to that of standard triple eradication therapy. A combination of azithromycin, amoxicillin and a PPI constitutes an encouraging empirical first-line strategy. Furthermore, azithromycin-containing triple therapy showed a lower occurrence of drug-related side effects.

## COMMENTS

### Background

Colonization with *Helicobacter pylori* (*H pylori*) causes a wide range of upper gastrointestinal disorders in humans. Unfortunately, eradication therapy is not always successful, and can even induce several side effects. Azithromycin has some special attributes that make it a promising compound in the regimens for *H pylori* eradication.

### Research frontiers

In first-line therapy, *H pylori* eradication rates using proton-pump inhibitor (PPI)-based triple therapy are about 80%. This signifies that up to 20% of patients are expected to be treatment failures and it could be even higher in areas with a high prevalence of resistant *H pylori* strains. In this study, the authors demonstrated that, for first-time treatment, azithromycin-containing triple therapy has equal efficacy to standard triple eradication therapy.

## Innovations and breakthroughs

Recent studies have shown that azithromycin is a promising compound in regimens for *H pylori* eradication. Our meta-analysis demonstrated that azithromycin-containing triple therapy has equal efficacy to standard triple eradication therapy, and has a lower occurrence of side effects. A combination of azithromycin, amoxicillin and a PPI constitutes an encouraging empirical first-line strategy.

## Applications

By understanding the effect of azithromycin in *H pylori* eradication, this study represents a new encouraging strategy for first-time treatment, and it could decrease the physiological and pharmacoeconomic burden of second courses of therapy.

## Terminology

Azithromycin is a new-generation macrolide and has some special attributes. It is able to reach high gastric concentrations that persist for several days, and therefore may be administered at a dose of 500 mg once daily for only 3 d during 7-d triple eradication therapy.

## Peer review

The authors performed a meta-analysis and demonstrated that azithromycin-containing triple therapy has equal efficacy to standard triple *H pylori* eradication therapy. This was an original and good study.

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## Performance value of high risk factors in colorectal cancer screening in China

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### Abstract

**AIM:** To analyze the performance value of high risk factors in population-based colorectal cancer (CRC) screening in China.

**METHODS:** We compared the performance value of the immunochemical fecal occult blood test (iFOBT) and other high risk factors questionnaire in a population sample of 13214 community residents who completed both the iFOBT and questionnaire investigation. Patients with either a positive iFOBT and/or questionnaire were regarded as a high risk population and those eligible were asked to undergo colonoscopy.

**RESULTS:** The iFOBT had the highest positive predictive value and negative predictive value in screening for advanced neoplasia. The iFOBT had the highest sensitivity, lowest number of extra false positive results associated with the detection of one extra abnormality for screening advanced neoplasias and adenomas. A history of chronic cholecystitis or cholecystectomy, chronic appendicitis or appendectomy, and chronic diarrhea also had a higher sensitivity than a history of adenomatous polyps in screening for advanced

neoplasias and adenomas. The sensitivity of a history of chronic cholecystitis or cholecystectomy was highest among the 10 high risk factors in screening for non-adenomatous polyps. A history of chronic appendicitis or appendectomy, chronic constipation, chronic diarrhea, mucous and bloody stool, CRC in first degree relatives, malignant tumor and a positive iFOBT also had higher sensitivities than a history of adenomas polyps in screening for non-adenomatous polyps. Except for a history of malignant tumor in screening for non-adenomatous polyps, the gain in sensitivity was associated with an increase in extra false positive results associated with the detection of one extra abnormality.

**CONCLUSION:** The iFOBT may be the best marker for screening for advanced neoplasias and adenomas. Some unique high risk factors may play an important role in CRC screening in China.

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**Key words:** Colorectal cancer; Cancer screening; Feces; Occult blood; Risk factors; Predictive value of tests

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Meng W, Cai SR, Zhou L, Dong Q, Zheng S, Zhang SZ. Performance value of high risk factors in colorectal cancer screening in China. *World J Gastroenterol* 2009; 15(48): 6111-6116 Available from: URL: <http://www.wjgnet.com/1007-9327/15/6111.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.6111>

### INTRODUCTION

The incidence of colorectal cancer (CRC) is increasing rapidly, and there is a similar incidence in some Asian populations to that in Western countries because of a more "Westernized" lifestyle and dietary habits<sup>[1]</sup>. A relatively long time for malignant transformation, together with improved survival associated with early detection of cancer, makes CRC an ideal target for screening. In source-limited Asian countries, the fecal occult blood test (FOBT) is the first choice for CRC screening because of its better population impact<sup>[1]</sup>. However, bleeding from cancers and precancerous polyps may be intermittent and

most small colorectal neoplasias do not tend to bleed<sup>[2]</sup>. Therefore, the immunochemical FOBT (iFOBT) alone inevitably misses some important lesions that do not bleed, or bleed intermittently. The iFOBT and a high risk factors questionnaire approach as primary screening followed by full colonoscopy examination as follow-up screening, has been recommended by the Department of Disease Control, the Ministry of Health of China as the protocol for population-based CRC screening in China<sup>[3]</sup>. However, the performance value of the iFOBT and a high risk factors questionnaire is still unknown in CRC screening practice in China. According to the definition of high risk factors by American Cancer Society<sup>[4]</sup>, individuals at higher risk for CRC include "individuals with a history of adenomatous polyps (HAP)". Therefore we used the data available in CRC screening practice in China to examine the performance value of each high risk factor using an acknowledged high risk factor - HAP - as a reference in CRC screening practice in China.

## MATERIALS AND METHODS

### CRC screening protocol in China

The CRC screening protocol of China has been published in a recent study<sup>[5]</sup>. Subjects (age should be defined as  $\geq 40$  years and  $\leq 74$  years) who have one or more of the following items are considered to be at high risk of CRC and should undergo colonoscopy: (1) Positive results from the iFOBT; (2) First-degree relatives with CRC; (3) A personal history of cancers or intestinal polyps; (4) 2 or more of the following items: (a) chronic diarrhea; (b) chronic constipation; (c) mucous and bloody stool; (d) history of appendicitis or appendectomy; (e) history of chronic cholecystitis or cholecystectomy; (f) history of psychiatric trauma (e.g. divorce, death of relatives).

### Study population

From July 2006 to December 2008, a screening program was implemented following the CRC screening protocol recommended by the Ministry of Health of China, for individuals aged 40-74 years in Xiacheng District, Hangzhou City, China. Among 33778 targeted residents, 16918 declined, 3646 participated only in the questionnaire investigation, and 13214 (39.1%) undertook both the iFOBT and questionnaire investigation.

### Study design

The 33778 subjects, aged 40-74 years, who lived in Xiacheng District were enrolled as the target population for our CRC screening practice. Therefore the targeted population can be classified into average, intermediate or high risk individuals. The targeted population was contacted by Chronic Disease Control (CDC) staff to explain the aim of the study, with an invitation to undergo both tests.

The aim of primary screening was to determine the high risk population among the targeted population by the iFOBT and questionnaire approach. Therefore the primary screening test kits included an iFOBT kit (Acon Biotech Co. Ltd., Hangzhou, China), a detailed instruction sheet, a consent form, and a questionnaire containing high

risk items. The iFOBT kit used is a qualitative method, with a hemoglobin detection threshold of 200 ng/mL. Participants were asked to prepare a fecal sample from 3 areas of a stool specimen. No specific dietary restriction was stipulated.

The study was approved by the local ethics committee and all participants gave written informed consent.

### Identification of high risk subjects

All participants learned how to use the iFOBT kit and how to fill in the questionnaire sheet under guidance of CDC staff. Feces samples were processed and results were obtained at the central laboratory of the local CDC. Processing and evaluation were not automated but were performed by trained staff and under strict quality control (double reading, control of frequency of positive tests, reproducibility). Scrutineers of the iFOBT were blinded to the subject's medical records. The screening procedure was considered positive when at least one of the tests was positive. All positive cases resulting from the primary screening were regarded as high risk subjects and those eligible were invited to the follow-up colonoscopy examinations. The CDC staff and primary care managers were responsible for inviting eligible high risk subjects for further colonoscopy examination.

### Colonoscopy examination

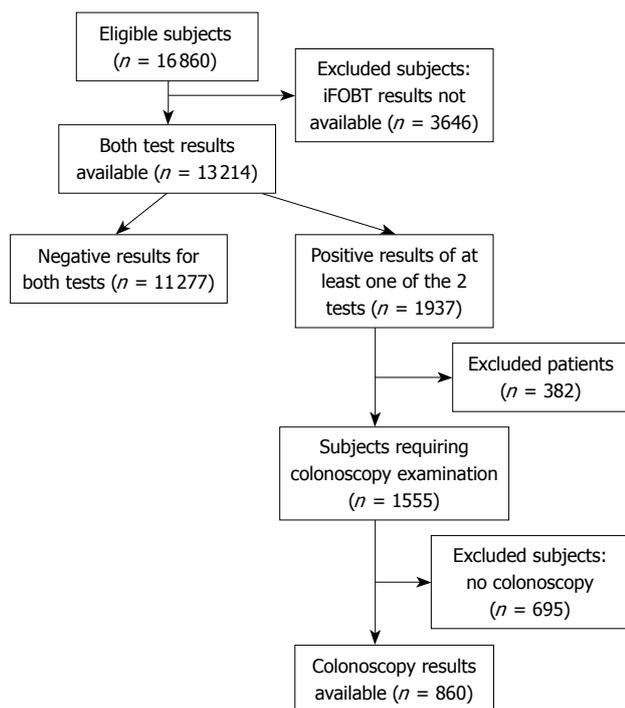
Colonoscopy examination was performed by gastroenterologists in endoscopy units of local hospitals and all participants gave written informed consent. The gastroenterologists recorded data using a standard form, including the quality of bowel preparation, the completeness of the colonoscopy, the number, size, and localization of any detected lesions, and the occurrence of complications. All polyps detected during the colonoscopy were immediately removed and/or biopsied for histologic diagnosis by pathologists. Those who were suspected of having CRC or had polyps that could not be removed endoscopically were referred for surgery. If a colonoscopy examination failed because of inadequate bowel preparation, inaccessibility of the cecum, or lack of satisfactory colonoscopy results, a subsequent colonoscopy would be performed within 1 mo.

### Pathologic examination

In subjects with more than one polyp, the most advanced pathological lesions or the largest lesion was included in the analysis. An advanced neoplasia was comprised of advanced adenomas (an adenoma measuring 10 mm or more in size, adenomas with high grade dysplasia, or an adenomas with villous component  $\geq 25\%$ ) and invasive cancer<sup>[6,7]</sup>. Non-adenomatous polyps included juvenile polyps, inflammatory polyps and hyperplastic polyps. Invasive cancer was defined as invasion by malignant cells through the muscularis mucosae. Intramucosal carcinoma and carcinoma *in situ* were categorized as high grade dysplasia. Pathologic slides of positive lesions were re-examined and diagnosed by consensus by pathologists.

### Statistical analysis

The population of participants in the primary screening



**Figure 1** Flow diagram of the study. iFOBT: Immunochemical fecal occult blood test.

comprised all patients who had given written consent ( $n = 16860$ ). Subjects who accepted only questionnaire investigation ( $n = 3646$ ) in the primary screening were excluded from the study. Subjects with at least one test positive ( $n = 1937$ ) were regarded as positive. A colonoscopy examination was not conducted in 382 subjects because of death, health problems, moving or other reasons. A further 695 subjects rejected a colonoscopy examination. Figure 1 provides a flow diagram of the study.

As the confirmatory procedure (colonoscopy examination) was restricted to subjects classified as positive in at least one of 2 tests (iFOBT and questionnaire examination) positive, the sensitivity of each high risk item could not be directly estimated. According to the theory originally suggested by Schatzkin *et al*<sup>81</sup>, we therefore compared the relative sensitivity (RSN) by calculating the ratio using HAP as reference. For example, if the number of true positive subjects for one high risk factor is denoted by  $m$  and the number of true positive subjects for HAP by  $n$ , RSN is calculated as  $m/n$ . Confidence intervals (95%) were calculated according to the formulae suggested by Cheng *et al*<sup>91</sup>. Using the theory recommended by Chock, the number of extra false positives associated with the detection of one extra true positive was denoted FP:TP, which was calculated as the ratio between the difference in the number of false positive subjects with one high risk factor versus HAP and the difference in the number of true positive subjects with one high risk factor versus HAP<sup>101</sup>.

## RESULTS

### Colonoscopic results of the iFOBT and questionnaire

A total of 21 CRC (2.4%) cases, 48 (5.6%) subjects with advanced adenomas, 147 (17%) subjects with adenomas,

**Table 1** Colonoscopy results of iFOBT and questionnaire  $n$  (%)

	Colorectal cancer	Advanced adenomas	Adenoma	Non-adenomatous polyps
iFOBT positive only	13 (61.9)	22 (45.8)	44 (29.9)	9 (16.7)
Both positive <sup>1</sup>	4 (19)	5 (10.4)	12 (8.2)	2 (3.6)
Only questionnaire positive	4 (19)	21 (43.8)	91 (61.9)	43 (79.7)
Total	21 (100)	48 (100)	147 (100)	54 (100)

<sup>1</sup>Both iFOBT and questionnaire positive. iFOBT: Immunochemical fecal occult blood test.

and 54 (6.3%) subjects with non-adenomatous polyps were detected in 860 colonoscopies. Table 1 shows colonoscopic results of the iFOBT and questionnaire. The iFOBT alone diagnosed 13 cases of cancer, 22 cases of advanced adenomas, 44 cases of adenomas, and 9 cases of non-adenomatous polyps while the questionnaire alone found 4 cases of CRC, 21 cases of advanced adenomas, 91 cases of adenomas, and 43 cases with non-adenomatous polyps. Four cases of CRC, 5 of advanced adenomas, 12 of adenomas, and 2 of non-adenomatous polyps were found in both positives. Table 2 shows the results of colonoscopy according to each high risk item. One perforation was recorded after colonoscopy (0.1%).

### The characteristics of the study population

Table 3 shows the characteristics of the study population. Of 13214 subjects who completed both the iFOBT and questionnaire investigation, 1937 had at least one positive test. The positive rate of the questionnaire investigation was markedly higher than that of the iFOBT (11.7% *vs* 3.6%). A colonoscopy examination was not conducted in 382 subjects (19.7%) because of death, health problems, or other reasons. A total of 860 (55.3%) subjects underwent colonoscopy. In subjects undergoing endoscopic examination, 60.3% were iFOBT positive only, 53.2% were questionnaire positive only, 64.9% were positive for both the iFOBT and questionnaire.

### Performance of high risk factors in screening for advanced neoplasia

Using HAP as the reference, the sensitivity of iFOBT was highest among all high risk factors. The sensitivities of history of chronic cholecystitis or cholecystectomy, chronic appendicitis or appendectomy, history of chronic diarrhea were also higher than that of HAP. The positive predictive value (PPV) and negative predictive value (NPV) of iFOBT were highest among all high risk factors for advanced neoplasias, but the gain in sensitivity was accompanied by an increase in FP:TP. The iFOBT had the lowest FP:TP ratio (Table 4).

### Performance comparison among high risk factors in screening for adenomas

Using HAP as standard, the sensitivity of iFOBT was also highest among 10 high risk factors. Higher sensitivities were also found in history of chronic appendicitis or appendectomy, chronic diarrhea, CRC in first degree relatives, and chronic cholecystitis or cholecystectomy. The

Table 2 Colonoscopy results of high risk questionnaire items

	Cancer	Advanced adenomas	Adenomas	Non-adenomatous polyps	Normal results
iFOBT	17	27	56	11	128
History of malignant tumor	1	3	9	7	41
Colorectal cancer (CRC) in first degree relatives	2	7	29	11	81
History of adenomatous polyps (HAP)	0	9	25	6	48
History of mucous and bloody stool	5	4	15	10	101
History of chronic diarrhea	2	10	29	10	120
History of chronic constipation	0	8	24	13	102
History of chronic appendicitis or appendectomy	3	9	33	14	122
History of chronic cholecystitis or cholecystectomy	3	14	50	19	169
History of psychiatric trauma	4	5	18	2	73

Table 3 Characteristics of the study population *n* (%)

	Subjects with 2 analyzable tests ( <i>n</i> = 13214)
Sex	
Male	5391 (40.8)
Female	7823 (59.2)
Age (yr)	
40-49	2711 (20.5)
50-59	4704 (35.6)
60-69	3683 (27.9)
70-74	2116 (16.0)
Positive items	
iFOBT	481 (3.6)
History of malignant tumor	172 (1.3)
CRC in first degree relatives	367 (2.8)
HAP	158 (1.2)
History of mucous and bloody stool	430 (3.3)
History of chronic diarrhea	709 (5.4)
History of chronic constipation	902 (6.8)
History of chronic appendicitis or appendectomy	1126 (8.5)
History of chronic cholecystitis or cholecystectomy	1538 (11.6)
History of psychiatric trauma	655 (5)

PPV of the iFOBT was 22.5%, just behind that of HAP (26%). The NPV of iFOBT was highest among all high risk factors. The gain in sensitivity was also accompanied by an increase in FP:TP ratio (Table 4).

### Performance comparison of high risk factors in screening for non-adenomatous polyps

Using HAP as standard, a history of chronic cholecystitis or cholecystectomy was the most sensitive marker in screening for non-adenomatous polyps. The sensitivities of other high risk factors except history of psychiatric trauma were also higher than that of HAP. The PPV of history of malignant tumor (10.6%) was highest among all high risk factors in screening for non-adenomatous polyps. Except for history of malignant tumor, the gain in sensitivity was accompanied by increase in the FP:TP ratio (Table 4).

## DISCUSSION

Colonoscopy is often regarded as the "gold standard" for detection of CRC<sup>[11,12]</sup>. Direct colonoscopy screening is the most accurate test for CRC. However because of its potential harm, acceptability<sup>[13]</sup>, availability, and expense<sup>[14]</sup>,

the use of colonoscopy as a one-step screening method for the whole targeted population is unpractical in China. The use of noninvasive screening tests in primary screening, such as iFOBT and questionnaire investigation, have been adopted as the large scale population screening program in China<sup>[15]</sup>. The iFOBT and questionnaire investigation focused on different aspects. The iFOBT can detect bleeding lesions and the questionnaire can find lesions which do not bleed or bleed intermittently. Thus they may have different performance in screening colorectal abnormalities. To the best of our knowledge, the current study is the first analysis comparing the performance value of high risk factors in mass CRC screening in China. Because confirmatory examination was limited to subjects who had at least one positive test, studies calculated the RSN and relative false-positive rate in comparing the 2 screening methods<sup>[16,17]</sup>.

Colorectal adenomatous polyps are recognized as pre-cancerous lesions and are responsible for most cases of CRC<sup>[18]</sup>. Thus far, the important indicator for transition from adenomas to cancer has been the pathologic characteristics of the advanced adenomas. Thus it is important to find advanced adenomas and block the adenoma-carcinoma sequence in CRC screening. The iFOBT had the highest PPV, NPV and RSN, and the lowest FP:TP ratio in screening for advanced neoplasias, indicating that the iFOBT may be superior to other factors in screening for advanced neoplasias. Though some studies in Asian countries have shown that iFOBT is effective in CRC screening<sup>[19,20]</sup>, iFOBT alone may not be enough in CRC screening, because iFOBT inevitably misses some important lesions which do not bleed or bleed intermittently. A history of chronic cholecystitis or cholecystectomy, chronic appendicitis or appendectomy, and chronic diarrhea also had higher sensitivity than HAP, indicating that these unique Chinese high risk factors can detect a larger number of advanced neoplasias. Some studies have found an increase in the risk of CRC following cholecystectomy for gallstones<sup>[21-27]</sup>. Cholecystectomy also influences the adenoma to cancer transition, ultimately predisposing to the development of CRC<sup>[28]</sup>. A study from France supported the hypothesis that the appendix, as a lymphoid organ, plays a protective role in colon carcinogenesis<sup>[29]</sup>. These unique Chinese high risk factors for CRC may play an important role in screening for advanced neoplasias because of their higher sensitivity, which contributed to detection of a greater number of advanced neoplasias.

**Table 4** Comparison of the performance of high risk factors in screening advanced neoplasias, adenomas, non-adenomatous polyps using HAP as reference

	Advanced neoplasias				Adenomas				Non-adenomatous polyps			
	PPV (%)	NPV (%)	RSN	FP:TP	PPV (%)	NPV (%)	RSN	FP:TP	PPV (%)	NPV (%)	RSN	FP:TP
iFOBT	17.70	95.70	4.9 (2.42-9.87)	2.29 (1.36-3.83)	22.50	85.10	2.24 (1.4-3.52)	2.58 (1.37-4.87)	4.40	93	1.8 (0.71-4.58)	16 (3.39-75.58)
HMT	6.10	91.70	0.4 (0.17-1.19)		13.60	82.60	0.36 (0.18-0.7)		10.60	94	1.17 (0.39-3.48)	
FDR	5.70	91.30	1 (0.4-2.519)		18.50	83.20	1.16 (0.69-1.96)	8.25 (0.44-154.47)	7	93.90	1.8 (0.67-4.88)	6.6 (1.16-37.6)
HAP	9.40	92	1		26	84	1		6.30	93.70	1	
MBS	5.70	91.30	1 (0.42-2.39)		9.50	81.20	0.6 (0.33-1.08)		6.30	93.70	1.7 (0.66-4.38)	13.25 (2.02-86.77)
HCD	5.40	91	1.2 (0.54-2.63)	24 (1.87-307.35)	13.90	82	1.16 (0.72-1.88)	18 (1.1-293.1)	4.90	93.30	1.7 (0.71-4.08)	18 (3.2-101.33)
HCC	4.80	91	0.9 (0.35-2.33)		14.30	82.20	0.96 (0.56-1.65)		7.70	94.10	2.2 (0.88-5.49)	7.7 (2.26-26.21)
CAA	5.50	91	1.3 (0.59-2.83)	24.6 (1.63-370.18)	15.10	82.20	1.32 (0.8-2.16)	9.25 (1.52-55.7)	6.40	93.80	2.3 (0.88-5.99)	9.25 (2.96-28.79)
CCC	5.80	87.40	1.9 (0.87-4.12)	15.13 (4.47-51.23)	16.90	82.80	2 (1.26-3.16)	4.84 (2.43-9.65)	6.40	93.80	3.2 (1.38-7.42)	9.3 (4.5-19.22)
HPT	7.60	91.80	1 (0.42-2.39)		15.30	82.60	0.72 (0.43-1.2)		1.70	93	0.33 (0.07-1.63)	

HMT: History of malignant tumor; FDR: History of CRC in first degree relatives; MBS: History of mucous and bloody stool; HCD: History of chronic diarrhea; HCC: History of chronic constipation; CAA: History of chronic appendicitis or appendectomy; CCC: History of chronic cholecystitis or cholecystectomy; HPT: History of psychiatric trauma; PPV: Positive predictive value; NPV: Negative predictive value; RSN: Relative sensitivity; FP:TP ratio: The ratio between the difference in the number of false positive subjects with one high risk factor *vs* HAP and the difference in the number of true positive subjects with one high risk factor *vs* HAP. Values for RSN and FP:TP are mean (95% CI). RSN > 1: Sensitivity of the high risk factor is greater than that of HAP.

It would be unsafe to ignore adenomas < 10 mm because 30% of cancer is derived from 6-9 mm adenomas<sup>[30]</sup>. The questionnaire detected a greater number of adenomas than the iFOBT in our screening study. We also found that the iFOBT still had the highest sensitivity among all high risk factors, followed by history of chronic cholecystitis or cholecystectomy, chronic appendicitis or appendectomy, and chronic diarrhea. The iFOBT may also be superior to other factors in screening for adenomas because of high PPV and NPV, high RSN and low FP:TP ratio. Higher sensitivities indicated the important performance value of these unique Chinese high risk factors.

A history of chronic cholecystitis or cholecystectomy was the most sensitive marker in screening for non-adenomatous polyps, followed by history of chronic appendicitis or appendectomy, and chronic constipation. Though subjects with non-adenomatous polyps were not regarded as having increased risk of CRC, these polyps do not require surveillance colonoscopy, they may serve as a precursor to CRC in subjects with specific genetic and other molecular characteristics<sup>[31-34]</sup>. Thus it would be unsafe to ignore these polyps.

The study had several drawbacks. Firstly, to evaluate screening test performances among the general population, the ideal is to obtain sensitivity and specificity for all individuals. Because only eligible high risk subjects were invited and only about 55% of population accepted the colonoscopy examination, these results may not be completely representative of the general population. Secondly, although all the study population accepted both the iFOBT and questionnaire investigation, the colonoscopy uptake rate of the iFOBT positive only was

higher than that of the questionnaire positive only. This would slightly overestimate the RSN of the iFOBT.

HAP, an acknowledged high risk factor was used as the reference to calculate the relative ratio in this study. Therefore the other variables being compared may be underestimated. Even so, the iFOBT and some unique Chinese high risk factors - history of chronic cholecystitis or cholecystectomy, chronic appendicitis or appendectomy, and history of chronic diarrhea - still play an important role because of the higher sensitivities than that of HAP. The iFOBT may be superior to other factors in screening for advanced neoplasias and adenomas.

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## COMMENTS

### Background

The immunochemical fecal occult blood test (iFOBT) and high risk factors questionnaire approach as primary screening followed by full colonoscopy examination as follow-up screening, has been recommended as the colorectal cancer (CRC) screening guideline for population-based CRC screening in China. The performance value of the iFOBT and the high risk factors questionnaire is still unknown in CRC screening practice in China.

### Research frontiers

The limitation of the iFOBT is its low sensitivity for CRC. High risk factors for CRC among a Chinese natural population have been identified through a meta-analysis. The major advantage of the high risk factors questionnaire investigation is that it can detect lesions that do not bleed or bleed intermittently.

### Innovations and breakthroughs

This is believed to be the first study comparing the performance value of high risk factors in mass CRC screening in China. In this study, because participants with at least one positive factor were asked to undergo colonoscopy, the sensitivity of each high risk item could not be directly estimated. The authors therefore compared sensitivities by calculating the relative sensitivity using HAP (history of adenomatous polyps, an acknowledged high risk factor) as a reference.

### Applications

The study suggests that the iFOBT may be the best marker for screening advanced neoplasias and adenomas. Some unique Chinese high risk factors (history of chronic cholecystitis or cholecystectomy, chronic appendicitis or appendectomy, and history of chronic diarrhea) may play an important role in CRC screening in China because of higher sensitivities than that of HAP.

### Peer review

The authors have done much work in this study. The study is worthwhile and well performed.

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## High expression of osteoglycin decreases gelatinase activity of murine hepatocarcinoma Hca-F cells

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### Abstract

**AIM:** To investigate the possible correlation between osteoglycin expression and gelatinase activity of mouse hepatocarcinoma Hca-F cells.

**METHODS:** A eukaryotic expression plasmid pIRESpu-ro3 osteoglycin(+) was constructed and transfected into Hca-F cells to investigate the possible correlation between osteoglycin expression and gelatinase activity of Hca-F cells cultured with extract of lymph node, liver, spleen or in DMEM medium. The activity of gelatinases was examined through zymographic analysis.

**RESULTS:** High expression of osteoglycin attenuated the gelatinase activity of Hca-F cells cultured with extract of lymph node, and at the same time, decreased the metastatic potential of Hca-F cells to peripheral lymph nodes *in vivo*.

**CONCLUSION:** High expression of osteoglycin decreases the gelatinase activity of Hca-F cells cultured with extract of lymph node; regulation of gelatinase activity might be one of mechanisms that osteoglycin contributes to lymphatic metastasis suppression.

**Key words:** Osteoglycin; Transfection; Hepatocellular carcinoma; Neoplasm metastasis; Genes; Gelatinases

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### INTRODUCTION

Most cancer lesions metastasize through the lymphatic system and the status of regional lymph nodes is the most important indicator of a patient's prognosis<sup>[1]</sup>. But the molecular mechanism of lymphatic metastasis remains unclear. Hca-P and Hca-F are syngeneic mouse hepatocarcinoma cell lines, when inoculated subcutaneously in 615-mice, they metastasized only to the lymph nodes but not to other organs, Hca-P cells illustrated a low metastatic potential (lymphatic metastasis rate < 30%), while Hca-F cells showed a high metastatic potential (lymphatic metastasis rate > 80%)<sup>[2,3]</sup>. In our previous study, we found that osteoglycin was highly expressed in Hca-P cells and lowly expressed in Hca-F cells with suppressively subtracted hybridization (SSH) technique. Osteoglycin (OGN) is a member of proteoglycans (PGs) called small leucine-rich proteoglycans (SLRPs) residing in the extracellular matrix of connective tissues which are involved in matrix assembly, cellular growth and migration<sup>[4]</sup>. There are few reports about the relationship between osteoglycin and tumor metastasis. We subsequently transfected osteoglycin into Hca-F cells and found that high expression of osteoglycin inhibited the metastatic behavior of Hca-F cells<sup>[5]</sup>. However, the mechanism of osteoglycin regulating metastasis is elusive.

Gelatinases/type IV collagenases belong to matrix metalloproteinase (MMP) family, including gelatinase A (also known as MMP2, 72 kDa) and gelatinase B (also known as MMP9, 92 kDa), they are secreted in a proenzyme form and activated extracellularly<sup>[6]</sup>. Gelatin-

ases mainly degrade collagen IV and a number of other ECM proteins, such as Col I, V, VII, IX, fibronectin, laminin, elastin and vitronectin<sup>[7]</sup>. As the most frequently studied MMPs in tumor research, gelatinases are suggested to play critical roles in tumor invasion and metastasis<sup>[8]</sup>.

In this study, we resorted to gene transfection technique to explore the possible correlation between osteoglycin expression and gelatinase activity of murine hepatocarcinoma Hca-F cells with a high metastatic potential. We found that high expression of osteoglycin decreased the gelatinase activity of Hca-F cells cultured with extract of lymph node, and at the same time, decreased the metastatic potential of Hca-F cells to peripheral lymph nodes *in vivo*; regulation of gelatinase activity might be one of mechanisms that osteoglycin contributes to lymphatic metastasis suppression.

## MATERIALS AND METHODS

### Cell culture and animals

Mouse hepatocarcinoma Hca-P cells and Hca-F cells (established by Department of Pathology, Dalian Medical University) were cultured in DMEM (Invitrogen) supplemented with antibiotics (1 × penicillin/streptomycin 100 U/mL, Invitrogen), 10% FBS (Invitrogen) and cultured in a humidified incubator at 37°C with 50 mL/L CO<sub>2</sub>; inbred 615-mice (male, 8 wk old) were provided by Animal Facility of Dalian Medical University.

### Construction of targeting vector

The osteoglycin coding sequence was amplified by polymerase chain reaction (PCR). Briefly, total RNA from 1 × 10<sup>7</sup> Hca-F cells was isolated with Trizol (Invitrogen). A High Fidelity PrimeScript RT-PCR kit (TaKaRa) was used to synthesize the cDNA according to the manufacturer's protocol. PCR was carried out with primer sets P1, 5'-GAATTCATGGAGACTGTGCACTCTA-3' (forward), and P2, 5'-GCCGCCGCTTAGAAGTATGACCCTA-3' (reverse), containing *Eco*R I and *Not* I sites, respectively (underlined). Using obtained cDNA as a template, PCR was carried out under the following conditions: 30 cycles of denaturation for 10 s at 98°C, annealing for 15 s at 55°C, and extension for 60 s at 72°C. After digestion by *Eco*R I and *Not* I enzymes, the PCR product was cloned into pIRESpuro3 vector digested by the same enzymes and designated as pIRESpuro3 osteoglycin(+). Sequence and orientation were confirmed by DNA sequencing using a BigDye Terminator V3.1 cycle sequencing kit (Applied Biosystems).

### Cell transfection and screening

Hca-F cells incubated in antibiotic-free medium with 10% FBS (Invitrogen) were transferred to a 6-well culture plate and incubated at 37°C, CO<sub>2</sub> incubator to obtain 60%-80% confluence, and then were stably transfected with pIRESpuro3 and pIRESpuro3 osteoglycin(+) using *TransIT-LT1* Transfection Reagent (TaKaRa) according to the protocol provided by the manufacturer. Two µg plasmid DNA was added to each

transfection. The transfected Hca-F cells were selected by puromycin (Clontech) for 2 wk and maintained in medium containing 0.5 mg/L puromycin.

### RT-PCR analysis

For RT-PCR analysis of osteoglycin mRNA levels, total RNA was isolated from cells using Trizol (Invitrogen) and cDNA was synthesized with High Fidelity PrimeScript<sup>TM</sup> RT-PCR Kit (TaKaRa) according to the manufacturer's instruction. The sequences of the primers were as follows: F1: 5'-TTCTCCTGCTACTCTTCGTG-3' and R1: 5'-AAGCAGACACACAACAGGCA-3' for osteoglycin; and F1: 5'-CGGGACCTGACAGACTACC T-3' and R1: 5'-AGCACTGTGTTGGCATAGAG-3' for β-actin, respectively. PCR analysis was performed under the following conditions: 30 cycles of denaturation for 10 s at 98°C, annealing for 15 s at 55°C, and extension for 30 s at 72°C. The amplified products were analyzed by agarose gel electrophoresis using 1.6% gel, followed by ethidium bromide staining. The bands were analyzed with LabWorks (UVP GDS-800 Version 4.0).

### Western blotting analysis

Western blotting analysis was carried out to evaluate osteoglycin protein levels. Cellular protein was extracted with lysis buffer [20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L MgCl<sub>2</sub>, 2 mmol/L EGTA, 10% glycerol, 0.15% sodium dodecylsulfate, 1% deoxycholate, 1% Triton X-100, and 1% anti-protease cocktail (Sigma)]. The extracted proteins were subjected to 10% sodiumdodecylsulfate-polyacrylamide gel electrophoresis, blotted onto polyvinylidene difluoride membranes (Invitrogen), then probed with goat anti-mouse osteoglycin polyclonal antibody and β-actin monoclonal antibody (Santa Cruz) followed by secondary antibody conjugated to horseradish peroxidase (Santa Cruz) and detected by enhanced chemiluminescence (Amersham Biosciences). The bands were analyzed with LabWorks (UVP GDS-800 Version 4.0).

### In vivo tumor metastasis assay

Ninety inbred 615-mice were randomly divided into 3 groups. Hca-F cells (F), Hca-F cells transfected with pIRESpuro3 (F0), or Hca-F cells transfected with pIRESpuro3 osteoglycin(+) [F(+)] were inoculated subcutaneously at 2 × 10<sup>6</sup> tumor cells of approximately 0.05 mL cell suspension into the left foot of each mouse in each group. They were terminated on the 28th day after inoculation, the implanted tumor and their axillary lymph nodes, inguinal lymph nodes, and popliteal lymph nodes were hematoxylin eosin (HE) stained and examined under microscope. The mouse which had at least one metastatic axillary lymph node or one metastatic inguinal lymph node or one metastatic popliteal lymph node was considered as a metastatic mouse. The lymph node metastatic rate of tumor-burden mice = metastatic mice/total mice.

The lymph node metastatic rates of F, F0 and F(+) cells burden mice were calculated. The number of positive lymph nodes per mouse was also evaluated.

### Zymographic analysis

The F, Hca-P (P), F0 and F(+) cells were put into different wells at  $5 \times 10^5$ , and then added 50 mg extract of lymph node, liver or spleen respectively. The Dulbecco's Modified Eagle Media (DMEM) was placed into each well up to 1 mL. DMEM medium containing only F, P, F0 or F(+) cells, and DMEM medium added only extracts of lymph node, liver or spleen served as controls. These cells were cultured at 37°C for 24 h. The supernatant of cultured cells was collected by centrifugation at  $3000 \times g$ . Gelatinases contained in supernatants of each cell with or without extracts of lymph node, liver or spleen were detected through zymographic analysis according to the method described by Fridman<sup>[9]</sup>. The bands were analyzed with LabWorks (UVP GDS-800 Version 4.0).

### Statistical analysis

Data were presented as means  $\pm$  SD and analyzed by the Student's *t* test, analysis of variance and  $\chi^2$  test using SPSS 11.5.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Osteoglycin expression at mRNA and protein level

The relative mRNA and protein levels of osteoglycin were determined by RT-PCR and Western blotting analysis, respectively. Compared with F and F0 cells, F(+) cells showed significantly higher expression of osteoglycin at both mRNA and protein levels; however, no significant difference of osteoglycin expression was found between F0 and F cells. Transfection of osteoglycin into Hca-F cells resulted in high expression of osteoglycin at both mRNA and protein levels. Osteoglycin was highly expression at both mRNA and protein levels in P cells (Figure 1).

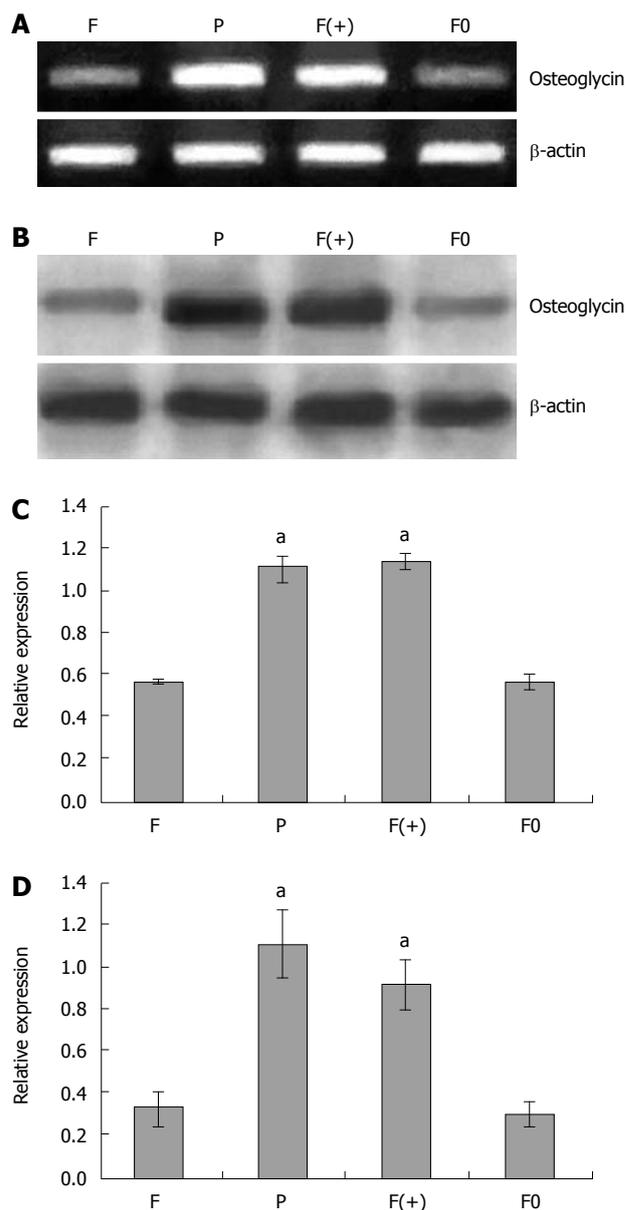
### In vivo tumor metastasis assay

F, F0 and F(+) cells were injected subcutaneously into the left foot of 615-mice. The implanted tumors were palpable on the 7th day after inoculation. On the 28th day after inoculation, 53.3% (16/30) F(+) cells burden mice developed lymphatic metastasis, while 80% (24/30,  $P < 0.05$ ) F cells burden mice and 83.3% (25/30,  $P < 0.05$ ) F0 cells burden mice developed lymphatic metastasis. Hca-F cells with transfected osteoglycin showed significant decrease in metastasis potential to lymph node (Figure 2). The result supported the fact that osteoglycin acted as a tumor lymphatic metastasis suppressed gene.

No significant difference was found in the number of positive lymph nodes per mouse in F(+), F and F0 cells burden mice.

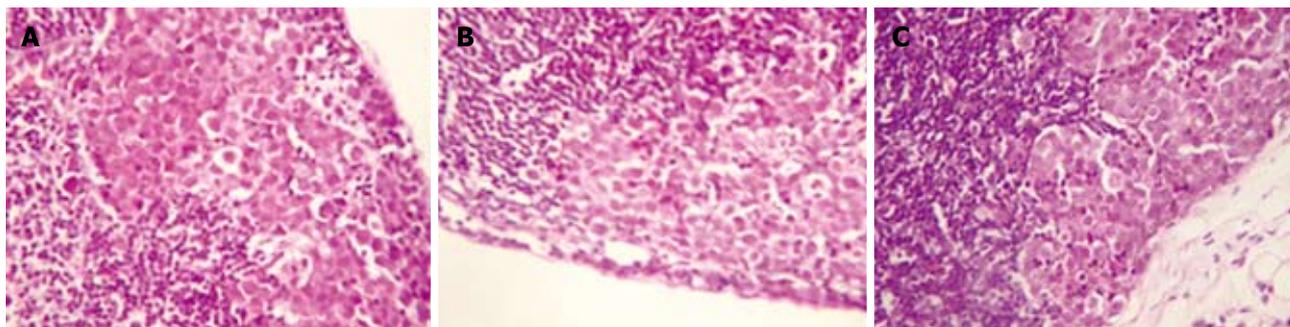
### Zymographic analysis

When cultured in DMEM, no cell produced any gelatinase (no gelatinase was detected in the supernatant of each cell). However, when cultured with extract of lymph node, all cells produced gelatinases (Pro-MMP-9, MMP-9 active, Pro-MMP-2 and MMP-2 active were detected in the supernatant of each cell). The quantity of gelatinases produced by tumor cells were closely associated with the metastatic potential of each tumor cell (quantity of

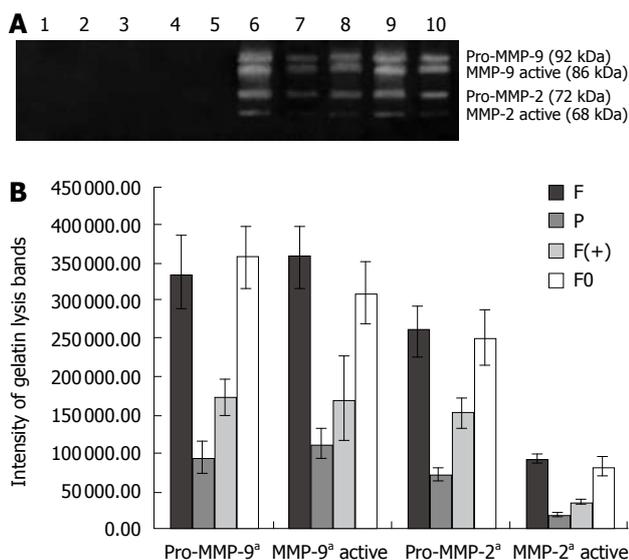


**Figure 1** Analysis of osteoglycin expression. RT-PCR analysis (A) and Western blot analysis (B) of osteoglycin expression in mouse hepatocarcinoma cells; relative signal intensities of osteoglycin mRNA (C) and protein (D) levels were normal as against those of  $\beta$ -actin by LabWorks (UVP GDS-800 Version 4.0) analysis (compared with F cells,  $^aP < 0.05$ ). F: Hca-F cells; P: Hca-P cells; F(+): Hca-F cells transfected with pIRESpuo3 osteoglycin(+); F0: Hca-F cells transfected with pIRESpuo3.  $\beta$ -actin was used as an internal control.

MMP2 and MMP9 detected in the supernatant of F and F0 cells were much higher than those detected in F(+) and P cells ( $P < 0.05$ ). High expression of osteoglycin *via* transfection of osteoglycin attenuated the secretion of gelatinases in Hca-F cells cultured with extract of lymph node (quantities of MMP2 and MMP9 detected in the supernatant of F(+) cells were much lower than those detected in F and F0 cells ( $P < 0.05$ ). The extract of lymph node did not contain any gelatinase (Figure 3). Gelatin lysis bands were found in the zymograms of the supernatant of all cells cultured with extract of liver, and the same gelatin lysis bands were found in the zymograms of the extract of liver, and their intensities were almost the same (Figure 4); gelatin lysis bands were also found in the



**Figure 2** Metastatic lymph nodes of tumor-burden mice inoculated with Hca-F cells (A), Hca-F cells transfected with pIRESpuro3 (B), or Hca-F cells transfected with pIRESpuro3 osteoglycin(+) (C). Lymph nodes of tumor-burden mice were HE stained and examined under microscope.

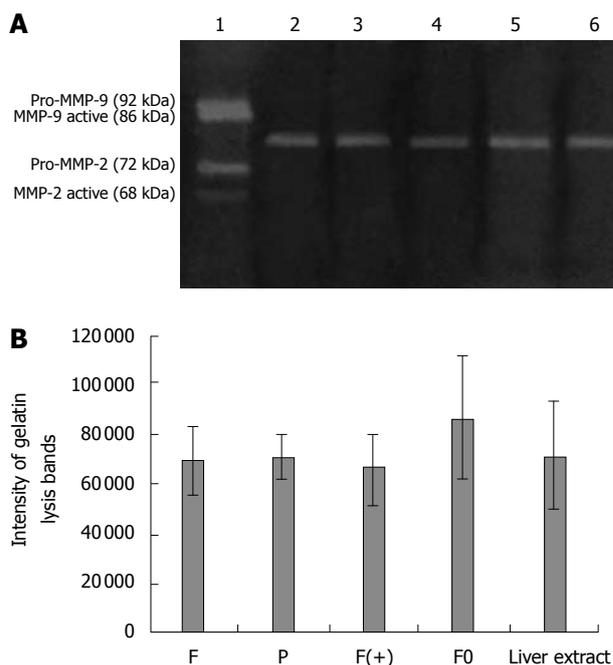


**Figure 3** Zymographic analysis of MMPs activity of tumor cells in DMEM with or without lymph node extract (A); the intensity of gelatin lysis bands obtained by scanning densitometry (LabWorks UVP GDS-800 Version 4.0, multiple comparisons, <sup>a</sup> $P < 0.05$ ) (B). 1. L; 2. F; 3. P; 4. F(+); 5. F0; 6. F; 7. P; 8. F(+); 9. F0. 10. Type IV collagenases. 1. L: lymph node extract; 2-5: cells in DMEM. 6-10: cells cultured with lymph node extract. F: Hca-F cells; P: Hca-P cells; F(+): Hca-F cells transfected with pIRESpuro3 osteoglycin(+); F0: Hca-F cells transfected with pIRESpuro3.

zymograms of the supernatant of all cells cultured with extract of spleen, and in the zymograms of the extract of spleen, with similar intensities (Figure 5). Therefore, we think that all cells in the liver and spleen did not produce any gelatinases.

## DISCUSSION

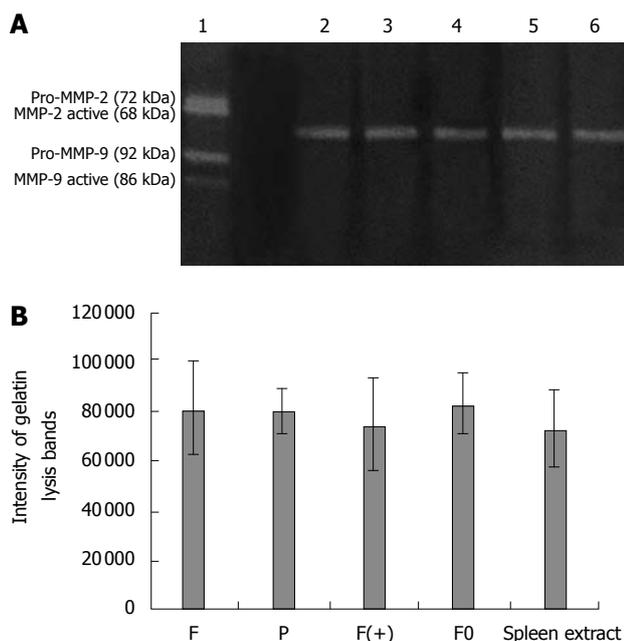
The metastatic potential of tumor cells is believed to be regulated by interactions between the tumor cells and their extracellular environment (extracellular matrix)<sup>[10,11]</sup>. Being a matrix molecule, osteoglycin participates in the organization and regulation of the extracellular matrix and might influence the tumor metastasis, as exemplified by studies *in vivo* that osteoglycin played a role in collagen fibrillogenesis<sup>[12,13]</sup>, a process essential in metastasis<sup>[11,12]</sup>. In addition to its extracellular matrix functions, osteoglycin, like other members of SLRPs, also plays a role



**Figure 4** Zymographic analysis of MMPs activity of tumor cells in liver extract (A); the intensity of gelatin lysis bands obtained by scanning densitometry (LabWorks UVP GDS-800 Version 4.0) (B). 1. Type IV collagenases; 2. F; 3. P; 4. F(+); 5. F0; 6. Liver extract. F: Hca-F cells; P: Hca-P cells; F(+): Hca-F cells transfected with pIRESpuro3 osteoglycin(+); F0: Hca-F cells transfected with pIRESpuro3.

in regulation of cell biological behavior<sup>[4]</sup>. As illustrated in the literature, the expression of mimecan was high at mRNA level in corneal keratocytes cultured in low-serum or serum-free media, but was attenuated if these cells were cultured in media containing serum<sup>[14]</sup>. Osteoglycin mRNA was absent or at a low level in the majority of cancer cell lines and tumors<sup>[15]</sup>. Bioactive such as p53, basic fibroblast growth factor, interferon- $\gamma$  and bone morphogenetic protein-1/tolloid-related metalloproteinases interacted with osteoglycin<sup>[16-20]</sup>. In the earlier studies, we found that osteoglycin was highly expressed in Hca-P cells and lowly expressed in Hca-F cells, and that osteoglycin acted as a tumor lymphatic metastasis suppressed gene<sup>[5]</sup>. However, no data identified intrinsic mechanism for osteoglycin regulation of tumor lymphatic metastasis.

Hca-P and Hca-F are syngenic mouse hepato-



**Figure 5** Zymographic analysis of MMPs activity of tumor cells in spleen extract (A); the intensity of gelatin lysis bands obtained by scanning densitometry (LabWorks UVP GDS-800 Version 4.0) (B). 1. Type IV collagenases; 2. F; 3. P; 4. F(+); 5. F0; 6. spleen extract. F: Hca-F cells; P: Hca-P cells; F(+): Hca-F cells transfected with pIRESpuo3 osteoglycin(+); F0: Hca-F cells transfected with pIRESpuo3.

carcinoma cell lines presenting a specific potential of lymphatic metastasis with a significant difference in their potential of metastasis<sup>[2,3]</sup>, which provide good experimental models for lymph node metastasis.

Cell adhesion to extracellular matrices is a determinant for cell migration and invasion<sup>[21,22]</sup>. Osteoglycin, being a matrix molecule, as we once assumed, would probably affect adhesive capacity of tumor cells, whereby influencing tumor migration and invasion. However, our previous work showed that adhesion was not responsible for the contribution of osteoglycin to lymphatic metastasis inhibition<sup>[5]</sup>. As the main mediators of extracellular matrix degradation, gelatinases play an important role in tumor metastasis as demonstrated in gastrointestinal cancer<sup>[23,24]</sup>, breast cancer<sup>[25]</sup>, hepatocarcinoma<sup>[26]</sup>, *etc.* Inhibition of the gelatinase activity can reduce the metastatic potential of cancer cells<sup>[27]</sup>. In the present study, high expression of osteoglycin *via* osteoglycin transfection attenuated the secretion of gelatinases (Pro-MMP-9, MMP-9 active, Pro-MMP-2 and MMP-2 active) in Hca-F cells cultured with extract of lymph node, and at the same time, decreased the metastatic potential of Hca-F cells to peripheral lymph nodes *in vivo*, which suggested that regulation of gelatinase activity might be one of mechanisms that osteoglycin contributes to lymphatic metastasis suppression. Moreover, osteoglycin expression only influenced gelatinase activity of Hca-F cells cultured with extract of lymph node, but failed to influence gelatinase activity of Hca-F cell cultured with extracts of liver and spleen or in DMEM medium, demonstrating a lymph node environment-selective metastasis suppression, which further supported the fact that osteoglycin acted as lymphatic metastasis suppression

gene. The mechanism of osteoglycin impact on gelatinases is unclear. Some of the SLRPs members bind and modulate TGF- $\beta$  and cytokines such as TNF- $\alpha$ <sup>[28,29]</sup> and play roles in EGFR activation pathway and the NF- $\kappa$ B signal transduction system as well<sup>[30,31]</sup>. And these bioactives (TGF- $\beta$ , TNF- $\alpha$ , EGF and NF- $\kappa$ B) are also the regulators of gelatinase activity<sup>[6,32]</sup>, which implicates that SLRPs might involve in the regulation of gelatinase activity. Further studies are needed to clarify the interaction between gelatinases and osteoglycin.

## COMMENTS

### Background

Lymphatic metastasis is responsible for the early stage of tumor metastasis and acts as the most important indicator of a patient's prognosis. But the molecular mechanism of lymphatic metastasis remains poorly understood.

### Research frontiers

The metastatic potential of tumor cells is believed to be regulated by interactions between the tumor cells and their extracellular environment (extracellular matrix). Matrix molecules play important roles in tumor metastasis. Osteoglycin, as one of matrix molecules, is suggested to play a part in matrix assembly, cell growth and migration. However, there has been no report on osteoglycin and tumor metastasis.

### Innovations and breakthroughs

The authors first report that osteoglycin acted as a tumor lymphatic metastasis suppression gene, and regulation of gelatinase activity might be one of mechanisms that osteoglycin contributes to lymphatic metastasis suppression.

### Applications

This study may help for therapeutic intervention in tumor metastasis.

### Terminology

Osteoglycin belongs to a small leucine-rich proteoglycan (SLRP) gene family, as one of the matrix molecules, it is reported to participate in the organization and regulation of the extracellular matrix. In addition to its extracellular matrix functions, osteoglycin, like other members of SLRPs, also plays a role in regulation of cell biological behavior, cell growth and migration, *etc.*

### Peer review

In this study, it was observed that osteoglycin upregulation, induced by its transfection into mouse hepatocarcinoma Hca-F cells, results in a decrease in gelatinase activity and metastatic potential of Hca-F cells. The effect of osteoglycin transfection on gelatinase activity has been convincingly demonstrated.

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## Meckel's diverticulum manifested by a subcutaneous abscess

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### Abstract

This case report describes an extremely rare complication of a Meckel's diverticulum: enterocutaneous fistula of the diverticulum. The presence of Meckel's diverticulum is a well known entity, but subcutaneous perforation of the diverticulum is very rare. Here we report the case of a patient with the complaint of a right lower quadrant abscess, preoperatively diagnosed as enterocutaneous fistula, which was determined intraoperatively to be a fistula resulting from Meckel's diverticulum.

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**Key words:** Meckel's diverticulum; Enterocutaneous fistula; Abscess

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### INTRODUCTION

Meckel's diverticulum is the most common congenital

anomaly of the gastrointestinal tract<sup>[1-4]</sup>. It is an outpouching of the distal ileum located at the antimesenteric border usually within 45-60 cm of the ileocecal valve<sup>[4]</sup>. The majority of Meckel's diverticulum cases are asymptomatic although they can, occasionally, cause complications such as bleeding, intestinal obstruction and/or inflammatory process<sup>[1-4]</sup>. The presentation as an abscess in the abdominal wall is a rare clinical entity. Because of insidious onset and subtle clinical signs of the resulting abscess, the diagnosis of such cases is often delayed. Meckel's diverticula such as those forming extensive abscesses may sometimes become complicated and require a prolonged treatment period. These complications should be kept in mind in order to avoid further sequelae. The diagnosis is difficult and is usually performed at surgery<sup>[2]</sup>. We here report a patient who developed a fistula between an inflamed Meckel's diverticulum and subcutaneous tissue, which is the first case in the literature.

### CASE REPORT

A 32-year-old woman with no previous abdominal surgery presented with a 48-h history of right abdominal pain, fever, nausea, vomiting and a palpable mass. There was no significant medical history. Examination of the abdomen showed a marked distension without peritonitis and a mass 8 cm × 5 cm in size. There were significant inflammatory signs such as local heat, swelling, edema and tenderness involving the entire right abdominal wall. Her body temperature was 38.5°C. Laboratory findings showed a white blood cell count of 28000, hemoglobin value of 115 gm/L, and a platelet count of 250000. The other laboratory investigations, including electrolytes and urinalysis, were within normal limits. Chest and abdominal X-ray revealed no abnormality. An abdominal ultrasound scan suggested a fluid collection in the right lower abdominal quadrant, which was evaluated to be an abscess. The magnetic resonance imaging (MRI) finding indicated a mass with a suspicious intraabdominal connection (Figure 1). The clinical, radiological and laboratory findings revealed the presence of a right lower abdominal quadrant abscess as the source of sepsis. The abdomen was opened immediately and the exploration revealed a Meckel's diverticulum with a connection to the right lower quadrant mass (Figure 2A-C). There was not any fluid contamination in the abdomen. A resection of the necrotic segment, Meckel's diverticulum and a functional end-to-

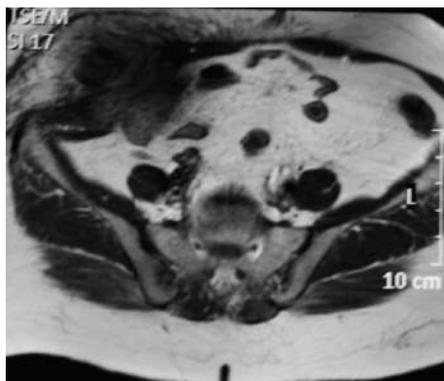


Figure 1 Pre-operative magnetic resonance imaging view of our case.

end anastomosis of the bowel were completed. Afterwards, debridement of the non-viable abdominal wall muscle groups and drainage of the abscess cavity were performed (Figure 2D). The bacterial culture revealed *Escherichia coli* and *Klebsiella pneumoniae* infection. The diverticulum was confirmed as Meckel's diverticulum by histological examination (Figure 3). The patient recovered without incident and was discharged after five days in hospital.

## DISCUSSION

Meckel's diverticulum is the most common end result of the spectrum of omphalomesenteric duct anomalies, which also include umbilical-ileal fistula, omphalomesenteric duct sinus, omphalomesenteric duct cyst, fibrous connection of the ileum to the umbilicus, and Meckel's diverticulum<sup>[1,2,5]</sup>. The lifetime risk of complications is estimated to be about 4%; 40% of which occur in children<sup>[1]</sup>. The presentation in children is most commonly with gastrointestinal bleeding from ectopic gastric or pancreatic mucosa, whereas adults more commonly develop obstruction, intussusception, ulceration, vesicodiverticular fistula, or tumor<sup>[1-6]</sup>. Regarding our case, we confronted a rare presentation of Meckel's diverticulum, which first emerged as enterocutaneous fistula. The etiopathogenesis of the disease can be explained by the direct contamination of the right anterior abdominal wall by an inflamed Meckel's diverticulum. The spread of resultant sepsis along the abdominal wall muscles, preperitoneal space and downward behind the inguinal ligament into the thigh, thus presented clinically as an abscess. A review of the literature suggests certain intra-abdominal inflammatory pathologies in the etiology of enterocutaneous fistula, such as diverticulitis, acute appendicitis, Crohn's disease, colorectal carcinoma, rectal trauma and primary staphylococcal abscess<sup>[7,8]</sup>. Therefore, we should define our case as a new reason for enterocutaneous fistula (Table 1). In the literature another interesting complication of Meckel's diverticulum has been described by Graziotti *et al*<sup>[9]</sup>. They presented a case where an ingested foreign body (chicken bone) entrapped in a Meckel's diverticulum eventually caused a vesicoenteric fistula.

Much rarer complications of Meckel's diverticula include neoplasms, with the most common being benign tumors reported as leiomyomas, angiomas and lipomas.

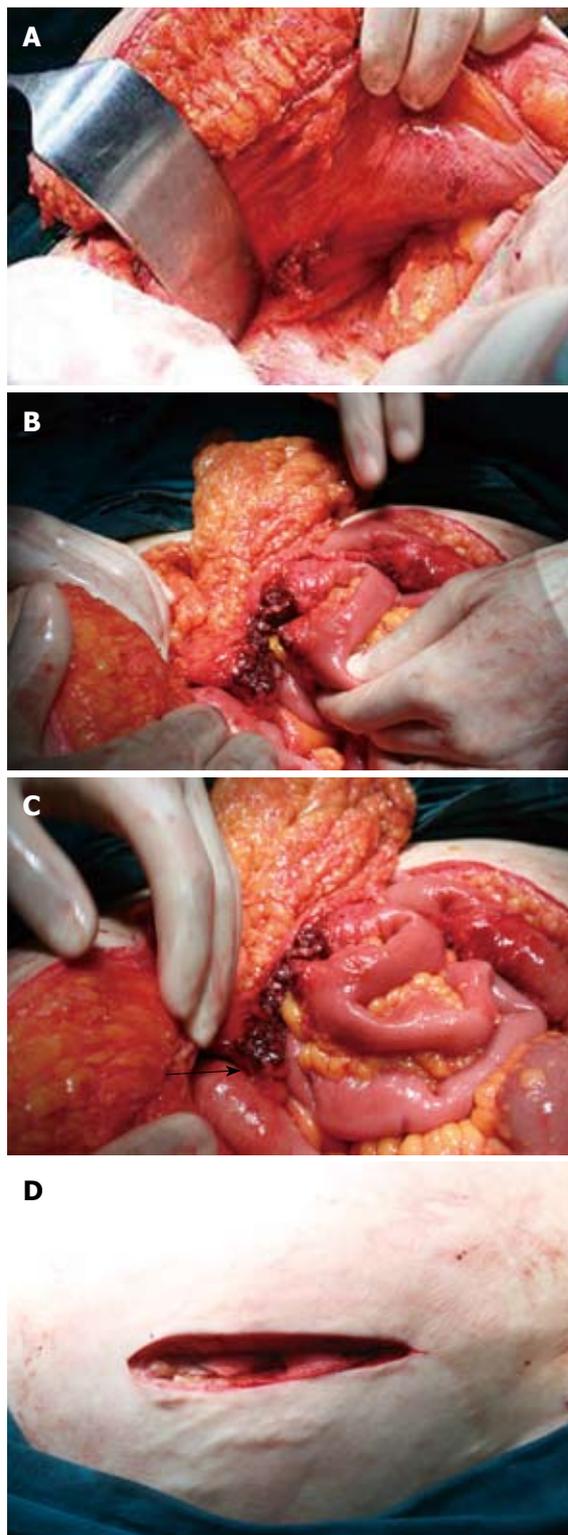
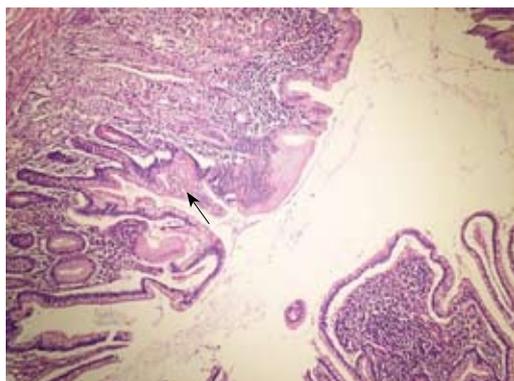


Figure 2 Intraoperative view of the meckel's diverticulum manifested by cutaneous abscess. A: The fistulization area of the diverticulum in the right lower abdominal wall; B: After retraction of the Meckel's diverticulum: intraabdominal view of the diverticulum and necrotic omentum; C: The intraoperative view of the perforated diverticulum (black arrow shows Meckel's diverticulum); D: The view of the abscess cavity.

Malignant neoplasms reported include adenocarcinomas, which commonly originate from the gastric mucosa, sarcoma, and carcinoid tumor<sup>[10]</sup>.



**Figure 3** Meckel's diverticulum (HE stain, × 100). Photomicrograph shows the diverticulum composed of all layers of intestinal wall. Normal small intestinal mucosa and a focus of (arrow) gastric mucosa line the diverticulum.

Author	Localization
Kundra <i>et al</i> <sup>[12]</sup> , 2001	Subphrenic
Hussien <i>et al</i> <sup>[11]</sup> , 2001	Liver
Wasike <i>et al</i> <sup>[13]</sup> , 2006	Mesenteric
Karatepe <i>et al</i> , 2009	Subcutaneous

Clinical diagnosis of Meckel's diverticulum is rarely possible; less than 10% are diagnosed preoperatively<sup>[1,2]</sup>. It is therefore critical for surgeons to rule out Meckel's diverticulum in patients undergoing surgical evaluation for chronic abdominopelvic pain<sup>[5]</sup>. The correct diagnosis of Meckel's diverticulum before surgery is often difficult because a complicated form of this condition is similar to many other abdominal pathologies<sup>[1-5]</sup>. Arteriography and technetium pertechnetate scanning may be especially useful when there is significant bleeding or ectopic gastric mucosa<sup>[5]</sup>. In children, the single most accurate diagnostic test for Meckel's diverticula is scintigraphy with sodium <sup>99m</sup>Tc-pertechnetate. The diagnostic sensitivity of this scan has been reported to be as high as 85%, with a specificity of 95% and an accuracy of 90% in the pediatric age group. In adults, however, <sup>99m</sup>Tc-pertechnetate scanning is less accurate because of reduced prevalence of ectopic gastric mucosa within the diverticulum<sup>[10]</sup>. However, these scans are not readily amenable in an emergency situation.

Contrast-enhanced CT scan may be helpful in patients with enigmatic clinical symptoms of enterocutaneous fistula caused by Meckel's diverticulum. In vesicoenteric fistulas, cystoscopy has a key role in visualization of the fistula<sup>[6]</sup>.

Briefly, the lesson from this case is that Meckel's diverticulum should be kept in mind as one of the differential diagnoses of enterocutaneous fistula.

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CASE REPORT

## Carcinoma of the papilla of Vater following treatment of pancreaticobiliary maljunction

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Watanabe M, Midorikawa Y, Yamano T, Mushiake H, Fukuda N, Kirita T, Mizuguchi K, Sugiyama Y. Carcinoma of the papilla of Vater following treatment of pancreaticobiliary maljunction. *World J Gastroenterol* 2009; 15(48): 6126-6128 Available from: URL: <http://www.wjgnet.com/1007-9327/15/6126.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.6126>

### INTRODUCTION

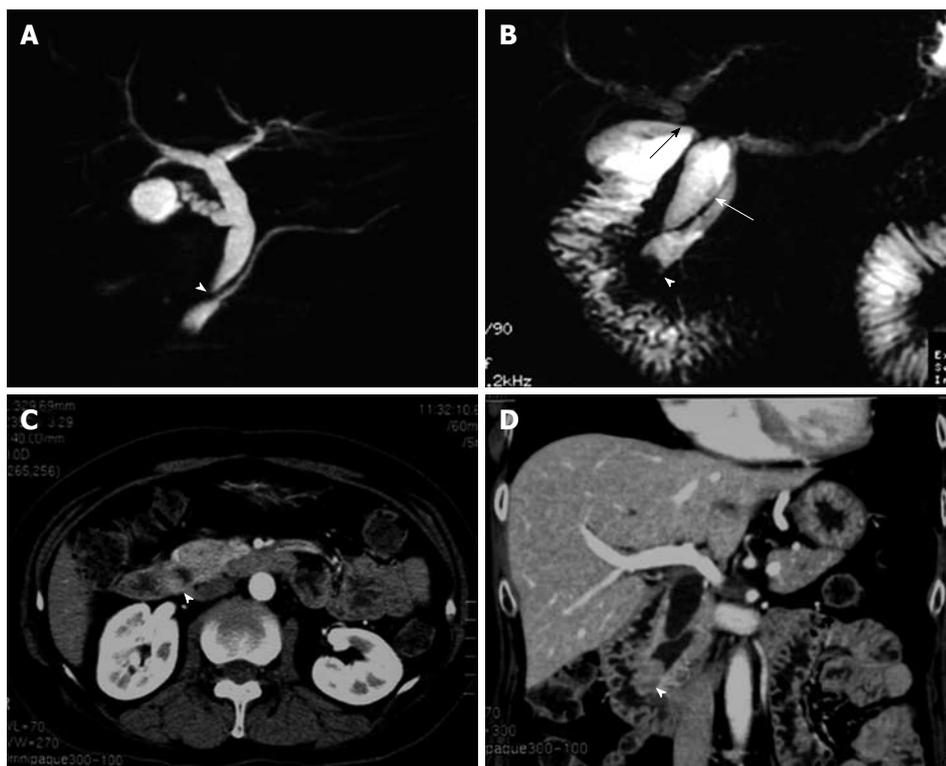
Pancreaticobiliary maljunction (PBM) is a congenital anomaly defined as a union of the pancreatic and biliary ducts outside the duodenal wall<sup>[1]</sup>. Due to the loss of control by the sphincter of Oddi, pancreatic enzymes reflux into the choledochus, especially in the C-P type of maljunction, in which the common bile duct joins the main pancreatic duct<sup>[1]</sup>. Consequently, PBM is frequently associated with choledochal cyst and sometimes biliary cancer; a large survey showed that the rate of developing biliary cancer was 10.6% and 37.9% in PBM with and without choledochal dilation, respectively<sup>[2]</sup>.

Once PBM is diagnosed, the development of cancer should be prevented by removal of the entire extrahepatic bile duct, even in patients without biliary dilation<sup>[3]</sup>. However, it has been reported that residual bile duct cancer could be found during the long-term follow-up period, even after termination of pancreatic enzyme reflux<sup>[4-6]</sup>, and Watanabe *et al*<sup>[6]</sup> reported that 23 (0.7%) of 1291 patients developed residual bile duct cancer after cyst excision. Besides residual bile duct cancer, other periampullary carcinomas have been reported, but they are very rare<sup>[7,8]</sup>.

In this paper, we report a case of carcinoma of the papilla of Vater that developed following treatment of PBM with choledochal dilation by choledochoduodenostomy. This is the first report of such a case, and particular attention is paid to the incidence, differential diagnosis, and treatment of periampullary neoplasms after treatment for PBM.

### Abstract

Pancreaticobiliary maljunction (PBM) is frequently associated with biliary cancer due to reflux of pancreatic enzymes into the choledochus, and even after surgery to correct the PBM such patients still have a risk of residual bile duct cancer. Here, we report the case of a 59-year-old female with carcinoma of the papilla of Vater which developed 2.5 years after choledochoduodenostomy for PBM. During the postoperative follow-up period, computed tomography obtained 2 years after the first operation demonstrated a tumor in the distal end of the choledochus, although she did not have jaundice and laboratory tests showed no abnormalities caused by the previous operation. As a result, carcinoma of the papilla of Vater was diagnosed at an early stage, followed by surgical cure. For early detection of periampullary cancer in patients undergoing surgery for PBM, careful long-term follow-up is needed.



**Figure 1** Imaging modalities for pancreaticobiliary maljunction and carcinoma of the papilla of Vater. A: Magnetic resonance cholangiopancreatography before first operation shows both maljunction of the main pancreatic duct and the choledochus (arrowhead) and the choledochal dilation, however there is no finding of neoplasm at the papilla of Vater; B: Magnetic resonance cholangiopancreatography at follow-up after first operation shows the tumor in the main pancreatic duct (arrowhead). In addition, the choledochoduodenostomy (black arrow) and the residual choledochus (white arrow) can be seen; C, D: Computed tomography obtained 2 years after choledochoduodenostomy for pancreaticobiliary maljunction shows the mass in the distal end of the common bile duct, which had not been detected before first operation (arrowheads).

## CASE REPORT

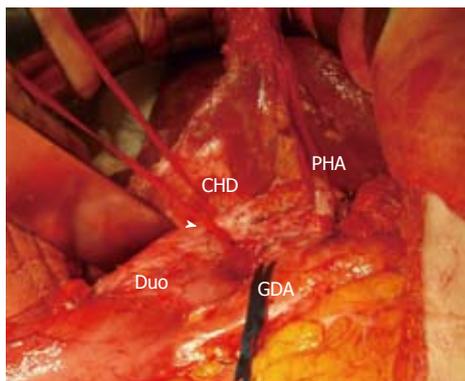
A 59-year-old female was referred to our hospital. She had undergone choledochoduodenostomy and cholecystectomy at another hospital 2.5 years earlier after diagnosis of PBM. Although imaging modalities obtained before surgery showed choledochal dilation, the minimally invasive operation was performed without excision of a choledochal cyst because the patient rejected blood transfusion preoperatively (Figure 1A). During the postoperative follow-up period, magnetic resonance cholangiopancreatography demonstrated a tumor in the main pancreatic duct and in the anastomotic site of the choledochus and duodenum from the first operation (Figure 1B). Computed tomography also showed a tumor in the distal end of the residual choledochus, which had not been observed before the first operation despite bile diversion (Figure 1C and D). Endoscopic retrograde pancreatography was then performed, and a 2-cm-diameter filling defect was demonstrated at the papilla of Vater, which was shown to be adenocarcinoma on cytology. Regardless of these findings, the patient did not complain of any symptoms such as jaundice or abdominal pain, and laboratory tests, including hepatobiliary enzymes and tumor markers, showed no abnormalities. With a diagnosis of carcinoma of the papilla of Vater after choledochoduodenostomy for PBM, the patient underwent pancreaticoduodenectomy. Despite moderate adhesions due to the previous operation, we were able to find common hepatic duct which was anastomosed to the duodenum (Figure 2). After Kocher maneuver, residual extrapancreatic choledochus was easily identified behind the anastomotic region and all the residual choledochus could be removed. Because the cancerous lesion was not found at the first operation or at preoperative examination, resection of all the residual bile

duct was thought to be sufficient for curative surgery. On pathology of the resected specimen, the tumor was found to be well-to-moderately differentiated adenocarcinoma of the papilla of Vater, with metastasis to the lymph nodes behind the head of the pancreas (Figure 3). The patient's postoperative course was uneventful, and there has been no cancer recurrence 1 year after the second surgery.

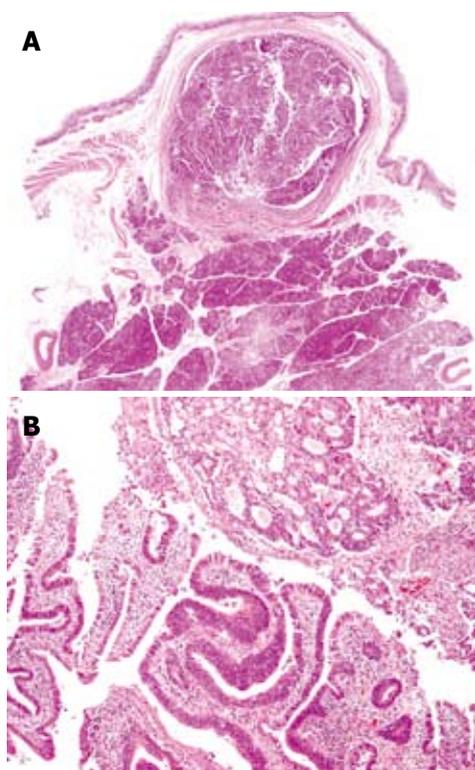
## DISCUSSION

Even after surgery that stops reflux of pancreatic enzymes into the choledochus and prevents the development of biliary cancer, patients with PBM still have a risk of developing residual bile duct carcinoma, both in the proximal duct and the distal end<sup>[6]</sup>. In the present case, carcinoma of the papilla of Vater was diagnosed 2 years after choledochoduodenostomy by computed tomography. In addition to complete excision of a choledochal cyst, careful long-term follow-up is necessary; the interval between excision of a choledochal cyst and cancer detection ranges from 1 to 19 years in several reports<sup>[6]</sup>.

Compared with other periampullary carcinomas, including those of the duodenum, bile duct, and pancreas, survival and resectability rates of carcinoma of the papilla of Vater are relatively high<sup>[9,10]</sup>. In addition to the fact that the rate of resection is one of the predictive factors for survival<sup>[9]</sup>, early detection is of great importance to provide benefit for patients with carcinoma of the papilla of Vater<sup>[11]</sup>. One of the most common manifestations at presentation in patients with carcinoma of the papilla of Vater is jaundice, as in the other periampullary carcinomas. However, jaundice is not usually observed in patients undergoing a diversion operation of the bile duct, as in the present case. Therefore, unless careful postoperative examinations of the periampullary region are performed,



**Figure 2** Intraoperative photograph of the second surgery. Common hepatic duct which was anastomosed to the duodenum was found. Behind this anastomotic region, residual choledochus was easily identified after Kocher maneuver. Arrow head indicates the region of choledochoduodenostomy. CHD: Common hepatic duct; Duo: Duodenum; PHA: Proper hepatic artery; GDA: Gastroduodenal artery.



**Figure 3** Pathological findings of the tumor. A: Close-up findings of the sliced specimen show a tumor growing mainly in the papilla of Vater. There is minimal invasion, which can hardly be seen at this magnification (HE stain,  $\times 1$ ); B: Histological findings of the tumor show adenocarcinoma composed of two different types of histology: moderately differentiated (upper) and well differentiated (lower) type (HE stain,  $\times 40$ ).

physicians may miss the chance to cure carcinoma of the papilla of Vater.

Compared to bile duct cancer, other residual biliary tree neoplasms are rare but can develop postoperatively. For example, a bile duct Schwannoma that developed 15 years after bypass operation and diversion of bile from a choledochal cyst has been reported<sup>[8]</sup>. Carcinoma of the papilla of Vater after choledochoduodenostomy

for PBM is also very rare and has never been previously reported. Though PBM is not a risk factor for carcinoma of the papilla of Vater<sup>[12,13]</sup>, which was detected only about 2 years after diversion of bile in the present case, inflammation due to the mixture of pancreatic enzymes with bile may have caused the carcinoma of the papilla of Vater in this case.

In summary, a case of carcinoma of the papilla of Vater that developed after choledochoduodenostomy for PBM was reported. In this present case, the lesion was detected at an early stage, and curative resection was performed, suggesting that careful, long-term follow-up of patients following surgery to treat PBM is necessary.

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## A case of hypereosinophilic syndrome presenting with intractable gastric ulcers

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### Abstract

We report a rare case of hypereosinophilic syndrome (HES) presenting with intractable gastric ulcers. A 71-year-old man was admitted with epigastric pain. Initial endoscopic findings revealed multiple, active gastric ulcers in the gastric antrum. He underwent *Helicobacter pylori* (*H. pylori*) eradication therapy followed by proton pump inhibitor (PPI) therapy. However, follow-up endoscopy at 4, 6, 10 and 14 mo revealed persistent multiple gastric ulcers without significant improvement. The proportion of his eosinophil count increased to 43% (total count: 7903/mm<sup>3</sup>). Abdominal-pelvic and chest computed tomography scans showed multiple small nodules in the liver and both lungs. The endoscopic biopsy specimen taken from the gastric antrum revealed prominent eosinophilic infiltration, and the liver biopsy specimen also showed eosinophilic infiltration in the portal tract and sinusoid. A bone marrow biopsy disclosed eosinophilic hyperplasia as well as increased cellularity of 70%. The patient was finally diagnosed with HES involving the stomach, liver, lung, and bone marrow. When gastric ulcers do not improve despite *H. pylori* eradication and prolonged PPI therapy, infiltrative gastric disorders such as HES should be considered.

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**Key words:** Gastric ulcer; Hypereosinophilic syndrome

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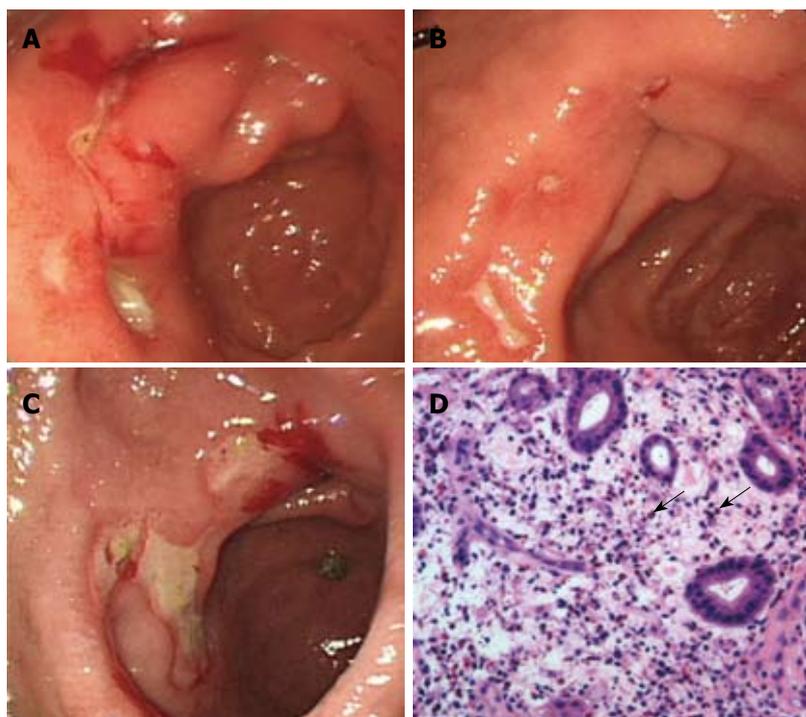
Park TY, Choi CH, Yang SY, Oh IS, Song ID, Lee HW, Kim HJ, Do JH, Chang SK, Cho AR, Cha YJ. A case of hypereosinophilic syndrome presenting with intractable gastric ulcers. *World J Gastroenterol* 2009; 15(48): 6129-6133 Available from: URL: <http://www.wjgnet.com/1007-9327/15/6129.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.6129>

### INTRODUCTION

Hypereosinophilic syndrome (HES) is a rare disorder characterized by the overproduction of eosinophils in the bone marrow with persistent peripheral eosinophilia, tissue infiltration, and end-organ damage by eosinophil infiltration and the secretion of mediators<sup>[1]</sup>. The diagnosis of HES is based on marked eosinophilia exceeding 1500/mm<sup>3</sup>, a chronic course longer than 6 consecutive months, exclusion of parasitic infestations, allergic diseases and other etiologies for eosinophilia, and signs and symptoms of eosinophil-mediated tissue injury<sup>[1,2]</sup>. While HES can involve multiple organ systems, including bone marrow, heart, lung, liver, lymph node, muscle, and nerve tissue<sup>[1]</sup>, gastrointestinal tract involvement is rare<sup>[1-3]</sup>. To date, only a handful of cases of HES presenting with gastritis or enteritis have been reported worldwide<sup>[4-9]</sup>, and HES presenting with intractable gastric ulcers has not been reported. We report our case of a 71-year-old male patient with HES presenting with multiple intractable gastric ulcers with a review of the literature.

### CASE REPORT

A 71-year-old man presented with epigastric pain. He underwent cholecystectomy 20 years previously due to acute cholecystitis with gallstones, and has intermittently taken nonsteroidal anti-inflammatory drugs (NSAID) and corticosteroids on account of degenerative arthritis



**Figure 1** Esophagogastroduodenoscopy (EGD) and biopsy findings. A: Initial EGD findings revealed several active gastric ulcers in the antrum of the stomach; B: In the EGD findings after 2 mo, multiple gastric ulcers were still noticeable with only slight improvement; C: In the EGD findings after 14 mo, multiple gastric ulcers were still found in the antrum; D: Biopsy findings revealed prominent eosinophilic infiltrations > 20 cells/HPF (arrows) (HE stain,  $\times 400$ ).

for 15 years. Other symptoms, as well as his past medical and family history, were otherwise unremarkable. The initial physical examination showed a flat, soft abdomen with normoactive bowel sounds with no sign of direct or rebound tenderness and no hepatosplenomegaly. Thoracic auscultation revealed no remarkable results. Routine complete blood count reported a leukocyte count of  $7790/\text{mm}^3$  with 5.3% eosinophils, hemoglobin level of 12.1 g/dL, and a platelet count of  $198000/\mu\text{L}$ . There were no noteworthy findings on simple chest and abdominal radiography. No specific cardiac abnormalities on standard 12-lead electrocardiogram (ECG) or Doppler echocardiogram were detected. ECG revealed normal sinus rhythm and the echocardiogram showed normal global left ventricular systolic function (estimated ejection fraction 70%).

Esophagogastroduodenoscopy (EGD) findings revealed several active gastric ulcers in the antrum of the stomach (Figure 1A). Biopsy findings showed an ulcer with *Helicobacter pylori* (*H. pylori*). He underwent *H. pylori* eradication therapy (lansoprazole 30 mg twice a day, clarithromycin 500 mg twice a day and amoxicillin 1000 mg twice a day for 7 d) followed by a proton pump inhibitor (PPI) and gastroprotective agent therapy for 2 mo. Follow-up EGD and biopsy performed after 2 mo showed that *H. pylori* was eradicated, whereas multiple gastric ulcers were still noticeable with only slight improvement (Figure 1B). Follow-up endoscopy at 4, 6, and 10 mo showed persistent multiple gastric ulcers in the antrum despite continuous PPI treatment. Therefore, he was readmitted after 14 mo for etiological evaluation of the intractable gastric ulcers.

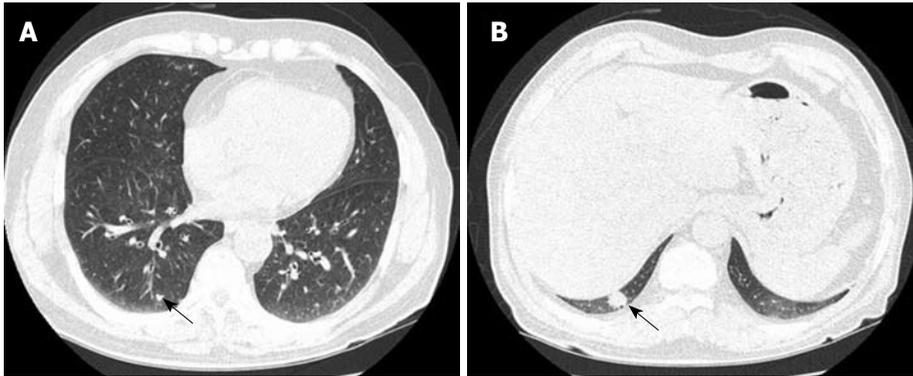
In the follow-up laboratory data, routine complete blood count showed a leukocyte count of  $18380/\text{mm}^3$  with 43% eosinophils, and an absolute eosinophil count of  $7903/\text{mm}^3$ . Serum chemistry showed: Aspartate

aminotransferase/alanine aminotransferase (AST/ALT), 39/97 IU/L; total bilirubin/direct bilirubin, 0.3/0.1 mg/dL; alkaline phosphatase, 235 IU/L; total protein/albumin, 7.3/3.2 g/dL; and BUN/Cr, 14/1.1 mg/dL. Serum immunoglobulin E level was elevated to 2147 kU/L. In pulmonary function tests, pre-bronchodilator FEV1 was 2090 mL (95% of predicted value) and the bronchodilator response was negative. The allergen skin test was negative. There were no parasites or ova in stool specimens. ELISA of paragonimiasis westermani, Clonorchis sinensis, cysticercus, and sparganum were negative. Anti-HIV antibody and anti-nuclear antibody were negative.

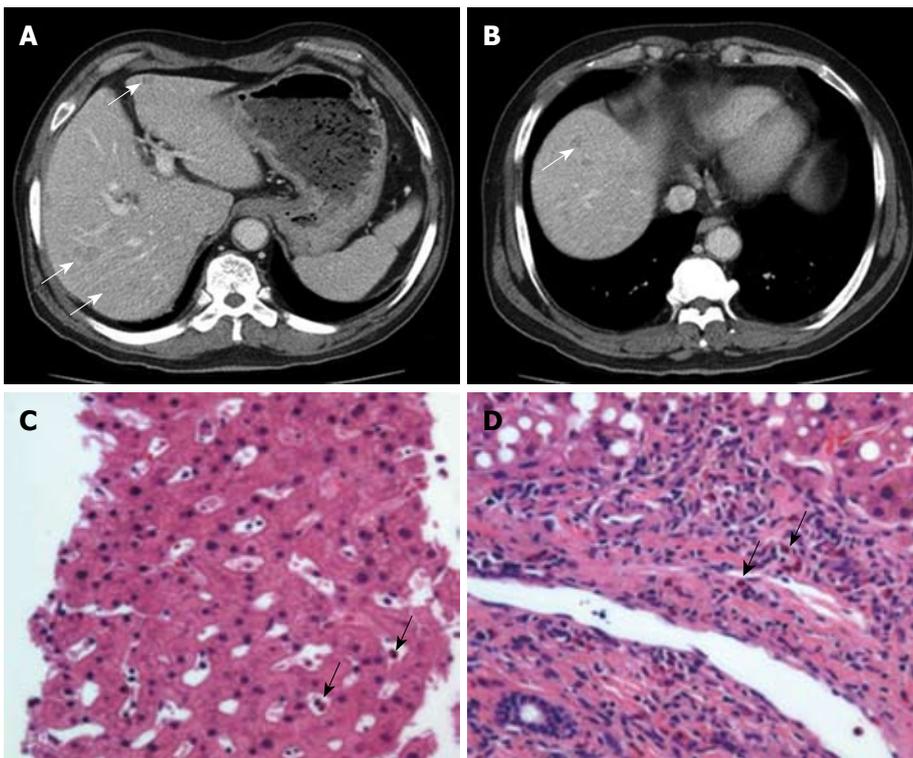
In the EGD findings, multiple gastric ulcers were still found in the antrum of stomach (Figure 1C). The endoscopic biopsy specimen revealed prominent eosinophilic infiltrations of > 20 cells/HPF (Figure 1D). A retrospective review of the previous endoscopic biopsy specimens disclosed eosinophilic infiltration at the antrum which was overlooked at the initial evaluation.

The chest computed tomography (CT) scan showed very tiny nodules in both lungs and approximately 15-mm-sized nodular lesions in the posterior basal segment of the right lower lobe (Figure 2A and B). In the abdominal-pelvic CT scan, multiple, small, and ill-defined low density lesions were found in both lobes of the liver (Figure 3A and B). The liver biopsy showed eosinophilic infiltration in the portal tract and sinusoid (Figure 3C and D).

The peripheral blood smear report showed that there were no immature or dysplastic cells or morphologically abnormal eosinophils. The bone marrow aspiration smear showed an M:E ratio of 3.8:1 and an elevated eosinophil count of 22.2% (Figure 4A). Bone marrow biopsy findings also indicated eosinophilic hyperplasia, with increased cellularity of 70% and normal distribution of erythroid, myeloid, and megakaryocytic cell lineages (Figure 4B). The Fip1-like 1-platelet-derived growth factor receptor A



**Figure 2** A chest CT scan showed very tiny nodules in both lungs (A) and approximately 15-mm-sized nodular lesions in the posterior basal segment of right lower lobe (B) (arrows).



**Figure 3** Abdominal-pelvic CT scan and liver biopsy findings. A, B: There were multiple, small, ill-defined low densities found in the liver (white arrows); C, D: Microscopically, eosinophilic infiltration was noted in the sinusoid and the portal tract (black arrows) (HE stain,  $\times 400$ ).

fusion gene (*FIP1L1-PDGFR4*) rearrangement was not detected and there were no cytogenetic abnormalities.

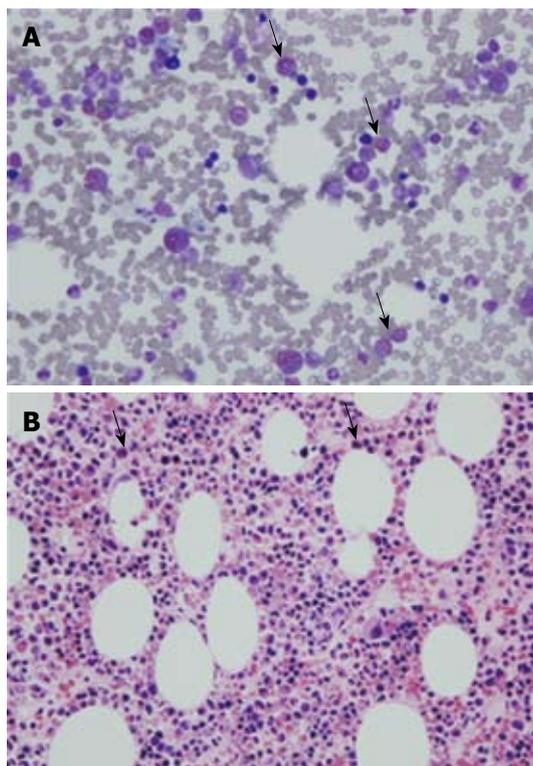
This patient was finally diagnosed with HES involving the stomach, liver, lung, and bone marrow. He was treated with oral prednisolone 60 mg/d and PPI. After two weeks of therapy, clinical manifestations rapidly improved and peripheral blood eosinophilia had subsided.

## DISCUSSION

HES is a rare disease characterized by unexplained persistent eosinophilia associated with multiple organ dysfunction<sup>[1,2]</sup>. In 1968, Hardy and Anderson<sup>[10]</sup> reported three patients with hypereosinophilia, hepatosplenomegaly, and cardiopulmonary symptoms, and first suggested that they had a nonmalignant disorder that belonged within the spectrum of disease termed hypereosinophilic syndrome. In HES, the degree of end-organ damage is heterogeneous, and there is often no correlation between the level or duration of eosinophilia and the severity of organ damage<sup>[1,3]</sup>. Also, the clinical manifestations are

variable from one patient to another, depending on target-organ infiltration by eosinophils<sup>[11]</sup>. Virtually any tissue or organ can be affected, but cardiac involvement is the major cause of the morbidity and mortality associated with HES<sup>[1,9,12]</sup>. We did not find cardiac involvement in our patient.

Since Chusid *et al*<sup>[13]</sup> reported the analysis of fourteen cases of HES in 1975, some cases of HES involving the gastrointestinal (GI) tract have been reported. Ichikawa *et al*<sup>[4]</sup> reported a case of probable HES with a gastric lesion, López Navidad *et al*<sup>[5]</sup> reported a case of HES presenting as a form of epithelioid leiomyosarcoma of gastric origin, and Levesque *et al*<sup>[6]</sup> reported two cases of HES with predominant digestive manifestations. In Korea, Jung *et al*<sup>[8]</sup> reported a case of HES presenting as colitis and You *et al*<sup>[9]</sup> reported a case of HES presenting with various GI symptoms. However, HES presenting with intractable gastric ulcers has not been reported. Our patient suffered from HES presenting with multiple intractable gastric ulcers as well as liver, lung, and bone marrow involvement. The exact mechanism of



**Figure 4 Bone marrow examination.** A: In the bone marrow aspiration smear, the M:E ratio was 3.8:1 and the eosinophil count was elevated to 22.2% (arrows); B: Bone marrow biopsy findings showed eosinophilic hyperplasia (arrows) with increased cellularity of 70% and erythroid, myeloid, and megakaryocytic cell lineages with a normal distribution pattern (HE stain,  $\times 400$ ).

eosinophil-related tissue damage, including gastric ulcer, is not known<sup>[3]</sup>, but the accumulation of eosinophils can have direct cytotoxicity through the local release of toxic substances, including cationic proteins, enzymes, reactive oxygen species, pro-inflammatory cytokines, and arachidonic acid-derived factors<sup>[14]</sup>.

The differential diagnosis of HES includes the disparate diseases associated with eosinophilia. Peripheral blood eosinophilia can be associated with allergic disorders, parasite infections, malignancies, and organ diseases, including eosinophilic gastroenteritis (EG) or eosinophilic pneumonitis due to eosinophilic infiltration<sup>[15]</sup>. In our patients, the bronchodilator response was negative and there was no symptom or sign of allergic disease. Even if allergic disease is present, the severe peripheral eosinophilia noted in our patient is unusual<sup>[15]</sup>. In addition, *FIP1L1-PDGFR $\alpha$*  gene rearrangement was not detected in bone marrow and there were no cytogenetic abnormalities. Therefore, we could rule out primary clonal eosinophilia such as eosinophilic leukemia.

HES may be confused with EG. The diagnosis of EG is based on the following three criteria: (1) the presence of gastrointestinal symptoms, (2) biopsies showing eosinophilic infiltration of one or more areas of the GI tract, or characteristic radiologic findings with peripheral eosinophilia, and (3) no evidence of parasitic or extraintestinal disease<sup>[16]</sup>. Because EG is also of unknown etiology, the distinction from HES must be made on clinical and pathologic bases<sup>[17,18]</sup>. Eosinophilic

gastroenteritis characteristically does not extend beyond the target organ<sup>[1,18]</sup>. Hence, EG lacks the multiplicity of organ involvement often found in HES and does not have the predilection to develop secondary eosinophil-mediated cardiac damage<sup>[1,18]</sup>. Thus, EG can usually be distinguished from HES, although individual patients may on occasion present with overlapping features that confound classification<sup>[1,18]</sup>. In our patient, multiple organ involvement was demonstrated, and there was no other possible cause of severe eosinophilia.

In patients with eosinophilia who lack evidence of organ involvement, specific therapy is not needed<sup>[1]</sup>. Such patients can have prolonged courses without the need for therapeutic intervention<sup>[1]</sup>. However, patients with vital organ involvement require treatment<sup>[1]</sup>. The goals in the management of HES are as follows: (1) reduction of peripheral blood and tissue levels of eosinophils; (2) prevention of end-organ damage; and (3) prevention of thromboembolic events<sup>[1,3]</sup>. Corticosteroids have been used for decades in the treatment of HES and, with the exception of PDGFRA-associated HES, remain the first-line treatment for most patients<sup>[17]</sup>. Typically high-dose prednisone (1 mg/kg per day or 60 mg/d in adults) can be initiated<sup>[1,3]</sup>. A good response to corticosteroid therapy is associated with a better prognosis<sup>[1]</sup>. If patients are refractory or intolerant to corticosteroids, alternative therapies must be considered. Cytotoxic agents, including hydroxyurea, can be considered as second-line therapy<sup>[1,3]</sup>. Immunomodulatory agents including IFN- $\alpha$ , cyclosporine, and alemtuzumab can also be used<sup>[17]</sup>. In patients with FIP1L1-PDGFR $\alpha$ -positive HES, imatinib mesylate (Gleevec<sup>®</sup>), which selectively inhibits a series of protein tyrosine kinases, is considered first-line therapy<sup>[17]</sup>.

In conclusion, we report a case of HES presenting with intractable gastric ulcers. The final diagnosis in this patient was HES involving the stomach, liver, lung, and bone marrow. Clinicians should bear in mind that gastric ulcers can develop in association with infiltrative disorders including HES. When gastric ulcers do not improve despite *H pylori* eradication and prolonged PPI therapy, an infiltrative gastric disorder, such as HES, should be considered.

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CASE REPORT

## A special growth manner of intrahepatic biliary cystadenoma

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### Abstract

We report a case of a 56-year-old woman with intrahepatic biliary cystadenoma (IBC) accompanying a tumor embolus in the extrahepatic bile duct, who was admitted to our department on October 13, 2008. Imaging showed an asymmetry dilation of the biliary tree, different bile signals in the biliary tree, a multiloculated lesion and an extrahepatic bile duct lesion with internal septation. A regular left hemihepatectomy *en bloc* was performed with resection of the entire tumor, during which a tumor embolus protruding into the extrahepatic bile duct and originating from biliary duct of segment 4 was revealed. Microscopically, the multiloculated tumor was confirmed to be a biliary cystadenoma with an epithelial lining composed of biliary-type cuboidal cells and surrounded by an ovarian-like stroma. An aggressive *en bloc* resection was recommended for the multiloculated lesion. Imaging workup, clinicians and surgeons need to be aware of this different presentation.

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**Key words:** Intrahepatic biliary cystadenoma; Growth manner; Tumor embolus

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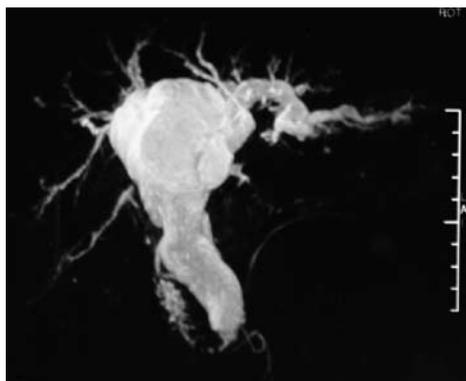
Yi B, Cheng QB, Jiang XQ, Liu C, Luo XJ, Dong H, Zhang BH, Wu MC. A special growth manner of intrahepatic biliary cystadenoma. *World J Gastroenterol* 2009; 15(48): 6134-6136 Available from: URL: <http://www.wjgnet.com/1007-9327/15/6134.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.6134>

### INTRODUCTION

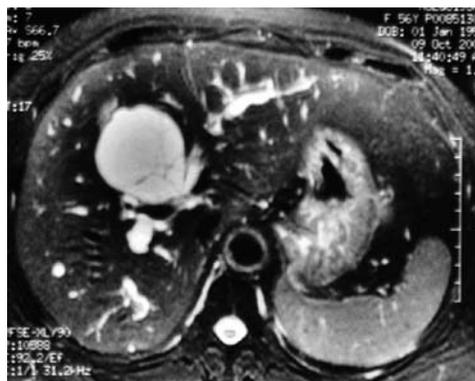
Intrahepatic biliary cystadenoma (IBC) is a rare benign epithelial tumor characterized by unicystic or multicystic growth, accounting for less than 5% of solitary non-parasitic cysts of biliary origin<sup>[1,2]</sup>. IBC was first described in 1892 by Keen<sup>[3]</sup>, followed by Edmondson as a multilocular cystic lesion lined by mucus-secreting cuboidal or columnar epithelium accompanying a densely cellular "ovarian-like" stroma<sup>[4]</sup>. The most common symptoms of IBC patients at presentation are abdominal pain and mass in which 35% have obstructive jaundice<sup>[1,5]</sup>. Knowledge about the pathogenesis of IBC is limited. IBC manifested as a tumor embolus in the extrahepatic bile duct occurs rarely. In addition, no other case report has yet described its clinicopathological features. We report a case of IBC with a tumor embolus in the extrahepatic bile duct with its clinicopathological features described and its diagnostic approaches and surgical procedures discussed.

### CASE REPORT

A 56-year-old woman with hypertension, diabetes and chronic B hepatitis was transferred to our hospital on October 13, 2008 due to a 2-wk history of right hypochondrial pain and spontaneously remitted jaundice. She denied nausea, vomiting, fever, shoulder or back pain. She was admitted to a local hospital but not received surgical treatment. Before admission, her peak bilirubin level was 434  $\mu\text{mol/L}$  and magnetic resonance cholangiopancreatography (MRCP) demonstrated a multiloculated cystic lesion in segment 4, measuring 5.5 cm in diameter, and an obviously dilated left intra- and extra-hepatic biliary tree (Figure 1) but no evidence of choledocholithiasis. The signal was different between extrahepatic and marginal bile ducts on T2-weighted magnetic resonance images (MRI)



**Figure 1** Magnetic resonance cholangiopancreatography (MRCP) showing the dilation of extrahepatic and intrahepatic bile ducts lacking of asymmetry.



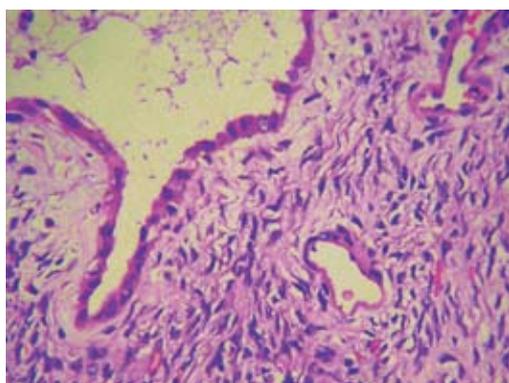
**Figure 2** Magnetic resonance imaging (MRI) showing different bile signals between the right and left intrahepatic ducts in a multiloculated lesion with internal septation.



**Figure 3** A tumor embolus originating from left intrahepatic duct with smooth surface protrudes the extrahepatic bile duct.



**Figure 4** Macroscopy revealing a multilocular cystic lesion containing serous fluid with tumor embolus protruding into the left hepatic duct and the extrahepatic bile duct.



**Figure 5** Postoperative pathology showing epithelial lining composed of biliary-type cuboidal cells surrounded by an ovarian-like stroma (400 ×).

showing thin septa in bile duct (Figure 2). The patient had no history of tobacco smoking, alcohol abuse or recent travel or previous surgery.

After admission, she demonstrated obstructive jaundice features, her bilirubin level declined spontaneously to 103.8  $\mu\text{mol/L}$ , carbohydrate antigen 19-9 (CA19-9) was elevated to 92.1  $\mu\text{g/L}$ , and carcinoembryonic antigen (CEA) level was normal. Abdominal ultrasound showed a dilated biliary tree and a multilocular cyst in segment 4. The extrahepatic bile duct (1.8 cm in diameter) was filled with fluid-like matters. Prior to a planned

left hepatectomy, percutaneous transhepatic biliary drainage (PTBD) of the right lobe was performed to decompress the right biliary system with an 8.5F tube. The TB level was 71  $\mu\text{mol/L}$  and 35.8  $\mu\text{mol/L}$ , respectively, before PTBD and hepatectomy. Volumetric computed tomography (CT) scan revealed that the ratio of remnant liver to total liver volume was 63.2%, with no change in cystic tumor size and no dilation of the right bile ducts, and CT scans showed a small septum in the extrahepatic bile duct, thus the cystic tumor was diagnosed as an intrahepatic tumor infiltrating the extrahepatic bile duct.

The patient underwent choledochotomy under general anesthesia on November 5, 2008, during which no palpable mass was found on the liver surface, but a tumor embolus originating from the intrahepatic duct of segment 4 with smooth surface that protruded into the extrahepatic bile duct was observed without mucus-secretion (Figures 3 and 4). Coexistence of a confluence variation in separate posterior right bile ducts and a variation in the replaced right hepatic artery from superior mesenteric artery was observed. A multiloculated cyst in segment 4 adhering to the bile duct wall of the anterior right lobe was found. So a regular left hemihepatectomy *en bloc* was performed with resection of the entire tumor, segment 5 and the wall of 8 bile ducts. The common hepatic duct was transected to facilitate reconstruction of

the orifice wall of the anterior right bile duct, and Roux-en-Y anastomosis between the hilar bile duct and jejunum was performed. Microscopically, the multiloculated tumor infiltrating the left hepatic duct was confirmed to be a biliary cystadenoma with an epithelial lining composed of biliary-type cuboidal cells and surrounded by an ovarian-like stroma (Figure 5). The bile duct margin was negative. The patient had an uneventful postoperative recovery. Follow-up imaging within twelve months showed no signs of local or distant tumor recurrence.

## DISCUSSION

IBC is an uncommon tumor of biliary tract, but IBC with a tumor embolus in the extrahepatic bile duct is rare. The clinical features of IBC, especially with a tumor embolus in the extrahepatic bile duct, are unclear. No risk factors for IBC have been identified, although its predominance in females suggests a hormonal factor for its etiology. The lesion grows slowly but it is believed to be premalignant<sup>[6]</sup>. Clinically, it can be differentially diagnosed from other cystic hepatic lesions, such as simple cysts, liver abscesses, cystic degeneration of a liver neoplasm, Caroli's disease, etc.<sup>[6,7]</sup>.

Imaging studies are of great importance in its diagnosis. Characteristic ultrasound, CT and MRI findings, including a multiloculated lesion with internal septation, a thickened and irregular wall, mural nodules and papillary projections, calcifications, and wall enhancements, have been well described<sup>[7,8]</sup>. However, IBC with a tumor embolus extending into the extrahepatic bile duct is uncommon and demonstrates some specific clinicopathological features, such as jaundice with spontaneous remission or recurrence<sup>[9]</sup>. Generally, jaundice often occurs when a big tumor embolus fills the extrahepatic bile duct. When the pressure of intra-biliary tract is high, the bile duct is dilated and the pressure of cystadenoma is counteracted, remitting the obstructive jaundice. The MRCP imaging manifestations of IBC, with a tumor embolus protruding into the extrahepatic bile duct, are different from those of IBC without a tumor embolus. In our case, MRCP showed different bile signals in the peripheral and extrahepatic bile ducts, while MRI and CT showed distinctive thin septa in the extrahepatic bile duct. In IBC with a tumor embolus in the extrahepatic bile duct, the extrahepatic bile duct was dilated much greater than the intrahepatic bile duct, and septa in the extrahepatic bile duct should be the main feature which is different from that of mucin-producing IBC without a tumor embolus, although their manifestations on MRCP are similar. Cystadenoma with an epithelial lining composed of biliary-type cuboidal cells is surrounded by an ovarian-like stroma. Before surgery for our patient, PTBD was performed with no mucin or serous fluid observed in the drainage. Since the tumor did not communicate with the bile duct tree, and was filled with serous fluid, intraductal papillary neoplasm of bile duct (IPNB) and biliary papillomatosis were excluded. This tumor develops intraductally but rarely infiltrates the distal bile duct. The base point of this tumor infiltrating the extrahepatic bile duct is intrahepatic, and the lower end

of its embolus is dissociated, thus facilitating complete resection of IBC with a tumor embolus as that without a tumor embolus<sup>[10-12]</sup>. Complete resection of the tumor provides the chance of cure. An early preoperative diagnosis of IBC is essential to improve the prognosis and survival of such patients.

In conclusion, IBC has a special growth manner<sup>[9,10,13-15]</sup>, but its clinical features have not been fully illustrated. Imaging workup, clinicians and surgeons need to be aware of its different presentations, such as recurrent jaundice, different bile signals on MRI, distinctive thin septa in extrahepatic bile duct, and asymmetry dilation of bile ducts. An aggressive complete resection of the lesion is recommended. Large randomized controlled trials are warranted.

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## Comments on the editorial by Riggio & Ageloni on the ascitic fluid analysis

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**Author contributions:** Koulaouzidis A reviewed the literature, analyzed the data, and wrote the manuscript.

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### Abstract

Angeloni *et al* published a landmark study on the use of Coulter counters in spontaneous bacterial peritonitis (SBP) diagnosis. Riggio and Angeloni have recently published an editorial on the ascitic fluid analysis in diagnosis and monitoring of SBP. Herein, some points of interest are discussed.

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**Key words:** Peritonitis; Reagent; Dipstick; Paracentesis; Guidelines; Ascites

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### TO THE EDITOR

I read with great interest the editorial of Riggio & Angeloni on “The ascitic fluid analysis for diagnosis and monitoring of spontaneous bacterial peritonitis”<sup>[1]</sup>. In 2003, Angeloni *et al*<sup>[2]</sup> published the landmark paper, which set a new era in the diagnostic algorithm of spontaneous bacterial peritonitis (SBP), allowing many clinicians and laboratory staff to feel secure in switching from polymorphonuclear (PMN) manual count to the automated one.

I would like to comment on a few points presented in this editorial. First of all, I would appreciate if the authors could clarify the statement on the need for collection of 10 mL ascitic fluid (AF) in ethylenediaminetetraacetic acid (EDTA) containing tube. Universally, most of the EDTA tubes (“purple-top or red-top tubes”, used for blood collection) have a maximum capacity of 2.5-3.0 mL. If Riggio & Angeloni meant the universal containers, it is my understanding that these tubes, except for being sterile, they do not contain any anticoagulant. On top of that, only 1 mL of fluid is enough for most laboratories to do the differential diagnosis. I disagree with the statement that “following hospitalization of any cirrhotic patient with newly diagnosed ascites, a diagnostic paracentesis is advised”. In fact, all cirrhosis with ascites should have diagnostic paracentesis on hospital admission<sup>[3]</sup>.

There are indeed 4 well-disseminated practical guidelines and expert’s consensus reports, but many other national guidelines have been produced as well<sup>[4]</sup>.

Riggio & Angeloni’s comprehensive “Table 2” should list 90 AF samples and not 47 in the study by Wisniewski *et al*<sup>[5]</sup>, 2123 samples and not 1041 in the study by Nousbaum *et al*<sup>[6]</sup>, and 78 samples and not 72 in the study by Vanbiervliet *et al*<sup>[7]</sup>, although three studies have not been included<sup>[8-11]</sup>. In addition, Castellote *et al*<sup>[12]</sup> in a recently published paper argued that the leucocyte reagent strips may have a role in repeated paracentesis and hence management of SBP.

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## Relationship between abdominal trauma or surgery and mesenteric panniculitis

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### Abstract

Mesenteric panniculitis is a rare disease characterized by chronic non-specific inflammation of mesenteric fat tissue. Several etiologic and/or associated factors have been reported in the literature so far. Although trauma or surgery is one of the potential etiologic factors for mesenteric panniculitis, to the best of our knowledge, no strong correlation has been shown in the literature until now.

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**Key words:** Mesentery; Pathology; Mesenteric panniculitis; Trauma; Surgery

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### TO THE EDITOR

We read with a great interest the article “Mesenteric panniculitis: Various presentations and treatment regimens” by Issa *et al*<sup>[1]</sup> in the August issue of the *World Journal of Gastroenterology*.

Mesenteric panniculitis is a rare chronic inflammatory disorder of adipose tissue of the intestinal mesentery. This entity has several different names, such as mesenteric Weber-Christian disease, fibrosing mesenteritis, retractile mesenteritis, mesenteric lipodystrophy, and sclerosing mesenteritis. Its etiology still remains unclear, although a variety of possible causative and associated factors, such as infective and autoimmune causes, vascular insufficiency, prior abdominal surgery, and malignancy, have been reported<sup>[1-4]</sup>.

Issa *et al*<sup>[1]</sup> reported that 84% of patients with mesenteric panniculitis have a history of abdominal trauma or surgery as its etiological factor<sup>[2]</sup>. However, the actually reported rate of trauma or surgery as an etiologic factor is 4.76% rather than 84%<sup>[2]</sup>. A same mistaken rate of 84% has also been reported in a case series<sup>[3]</sup>, showing that trauma and surgery are closely correlated with mesenteric panniculitis. Upon reviewing the literature, we were not able to find this strong correlation in any study.

Several studies are available on the etiology of mesenteric panniculitis<sup>[1-4]</sup>. Daskalogiannaki *et al*<sup>[4]</sup> reported that mesenteric panniculitis is associated with 69.3% of malignancies, such as lymphoma, breast cancer, colon cancer, lung cancer and melanoma, demonstrating that mesenteric panniculitis is an associated and/or causative factor for malignancies. Although trauma or surgery is one of the potential etiologic factors, to the best of our knowledge, no strong correlation has been shown in the literature until now.

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## Meetings

### Events Calendar 2009

January 12-15, 2009  
Hyatt Regency San Francisco, San Francisco, CA  
Mouse Models of Cancer

January 21-24, 2009  
Westin San Diego Hotel, San Diego, CA  
Advances in Prostate Cancer Research

February 3-6, 2009  
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)  
Second AACR Conference  
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009  
Hyatt Regency Boston, Boston, MA  
Translation of the Cancer Genome

February 8-11, 2009  
Westin New Orleans Canal Place, New Orleans, LA  
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009  
Hong Kong Convention and Exhibition Centre, Hong Kong, China  
19th Conference of the APASL  
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009  
Orlando, Florida  
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009  
Vienna, Austria  
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease  
[www.easl.ch/vienna2009](http://www.easl.ch/vienna2009)

March 13-14, 2009  
Phoenix, Arizona  
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009  
Marriott Wardman Park Hotel  
Washington, DC  
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009  
Glasgow, Scotland  
British Society of Gastroenterology (BSG) Annual Meeting  
Email: [bsg@mailbox.ulcc.ac.uk](mailto:bsg@mailbox.ulcc.ac.uk)

April 8-9, 2009  
Silver Spring, Maryland  
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009  
Colorado Convention Center, Denver, CO  
AACR 100th Annual Meeting 2009

April 22-26, 2009  
Copenhagen, Denmark  
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)  
<http://www.easl.ch/>

May 17-20, 2009  
Denver, Colorado, USA  
Digestive Disease Week 2009

May 29-June 2, 2009  
Orange County Convention Center  
Orlando, Florida  
45th ASCO Annual Meeting  
[www.asco.org/annualmeeting](http://www.asco.org/annualmeeting)

May 30, 2009  
Chicago, Illinois  
Endpoints Workshop: NASH

May 30-June 4, 2009  
McCormick Place, Chicago, IL  
DDW 2009  
<http://www.ddw.org>

June 17-19, 2009  
North Bethesda, MD  
Accelerating Anticancer Agent Development

June 20-26, 2009  
Flims, Switzerland  
Methods in Clinical Cancer Research (Europe)

June 24-27 2009  
Barcelona, Spain  
ESMO Conference: 11th World Congress on Gastrointestinal Cancer  
[www.worldgicancer.com](http://www.worldgicancer.com)

June 25-28, 2009  
Beijing International Convention Center (BICC), Beijing, China  
World Conference on Interventional Oncology  
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009  
Snowmass, CO, United States  
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009  
Aspen, CO, United States  
Molecular Biology in Clinical Oncology

August 1-7, 2009  
Vail Marriott Mountain Resort, Vail, CO, United States  
Methods in Clinical Cancer Research

August 14-16, 2009  
Bell Harbor Conference Center, Seattle, Washington, United States  
Practical Solutions for Successful Management  
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009  
Beijing International Convention Center (BICC), Beijing, China  
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists (IASGO)  
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009  
Taipei, China  
Asian Pacific Digestive Week  
<http://www.apdwcongress.org/2009/index.shtml>

October 7-11, 2009  
Boston Park Plaza Hotel and Towers, Boston, MA, United States  
Frontiers in Basic Cancer Research

October 13-16, 2009  
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States  
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009  
Versailles, France  
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009  
Boston, MA, United States  
The Liver Meeting

November 15-19, 2009  
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States  
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009  
London, UK  
Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

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- Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

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- Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of

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- 5 **Vallancien G,** Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 7 **Geraud G,** Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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- 10 **Sherlock S,** Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 11 **Lam SK.** Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 12 **Breedlove GK,** Schorfheide AM. Adolescent pregnancy. 2nd ed. Wicczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- 13 **Harnden P,** Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 15 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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