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### EDITING

Editorial Board of *World Journal of Gastroenterology*, Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China  
Telephone: +86-10-5908-0039  
Fax: +86-10-8538-1893  
E-mail: [wjg@wjgnet.com](mailto:wjg@wjgnet.com)  
<http://www.wjgnet.com>

### PUBLISHING

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Telephone: +86-10-8538-1892  
Fax: +86-10-8538-1893  
E-mail: [baishideng@wjgnet.com](mailto:baishideng@wjgnet.com)  
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### SUBSCRIPTION

Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China  
Telephone: +86-10-8538-1892  
Fax: +86-10-8538-1893  
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Telephone: +86-10-5908-0039  
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## Hepatocyte cryopreservation: Is it time to change the strategy?

Xavier Stéphenne, Mustapha Najimi, Etienne M Sokal

Xavier Stéphenne, Mustapha Najimi, Etienne M Sokal, Catholic University of Louvain and St Luc Clinics, Paediatric Department (HPED), PEDI unit, Laboratory of Paediatric Hepatology and Cell Therapy, Hippocrate Avenue 10, 1200 Brussels, Belgium

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**Correspondence to:** Etienne M Sokal, MD, PhD, Professor, Catholic University of Louvain and St Luc Clinics, Paediatric Department (HPED), PEDI unit, Laboratory of Paediatric Hepatology and Cell Therapy, Hippocrate Avenue 10, B-1200 Bruxelles, Belgium. [etienne.sokal@uclouvain.be](mailto:etienne.sokal@uclouvain.be)

Telephone: +32-2-7641387 Fax: +32-2-7648909

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### Abstract

Liver cell transplantation presents clinical benefit in patients with inborn errors of metabolism as an alternative, or at least as a bridge, to orthotopic liver transplantation. The success of such a therapeutic approach remains limited by the quality of the transplanted cells. Cryopreservation remains the best option for long-term storage of hepatocytes, providing a permanent and sufficient cell supply. However, isolated adult hepatocytes are poorly resistant to such a process, with a significant alteration both at the morphological and functional levels. Hence, the aim of the current review is to discuss the state of the art regarding widely-used hepatocyte cryopreservation protocols, as well as the assays performed to analyse the post-thawing cell quality both *in vitro* and *in vivo*. The majority of studies agree upon the poor quality and efficiency of cryopreserved/thawed hepatocytes as compared to freshly isolated hepatocytes. Intracellular ice formation or exposure to hyperosmotic solutions

remains the main phenomenon of cryopreservation process, and its effects on cell quality and cell death induction will be discussed. The increased knowledge and understanding of the cryopreservation process will lead to research strategies to improve the viability and the quality of the cell suspensions after thawing. Such strategies, such as vitrification, will be discussed with respect to their potential to significantly improve the quality of cell suspensions dedicated to liver cell-based therapies.

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**Key words:** Hepatocyte; Cryopreservation; Quality; Mitochondria; Intracellular ice formation

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### INTRODUCTION

Liver cell transplantation (LCT) is able to correct inborn errors of liver metabolism by supplying viable and functional hepatocytes<sup>[1-5]</sup>. This innovative therapeutic approach is currently accepted as a bridge to transplantation during the waiting time for a graft. The efficacy of

this alternative treatment varies according to the etiology of the disease and remains principally dependent on the quality of the initial liver cell suspension used.

The current great challenge of LCT is the constant availability of the cell suspension. Besides sterility, the cell suspension should present high viability and metabolic activity levels. If freshly isolated cells can be ready to use 5-8 h after the treatment of the organ, data of sterility tests are rarely available before transplantation. Nevertheless, most of the quality control tests performed and documented in the literature reveal a good quality profile of freshly isolated cells, both *in vitro* and *in vivo* after transplantation. However, their availability remains significantly limited by ongoing organ shortages. Furthermore, even if freshly isolated cells could be transplanted the same day, we are limited by the quantity of cells that can be infused in one single session. Extensive research based on two different strategies has been developed to efficiently store the isolated liver cells: cold preservation, which could happen during the first 24 h post-isolation and cryostorage. This later strategy remains the sole practical method for the long-term storage of hepatocytes and leads to (1) the development of a readily available cell bank, even in emergency cases, such as metabolic decompensation; (2) the use of fully analysed cell suspensions, including bacterial and viral safety assays; and (3) an efficient planning of future transplantation. In 1999 an “international panel of experts” recognized that “research should continue to improve the liver cell cryopreservation procedures”<sup>[6]</sup>. Ten years later, only a few cryopreservation protocol improvements have been documented, and hepatocyte post-thawing quality remains poor.

The aim of this review is to discuss current developments regarding the major cryopreservation/thawing (C/T) protocols used in the field. Pre-C/T management of the cell suspension and post-thawing *in vitro* and *in vivo* analyses will be discussed and reviewed. Understanding the biophysical properties of the cryopreservation protocol might supply key and useful information to build efficient strategies. Intracellular ice formation (IIF) or exposure to hyperosmotic solutions, which remain the major C/T damages initiators will be reviewed in detail, regarding their effects on the decrease or loss of cell function, and on cell damages and cell death. Finally, technological developments, such as vitrification, which avoids the crystalline state, or encapsulation, which confers mechanical protection, are currently considered to be exciting new perspectives for the improvement of the cell suspension quality dedicated to clinical LCT.

## PRE-CRYOPRESERVATION/THAWING CRITICAL FACTORS

### **Donor organ and isolation step**

An initial high quality cell suspension after isolation remains essential prior to cryopreservation. Indeed, key factors that compromise the quality of the isolated hepatocytes include high liver fat content, prolonged warm

ischemia and/or storage of the organ<sup>[6]</sup>.

Liver cell isolation is mainly performed using the two-step collagenase perfusion protocol. At 37°C, the first solution, which contains a calcium chelating agent, is perfused to weaken the intercellular junctions of liver cells by removing extracellular calcium ions. The second solution contains collagenase and calcium, essential for the collagenase activity, and disaggregates the extracellular compartment to easily release both non-parenchymal and parenchymal cell fractions. The isolated hepatocyte suspension is obtained after mechanical dissociation, filtration and low speed centrifugation<sup>[7]</sup>.

Isolation is thus the first cause of cell trauma, probably due to oxidative stress, as demonstrated in ischemia/reperfusion of the liver, with impaired mitochondrial functions, consequent intracellular adenosine triphosphate (ATP) depletion (personal unpublished data), and production of reactive oxygen species, leading to hepatocyte death. Addition of anti-oxidant molecules to the isolation medium, such as curcumin, ameliorates the post-isolation quality in terms of metabolic activity and plating. However, such compounds did not show any beneficial effect after cryopreservation/thawing<sup>[8]</sup>.

Detachment from the extracellular matrix has also been shown to promote apoptosis, called anoikis (loss of adhesion molecule). This early cell death could not be totally reversed after *in vitro* culture of hepatocytes because cells already engaged in this process will die in the hours following the isolation procedure<sup>[9,10]</sup>. Anoikis is possibly a consequence of the recently described isolation oxidative stress. In conclusion, cell damage, due to the isolation process itself, is already evidenced prior to C/T. However, if plated, the cells have the opportunity in culture to recover and maintain a good metabolic activity.

### **Post-isolation management of the cell suspension**

**Suspension pre-culture:** To allow the hepatocytes to recover from the isolation stress and improve their quality post-isolation, authors proposed culturing (no attachment culture conditions) freshly isolated hepatocytes prior to C/T. Cold (4°C) or warm (37°C) non-attached, stirred, culture conditions (bio-artificial liver) were developed to avoid later addition deleterious detachment of plated cells. Using pig hepatocytes, Darr *et al.*<sup>[11,12]</sup> demonstrated that 24 h pre-culture in a spinner bioreactor at 37°C leads to a detectable increase in albumin production after C/T as compared to non pre-cultured hepatocytes. This beneficial effect decreased after 48 h of pre-culture, showing that the recovery of cell quality post-thawing remains difficult. Indeed, non-attached culture conditions might, over time, lead to additional cell damage. Furthermore, albumin production levels remained markedly lower following cryopreservation as compared to freshly isolated cells, even with 24 h pre-incubation. The utility of pre-culture was confirmed by Gómez-Lechón *et al.*<sup>[13]</sup> who demonstrated that high post-thawing quality hepatocytes of other species (rat, dog and human hepatocytes) were possible using non-attached pre-culture. Criteria such as viability, adaptation of hepatocytes



to culture, drug-metabolizing capability and cytochrome P450 (CYP) activity were assessed. The influence of a non-supplemented pre-culture step on hepatocyte quality was not confirmed by data published by Lloyd *et al.*<sup>[14]</sup> with pig hepatocytes cultured in a bioartificial liver.

**Pre-incubation with antioxidants:** ATP cellular boosters or antioxidants have been proposed to supplement pre-incubation medium and to potentialize the beneficial effects of pre-culture. Several pre-culture conditions, type of ATP booster and culture at 4°C or 37°C, were evaluated by Terry *et al.*<sup>[15,16]</sup>. Two hours hyperosmotic glucose 100-300 mmol/L pre-incubation has been shown to improve the viability and attachment efficiency of rat hepatocytes, as well as the viability of human hepatocytes post-thawing. Fructose 100-300 mmol/L pre-incubation also improved the viability and attachment efficiency of rat hepatocytes. On human hepatocytes, fructose improved their attachment efficiency, but not their viability. Pre-incubation with the anti-oxidant alpha-lipoic acid at 0.5-5 mmol/L improved the viability and attachment efficiency of both rat and human hepatocytes. The beneficial effects of this pre-treatment (at lower concentration: 15 mmol/L glucose for 30 min at 37°C) in human hepatocytes were demonstrated by Silva *et al.*<sup>[17]</sup>. They found that the response of CYP enzymes to typical inducers was significantly improved in the pre-incubated rat and human hepatocytes. The pre-incubated hepatocytes showed a significantly higher plating efficiency compared with hepatocytes cryopreserved without pre-incubation. Finally, Gómez-Lechón *et al.*<sup>[18]</sup> recently demonstrated that the optimal preservation of isolated cells (cell viability, attaching capacity, and functionality, particularly GSH and glycogen levels, as well as drug-metabolizing cytochrome P450 enzymes) was found in media supplemented with 2 mmol/L N-acetyl-cystein (anti-oxidant molecule) and 15 mmol/L glucose, confirming the importance of anti-oxidant protection after isolation.

In conclusion, based on the literature and on our experience, we believe that liver cell isolation represents an important oxidative stress, potentially controlled by the addition of anti-oxidants to the isolation media and/or by non-attached time-limited culture in an anti-oxidant supplemented medium. Quality of cells prior to C/T is therefore increased. However, all the damage related to C/T is not avoided by these pre-C/T steps. Furthermore, in clinical settings, pre-culture adaptation is difficult.

## STANDARDISED HEPATOCYTE CRYOPRESERVATION/THAWING PROTOCOL: STATE OF THE ART

After isolation and related oxidative stress, the obtained cell population is re-suspended in cryopreservation media and distributed at specific concentrations in special freezing vials. The hepatocytes are then ready for the cooling process and storage in liquid nitrogen. In this

chapter, we will review the literature data for liver cell freezing solution as well the documented cooling and thawing process.

### Concentration of hepatocytes and type of vials

In most studies, the hepatocyte concentration varied from  $10^6$  to  $10^7$  cells/mL<sup>[19]</sup>. In this range, Lloyd *et al.*<sup>[14]</sup> did not find any significant superiority of cell concentrations investigated, when evaluating porcine hepatocytes after thawing. This was confirmed by analyzing, hepatocyte attachment, lactate dehydrogenase (LDH) leakage, bilirubin conjugation, and CYP3A4 activity. However, De Loecker *et al.*<sup>[20,21]</sup> revealed that a decreased cell density of rat hepatocytes correlated with an increased post-thawing viability, as estimated by viability trypan blue exclusion assay. These data suggest that higher cell densities might increase membrane-membrane contacts and subsequent cell damage. Therefore, unless high cell density will save space and is useful for the development of cell banks, cryopreservation at a low cell density (less than  $10^7$ /cell) is recommended.

Besides cell density, type and volume of vials used for liquid nitrogen storage might also influence post-thawing cell quality; however, few data are available in the literature. Based on the trypan blue exclusion test, cytochrome activity, and tetrazolium inner salt assays, bags of 50 mL seem to give better quality pig hepatocytes, post-thawing, than bags of 100 mL<sup>[22]</sup>.

The lack of recently published data on the concentration of hepatocytes is considered as a minor point for reaching the best post-thawing quality.

We also confirm that, in our hands, in several different volumed vials (from 2 to 100 mL) and varying cell densities, cell density does not influence post-thawing cell quality.

### Freezing solution

**Cryopreservation medium:** University of Wisconsin solution (UW) is the gold standard cryopreservation medium for isolated hepatocytes. It was originally developed as a cold storage solution for transplant organs. The principal cryoprotectants of the UW solution are Lactobionate (100 mmol/L), a large molecular weight anion impermeable to most membranes and supposed to suppress hypothermia-induced cell swelling, and Raffinose (30 mmol/L), which allows additional osmotic support<sup>[23]</sup>. Dexamethasone, another compound in UW solution, is used to stabilise cell membranes<sup>[24]</sup>.

The superior beneficial effect of UW solution was demonstrated by comparing UW to three other freezing media [all were supplemented with 12% dimethylsulfoxide (DMSO)], Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and commercial solutions, Cell Banker 1 and Cell Banker 2. Parameters including viability, plating efficiency, LDH release, ammonia removal test, and lentiviral gene transfer were shown to be highly maintained in hepatocytes cryopreserved with UW solution<sup>[25]</sup>.

The effectiveness of UW solution as a cryoprotectant

agent suggests that metabolic, as well as ultrastructural, factors might be important in the effective cryopreservation of isolated hepatocytes<sup>[26]</sup>. However, UW solution also has limitations, principally its cost and, as demonstrated in the “Parameters for the evaluation of hepatocytes quality after cryopreservation/thawing” paragraph, its incomplete cell protection.

HypoThermosol (HTS), a recently developed freezing solution, is a dextran-based intracellular-type solution. It is used as the carrier solution of the freezing medium. Freshly isolated rat hepatocytes cryopreserved in HTS supplemented with 10% DMSO have been shown to present high viability levels, good long-term maintenance of hepatospecific functions, and good quality response to cytokine challenge at the post-thawing level compared to other supplemented culture media<sup>[27]</sup>. Further studies confirmed the utility of HTS, allowing a decrease in the DMSO levels within the cryopreservation solution. However, no data comparing UW to HTS are available in the literature<sup>[28,29]</sup>.

**Cryoprotectants:** Cryoprotectants are essential components of freezing solutions. Two classes of cryoprotectants are described, those that permeate the cell membrane (DMSO, Glycerol) and those that do not such as polymers (Dextran), oligosaccharides (Trehalose), and sugars (Glucose, sucrose or fructose).

DMSO is an important polar permeating aprotic solvent which is less toxic than other members of this class. The use of DMSO in medicine dates from around 1963, when an University of Oregon Medical School team discovered it could penetrate the skin and other membranes without damaging them and could carry other compounds into a biological system. It is able to enter cells and reduce injury by moderating the increase in solute concentration during freezing. In most studies, DMSO is the ideal cryoprotectant, notably giving the best plating efficiency<sup>[27,30,37]</sup>. Classically, a final concentration of 10% DMSO is described in many protocols, with some exceptions, although higher levels are potentially toxic due to high osmolarity<sup>[38]</sup>. The rate of addition of the cryoprotectant also appears important to the outcome of cryopreservation. Freezing must be commenced as soon as possible after addition of the cryoprotectant to reduce the possibility of toxicity at ambient temperatures. Hence, DMSO should be added at 4°C, as toxicity of DMSO was demonstrated at 25°C or 37°C. Some authors proposed adding permeating cryoprotectants slowly to the cell suspension to avoid damages related to osmotic shock and cellular dehydration<sup>[33,39]</sup>. However, this seems to be a minor point<sup>[38]</sup>.

The use of oligosaccharides with higher molecular weights resulted in greatest improvement in viability. Their combination with DMSO has been shown to allow efficient hepatocyte cryopreservation. Both rat and human hepatocytes exhibit significantly higher viability (as estimated by trypan blue exclusion assay) than hepatocytes previously cryopreserved without oligosaccharides. Moreover, attachment and survival rates in plastic dishes of rat

hepatocytes were greater after freezing in the presence of di-, tri-, and tetrasaccharides. Such plating amelioration was not confirmed with human hepatocytes<sup>[40]</sup>. Metabolic activity was also evaluated after cryopreservation with oligosaccharides. When trehalose was combined with DMSO for the cryopreservation of human hepatocytes, a significant increase in total protein level and secretion of albumin was observed after thawing, as well as decreased levels of aspartate aminotransferase<sup>[41]</sup>.

Those works were inspired from data demonstrating the influence of trehalose on cell quality on bull sperm<sup>[42,43]</sup>. Similarly, several authors recommended adding sucrose to the cryopreservation medium, with or without trehalose, to ameliorate the quality of the cells after thawing. This allows the concentration of DMSO to be decreased while ameliorating the quality of cells. However, it was evaluated on hematopoietic stem cells and fetal liver hematopoietic stem/progenitor cells<sup>[44,45]</sup>, not on hepatocytes.

The beneficial role of a non-metabolizable glucose derivative as a cryoprotectant that mimicked the natural cryoprotective adaptations observed in freeze-tolerant frogs was also investigated. Primary rat hepatocytes were loaded with 3-O-methyl glucose (3OMG) through endogenous glucose transporters without evident toxicity and cryopreserved according to a controlled rate freezer program down to -80°C before storage in liquid nitrogen. In this study, hepatocytes cryopreserved with a relatively small amount of intracellular 3OMG (< 0.2 mol/L) showed high post-thaw viability and maintained long-term hepatospecific functions, including synthesis, metabolism, and detoxification. Metabolite uptake and secretion rates were also largely preserved in the cryopreserved hepatocytes, showing that 3OMG must be considered as an interesting cryoprotectant<sup>[46]</sup>.

An interesting report proposed that wheat protein extracts permitted long-term storage and recovery of large quantities of healthy cells that maintain high hepatospecific functions, *via* an osmotic modulation effect<sup>[47]</sup>. In post-thawing culture, the morphology of hepatocytes cryopreserved with wheat protein extracts was similar to that of fresh cells. Furthermore, hepatospecific functions, such as albumin secretion and biotransformation of ammonium to urea, were well maintained during four-days post-plating. Inductions of CYP1A1 and CYP2B in hepatocytes cryopreserved with wheat extracts were similar to those in fresh hepatocytes. Additional data confirmed the utility of wheat extracts as efficient, non-toxic, economic natural cryoprotectants, superior to DMSO, which has limitations due to potential cellular toxicity<sup>[47-49]</sup>.

Finally, human application does not tolerate the use of animal origin products because of possible zoonosis contamination and/or immune response to animal proteins<sup>[50]</sup>. Fetal calf serum (FCS) or human albumin, are classical ingredients of the cryopreservation solution, in a proportion of 10% to 90 %. No significant differences in classical viability or drug metabolizing enzyme activities were noted while varying the percentage of serum for (human, pig, and rat) hepatocyte cryopreservation in most

Table 1 Summary of freeze-rate comparison studies

Species	Cryoprotectant	Freeze rate	Storage temperature	Ref.
Human	DMSO	-1°C/min	-80°C	[55]
Rat	DMSO	-1°C/min to -38°C (with cooling shock) then liquid nitrogen	Liquid nitrogen	[61]
Dog, monkey, human	DMSO	-1.9°C/min from 4°C to -30°C, then -30°C from -30°C to -150°C	Liquid nitrogen	[57]
Rat	DMSO	-38°C/min	Liquid nitrogen	[59]
		-2°C/min		
		Slow variable		
		Optimized variable rate		
Human	DMSO	-1.9°C/min from 4°C to -30°C, then -30°C from -30°C to -150°C	Liquid nitrogen	[51]
Rat	DMSO	Cooling in 10 min down to 0°C, 8 min at 0°C, in 4 min down to -8°C, in 0.1 min down to -28°C, in 2 min down to -33°C, in 2 min up to -28°C, in 16 min down to -60°C, in 4 min down to -100°C (variable rate)	Liquid nitrogen	[60]
Human	DMSO	Variable rate	Liquid nitrogen	[6]
Dog, monkey, human	DMSO	-1.9°C/min from 4°C to -30°C, then -30°C from -30°C to -150°C	Liquid nitrogen	[58]
Rat	DMSO	Variable rate	Liquid nitrogen	[59]
Pig	DMSO	Optimized <sup>[59]</sup>	Liquid nitrogen	[22]
		Modified variable		

DMSO: Dimethylsulfoxide.

of the published studies<sup>[26,30,33,36,51-53]</sup>. We think that a minimal concentration of serum is required for optimal cryopreservation, even if some authors have also successfully cryopreserved porcine hepatocytes without serum. They showed that, after thawing, in appropriate conditions and without serum, the addition of conditioned medium derived from hepatic non-parenchymal cells improved attachment and function of hepatocytes (urea production and CYP activity)<sup>[54]</sup>.

In conclusion, UW solution remains the best and most studied freezing medium and must be supplemented with a permeating cryoprotectant; DMSO remains the gold standard. The addition of a non-permeating cryoprotectant to this solution must also be considered.

### Cooling process

Slow freezing protocols are considered to be the best strategy for cryopreserving mature isolated hepatocytes. All the protocols described in this paragraph were developed using DMSO as the cryoprotectant. First protocols included the use of an isopropanol cooler device, placed in a -80°C freezer, giving a constant temperature decrease of -1°C/min down to -70°C or -80°C, before storing in liquid nitrogen<sup>[55]</sup>. Other slow freezing protocols are described in the literature, varying from -1°C/min to -5°C/min up to -40°C or -80°C, before storing at -196°C<sup>[56]</sup>. A decrease in temperature at -1.9°C/min from 4 to -30°C and then -30°C/min from -30°C to -150°C was also adopted by many authors<sup>[51,57,58]</sup>. More specific protocols were developed by Diener *et al* and by Hengstler *et al*<sup>[39,59,60]</sup>. Several cooling process protocols, where the temperatures of the vial and of the cryopreserving solution were controlled, were tested on rat hepatocytes. Firstly, shock freezing in liquid nitrogen dramatically decreased cell viability, despite the presence of 10% DMSO. Secondly, a slow freezing protocol with -2°C/min led to much better recovered viability than a cooling rate of -38°C/min. While using the slow freezing protocol, the authors determined that the cell suspension becomes

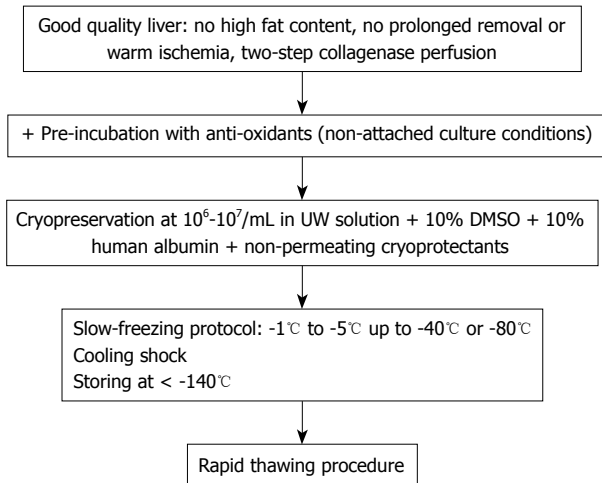
supercooled around -20°C. Indeed, when crystallisation starts, the latent heat of fusion is released and the cell sample is warmed. This heat release may be deleterious; therefore, a freezing program with shock cooling was developed. Analysis of post-thawing viability did not show significant differences of hepatocyte viability (86% viability *vs* 79% according to the slow linear protocol). The same cooling shock can be obtained by clamping the vials, with forceps cooled in liquid nitrogen<sup>[61]</sup>. However, studies from Lloyd *et al*<sup>[14]</sup> (measuring LDH release, cell return, attachment, and biochemical assays) and from our team<sup>[62]</sup> did not show any difference between computer-controlled freeze rate (without frozen shock), the Nalgene propan-2-ol device or simply using -20°C and -80°C freezers.

Storage of hepatocytes at -20°C or -80°C remains deleterious for cells functions as several proteases might be active at those temperatures. At -130°C, no chemical reaction can occur as there is no more thermal energy. Furthermore, at this temperature, no water, which is at the vitreous or crystalline state, is present at the liquid state. Therefore, -140/-150°C is the minimum acceptable temperature for long-term storage of cryopreserved hepatocytes<sup>[6,34,57,63]</sup>. At -140°C (the vapour phase of liquid nitrogen) or -196°C (the liquid phase of liquid nitrogen), cells can be stored for long periods<sup>[6,34,51,57]</sup>. A summary of freeze rate comparison studies is presented in Table 1. The passage of water from one state to another, IIF, is the critical point that might modulate the cell quality. The limitations of these cooling processes will be discussed later in the IIF paragraph.

### Thawing procedure

The critical point of this procedure is to avoid the deleterious phenomenon, IIF. Rapid thawing at 37°C to minimize cellular damage due to reformation of intracellular ice will significantly enhance cell viability. As for cooling, a slow dilution of the cryoprotectant at 4°C is recommended, to avoid osmotic shock and the toxicity





**Figure 1 Standardized cryopreservation protocol.** UW: University of Wisconsin solution; DMSO: Dimethylsulfoxide.

of the cryoprotectant<sup>[38]</sup>.

The standardized cryopreservation/thawing protocol is summarized in Figure 1.

## PARAMETERS FOR EVALUATING HEPATOCYTE QUALITY AFTER CRYOPRESERVATION/THAWING

The aim of this paragraph is to review the principal assays that may help to standardize the post-thawing evaluation step process.

The viability assays (Trypan blue exclusion test, LDH release, mitochondrial functions and necrotic/apoptotic markers) are important quality markers. Most of the “metabolic” assays investigate only some specific hepatocyte functions, notably drug metabolizing enzymes activities, essential for drug industry application; but also the hepatocytes’ capacity to plate to collagen coated dishes. Other assays should be developed to allow a rapid evaluation of LCT-related critical parameters.

### Viability assays

Necrosis was first described to occur following C/T<sup>[31]</sup>, whereby intracellular organelles, most notably the mitochondria, and the entire cell swell and rupture (cell lysis). This phenomenon begins with an impairment of the cell’s ability to maintain homeostasis, leading to an influx of water and extra-cellular ions. Due to the breakdown of the plasma membrane, the cytoplasmic contents, including lysosomal enzymes, are released into the extracellular fluid. This can be tested by LDH release from the cytoplasm to the extracellular medium and reflects cell membrane integrity. However, we found that LDH release was unaffected after C/T of both human and mice hepatocytes and does not adequately assess viability after C/T<sup>[62]</sup>.

Apoptosis, a programmed cell death which has been well characterized, both at the morphological and biochemical levels, was also described following C/T. Annexin V staining, *in situ* TUNEL assay combined with

confocal laser scanning microscopy, or deoxyribonucleic acid (DNA) fragmentation are the most popular apoptosis assays<sup>[28,31,64]</sup>. These assays should be carefully interpreted if performed *in vitro* or *in vivo*.

The mitochondrion is a key player in the initiation of apoptosis and recent studies highlighted its role in C/T-induced cellular damage<sup>[65]</sup>. Disruption of mitochondrial membrane potential ( $\Delta\Psi$ ) was reported following C/T, which is followed, within hours after thawing, by cytochrome c extra-mitochondrial release, caspase-3 activation, and DNA fragmentation. Addition of caspase inhibitors (IDN-1965 or ZVAD-fmk) to the medium during cryopreservation and static culture rescued cells from apoptosis and was associated with increased phase 1 and phase 2 metabolism<sup>[64,66]</sup>.

As mitochondria are the major source of reactive oxygen species (ROS), induction of apoptosis by oxidative stress was also proposed to be involved in the impairment of hepatocytes after C/T<sup>[67,68]</sup>. In fact, the combination of antioxidant medium containing a caspase inhibitor allowed significant improvements in viability and function in treated rat hepatocytes<sup>[68]</sup>. Similarly, other authors proposed the addition of S-adenosylmethionine to the cryopreservation medium, to avoid glutathione and viability decrease during cold preservation or cryopreservation in liquid nitrogen<sup>[69]</sup>. Finally, C/T decreased mitochondria-related cellular respiration and oxygen consumption rate. This effect was evidenced on oligomycin (ATP synthase inhibitor)-sensitive respiration, suggesting that it could result from alteration of a mitochondrial process linked to ATP synthesis, rather than an intrinsic modification of mitochondrial membrane proton permeability (leak). In permeabilized hepatocytes, a marked impairment of mitochondrial oxidative phosphorylation following C/T was observed in *in situ* mitochondria with substrates for complex 1, under basal mitochondrial respiratory rate. Interestingly, the inhibition of basal mitochondrial respiration was not revealed with complex 2 substrates<sup>[62]</sup>. The respiratory-chain complex 1 is one of the largest known membrane protein complexes, and is also the major source of mitochondrial ROS<sup>[70,71]</sup>. Thus, specific alterations of complex 1 subunit(s), which comprise the hydrophilic domain containing the redox centres of the enzyme, and/or deregulation of ROS production leading to oxidative stress, could constitute one of the start-points of the C/T-induced damage. This could explain the oxygen consumption and  $\Delta\Psi$  decrease, leading to ATP depletion and later cytochrome c release. The intracellular ATP concentration, which is an indirect mitochondrial marker, is probably the easiest and most rapidly measurable parameter for detecting early cellular damages related to cryo-storage.

Detailed information regarding cryopreserved hepatocyte death *in situ* after transplantation remains poorly investigated in LCT *in vivo* models.

### Plating

Attachment of isolated hepatocytes *in vitro* (collagen coated dishes) is widely used for the evaluation of their quality. The low plating efficiency is often documented

in cryopreserved cells. This remains a major problem because engraftment of the transplanted hepatocytes in the recipient liver parenchyma is also dependent on the proteins involved in extracellular matrix adhesion mechanisms<sup>[17,23,25,63,72]</sup>. Structural membrane damage observed after cryopreservation might contribute to such alterations. Recently, it was demonstrated that the process of cryopreservation leads to down-regulation of cell adhesion at the gene and the protein level ( $\beta$ 1-integrin and E-cadherin, amongst others)<sup>[73]</sup>. This is relevant and probably begins to explain the observed low plating efficiency. Another team was able to demonstrate that when hepatocytes are cryopreserved with wheat extracts instead of DMSO, there was a clear protective effect against loss of  $\beta$ 1-integrin, E-cadherin, and  $\beta$ -catenin<sup>[74]</sup>. We must also recognize the high plating variability from one liver to another.

### Hepato-specific functions

Conjugation and secretion of biliary acids seems to be maintained following C/T of human hepatocytes. The uptake of taurocholate in cryopreserved hepatocytes of was found to range from 10% to 200% of that observed in freshly isolated cells immediately after thawing at 37°C<sup>[75]</sup>.

The characterization of freshly isolated and C/T monkey hepatocytes demonstrated the maintenance of various hepato-specific functions, but at a low level. The ability to synthesize proteins, glucose, and glucose-6 phosphatase activity was decreased after deep-freeze storage<sup>[30]</sup>. Concerning protein synthesis, data from the literature show that this important hepatic function is often impaired in hepatocytes after C/T. De Loecker *et al.*<sup>[61]</sup> demonstrated that cryopreserved human hepatocytes albumin production was reduced to half that of freshly isolated hepatocytes. Glycogen synthesis in cryopreserved porcine hepatocytes was reduced by about 30% after 24 h of culture and about 47% after 48 h of culture compared to freshly isolated hepatocytes. Reduced basal levels of glycogen and of glycogen synthesis could be explained by an increased energy demand in cryopreserved hepatocytes to repair damage caused by cryopreservation. Glycogenolysis was reduced to about 50% in cryopreserved hepatocytes and gluconeogenesis to about 40% of the glucose production in freshly isolated hepatocytes at day 1 and 2 post-thawing. Incubation with glucagon (90 min) increased the glucose production from glycogenolysis and gluconeogenesis in both freshly isolated and cryopreserved hepatocytes<sup>[76]</sup>.

Urea production also seems to be reduced following C/T, according the majority of the papers<sup>[30]</sup>.

There is no apparent significant change in drug metabolizing enzyme activities between freshly isolated and cryopreserved hepatocytes for the major drug-metabolizing pathways. The cryopreservation of human hepatocytes isolated from 17 donors was shown not to alter their capability to metabolize substrates for the major CYP isoforms (CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4), as well as the phase II enzymes UDP glucuronyltransferase, and 7-HC sulfation for sulfotransferase<sup>[77]</sup>.

Steinberg *et al.*<sup>[78]</sup> showed that phase I drug-metabolizing enzyme activities analyzed in cryopreserved human, rat, and mouse hepatocytes were very similar to those of freshly isolated hepatocytes; while phase II enzyme activities were affected by cryopreservation.

Other studies show better stability of drug metabolizing activities in monkey than in rodent hepatocytes. After thawing, Phase I and Phase II activities (CYP, ethoxycoumarin-O-deethylase, aldrin epoxidase, epoxide hydrolase, glutathione transferase, glutathione reductase, and glutathione peroxidase) were well preserved<sup>[79]</sup>. The decrease in the activity of phase II enzymes, documented in several studies, might be related to the loss of the corresponding cofactors; however, the hypothesis of physical cell alteration is not excluded<sup>[78]</sup>. The cytosolic enzymes, notably glutathione S-transferase, are more exposed to intracellular ice formation and related C/T damages, even if some mechanical protection can be given by microsomal membranes<sup>[55]</sup>.

Finally, if thawed hepatocytes cultures are sensitive to CYP inducers (rifampicin, rifabutin, phenobarbital, omeprazole, and  $\beta$ -naphthoflavone) the induced activity remains lower as compared to freshly isolated cells, with an increased delay induction time<sup>[6,17,60,80-83]</sup>. This is summarized in Table 2.

In conclusion, the standardized “literature based” C/T protocols have limitations, as demonstrated by the collected data in the last four paragraphs. If some progress were made in the assessment of specific hepatic functions, then the true effects of pre-C/T incubation with anti-oxidants or the addition of non-permeating cryoprotectants to the freezing solution on the poor quality of hepatocyte post-C/T could be properly tested. Finally, cell death, probably not a reversible mechanism following C/T, is initiated due to mitochondrial impairment. ATP concentration evaluation, as a mitochondrial operation marker, is a crucial test to evaluate the quality of C/T cells.

## INTRA-CELLULAR ICE FORMATION: THE START POINT OF THE OBSERVED CELL DAMAGE?

Post-thawing cell quality remains poor. How can we explain the observed damages? The passage from a liquid stage of the intracellular and extracellular water, to a crystalline state probably holds the key to understanding C/T damages. In the cryopreservation of cells or tissues, each system has its specific optimal cooling rate, showing a decreased survival at both too low (slow cooling damage) and too high cooling rates (fast cooling damage). During freezing, the transition phase of water leads to a decrease of the extracellular water content. Water can pass through the plasma membrane, which will in turn lead to water efflux and cell dehydration. Slow cooling damage has been attributed to such phenomena as the increase in the external and internal solute (salt) concentration, the small size of the

Table 2 *In vitro* evaluation of post-thawing quality of hepatocytes

Hepatocyte <i>in vitro</i> model	Cryopreservation protocol	Parameters evaluated: impairment following C/T	Parameters evaluated: no impairment following C/T	Ref.
Rat and human	Pre-incubation -20°C, -70°C, liquid nitrogen	Plating	CYP induction	[17]
Porcine	Slow freezing protocol up to -80°C	Trypan blue exclusion test		[23]
		Plating		
		Ammonia clearance		
Rat	Slow freezing protocol up to -80°C	Trypan blue exclusion test		[25]
		Plating		
		Ammonia clearance		
Human	20% DMSO, 40% FCS Slow freezing protocol	Trypan blue exclusion test	ATP	[63]
		Plating	Urea synthesis	
		LDH release		
		MTT		
Porcine	Freezing boxes or slow freezing protocol	CYP	Plating	[72]
		Glycogen synthesis		
		Glycogenolysis		
		Gluconeogenesis		
Rat and mouse	Slow freezing protocol	Plating		[41]
		Uptake of neutral red		
		Protein synthesis		
Porcine	Immediate cryopreservation Serum free	Protein synthesis	Trypan blue exclusion test	[30]
		Gluconeogenesis		
		CYP activity		
		Urea synthesis		
		Protein synthesis		[61]
Rat (monolayer culture post-thawing)	Not available			[75]
Human	Storage in liquid nitrogen		Conjugation and secretion of biliary acids	
			CYP activity	[77]
Human, rat, rabbit, dog and monkey	Slow freezing protocol		Phase 2 enzymes	[78]
Human, rat and mouse	Slow freezing protocol	Phase 2 enzymes	Phase 1 enzymes	[79]
Monkey	Slow freezing protocol	LDH release	Phase 1 enzymes	
		Plating	Phase 2 enzymes	
Human	Storing at -80°C	Cytosolic enzymes: glutathione S-transferase	CYP activity	[55]
Rat	Slow freezing protocol	CYP induction		[60]
Human	Slow freezing protocol	CYP induction		[80]
Rat	Slow freezing protocol		CYP induction	[81]
Human	Not available	CYP induction		[82]
Human	Not available	CYP induction		[83]

C/T: Cryopreservation/thawing; FCS: Fetal calf serum; LDH: Lactate dehydrogenase; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide; CYP: Cytochrome P450.

channels of unfrozen solution, or the mechanical stress of cell shrinkage and destabilisation of membranes and proteins at low water potential. At high cooling rates, the intracellular dehydration (by water efflux) cannot keep pace with the extracellular dehydration (by phase transition of water). As a consequence, higher cooling rates result in higher levels of intra-cellular supercooling, higher trans-membrane differences in osmotic pressure and solute concentrations, and higher rates of water efflux through the membrane. This fast cooling damage seems to be due particularly to IIF<sup>[84-89]</sup>.

We recently demonstrated that cryopreservation at -20°C for 20 min followed by rapid thawing induced a dramatic drop of ATP levels in cryopreserved/thawed hepatocytes, correlated with a decreased oxygen consumption rate and altered mitochondrial complex 1 activity (personal unpublished data).

These results suggest that during the cryopreserva-

tion damage, complex 1 impairment occurred early in the cryopreservation process by mechanical alteration of mitochondria due to IIF or exposure to hyperosmotic solutions. However, IIF might also occur during the thawing process.

## CRYOPRESERVED/THAWD HEPATOCYTES FOR LIVER CELL TRANSPLANTATION

### Animal

According to the data available in the literature, the ability of cryopreserved/thawed hepatocytes to engraft and to repopulate the recipient liver is not definitively demonstrated. In the eighties, Fuller *et al.*<sup>[90]</sup> described that fewer cryopreserved (slow freezing protocol in DMSO) autologous hepatocytes cells were detected one month

post-transplantation in the recipient liver as compared to freshly isolated cells. David *et al*<sup>[91]</sup> in Nagase albuminemic transplanted rats, found few clusters of C/T cells three months post-transplantation as compared to freshly isolated hepatocytes, with no significant production of albumin. Dunn *et al*<sup>[92]</sup> showed in a Dalmatian dog model that sequential intrasplenic LCT can provide a significant but transient, 22 d, correction of urinary uric acid excretion that was similar with either freshly isolated or C/T (slow freezing protocol in DMSO, post-thawing viability around 60%) hepatocytes. The protocol of Papalois *et al*<sup>[93]</sup> has demonstrated that cryopreserved pig hepatocytes, at -20°C without cryoprotective medium in Hank's solution, have adequate viability (around 60%) after one month of storage to support hepatic function in animals with severe acute liver failure by hepatoproliferative factors produced by the hepatocytes engrafted in the spleen.

Besides metabolic supply, intrasplenic transplantation of C/T hepatocytes (at -80°C in UW and DMSO) in rats pre-treated with D-galactosamine improved survival to 60% after seven days (as compared to 100% obtained using freshly isolated hepatocytes)<sup>[23]</sup>.

However, in a transgene-induced liver disease model, an environment that is permissive for clonal expansion of donor cell populations, C/T hepatocytes (stored up to 32 mo in liquid nitrogen) have been shown to possess clonal replicative potential identical to that of freshly isolated hepatocytes. C/T hepatocytes constituting 0.1% of the total adult hepatocyte number in the recipient could repopulate a mean of 32% of recipient liver parenchyma<sup>[94]</sup>. Furthermore, transplantation of woodchuck hepatocytes into the liver of urokinase-type plasminogen activator/recombination activation gene-2 mice demonstrated that cryopreserved (slow freezing protocol in DMSO, viability up to 70%-80%) cells, retained the ability to divide and to repopulate a xenogenic liver three months post-transplantation. Notably, *in vivo* susceptibility to infection with woodchuck hepatitis B virus and the proliferative capacity of frozen/thawed woodchuck hepatocytes in recipient mice were identical to those observed by transplanting freshly isolated hepatocytes<sup>[95]</sup>.

The efficiency of C/T (slow freezing protocol in DMSO and HTS, post-thawing viability around 60%) for human hepatocytes was also evaluated in an animal NOD/SCID mice model. Cho *et al*<sup>[96]</sup> demonstrated that transplanted cryopreserved human liver cells engrafted in the peritoneal cavity as well as the liver, retained hepatic function (glycogen storage and Glucose-6 phosphatase activity) and proliferated in response to liver injury by carbon tetrachloride. This effect was greater two hours and three days post-transplantation as compared to 7, 14 and 40 d post-transplantation, suggesting some loss of transplanted cells at later times.

Based on the above data, we may conclude that C/T hepatocytes, in a favourable environment, are transiently able to maintain hepatocyte function *in vivo*, engraft the liver and proliferate at low levels, as compared to freshly isolated cells.

## Human

**Metabolic diseases:** Immediate and medium term metabolic efficacies, decrease of the ammonia levels and urea synthesis were observed in our hands in two urea cycle disorder patients using C/T hepatocytes<sup>[97,98]</sup>. This was correlated, in one case, with effective demonstration of engraftment up to one year after cell infusion, using Fluorescence *In Situ* Hybridization (FISH) for the Y chromosome. This four year-old arginosuccinate-lyase deficiency girl was transplanted with C/T cells and underwent a first liver biopsy after infusion of C/T male hepatocytes, which showed a XX/XXYY chimerism, with 4.7% Y-positive cells. This cell lineage was further described on several post-transplant biopsies, reaching more than 10% of the recipient cells, while she received additional fresh and C/T hepatocyte infusions. At King's College Hospital, one patient with an inherited factor VII deficiency was entirely transplanted with C/T hepatocytes, which led to a transient reduction to 20 percent of the requirements for factor VII therapy<sup>[99]</sup>. To our knowledge, all other published case reports used an infusion protocol at least partially comprising freshly isolated hepatocytes, preventing any conclusion regarding the respective efficiency of C/T *vs* freshly isolated cells.

In conclusion, as in animal models and based on few reported data, C/T hepatocytes seem able to transiently support deficient hepatocyte function, justifying their use to stabilise metabolically unstable patients while waiting for a liver graft.

## PERSPECTIVES ON HEPATOCYTE CRYOPRESERVATION

In this final section, we will analyze and discuss several ways to ameliorate the C/T protocols. We think that these new techniques applicable to C/T protocols are the best hopes for changing the future of cryopreservation/thawing.

### New hepatocyte culture configurations

**Encapsulation-*in vitro*, *in vivo*:** Encapsulation, by conferring a mechanical protection, was investigated with success for hepatocyte cryopreservation protocols.

Firstly, and before considering LCT, several *in vitro* studies showed that encapsulation of freshly isolated hepatocytes in specially designed multi-component capsules (alginate, cellulose sulphate, and poly (methylene-co-guanidine) hydrochloride) retained their specific functions (transaminase activity, urea synthesis and protein secretion) over the first days of culture. Furthermore, most detoxifying enzymes were also expressed (in cryopreserved alginate-entrapped hepatocytes) at levels close to those in unfrozen encapsulated hepatocytes<sup>[100]</sup>. Long-term, up to 120 d of cryopreservation, preservation of drug metabolism and transport activities was demonstrated using microencapsulated rat hepatocytes<sup>[101]</sup>. Moreover, cold-induced apoptosis in hepatocytes can be significantly reduced following their entrapment within alginate gel beads, as demonstrated by measurement of



caspase-3-like activity<sup>[102]</sup>. Finally, cryomicroscopy studies showed that the alginate microencapsulation technique protected the hepatocytes from physical damage caused by the growth of extracellular ice crystals<sup>[103]</sup>.

How can we adapt the encapsulation of cells to the LCT protocol? In 1993, in the Gunn rat model, the authors proposed intraperitoneal transplantation of cryopreserved alginate-encapsulated hepatocytes, allowing significantly reduced hyperbilirubinemia, as well as freshly isolated encapsulated hepatocytes, up to 28 d following transplantation<sup>[104]</sup>. In a severe liver failure model, two-stage 95% hepatectomy, with xenogenic hepatocytes and without immunosuppression, the authors demonstrated the utility of intrasplenic encapsulated hepatocytes<sup>[105]</sup>.

Intraperitoneal transplantation of cryopreserved or fresh encapsulated rat hepatocytes significantly increased the survival rate to 66% and 80% in the ALF model (acetaminophen administration and 30% hepatectomy). Intraperitoneal transplantation of cryopreserved or fresh encapsulated immortalized hepatocytes improved survival, in this model, to 50% and 55%, respectively. Histopathology revealed that encapsulated hepatocytes were viable, but for a limited period (up to two weeks post-transplantation)<sup>[106]</sup>. Recently, Baldini *et al.*<sup>[107]</sup> showed the retention of biological activity and significant viability of porcine encapsulated hepatocytes transplanted intraperitoneally in rats without immunosuppression, confirming the utility of encapsulation to avoid rejection. Moreover, Aoki *et al.*<sup>[108]</sup> demonstrated that poly-L-lysine entrapped cryopreserved human hepatocytes survived and expressed albumin in rat spleen after transplantation. Finally, Mei *et al.*<sup>[109]</sup> confirmed these data by showing an increased rate of survival in a mouse model of fulminant hepatic failure after xenogenic transplantation of pig hepatocytes.

In conclusion, cryopreservation of encapsulated hepatocytes is a promising tool for the establishment of banks for the supply and storage of hepatocytes, by mechanically conferring protection. However, the main problem of this technique remains the adaptation to LCT, the problem of injection site and adaptation to the treatment indication (size of capsule pore). Furthermore, this can be only be proposed for ALF or metabolic unstable patients, as the efficacy of the transplantation remains time-limited. Repeated injections must therefore be considered. The time-limited effect is notably due to the hepatocyte de-differentiation, observed with freshly isolated or C/T cells, in this kind of configuration. New projects must evaluate the utility of co-encapsulation of hepatocytes with mesenchymal bone marrow cells or pancreatic islets, as a new type of feeder cells to avoid de-differentiation.

### Vitrification

Vitrification (from the Latin, vitreus, glassy) is essentially the solidification of a supercooled liquid by adjusting the composition (high concentration of cryoprotectant) and cooling rate (fast freezing protocol) such that the crystal phase is avoided. The process involves a progressive and marked increase in viscosity during cooling and

prevention of ice nucleation and growth. The system is stabilized in the glassy state as translational molecular motion is essentially halted. Vitrification eliminates the biologically damaging effects associated with freezing. No appreciable degradation occurs over time in living matter trapped within a vitreous matrix. Vitrification is potentially applicable to all biologic systems. As the major problem with the current protocols remains IIF, alternatives such as vitrifying hepatocytes are an interesting strategy for attaining the best post-thawing cell quality. Vitrification of precision cut-slices, tissue engineered pancreatic substitute, jugular veins/vessels constructs, and embryonic kidneys has already been performed, allowing the absence of ice into the vitrified samples and an excellent post-thawing quality and/or viability<sup>[110-115]</sup>.

Classically, tissues (vessels constructs and embryonic kidneys) are vitrified at cooling rates of  $> 40^{\circ}\text{C}/\text{min}$  in a specific solution, comprising DMSO, formamide and 1,2-propanediol in EuroCollins solution (VS55) or a polyethylene formulation consisting of propanediol, DMSO and polyethyleneglycol 400<sup>[110,113]</sup>. Best viability results were obtained with the VS55 solution. To obtain cooling rates of  $> 40^{\circ}\text{C}$ , tissues contained in vials, are cooled to  $-100^{\circ}\text{C}$  in an isopentane bath (conductive cooling, freezing point  $-160^{\circ}\text{C}$ ) placed in a  $-135^{\circ}\text{C}$  freezer, removed from the 2-methylbutane bath and vitrified to  $-120^{\circ}\text{C}$  in the  $-135^{\circ}\text{C}$  freezer (convective cooling).

Re-warming is performed under controlled conditions, and the chemicals removed in a stepwise manner. However de-vitrification might occur during warming from the vitrified state. To prevent de-vitrification, the vitrified material must be warmed uniformly as fast as possible [slowly re-warmed to  $-100^{\circ}\text{C}$  using convection followed by rapid re-warming achieved by placing the vial in a DMSO/H<sub>2</sub>O mixture at room temperature ( $225^{\circ}\text{C}/\text{min}$ )] so that ice does not have the opportunity to form in significant quantities.

Vitrification of encapsulated hepatocytes in M or G-collagen was recently proposed as an alternative freezing protocol<sup>[116]</sup>. Wu *et al.*<sup>[117]</sup> proposed a rapid stepwise introduction of microencapsulated hepatocytes to vitrification solution (40 % v/v ethylene glycol, 0.6 mol/L sucrose in the medium) and their direct immersion in liquid nitrogen. Using this technique, they obtained 100% retention of hepatocyte functions, correlated with excellent viability, and no detectable damage to the microcapsules. If vitrification was also proposed as successful cryopreservation protocol for isolated cells, as has been done for human amnion derived mesenchymal stem cells<sup>[118]</sup>; however, vitrification of non-encapsulated hepatocytes has not yet been studied. Therefore, further investigations are needed to confirm the potential of vitrification for LCT protocols.

### CONCLUSION

Using current protocols, C/T of hepatocytes induces cell alteration. *In vitro* functions of C/T hepatocytes remain poorer than those of freshly isolated hepatocytes, while

the efficacy *in vivo* seems to be time-limited, both in animal models and in humans. Hepatocyte mitochondria are very sensitive to C/T, with marked complex 1 activity impairment following thawing. This leads to low intracellular ATP concentration, an excellent and easily obtaining post C/T viability marker. Related cytochrome c release induces cell death within hours by apoptosis.

The IIF or exposure to hyperosmotic solutions are probably the start point of the observed damage. New adapted cryopreservation protocols have therefore to be urgently developed. Interesting perspectives such as vitrification, to avoid the crystalline state, with or without encapsulation, conferring a mechanical protection, must be validated in the future, while considering the problem of their clinical translation.

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Hugh James Freeman, MD, FRCPC, FACP, Series Editor

## Limitations in assessment of mucosal healing in inflammatory bowel disease

Hugh James Freeman

Hugh James Freeman, Department of Medicine, University of British Columbia, Vancouver, BC, V6T 1W5, Canada

Author contributions: Freeman HJ contributed all to this paper. Correspondence to: Dr. Hugh James Freeman, MD, CM, FRCPC, FACP, Department of Medicine, University of British Columbia Hospital, 2211 Wesbrook Mall, Vancouver, BC, V6T 1W5, Canada. [hugfree@shaw.ca](mailto:hugfree@shaw.ca)

Telephone: +1-604-8227216 Fax: +1-604-8227236

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### Abstract

An emerging parameter to define the effectiveness of new therapeutic agents in clinical trials, and by extension, for use in day-to-day clinical practice has been labeled mucosal healing. It has been hypothesized that complete healing of the intestinal mucosa in inflammatory bowel diseases should result in reduced disease complications, reduced hospitalization and reduced surgical treatment. By implication, the natural history of inflammatory bowel disease might then be altered. Measurement of mucosal healing, however, is largely observational, requiring repeated invasive endoscopic examinations, sometimes with mucosal biopsies. Other indirect imaging methods may play a role in this assessment along with other surrogate markers, including intestinal permeability. These measurements may have significant limitations that prohibit precise correlation with symptom-based disease activity indices in clinical trials. This likely reflects the dynamic nature of this evolving and individualized inflammatory process that tends to be focused, but not limited, to the mucosa of the intestinal tract.

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**Key words:** Intestinal mucosa; Digestive system endoscopy; Clinical trials

### INTRODUCTION

Ulcerative colitis and Crohn's disease are inflammatory bowel disorders; both with no known cause. Curative treatment is still needed. As such, management has focused largely on ameliorating symptoms, and reducing hospitalization and the need for surgical treatment. In clinical trials, reductions of symptom-related numerical endpoints have been used [e.g. the Crohn's Disease Activity Index (CDAI)] as evidence of treatment effectiveness and their possible role in translation to clinical practice has been discussed previously<sup>[1-5]</sup>. Another treatment goal for these diseases is improving quality of life, based upon any means that this parameter might be clinically defined or measured. Now, an emerging measurement to define the effectiveness of new therapeutic agents in clinical trials, and by extension, for use in day-to-day clinical practice has been popularly labeled "mucosal healing".

In practical terms, the assessment of mucosal healing is based largely on observational evaluation, which requires the use of repeated endoscopic studies before and after a defined treatment period, sometimes in conjunction with histological examination of mucosal biopsies, or other more indirect imaging methods, other surrogate markers or miscellaneous methods, such as measurements of intestinal permeability. Logically, however, but not yet conclusively shown, complete healing of the intestinal mucosa should result over the long term in

reduced disease complications, hospitalization and surgical treatment. This proposed hypothesis further suggests that, if mucosal healing can be induced by treatment, then hopefully, the natural course and history of the disease in an individual patient might be modified, and by implication, improved. For example, in a Norwegian study<sup>[6]</sup>, Crohn's disease or ulcerative colitis first diagnosed between 1990 and 1994 (before the use of biological agents) were examined endoscopically for up to 5 years. Mucosal healing after 1 year of treatment was reported in almost 50% of 495 treated patients that could be followed. Mucosal healing also appeared to predict reduced subsequent disease activity and a decreased need for active treatment in ulcerative colitis, but not Crohn's disease. Of note, the study also has demonstrated that other environmental factors may play an important role in mucosal healing (e.g. smoking, level of education).

## ENDOSCOPIC INDICES

Earlier historical studies from Europe remain very important. These have shown considerable variability in endoscopic changes detected by experienced observers caring for patients with inflammatory bowel disease<sup>[7]</sup>. Moreover, the correlation between the patient's clinical status and endoscopic (and histopathological) changes in the colorectal mucosa was limited<sup>[8]</sup>. Later, using more modern measurements of disease activity (e.g. CDAI), there was a poor correlation between colonoscopic (or histological) findings and indices of disease activity, which implies that these were not reliable measures of disease severity or extent<sup>[9]</sup>. Similar results have been published by French investigators in a prospective evaluation of ileocolonic and colonic Crohn's disease<sup>[10]</sup>. In a later study<sup>[11]</sup>, however, specific lesions were identified for evaluation that included: erythema, superficial and deep ulceration, stenoses and pseudopolypoid changes. Then, an index was calculated (Crohn's Disease Endoscopic Index of Severity; CDEIS), based on the percentage of involvement of different ileocolonic segments, for use in clinical trials of new therapeutic agents. A good correlation with lesion severity was reported with positive inter-observer agreement, but these investigators were very experienced and well trained for their study<sup>[11]</sup>. In routine day-to-day clinical practice, however, the reproducibility of this measurement seemed to be less helpful. As a result, other simplified endoscopic activity measures were proposed and applied in some clinical trials for Crohn's disease<sup>[12]</sup> and ulcerative colitis<sup>[13,14]</sup>. A detailed and excellent review of treatment indices, including endoscopic endpoints used in inflammatory bowel disease, specifically ulcerative colitis, has appeared elsewhere<sup>[15]</sup>.

Definition of mucosal ulcers or erosions (or their apparent complete absence as a marker of mucosal healing) has been viewed by some clinicians with skepticism, given the highly fluid and dynamic nature of the inflammatory process in inflammatory bowel disease. Also, other factors may influence endoscopic evalua-

tion, particularly for inflammatory bowel disease and its treatment (e.g. bowel preparation effects on the inflamed intestinal mucosa may differ from non-inflamed mucosa). In addition, the depth or extent of small-intestinal penetration at the time of visualization during ileocolonoscopy may not be well defined in some studies. For example, capsule endoscopy has demonstrated mucosal erosions or ulcerations distributed throughout the small intestine in Crohn's disease that are not appreciated well by other imaging modalities, including routine ileocolonoscopy<sup>[16]</sup>. Finally, a recent prospective evaluation in Crohn's disease confirmed that clinical response of the patient seemed to correlate poorly with capsule evaluation of the surface mucosa for assessment of healing<sup>[17]</sup>.

Similarly, for ulcerative colitis, few well validated and well accepted endoscopic criteria for endoscopic mucosal healing have been evaluated for clinical trials. A large degree of overlap is evident within historical definitions of mild, moderate and severe endoscopic changes and, the degree of intra- and inter-observer error has been validated poorly in clinical trials, especially in multicenter studies with multiple observers involved in the evaluation of oral, intravenous or topical treatment regimens. In contrast, some studies have reported good inter-observer agreement for some, but not all endoscopic changes in ulcerative colitis, with experienced<sup>[18]</sup> as well as well-trained observers<sup>[19]</sup>.

## HISTOPATHOLOGICAL EVALUATION

In theory, microscopic definition of the mucosa provides precise evaluation of mucosal healing in response to treatment. However, this microscopic evaluation is not only dependent on endoscopic (or macroscopic) evaluation (for selection of the biopsy site), but is also prone to the impact of pathological inter- and intra-observer error. In Crohn's disease, this may be an especially significant problem owing to the focal or segmental nature of the inflammatory process. Even in ulcerative colitis, a disorder often characterized as a continuous inflammatory process, there may be a non-uniform pattern of mucosal healing. Little information is available on the temporal resolution of the inflammatory process, but it not likely to be uniform.

Moreover, the evaluation of the depth of inflammation may also be crucial to precise monitoring of treatment response. In Crohn's disease, this transmural dimension makes complete histopathological definition virtually impossible because endoscopic biopsies provide only mucosa for pathological evaluation. After treatment, this transmural pattern in Crohn's disease may be especially difficult to evaluate since medications may not affect the inflammatory process in a consistent or uniform fashion. Even with ulcerative colitis, a process thought to demonstrate a more continuous and mucosally based pattern of inflammation, variability in the histopathological severity within the colonic mucosa occurs. More precise studies are still needed that define the



mucosal response to different forms of injury and the healing response to different forms of treatment.

## OTHER IMAGING METHODS

Invasive imaging studies, particularly repeated endoscopic studies, are normally not appealing to patients, and potentially, although rare, can still result in a procedure-related complication. Indeed, complications in patients with active inflammatory disease may exceed reported rates in otherwise healthy individuals undergoing screening procedures, and have been studied or reported poorly, particularly from treatment trials of new agents. Other less invasive approaches have often also been used in clinical practice, especially for repeated evaluations to assess the effects of therapy. These include imaging methods, such as computerized tomography (CT) and magnetic resonance imaging (MRI), usually with complete enterography. As with older barium imaging, however, there may be some inherent limitations. For example, these more modern imaging methods still have difficulty differentiating the inflammatory component of an intestinal stricture from its more established fibrotic component. CT may correlate with endoscopic evaluation for detection of ileal disease, but substantially increased radiation exposure results with repeated studies<sup>[20,21]</sup>. While both CT and MRI have limitations, multi-detector spiral CT enteroclysis may be more sensitive than MR enteroclysis for suspected bowel disease. In contrast, pelvic MRI has emerged as a standard for evaluation of perianal inflammatory disease or sepsis, particularly for fistula assessment and treatment<sup>[22]</sup>. Further correlation of these imaging modalities with other measures of intestinal healing are still needed.

## OTHER NONINVASIVE METHODS

A number of surrogate markers have been promoted, including leukocytosis, thrombocytosis and C-reactive protein levels<sup>[23,24]</sup>, but these are more clearly systemic rather than intestinal markers of the inflammatory process. Some of these markers also have been correlated with other indices. Other luminal markers, such as fecal lactoferrin or calprotectin<sup>[25]</sup>, along with functional permeability measurements are available, and may provide a potentially important option for evaluation of healing, but need further evaluation.

## TREATMENT ASSESSMENT

### Placebo response and remission

In patients with inflammatory bowel disease, spontaneous clinical improvement or remission without treatment may occur. As a result, randomized placebo-controlled trials are done to determine if the investigative agent is superior to placebo treatment. Both patient and investigator are blinded to obviate bias. Placebo-based trials usually produce a positive effect even with placebo, in

part, because of repetitive attention provided by caregivers to the trial subject. The placebo response is known to be powerful and, in a meta-analysis of placebo rates for inflammatory bowel disease clinical trials, rates up to 40% have been noted<sup>[26]</sup>. A superimposed issue in a clinical trial is the need to provide a proven form of therapy (while also testing the trial treatment). As a result, the placebo may, by necessity, be a standard therapy, not an inert treatment, while the treatment may include the standard therapy plus the trial treatment. For some medications, it may be difficult to hide the treatment because of known systemic effects (e.g. sulphasalazine or steroids). As noted elsewhere<sup>[26]</sup>, placebo remission rates may also be influenced by trial length, number of study visits, use of strict remission definitions and enrollment favoring patients with more active disease.

### Historical steroid studies

Early clinical trials with steroids have noted reduced clinical symptoms and improved appearances of the colonic mucosa<sup>[27,28]</sup>. Later trials with steroids have shifted the emphasis to the persistence of inflammatory changes, even though reduced symptoms were evident<sup>[29,30]</sup>. Unfortunately, the longer term role, if any, of steroids in mucosal healing and curbing the inflammatory process is understood poorly. In clinical practice, physicians limit the duration and dosage of systemic corticosteroids and taper these rapidly within weeks. This may not permit sufficient time for steroids to cause complete restitution of the mucosal surface. In a pooled treatment analysis of a first-pass metabolized steroid, budesonide, mucosal healing was reported to be limited in Crohn's disease after 1 year<sup>[31]</sup>. Budesonide, however, differs substantially in its chemical structure, metabolism and other properties from other steroids, therefore, generalization to other steroids may be premature. Some have hypothesized that steroids *per se* might be potentially deleterious to the mucosal healing process<sup>[32]</sup>, but there is no evidence to support this view. It is possible that the observed healing effects of steroids only reflect the clinical tendency to minimize duration and dosage of systemic steroids because of fear of potential side effects.

### Studies with other agents

Other agents used to treat inflammatory bowel disease, recently summarized in detail elsewhere for ulcerative colitis<sup>[33]</sup>, also have been reported to cause endoscopic mucosal healing. These include 5-aminosalicylates, including a modernized formulation MMX mesalamine<sup>[34,35]</sup>, immunosuppressant agents in Crohn's disease, such as azathioprine and methotrexate<sup>[36-40]</sup>, antibiotics<sup>[41,42]</sup>, and even prolonged courses of anti-mycobacterial treatment in Crohn's disease<sup>[43]</sup>. Similarly, biological agents are now being evaluated and mucosal healing has been reported as an important endpoint of treatment in the clinical trials<sup>[44-46]</sup>. Most of these studies, along with initial reports of other biological agents, have been conducted over only limited time frames, relative to the

natural duration of the disease, so positive and negative effects over the long term are not evident. In a recent report from a cohort in a treatment trial that has compared infliximab and azathioprine to conventional therapy with steroids, complete mucosal healing, defined as a simple endoscopic score<sup>[12]</sup> of 0 after 2 years of treatment predicted a sustained remission 3 and 4 years after therapy in > 70% of patients, compared to almost 30% of those with endoscopic lesions<sup>[47]</sup>. Of note, the authors also have concluded that achieving mucosal healing (defined by endoscopy) was the sole determining predicting factor and not the treatment *per se*.

## FUTURE DIRECTIONS

A number of issues need to be addressed carefully in the near future. Therapeutic trials of differing pharmacological and biological agents in inflammatory bowel disease have shown that mucosal healing may occur with most of the traditional drugs, as well as the emerging biological agents, to a greater or lesser degree, but correlation with the patient's symptoms or other measures of disease activity appear to be limited. The current technology to assess mucosal healing in clinical trials and clinical practice remains limited, tends to be observational, and is not ideal because it does not evaluate transmural inflammation precisely, only the luminal surface mucosa. Repeated invasive endoscopic evaluations may not be optimal, particularly since these are largely one-dimensional. Possibly, this will be improved with the future evolution of confocal endoscopy. The inflammatory process is not a static target and the measured impact of one or the other agent may reflect, in part, this fluidity of the inflammatory process *per se*. As a result, assessing the longer-term effects of old and emerging agents is needed urgently, but may also prove to be particularly challenging. Genome-wide expression differences have been defined using endoscopic pinch biopsies in both ulcerative colitis and Crohn's disease<sup>[48]</sup>. These ultimately may provide a means for selecting individuals with either ulcerative colitis or Crohn's disease that might be managed optimally with a specific therapy, because multiple genes appear to be involved<sup>[49]</sup>. New studies have appeared employing microarray technology in animal and human colitis, which have increased our understanding of the basic inflammatory process, along with possible mediators that might be regulated<sup>[50-53]</sup>. Indeed, very recent genome-wide association studies in ulcerative colitis have identified new susceptibility loci that suggest that changes in the integrity of the mucosal barrier are important in pathogenesis<sup>[54]</sup>. By recognizing the limitations of current methodology used in clinical trials to assess mucosal healing, the modern day clinician will still have to rely on his or her clinical evaluation and best judgment whenever a new treatment paradigm is contemplated, or a change or cessation in therapy is indicated. Fortunately, however, emerging gene-based technology is likely to lead to better end points for more precise assessment of available treatments.

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## Loss of CD103<sup>+</sup> intestinal dendritic cells during colonic inflammation

Ulrike G Strauch, Nicole Grunwald, Florian Obermeier, Sonja Gürster, Heiko C Rath

Ulrike G Strauch, Nicole Grunwald, Florian Obermeier, Sonja Gürster, Heiko C Rath, Department of Internal Medicine I, University of Regensburg, D-93053 Regensburg, Germany  
**Author contributions:** Strauch UG designed the research and wrote the paper; Grunwald N and Gürster S performed the majority of experiments; Obermeier F and Rath HC provided materials and reagents, gave vital advice and were involved in editing the manuscript.

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Correspondence to: Dr. Ulrike G Strauch, Department of Internal Medicine I, University of Regensburg, D-93053 Regensburg, Germany. [ulrike.strauch@klinik.uni-regensburg.de](mailto:ulrike.strauch@klinik.uni-regensburg.de)  
 Telephone: +49-941-9447001 Fax: +49-941-9447002

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### Abstract

**AIM:** To investigate possible differences in dendritic cells (DC) within intestinal tissue of mice before and after induction of colitis.

**METHODS:** Mucosal DC derived from intestinal tissue, as well as from mesenteric lymph nodes and spleen, were analyzed by fluorescence activated cell sorting (FACS) analysis. Supernatants of these cells were analyzed for secretion of different pro- and anti-inflammatory cytokines. Immunohistochemistry and immunofluorescence were performed on cryosections of mucosal tissue derived from animals with colitis as well as from healthy mice.

**RESULTS:** It was shown that DC derived from healthy intestinal lamina propria (LP) represented an immature phenotype as characterized by low-level expression of costimulatory cytokines. In contrast to DC from spleen and mesenteric lymph nodes (MLN) that secreted proinflammatory cytokines, LP-DC produced high levels of the anti-inflammatory cytokine IL-10. After induction of mu-

rine colitis in a CD4<sup>+</sup>CD62L<sup>+</sup> transfer model or in chronic Dextran sulfate sodium-colitis, a marked increase of activated CD80<sup>+</sup> DC could be observed within the inflamed colonic tissue. Interestingly, in contrast to splenic DC, a significant population of DC within MLN and colonic LP expressed the mucosal integrin CD103 which was lost during colitis.

**CONCLUSION:** The constitutive secretion of anti-inflammatory cytokines by immature DC within the intestinal LP might regulate the homeostatic balance between mucosal immunity and tolerance. CD103<sup>+</sup> DC could mediate this important function.

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**Key words:** Dendritic cell; Colitis; Cytokines; Integrin; CD103

**Peer reviewers:** Dr. Wang-Xue Chen, Institute for Biological Sciences, National Research Council Canada, 100 Sussex Drive, Room 3100, Ottawa, Ontario K1A 0R6, Canada; Hartmut Jaeschke, Professor, Liver Research Institute, University of Arizona, College of Medicine, 1501 N Campbell Ave, Room 6309, Tucson, AZ 85724, United States

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### INTRODUCTION

The intestinal mucosa is continuously challenged by innocuous antigens and potentially harmful pathogens. Therefore, the local immune system has to mount an efficient response towards pathogenic bacteria but must keep the immunological balance during exposure to commensal antigens. Dendritic cells (DC) are most likely

involved within this dual functionality. However, so far only limited data are available regarding intestinal DC (reviewed in<sup>[1,2]</sup>). Mucosal DC are not only found within Peyer's patches (PP) and mesenteric lymph nodes (MLN) but are also located within smaller isolated lymphoid follicles and within the lamina propria, distributed throughout the wall of the small and large intestine<sup>[3-5]</sup>. Unusual phenotypic subsets of DC have been described within MLN and PP<sup>[6-9]</sup> that preferentially stimulate antigen-specific CD4<sup>+</sup> T cells to produce IL-10 and/or TGF- $\beta$ <sup>[9,10]</sup>. This cytokine pattern is similar to that of TR<sub>1</sub> or TH<sub>3</sub> regulatory T cells which have been identified in gut-associated lymphoid tissue of mice fed tolerogenic doses of proteins, and are thought to play an important role in oral tolerance<sup>[11]</sup>.

Considerably more is known about DC in PP and MLN than about DC within the lamina propria of the gut. However, these cells are ideally situated to pick up any material that is transported between or through epithelial cells and have been shown to sample luminal antigens directly by sending dendrites outside the epithelium<sup>[12]</sup>. It is thought that DC as mobile cells migrate to MLN after antigen-uptake and interact with naïve T cells mainly within lymphatic organs, rather than in the mucosa itself. This rapid and constitutive trafficking of DC from lamina propria to MLN is increased by the presence of inflammatory stimuli<sup>[13]</sup>. As shown recently, interaction of mucosal DC with T cells generates gut-tropic CD8<sup>+</sup> effector T cells that express CCR9 and  $\alpha$  $\beta$ <sup>[14]</sup>. Additionally, DC expressing the mucosal integrin CD103 promote the development of regulatory Foxp3<sup>+</sup> T cells through a TGF- $\beta$  and retinoic acid-dependent mechanism<sup>[15]</sup>. Together, these findings indicate that lamina propria DC (LP-DC) might be more important for the surveillance of the intestinal milieu and the shaping of intestinal immune responses than previously thought.

Animal model systems of colitis have been used extensively in an effort to determine possible mechanisms that contribute to the initiation and perpetuation of colitis in humans<sup>[16]</sup>. One model in particular which has been well characterized involves the transfer of CD4<sup>+</sup>CD45RB<sup>hi</sup> T cells. Here, transfer of naïve (CD4<sup>+</sup>CD45RB<sup>hi</sup> or CD4<sup>+</sup>CD62L<sup>+</sup>) T cells into immunodeficient SCID mice induces chronic intestinal inflammation<sup>[17,18]</sup>. Key factors that drive the pathogenic TH<sub>1</sub>-biased mucosal T cell responses within this model are still unknown<sup>[19]</sup>. However, as described for various other experimental colitis models<sup>[20-25]</sup>, antigen presentation of bacterial antigens plays a role, as only mild colitis develops when lymphocytes are transferred into animals with a restricted enteric flora and no intestinal inflammation is observed after transfer into germ-free mice<sup>[26]</sup>. Additionally, intestinal bacterial antigens and their presentation were shown to be crucial for the generation and expansion of regulatory T cells in a healthy individual<sup>[18]</sup>, and disruption of the interaction of mucosal DC with activated T lymphocytes by administration of a receptor-blocking antibody against OX40L was shown to ameliorate colitis<sup>[27]</sup>. Overall, the data indicate that antigen presentation, presumably *via* mucosal DC, plays a role in the pathogenesis of chronic intestinal inflammation. However, the properties of these

cells during intestinal inflammation are only beginning to be explored.

The aim of our study was to investigate differences between intestinal DC populations under healthy conditions and after induction of colitis.

## MATERIALS AND METHODS

### Mice

Balb/c mice and *scid* mice (C.B.-17 SCID) (H2<sup>d</sup>) were obtained from Charles River (Germany). Animals were housed under conventional animal facility conditions and were generally used at 6-8 wk of age weighing 20-22 g. The animal studies were approved by the local institutional Review Board.

### Monoclonal antibodies

The following experimental monoclonal antibodies (mAbs) were purchased from BD Pharmingen (Heidelberg, Germany): anti-CD8, anti-MHC-II, anti-B220, anti-CD11b, anti-CD3, anti-CD11c, anti-CD80, anti-CD86, anti-CD40, anti-CD103 ( $\alpha$ E $\beta$ 7), anti-CD16/CD32. Directly PE- or FITC-conjugated mAbs were used for FACS analysis. For immunofluorescent staining directly FITC-conjugated anti-CD103 mAb was used in addition to Alexa 546-conjugated tyramide (Invitrogen, Germany).

### Isolation of primary dendritic cells

DC were isolated from spleen, MLN and intestinal lamina propria by enzymatic digestion of tissue using collagenase I (Worthington, UK), hyaluronidase and DNase I (both from Sigma-Aldrich, Germany) followed by immunomagnetic selection with anti-CD11c coated microbeads (Miltenyi Biotech, Germany). For intestinal DC, mononuclear lamina propria cells were isolated from digested tissue as described previously<sup>[28]</sup>, followed by enrichment of CD11c<sup>+</sup> DC using microbeads. Purity was generally > 85%. Routinely, isolated cells were stained for contaminating T cells and B cells, however virtually no cells could be detected by FACS analysis in the DC preparations.

### Colitis models

We adapted a previously described transfer model that resembles the CD4<sup>+</sup>CD45RB<sup>high</sup> model and uses the expression of L-selectin (CD62L) to select for naïve splenic T lymphocytes<sup>[18]</sup>. Briefly, CD4<sup>+</sup> T cells were purified from spleen mononuclear cells of healthy mice by negative depletion of other cell types using anti-CD8, anti-MHC-II, anti-B220 and anti-CD11b mAbs and anti-rat-IgG immunomagnetic microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). The resulting CD4<sup>+</sup> lymphocytes were separated further into CD62L<sup>+</sup> and CD62L<sup>-</sup> T cells by CD62L-conjugated microbeads (Miltenyi Biotech). Recipient SCID mice were reconstituted with  $0.25 \times 10^6$  CD4<sup>+</sup>CD62L<sup>+</sup> lymphocytes in 200  $\mu$ L of sterile PBS by intraperitoneal injection. Colitis activity was monitored by changes in weight over time and by histological analysis.

For chronic DSS-colitis, dextran sodium sulfate

(DSS; MW 40000) was purchased from ICN (Eschwege, Germany) and intestinal inflammation was induced by feeding 3% DSS over 7 d followed by a period of 10 d of water without DSS. Mice received 4 cycles of DSS treatment and animals were sacrificed on day 8 after completion of the 4th cycle<sup>[29]</sup>.

### Cytokine ELISA

Dendritic cells from different organs were isolated as described above and  $2 \times 10^5$  cells/well were incubated in 200  $\mu$ L complete medium (RPMI-1640, 10% FCS, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, all from GIBCO-BRL, Eggenstein, Germany; and  $3 \times 10^{-5}$  mol/L  $\beta$ -mercaptoethanol, Sigma) for 24 h. Cells were partly stimulated with 5  $\mu$ g/mL CpG-ODN (Metabion, Martinsried, Germany) or with 1  $\mu$ g/mL *Salmonella typhimurium*-derived lipopolysaccharide (LPS) (Sigma, Deisenhofen, Germany). Cytokine levels were measured in the supernatant by ELISA (all from Endogene, Woburn, MA, USA), according to the manufacturer's instructions.

### FACS analysis

Samples were analyzed using two-color staining as described previously<sup>[18]</sup>. Briefly, isolated DC were preincubated with 20  $\mu$ g/mL of anti-CD16/CD32 and 10% FCS to block Fc-Receptors and stained with both FITC- and PE-conjugated mAbs. The cells were washed and analyzed by FACS using an EPICS-XL MCL Coulter.

### Immunohistochemistry and immunofluorescence

Tissue samples were snap-frozen in liquid nitrogen, embedded in OCT resin and 5 to 10- $\mu$ m cryostat-sections cut. For immunohistochemistry, primary antibody application was followed by biotinylated polyclonal anti-rat IgG or anti-hamster IgG (both Dianova, Germany) as secondary antibody. Tissue was stained using the ABC (avidin/biotin complex)-immunoperoxidase kit according to the manufacturer's instructions (Vector Laboratories) and developed with AEC. Sections were counterstained with hematoxylin. For immunofluorescence, sections were incubated with APC-conjugated anti-CD11c and with FITC-labeled anti-CD103 mAbs. The anti-CD11c mAb was visualized by applying horseradish peroxidase-labeled streptavidin followed by Alexa 546-conjugated tyramide, according to the manufacturer's recommendations (Invitrogen, Germany). Slides were counterstained with DAPI.

### Statistical analysis

Statistical analysis was performed using the two-tailed Mann-Whitney *U* test. Differences were considered statistically significant when  $P < 0.05$ .

## RESULTS

### **DC within the healthy intestinal lamina propria show an immature phenotype and produce constitutively the anti-inflammatory cytokine IL-10**

CD11c<sup>+</sup> DC are found within the lamina propria (LP) of

the small and large intestine of healthy mice. Whereas a dense network of cells underlining the epithelium can be detected by immunohistochemistry within the mucosa of the small intestine, only a few scattered cells are found within the colonic LP. To compare the phenotype of DC derived from colonic LP and mesenteric lymph nodes (MLN), CD11c<sup>+</sup> DC were isolated from intestinal tissue and MLN of healthy mice. As demonstrated in Figure 1A, no significant levels of costimulatory molecules (CD40, CD80 and CD86) could be detected on the cell surface of freshly isolated DC from the intestinal LP or MLN. This suggests that DC within mucosal tissues demonstrate a rather immature phenotype as compared to splenic DC which show low levels of costimulatory molecules (data not shown). Additionally, isolated primary DC were incubated either unstimulated or in the presence of CpG and secretion of cytokines was detected by ELISA. As shown, MLN-DC and splenic DC differed markedly from LP-DC with regard to their cytokine profile. Unstimulated MLN-DC or splenic DC did not secrete significant amounts of pro- or anti-inflammatory cytokines, however, LP-DC dramatically produced 30-fold higher levels of the anti-inflammatory cytokine IL-10 (MLN-DC:  $35.4 \pm 5.0$ ; splenic-DC:  $12.8 \pm 0.7$ , LP-DC:  $1035 \pm 270$  pg/mL,  $P = 0.0235$ , Figure 1B). In contrast, large amounts of IFN- $\gamma$  and IL-12 were secreted by MLN-DC and splenic-DC after stimulation with CpG, whereas LP-DC did not produce significant amounts of proinflammatory cytokines (MLN-DC:  $1398 \pm 407$ , splenic-DC:  $1087 \pm 30$ , LP-DC:  $24 \pm 11$  pg/mL,  $P = 0.0009$ ). The differences seen were independent from the stimulatory agent used as similar results were detected by using LPS to stimulate primary DC.

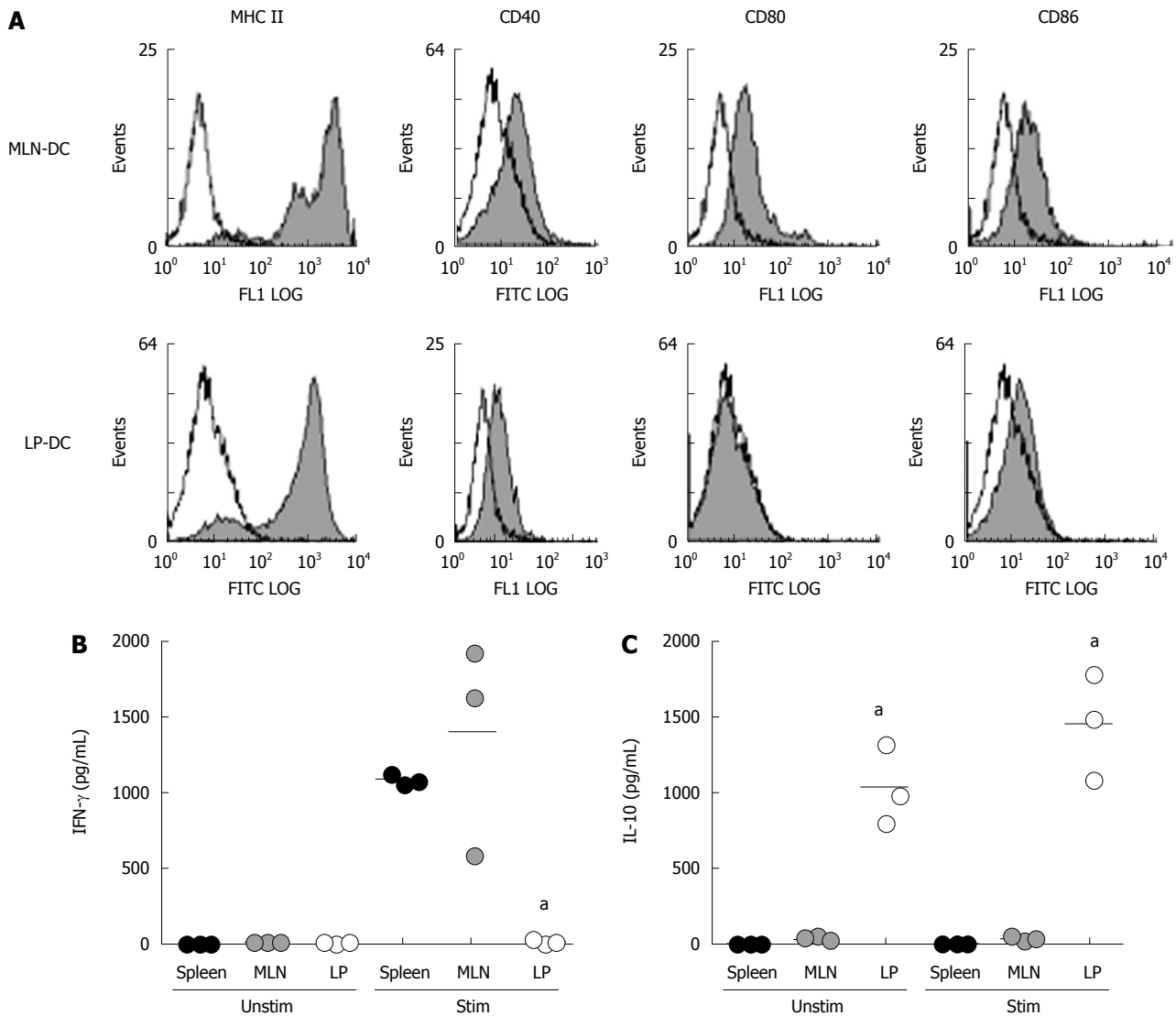
### **Diffuse infiltration of intestinal lamina propria with CD11c<sup>+</sup> DC during colitis**

As shown in Figure 2A, CD11c<sup>+</sup> DC were diffusely distributed throughout the non-inflamed colonic LP and were rarely detected in the submucosa. Chronic colitis was either induced by adoptive transfer of splenic CD4<sup>+</sup>CD62L<sup>+</sup> T lymphocytes from donor mice into immunodeficient SCID recipients or by cyclic administration of DSS in the drinking water of animals<sup>[18,29]</sup>. As analyzed by immunohistochemistry using an anti-CD11c antibody in both models of colitis, a dramatic increase in numbers of CD11c<sup>+</sup> DC could be detected within the inflamed mucosa of the colon of mice (Figure 2B). MLN of mice with colitis showed a slight increase in the number of DC (Figure 2C and D), whereas no changes in infiltrating DC were seen in Peyer's patches and the spleen (data not shown).

### **Infiltrating LP-DC in inflamed colonic tissue show a mature phenotype with high expression of CD80 cells and secretion of the regulatory cytokine IFN- $\alpha$ is decreased**

As shown above, mucosal DC in healthy tissue are immature. To investigate whether infiltrating CD11c<sup>+</sup> DC within the inflamed colon show an activated phenotype,





**Figure 1 Phenotypic and functional analysis of DC derived from MLN and LP of healthy mice.** A: Primary DC were isolated from MLN (MLN-DC) and colonic LP (LP-DC) of healthy mice. CD11c<sup>+</sup> DC were stained with FITC-conjugated mAbs for expression of MHC-class II and the costimulatory molecules CD40, CD80 and CD86; B: Isolated DC from spleen, MLN and LP were incubated for 24 h in complete medium partly stimulated with 5 µg/mL CpG (5 wells per situation). Cytokine levels were measured within the supernatant by ELISA. <sup>a</sup>*P* < 0.05. Data presented are representative of three independent experiments.

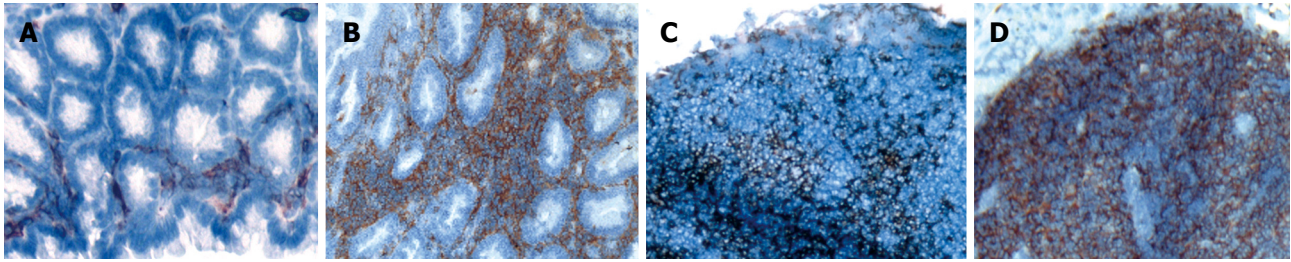
tissue sections were stained for the expression of costimulatory molecules. As demonstrated in Figure 3A, we were able to detect dramatically increased numbers of CD80<sup>+</sup> DC within the colonic LP as compared to healthy mucosa. On the other hand, no significant numbers of CD40<sup>+</sup> and CD86<sup>+</sup> cells were detected. We confirmed the results by FACS analysis after isolation of primary DC from inflamed tissue, demonstrating that LP-DC indeed showed increased cell-surface levels of CD80, whereas no difference in expression of CD40 and CD86 could be observed (Figure 3B).

To investigate whether the cytokine profile of isolated DC from different tissues was changed in animals with colitis we measured the cytokine secretion of primary DC. Secretion of IL-10 by LP-DC was reduced to only 27% of the amount secreted by LP-DC from healthy intestine (colitic LP-DC: 277 ± 27, healthy LP-DC: 1035 ± 270 pg/mL) (data not shown). Additionally,

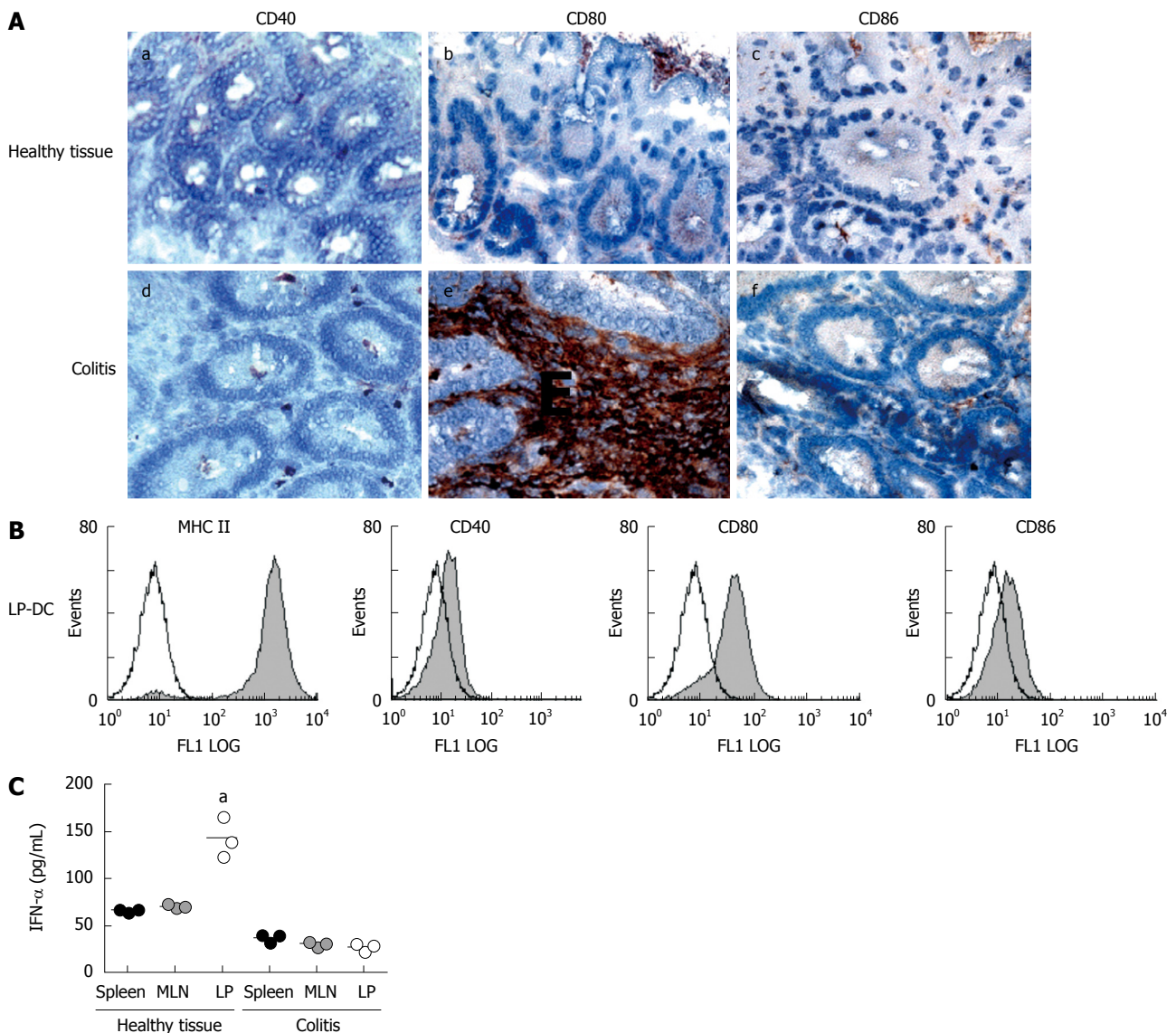
whereas LP-DC from normal colon did not secrete any proinflammatory cytokines as demonstrated above, DC from inflamed intestine were able to produce significant amounts of IFN-γ (397 ± 163 pg/mL) and TNF-α (650 ± 91 pg/mL) (data not shown). Furthermore, IFN-α, a cytokine attributed to plasmacytoid DC with regulatory function, was secreted to a greater extent by LP-DC from healthy mice as compared to splenic or MLN-DC which produced distinctly lower amounts of this cytokines (LP-DC: 143 ± 12, MLN-DC: 71 ± 2, splenic-DC: 67 ± 2 pg/mL, *P* = 0.0242). However, during colitis, production of IFN-α by LP-DC was significantly reduced to 19.6% (Figure 3C, colitic LP-DC: 28 ± 4 pg/mL, *P* = 0.0111).

#### Numbers of CD103<sup>+</sup> intestinal DC are dramatically reduced in colitic mice

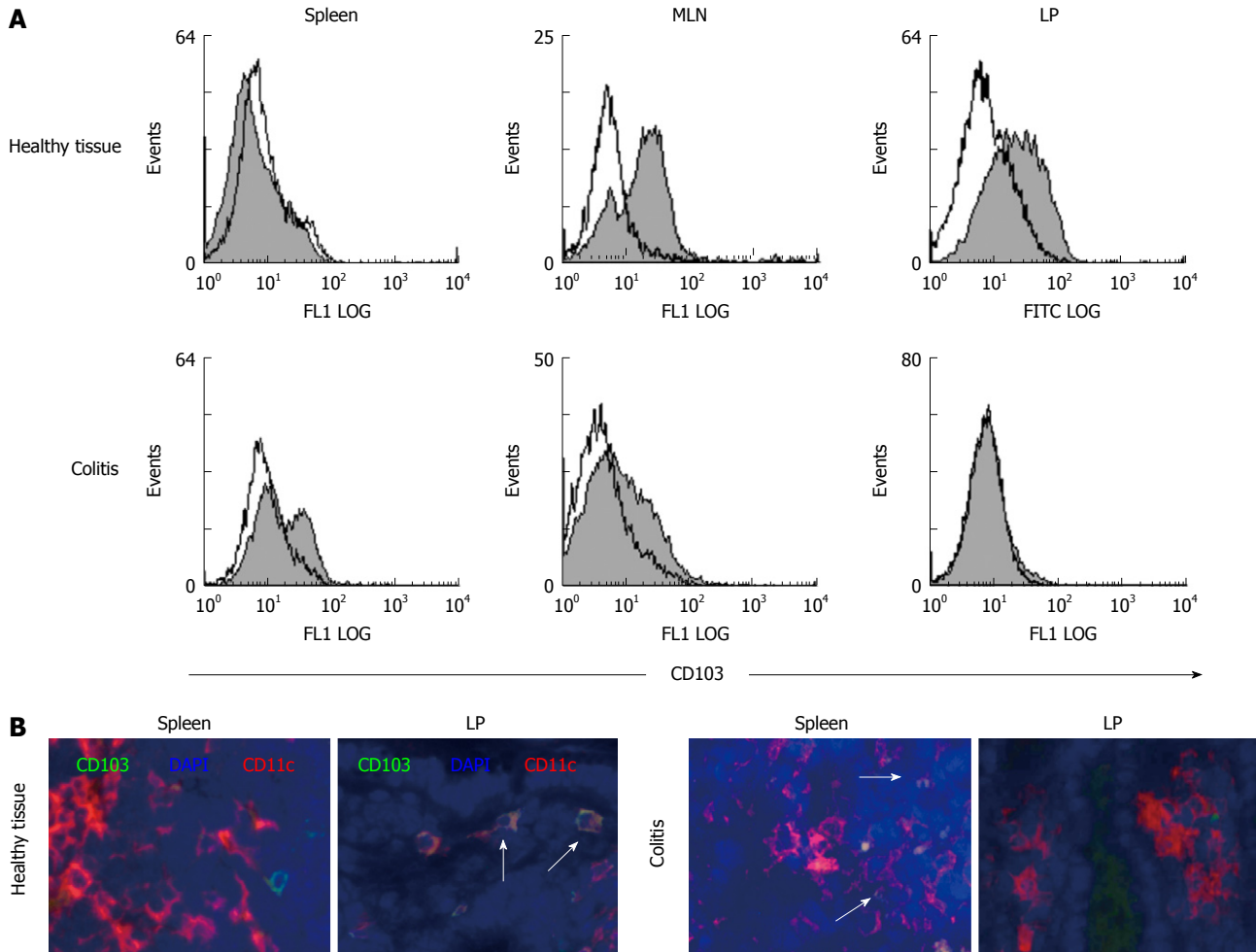
The mucosal integrin αEβ7 (CD103) is expressed on the cell surface of intraepithelial lymphocytes and mediates



**Figure 2 Inflammation induces infiltration of colonic LP and MLN with CD11c<sup>+</sup> DC.** Tissue was harvested from colonic LP (A/B) and MLN (C/D) of healthy animals (A/C) and mice with colitis (B/D). Immunohistochemistry was performed using an anti-CD11c<sup>+</sup> antibody to stain for intestinal DC. Staining with isotype control revealed no background staining (data not shown). Representative sections from 5 mice per group are shown (magnification 100 ×). Staining was performed on tissue derived from colitic animals with transfer colitis as well as chronic DSS colitis and revealed similar results. Sections shown within this figure are derived from animals with transfer colitis.



**Figure 3 Intestinal DC are activated after induction of colitis.** A: Tissue was harvested from LP of healthy animals and from mice with colitis. Immunohistochemistry was performed with antibodies against the following costimulatory molecules: CD40 (a/d), CD80 (b/e), CD86 (c/f). Representative sections are shown (magnification 100 ×). Experiments were performed from 5 animals each group. Sections shown within this figure are derived from animals with transfer colitis, however staining was also performed on tissues derived from colitic animals with chronic DSS colitis and revealed similar results; B: LP-DC were isolated from inflamed intestinal tissue and FACS analysis was performed. CD11c<sup>+</sup> DC were stained with FITC-conjugated mAbs for expression of MHC-class II and the costimulatory molecules CD40, CD80 and CD86. Data presented are representative of three independent experiments performed with cells derived from animals with transfer colitis. Similar results were generated in the DSS colitis model; C: Primary DC were isolated from different tissues (spleen, MLN, colonic LP) of healthy animals and mice with colitis. Isolated DC were incubated for 24 h in complete medium and levels of IFN- $\alpha$  were measured within the supernatants by ELISA (5 wells per situation). \* $P < 0.05$ . Data presented are from one of three independent experiments performed with cells derived from animals with transfer colitis. Similar results were generated in the DSS colitis model.



**Figure 4** CD103<sup>+</sup> DC are found in mucosal tissues of healthy mice and are lost during colitis. **A:** Primary DC were isolated from different tissues (spleen, MLN, colonic LP) of healthy animals and mice with colitis. FACS analysis was performed after staining of CD11c<sup>+</sup> DC with FITC-conjugated mAb against CD103, the integrin  $\alpha\text{E}\beta 7$ . Data presented are representative of three independent experiments that were carried out with cells derived from animals with transfer colitis. Similar results were generated in the DSS colitis model; **B:** Tissue was harvested from spleen and LP of healthy animals and mice with colitis. Immunofluorescence was performed using an anti-CD11c<sup>+</sup> mAb to stain for intestinal DC and an anti-CD103 mAb to recognize the integrin  $\alpha\text{E}\beta 7$ . DAPI was used to visualize nuclei. Images show overlays of CD103 (green), CD11c (red) and DAPI (blue). DC coexpressing CD103 appear yellow (indicated by arrows). Representative sections from 5 mice per group are shown (magnification 100  $\times$ ). Staining was performed on tissue derived from colitic animals with transfer colitis as well as chronic DSS colitis and revealed similar results. Sections shown within this figure are derived from animals with transfer colitis.

adhesion to epithelial cells *via* binding to E-cadherin<sup>[30]</sup>. Recently, it was shown that expression of CD103 characterizes an important subset of regulatory T cells<sup>[31]</sup>. Additionally, CD103<sup>+</sup> mucosal DC were suggested to play an important role for the generation of Foxp3<sup>+</sup> T lymphocytes within the gut<sup>[15]</sup>. As this integrin seems to play a role in regulatory immunological functions, especially in mucosal sites, we wanted to investigate whether CD103<sup>+</sup> intestinal DC change during intestinal inflammation. As demonstrated in Figure 4, freshly isolated DC from healthy spleen did not contain a significant population of CD103<sup>+</sup> DC. However, this was very different in mucosal sites of healthy animals. Here, a large subpopulation of MLN-DC expressed the integrin  $\alpha\text{E}\beta 7$  and even more strikingly, almost all LP-DC showed at least low levels of CD103 expression on the cell surface, demonstrating that LP-DC from healthy mucosa did not only differ dramatically in their cytokine secretion potential but also in their phenotype from DC

at peripheral sites. However, when we looked for CD103 expression on DC isolated from animals with colitis we observed a dramatic loss of this population in mucosal tissues. Within the chronically inflamed colonic LP, no CD103<sup>+</sup> DC were seen as demonstrated by FACS analysis, and numbers of integrin-positive DC in MLN were dramatically reduced. In contrast, we detected a significant subpopulation of CD103<sup>+</sup> DC within the spleen of colitic animals (Figure 4).

## DISCUSSION

Antigen-presenting cells are the key to maintaining the immunological balance between active immune responses and tolerance within the intestine and DC are most likely to participate importantly in this immunological homeostasis within the gut. However, data has recently started to be available regarding intestinal DC in normal and inflamed colon. Comparing DC populations from



different tissues we were able to demonstrate that mucosal DC represent an immature phenotype as characterized by the absence of CD40, CD80 and CD86 expression. Whereas CD80 was found in low levels on the cell surface of MLN-DC, the molecule was absent on LP-DC, indicating that LP-DC represent an even more immature phenotype than MLN-DC. It is thought that DC can be divided into tolerogenic immature and immunogenic mature differentiation stages<sup>[32]</sup>, as tolerance is mediated by partial- or semi-matured DC, whereas only full DC maturation is immunogenic<sup>[1,2,33]</sup>. Therefore, our observation of phenotypically immature (or semi-mature) LP-DC within the healthy gut supports the hypothesis that intestinal DC, which sample antigens without being fully activated, induce tolerance against antigens of the regular gut flora. Additionally, we were able to show that DC from spleen and MLN secrete proinflammatory cytokines such as IFN- $\gamma$  and IL-12 in response to the different strong inflammatory stimuli, CpG and LPS. In contrast, intestinal-derived CD11c<sup>+</sup> DC constitutively produced high levels of the anti-inflammatory cytokine IL-10 and did not release significant amounts of proinflammatory mediators after stimulation. As shown previously, pulmonary DC - situated within a mucosa that is similar to the intestine exposed to antigens - produce IL-10 in response to inhalative antigens and induce the development of regulatory T cells<sup>[34]</sup>. Therefore, it can be speculated that constitutive production of the anti-inflammatory cytokine IL-10 by LP-DC is also critical for the generation of regulatory T cells and the maintenance of tolerance towards luminal antigens within the normal gut.

However, during intestinal inflammation the cellular composition within the colonic lamina propria changes. As shown, gut inflammation in different murine models of colitis was accompanied by a marked infiltration of the colonic mucosa by CD11c<sup>+</sup> DC. LP-DC derived from the inflamed mucosa expressed high levels of CD80, a cell surface molecule thought to be involved with induction of TH<sub>1</sub> responses<sup>[35,36]</sup>, and cells resembled a phenotype of mature activated DC. Additionally, these cells produced dramatically lower levels of IL-10 and INF- $\alpha$ , cytokines necessary for anti-inflammatory responses<sup>[37]</sup>. Our observation is in concordance with a recent study that also demonstrated expansion of colonic LP-DC during murine colitis<sup>[5,38]</sup> and other data that showed up-regulated expression of activation markers on DC in diseased mucosal tissues from patients with inflammatory bowel disease<sup>[39]</sup>. It is likely that the infiltration of the intestinal mucosa with mature DC during intestinal inflammation leads to a continuous activation of T lymphocytes and a sustained overproduction of proinflammatory mediators within the lamina propria, which perpetuates colitis.

Additionally, we were able to show that LP-DC from normal colonic mucosa and MLN-DC contain a significant subpopulation of CD103<sup>+</sup> ( $\alpha\beta$ ) DC, whereas splenic-DC were negative for the mucosal adhesion molecule. This observation suggests the localization of specific DC subpopulations within the intestinal lamina

propria. So far, it is known that intraepithelial lymphocytes express the integrin  $\alpha\beta$ 7 which interacts with epithelial E-cadherin and is thought to mediate localization of T cells within the epithelial layer<sup>[30]</sup>. Additionally, it seems to characterize a specific subgroup of lymphocytes with regulatory function<sup>[31]</sup> and a recent study was able to show that CD103<sup>+</sup> DC promote expression of the gut-homing receptor CCR9 on T cells<sup>[14,40]</sup>, as well as generation of Foxp3<sup>+</sup> T lymphocytes with TGF- $\beta$  and retinoic-acid as cofactors<sup>[15]</sup>. Because in our study almost all intestinal LP-DC within the healthy mucosa express this integrin and show a tolerogenic phenotype and function, we hypothesize that CD103 mediates homing for tolerogenic DC into the intestinal mucosa or enables as adhesion molecule the crosstalk with other lamina propria cells, thereby influencing the balance between effector and regulatory T cell activity in the intestine. As we were not able to identify the CD103<sup>+</sup> LP-DC during colitis when tolerance is lost, this subgroup of DC could help to maintain the immunological balance within the normal intestinal mucosa. Surprisingly, during inflammation, CD103<sup>+</sup> DC were found within the spleen, suggesting that intestinal CD103<sup>+</sup> DC might migrate to systemic lymphatic tissues.

Overall, our results indicate that the specific localization of particular CD103<sup>+</sup> DC subpopulations within the intestinal mucosa may be an important mechanism of the immune system to determine between active immune responses and tolerance towards luminal antigens. Additionally, the constitutive secretion of anti-inflammatory cytokines by intestinal DC might regulate the homeostatic balance under healthy conditions. After induction of colitis, loss of CD103<sup>+</sup> intestinal DC and infiltration of mature DC that express the costimulatory molecule CD80 within the colonic mucosa would lead to a dysregulation of this balance. Antigen presentation *via* activated DC could be involved in the onset or/and chronification of colitis. Interrupting the activation of intestinal DC *in vivo* and promoting the preservation of presumably tolerogenic CD103<sup>+</sup> DC within the colonic mucosa may be key approaches to control the pathogenesis of inflammatory bowel disease.

## COMMENTS

### Background

Within the gut mucosa the immune system has the task of distinguishing between commensal bacteria and foreign antigens, to maintain tolerance or to mount an inflammatory response. Dendritic cells are very important for this process. However, even if in the last few years some important insights have been made, much still is unknown about these cells, especially about the changes they undergo during intestinal inflammation.

### Research frontiers

Previous data indicate that antigen presentation, presumably *via* mucosal dendritic cells, plays a role in the pathogenesis of chronic intestinal inflammation. However, the properties of these cells during intestinal inflammation are only beginning to be explored.

### Innovations and breakthroughs

Dendritic cells in normal intestinal lamina propria showed an immature phenotype and produced high levels of the anti-inflammatory cytokine IL-10 whereas



dendritic cells in the spleen and local lymph nodes secreted the proinflammatory cytokine IFN- $\gamma$ . Furthermore, the studies in mouse models of colitis showed that the development of colitis was associated with a marked increase of activated dendritic cells within the inflamed colonic tissue and the loss of CD103<sup>+</sup> dendritic cells in the colonic mucosa and local lymph nodes but not in spleen, suggesting that CD103<sup>+</sup> dendritic cells could play important roles in the regulation of homeostatic balance between mucosal immunity and tolerance in the gastrointestinal tract.

### Applications

Overall, these results indicate that the specific localization of particular CD103<sup>+</sup> dendritic cell subpopulations within the intestinal mucosa may be an important mechanism of the immune system in determining between active immune response and tolerance towards luminal antigens. Additionally, the constitutive secretion of anti-inflammatory cytokines by intestinal DC might regulate the homeostatic balance under healthy conditions. Interrupting the activation of intestinal dendritic cells *in vivo* and promoting the preservation of presumably tolerogenic CD103<sup>+</sup> dendritic cells within the colonic mucosa may be key approaches to control the pathogenesis of inflammatory bowel disease.

### Peer review

This is a well prepared manuscript and the experiments described were well designed, controlled and executed. The loss of CD103<sup>+</sup> CD11c<sup>+</sup> DCs in progression of colitis is a unique and novel finding which seems to coincide with other groups' findings relating to regulatory T cells. Overall the results could contribute to our understanding of the immunopathogenesis of human inflammatory bowel diseases.

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## Antibiotics and probiotics in chronic pouchitis: A comparative proteomic approach

Silvia Turroni, Beatrice Vitali, Marco Candela, Paolo Gionchetti, Fernando Rizzello, Massimo Campieri, Patrizia Brigidi

Silvia Turroni, Beatrice Vitali, Marco Candela, Patrizia Brigidi, Department of Pharmaceutical Sciences, University of Bologna, via Belmeloro 6, 40126 Bologna, Italy  
Paolo Gionchetti, Fernando Rizzello, Massimo Campieri, Department of Internal Medicine and Gastroenterology, University of Bologna, Polyclinic S. Orsola, via Massarenti 9, 40138 Bologna, Italy

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**Correspondence to:** Patrizia Brigidi, Professor, Department of Pharmaceutical Sciences, University of Bologna, via Belmeloro 6, 40126 Bologna, Italy. [patrizia.brigidi@unibo.it](mailto:patrizia.brigidi@unibo.it)

Telephone: +39-51-2099743 Fax: +39-51-2099734

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### Abstract

**AIM:** To profile protein expression in mucosal biopsies from patients with chronic refractory pouchitis following antibiotic or probiotic treatment, using a comparative proteomic approach.

**METHODS:** Two-dimensional polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight mass spectrometry were used to characterize the changes related to antibiotic therapy in the protein expression profiles of biopsy samples from patients with chronic refractory pouchitis. The same proteomic approach was applied to identify differentially expressed proteins in the non-inflamed pouch before and after probiotic administration.

**RESULTS:** In the first set of 2D gels, 26 different proteins with at least 2-fold changes in their expression levels between the pouchitis condition and antibiotic-

induced remission were identified. In the second set of analysis, the comparison between mucosal biopsy proteomes in the normal and probiotic-treated pouch resulted in 17 significantly differently expressed proteins. Of these, 8 exhibited the same pattern of deregulation as in the pouchitis/pouch remission group.

**CONCLUSION:** For the first time, 2D protein maps of mucosal biopsies from patients with ileal pouch-anal anastomosis were provided, and differentially expressed proteins following antibiotic/probiotic treatment were identified.

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**Key words:** Chronic disease; Pouchitis; Antibiotics; Probiotics; Proteins; Gene expression

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### INTRODUCTION

Total proctocolectomy with ileal J-pouch-anal anastomosis (IPAA) is the surgical treatment of choice for patients with refractory ulcerative colitis (UC) or UC with dysplasia. Although the surgery generally cures UC and has been shown to result in a significant improvement of health-related quality of life, complications can occur after IPAA<sup>[1]</sup>.

The most common long-term complication is pouchitis, an idiopathic inflammatory disease of the ileal reservoir. The reported incidence of pouchitis is variable, largely because of differences in the type and duration of follow-up. However, studies have shown that as many as 15%-46% of patients with UC develop at least 1 episode of pouchitis within 5 years after surgery<sup>[2]</sup>.

Clinically, pouchitis is characterized by variable symptoms, including increased stool frequency and fluidity, abdominal cramping, pelvic discomfort, bleeding, tenesmus, fever and weight loss, and extra-intestinal manifestations in more severe cases<sup>[3]</sup>. For an unequivocal diagnosis, endoscopic examination and histologic investigation are mandatory<sup>[4]</sup>. Pouchitis Disease Activity Index (PDAI) is the most commonly used diagnostic instrument and represents an objective and reproducible scoring system for pouchitis<sup>[5]</sup>. Active pouchitis is defined as a score  $\geq 7$  and remission is defined as a score  $< 7$ .

The etiology and pathophysiology of pouchitis are still poorly understood. However, the fact that pouchitis almost exclusively occurs in patients with underlying UC and that it generally responds to antibacterial therapy suggests a role for the gut microbiota and a genetic predisposition<sup>[6]</sup>.

The disease activity of pouchitis can be defined as remission, mild-moderate or severe based primarily on symptoms. Duration can be classified as acute ( $< 4$  wk) or chronic ( $\geq 4$  wk). Disease pattern can be infrequent (1-2 acute episodes), relapsing ( $\geq 3$  acute episodes) or chronic (a treatment-responsive form requiring maintenance therapy or a treatment-resistant form). Approximately 10%-15% of patients with pouchitis experience a chronic pouchitis, either treatment-responsive or treatment-refractory, and some of them require surgical excision or exclusion of the pouch because of impairment of reservoir function and poor quality of life<sup>[7]</sup>.

Treatment of pouchitis is largely empirical. Broad-spectrum antibiotics have been widely used and represent the mainstay of treatment. Small randomized trials have shown that both metronidazole and ciprofloxacin, alone, sequentially or in combination, are effective in reducing the PDAI score and achieving a significant improvement in clinical symptoms and endoscopic and histologic findings. However, metronidazole is poorly tolerated and treatment with systemically active antibiotics is not ideal from the perspective of the development of antibiotic resistance. In addition, in chronic pouchitis antibiotic-induced remission periods are often short and the condition is complicated by frequent relapses<sup>[8]</sup>.

Recently, several studies have suggested that altering the microbiota in the pouch by administering probiotic bacteria can be effective in maintaining remission and reducing the incidence of flare-ups in chronic pouchitis<sup>[9,10]</sup>. Moreover, the efficacy of probiotic therapy as prophylaxis to delay the first onset of pouchitis after pouch surgery, has been demonstrated<sup>[11,12]</sup>.

Comparative proteomic analysis represents an effective tool to identify proteins critical for functional pathways in normal cells and phenotype changes that

occur during disease development. Since biological and functional output of cells is governed primarily by proteins, the applications of proteomic technologies are beginning to have a profound impact on understanding of the molecular mechanisms underlying several disease processes, which, in turn, will help to reduce disease-related morbidity and mortality. However, despite their extensive use in proteomic profiling of gene expression in various diseases, the applications of such technologies in inflammatory bowel diseases are still in their infancy<sup>[13]</sup> and, so far, no proteomic study has been reported in IPAA research.

In the present study, we apply 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) to define the differential protein displays of mucosal biopsy samples from patients with chronic refractory pouchitis before and after antibiotic treatment. The same proteomic approach has also been applied to identify specific changes in protein expression in the non-inflamed *vs* probiotic-administered pouch in order to provide a picture of the intestinal mucosa protein modulation by probiotics.

## MATERIALS AND METHODS

### Patients and biopsy collection

Six patients who underwent restorative proctocolectomy with IPAA were recruited for this study and routinely followed up by the Department of Internal Medicine and Gastroenterology, University of Bologna, Polyclinic S. Orsola. Patients were included if they had a chronic refractory pouchitis, defined as no response to at least 4 wk of standard antibiotic therapies (ciprofloxacin 1 g twice daily (*bid*) or metronidazole 400 mg 3 times daily). They were divided in 2 groups according to PDAI score at study entry and treatment received. In the first group, 3 patients with PDAI  $\geq 7$  were orally administered with a combination of metronidazole (500 mg *bid*) and ciprofloxacin (500 mg *bid*) for 1 mo. The second group, including the other 3 patients with chronic refractory pouchitis but with a total PDAI  $< 7$  at study entry, received VSL#3 (VSL pharmaceuticals Inc., Ft. Lauderdale, FL, USA) 2 packets *bid* for 3 mo. VSL#3 contains 450 billion viable lyophilized bacteria per packet, comprised of 4 strains of lactobacilli (*Lactobacillus acidophilus*, *L. casei*, *L. delbrueckii* subsp. *bulgaricus* and *L. plantarum*), 3 strains of bifidobacteria (*Bifidobacterium breve*, *B. infantis* and *B. longum*) and one strain of *Streptococcus thermophilus*. Mucosal biopsies were collected during pouch endoscopy before and after antibiotic/probiotic therapy.

All samples were immediately snap frozen in liquid nitrogen. The institutional ethics committee approved all protocols and all enrolled subjects gave their informed consent.

### Protein extraction

Frozen mucosal biopsies (about 10-20 mg) were washed in 200  $\mu$ L of cold low salt washing buffer (3 mmol/L



KCl, 1.5 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 68 mmol/L NaCl, 9 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, with Complete Protease Inhibitor (Roche Molecular Biochemicals, Mannheim, Germany). After centrifugation at 13000 r/min for 2 min, tissue samples were homogenized in 1 mL of lysis solution (0.11 mol/L DTT, 0.11 mol/L CHAPS, 8 mol/L urea, 2 mol/L thiourea, 35 mmol/L Tris and Complete Protease Inhibitor) using an Ultra-Turrax® homogenizer (IKA Labortechnik, Staufen, Germany). Protein extraction was performed as previously described<sup>[14]</sup>. Total protein concentration of the cell extract was calculated using the PlusOne 2D Quant Kit™ (GE Healthcare, Uppsala, Sweden). The protein extract preparation was immediately used or aliquoted and frozen at -20°C.

## 2D-PAGE

Samples containing 100 µg of protein were diluted to 250 µL with rehydration solution (8 mol/L urea, 2% CHAPS, 10 mmol/L DTT, 2% (v/v) ampholine, pH 3.5-9.5 (GE Healthcare) and trace bromophenol blue) and applied to Immobiline DryStrips (13 cm, pH 3-10, GE Healthcare) for 12 h rehydration at 50 V. Isoelectric focusing was performed using IPGphor apparatus (GE Healthcare) to give a total of 19 kVh. IPG strips were then reduced and alkylated<sup>[15]</sup> prior to loading onto 15% acrylamide separating gels (20 cm long, 1 mm thickness). Electrophoresis was performed at 250 V for 7 h using Protean II xi Cell (Bio-Rad, Hercules, CA, USA). Protein spots were visualized with a MS-compatible silver-staining procedure<sup>[16]</sup>.

## Image analysis

Protein patterns in the gels were recorded as digitalized images using a GS-800 imaging densitometer (Bio-Rad). Spot detection, matching and the examination of differentially expressed proteins were performed by PDQuest v6.2 software (Bio-Rad). Three technical replicates were made per patient and condition and formed 1 replicate group with average normalized spot intensities. The comparison was carried out for each patient before and after antibiotic/probiotic therapy. Proteins that showed at least 2 times enhanced/decreased expression were selected for identification along with a few spots that showed a similar expression pattern in all 2D gels.

## Protein identification

Protein spots with conserved expression levels throughout the gels in all patients and conditions were identified. Two identification methods were employed: comparison of our reference proteome map with Swiss-2D PAGE (<http://www.expasy.ch/ch2d/>) and other published 2D proteome patterns<sup>[17-21]</sup> obtained under very similar experimental conditions, and MALDI-TOF MS analysis. Since both methods provided the same identification result for each spot, we used the gel matching method to identify the differentially expressed proteins in pouchitis/antibiotic-induced remission and normal pouch/

probiotic-treated pouch groups. When gel matching produced an unreliable and doubtful identification, because of excessive deviations in *pI* and *M<sub>r</sub>* values across gels, MALDI-TOF MS was employed.

Protein spots were manually excised from 2D gels, washed and in-gel digested as previously reported<sup>[22]</sup>. Crude digests were concentrated and desalted using mC18 ZipTips (Millipore, Bedford, MA, USA). Peptide extracts were mixed on the MALDI-TOF target (Applied Biosystems, Foster City, CA, USA) with an equal matrix volume of 5 mg/mL  $\alpha$ -cyano-4-hydroxycinnamic acid (Sigma-Aldrich, St. Louis, MO, USA) saturated with 50% acetonitrile/0.2% trifluoroacetic acid, and analyzed using a Voyager-DE Pro Biospectrometry Workstation (Applied Biosystems). All mass spectra were obtained in a reflectron mode, with an accelerating voltage of 20 kV and a delayed extraction of 40 ns. Internal mass calibration with peptides arising from trypsin autolysis was performed. Peptide masses were searched against Swiss-Prot, TrEMBL and NCBI non-redundant protein databases using ProFound (<http://prowl.rockefeller.edu/prowl-cgi/profound.exe>) and Aldente (<http://expasy.org/tools/aldente>) programs. Search parameters were set to allow up to one missed tryptic cleavage and a peptide mass tolerance of 50 ppm. Only protein hits with a significant probability score calculated by software and at least 3 matching peptide masses were considered.

## Statistical analysis

Statistical analysis of protein expression was performed using the Student's *t*-test carried out with SigmaStat v3.5 software (Systat Software, Point Richmond, CA, USA). A *P* value < 0.05 was considered as statistically significant. Bibliometric analysis for co-citation was performed using Biblisphere Pathway Edition from Genomatix (Genomatix Software, Munich, Germany).

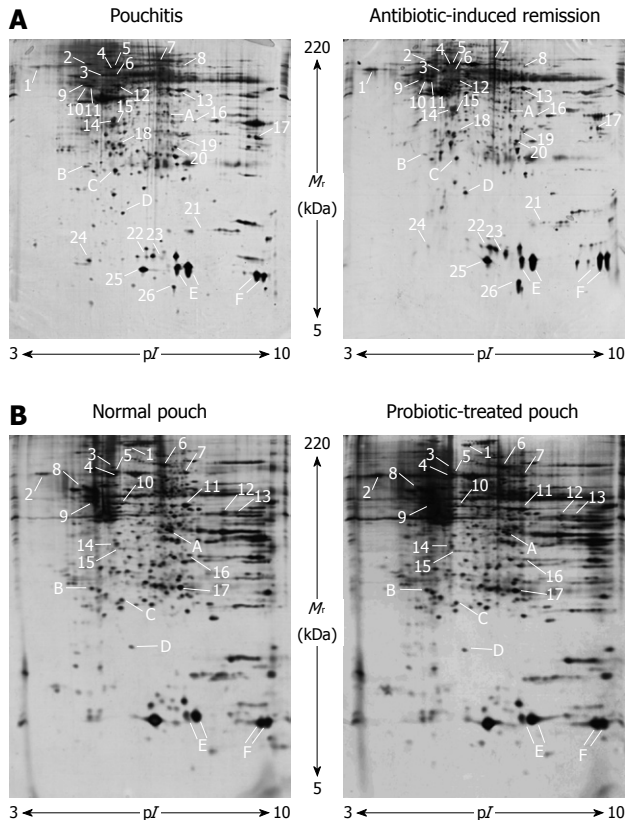
## RESULTS

### Clinical outcome of antibiotic/probiotic treatment

All the enrolled subjects completed the study. In the first group of patients, after 1 mo of antibiotic therapy, clinical and endoscopic remission was achieved with a significant decrease in both PDAI and median stool frequency (data not shown). In the second group, no episodes of active pouchitis were recorded during the probiotic administration. Both treatments were well tolerated and no side effects were recorded.

### Antibiotic administration-related effects on mucosal biopsy proteome in pouchitis

An example of 2D gels obtained from mucosal biopsies in pouchitis and pouch remission is provided in Figure 1A. Approximately 1200 protein spots per gel were detected within a *pI* range of 3-10 and a *M<sub>r</sub>* range of 5-220 kDa. The resolution of the polypeptides showed better quality in the low molecular mass area and toward the acidic side of the gels whereas increased streaking and precipitation,



**Figure 1** Representative 2D gel maps of the mucosal biopsy proteomes from a patient with chronic refractory pouchitis before (left) and after (right) antibiotic therapy (A) and from a subject with a non-inflamed pouch before (left) and after (right) probiotic administration (B). Proteins showing altered expression identified by gel matching and MALDI-TOF MS analysis are numbered and reported in Table 1. Identified spots with conserved expression levels in all patients and conditions are marked by letters and shown in Table 2.

a well known phenomenon observed in 2D-PAGE, were visible on the basic side.

For each patient, 2D patterns of mucosal biopsies collected before and after antibiotic administration were compared by PDQuest. Because of the high intrinsic variability among individuals, a stringent criterion was applied whereby only those proteins with at least 2 times increased or decreased expression and deregulation in the same way in all patients were considered. Out of 40 differentially expressed protein spots, 26 (65%) were identified, of which 15 were upregulated and 11 downregulated in antibiotic-induced remission of pouchitis (Figure 1A and Table 1). In addition, 6 protein spots with a similar expression pattern in all 2D gels were selected and identified (Figure 1 and Table 2).

The altered proteins were classified in terms of their subcellular location and biological function by information from Swiss-Prot, HPRD (Human Protein Reference Database, <http://www.humanproteinpedia.org>), and COGs (Cluster of Orthologous Groups of proteins, <http://www.ncbi.nlm.nih.gov/COG/>) (Figure 2A). The majority of the identified proteins were located in the cytoplasm (38%), mitochondria (27%) and endoplasmic reticulum (11%). Twenty-seven percent of

the altered proteins play a key role in post-translational modifications and protein turnover as chaperones, 15% are involved in energy production and conversion, and 11% are related to lipid transport and metabolism.

The results of a histogram data analysis carried out on the spot quantity values determined by PDQuest are displayed in Figure 3 together with representative gel images for each protein spot in each patient and clinical condition. A statistically significant increased expression in pouch remission was detected for tubulin  $\beta$ -2C chain (TUBB), ATP synthase subunit  $\beta$  (ATP5B) and calponin-2 (CNN2) in all patients, whereas calreticulin (CALR), 60 kDa heat shock protein (HSP60), heat shock cognate 71 kDa protein (HSPA8), and intestinal (FABP2) and liver fatty acid-binding proteins (FABP1) expression patterns showed an increase with statistical significance in only 1 or 2 out of the 3 patients enrolled. For ileal lipid binding protein (FABP6) and electron transfer flavoprotein subunit  $\alpha$  (ETF $\alpha$ ), *P* values of 0.07 and 0.06, respectively, near the threshold of significance were obtained. Among downregulated protein spots after antibiotic treatment, statistical significance was achieved in all patients for thioredoxin domain-containing protein 5 (TXNDC5), type I cytoskeletal keratin 20 (KRT20) and cathepsin D (CTSD). Pyruvate dehydrogenase E1 component subunit  $\beta$  (PDHB) showed a statistically significant decreased expression in only 1 patient.

#### **Probiotic administration-related effects on mucosal biopsy proteome in non-inflamed pouch**

Representative 2D gels obtained from mucosal biopsies in normal pouch and after probiotic therapy are shown in Figure 1B, confirming the protein maps reported in Figure 1A in terms of number, *M<sub>r</sub>* and *pI* of the spots.

For each of the 3 subjects enrolled, the comparison of the 2D patterns of non-inflamed mucosal biopsies before and after VSL#3 administration was performed by PDQuest as reported above. Seventeen spots, which represented 75% of total proteins recognized as differentially expressed, were identified, of which 7 were upregulated and 10 were downregulated in the probiotic-treated pouch (Figure 1B and Table 1). In addition, it was possible to identify 6 protein spots that showed a similar expression pattern in all 2D gels (Figure 1 and Table 2).

Pie charts representing the subcellular location and the functional distribution of the probiotic administration-altered proteins are reported in Figure 2B. The majority of the identified proteins were in the cytoplasm (41%), mitochondria (35%) and endoplasmic reticulum (12%). The functional classification indicated that 29% play a key role in energy production and conversion, 17% are related to post-translational modifications and protein turnover as chaperones and 12% are involved in carbohydrate transport and metabolism.

The spot quantity values determined by PDQuest are shown in the form of a histogram in Figure 4 together with representative gel images for each protein spot in each subject and condition. A statistically significant increased

Table 1 Differentially expressed proteins before and after antibiotic/probiotic administration

Spot ID	Swiss-Prot Acc. No.	Protein name	COG <sup>1</sup>	Subcellular location	Theoretical M <sub>r</sub> /pI	Experimental M <sub>r</sub> /pI	Method of identification <sup>2</sup>	Change in protein expression with AB/PB treatment <sup>3</sup>
Pouchitis/antibiotic-induced remission								
1	P27797	Calreticulin (CALR)	O	Endoplasmic reticulum	48.14/4.29	68.52/4.35	GM (Swiss-2D PAGE)	Up
2	P11021	78 kDa glucose-regulated protein (GRP78)	O	Endoplasmic reticulum	72.33/5.07	73.88/4.95	GM (Swiss-2D PAGE)	Down
3	P10809	60 kDa heat shock protein, mitochondrial precursor (HSP60)	O	Mitochondrial matrix	61.05/5.70	60.20/5.32	GM (Swiss-2D PAGE)	Up
4	P11142	Heat shock cognate 71 kDa protein (HSPA8)	O	Nucleolus	70.90/5.37	69.20/5.18	GM <sup>[20]</sup>	Up
5	P38646	Stress-70 protein, mitochondrial precursor (75 kDa glucose-regulated protein) (GRP75)	O	Mitochondrion	73.68/5.87	71.41/5.70	GM (Swiss-2D PAGE)	Down
6	Q9BU08	Putative uncharacterized protein, fragment (CCT5)	S	Undefined	59.47/5.45	60.46/5.58	GM <sup>[21]</sup>	Up
7	P02787	Serotransferrin precursor (TF)	P	Extracellular	77.05/6.81	79.49/7.09	GM (Swiss-2D PAGE)	Down
8	Q16822	Phosphoenolpyruvate carboxykinase (GTP), mitochondrial precursor (PCK2)	C	Mitochondrion	70.73/7.56	71.67/7.62	GM <sup>[19]</sup>	Up
9	P68371	Tubulin β-2C chain (TUBB)	Z	Cytoplasm	49.83/4.79	52.44/4.79	MALDI-TOF MS	Up
10	P06576	ATP synthase subunit β, mitochondrial precursor (ATP5B)	C	Mitochondrion	56.56/5.26	48.675.01	MALDI-TOF MS	Up
11	Q8NBS9	Thioredoxin domain-containing protein 5, precursor (TXNDC5)	R	Endoplasmic reticulum	47.63/5.63	49.43/5.09	GM <sup>[20]</sup>	Down
12	P35900	Keratin, type I cytoskeletal 20 (KRT20)	W	Cytoplasm	48.49/5.52	48.15/5.54	GM <sup>[19]</sup>	Down
13	P06733	α-enolase (ENO1)	G	Cytoplasm	47.17/7.01	46.80/7.57	MALDI-TOF MS	Down
14	P11177	Pyruvate dehydrogenase E1 component subunit β, mitochondrial precursor (PDHB)	C	Mitochondrion	39.25/6.20	32.96/5.64	MALDI-TOF MS	Down
15	P17707	S-adenosylmethionine decarboxylase proenzyme (AMD1)	T	Cytoplasm	38.34/5.71	31.91/5.74	MALDI-TOF MS	Up
16	P13804	Electron transfer flavoprotein subunit α, mitochondrial precursor (ETFA)	C	Mitochondrion	35.08/8.62	34.01/7.91	GM <sup>[20]</sup>	Up
17	P21796	Voltage-dependent anion-selective channel protein 1 (VDAC1)	P	Mitochondrion	30.77/8.62	30.60/9.20	GM <sup>[19]</sup>	Down
18	P07339	Cathepsin D, precursor (CTSD)	O	Lysosome	44.55/6.10	28.02/5.70	GM (Swiss-2D PAGE)	Down
19	Q99439	Calponin-2 (CNN2)	Z	Cytoplasm	33.70/6.94	29.64/7.55	MALDI-TOF MS	Up
20	P00915	Carbonic anhydrase I (CA1)	R	Cytoplasm	28.87/6.59	27.52/7.45	GM <sup>[19]</sup>	Down
21	P62937	Peptidyl-prolyl cis-trans isomerase A (PPIA)	O	Cytoplasm	18.01/7.68	16.42/8.09	GM <sup>[19]</sup>	Up
22	P12104	Fatty acid-binding protein, intestinal (FABP2)	I	Cytoplasm	15.21/6.62	14.07/6.99	MALDI-TOF MS	Up
23	P51161	Ileal lipid binding protein (FABP6)	I	Cytoplasm	14.37/6.29	13.58/7.22	MALDI-TOF MS	Up
24	P09382	Galectin-1 (LGALS1)	W	Extracellular	14.72/5.33	13.25/5.26	MALDI-TOF MS	Down
25	P07148	Fatty acid-binding protein, liver (FABP1)	I	Cytoplasm	14.21/6.60	12.13/6.80	MALDI-TOF MS	Up
26	Q5T1C5	Protein S100-A10 (S100A10)	R	Plasma membrane	11.20/6.82	10.52/7.25	MALDI-TOF MS	Up
Non-inflamed pouch/probiotic-treated pouch								
1	P18206	Vinculin (VCL)	Z	Cytoplasm	123.80/5.50	114.44/5.81	GM (Swiss-2D PAGE)	Up
2	P27797	CALR	O	Endoplasmic reticulum	48.14/4.29	68.52/4.35	GM (Swiss-2D PAGE)	Down
3	P28331	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial precursor (NDUFS1)	C	Mitochondrion	79.47/5.89	77.54/5.52	GM <sup>[21]</sup>	Down
4	P11142	HSPA8	O	Nucleolus	70.90/5.37	69.20/5.18	GM <sup>[20]</sup>	Up
5	P38646	GRP75	O	Mitochondrion	73.68/5.87	71.41/5.70	GM (Swiss-2D PAGE)	Down
6	P02787	TF	P	Extracellular	77.05/6.81	79.49/7.09	GM (Swiss-2D PAGE)	Down

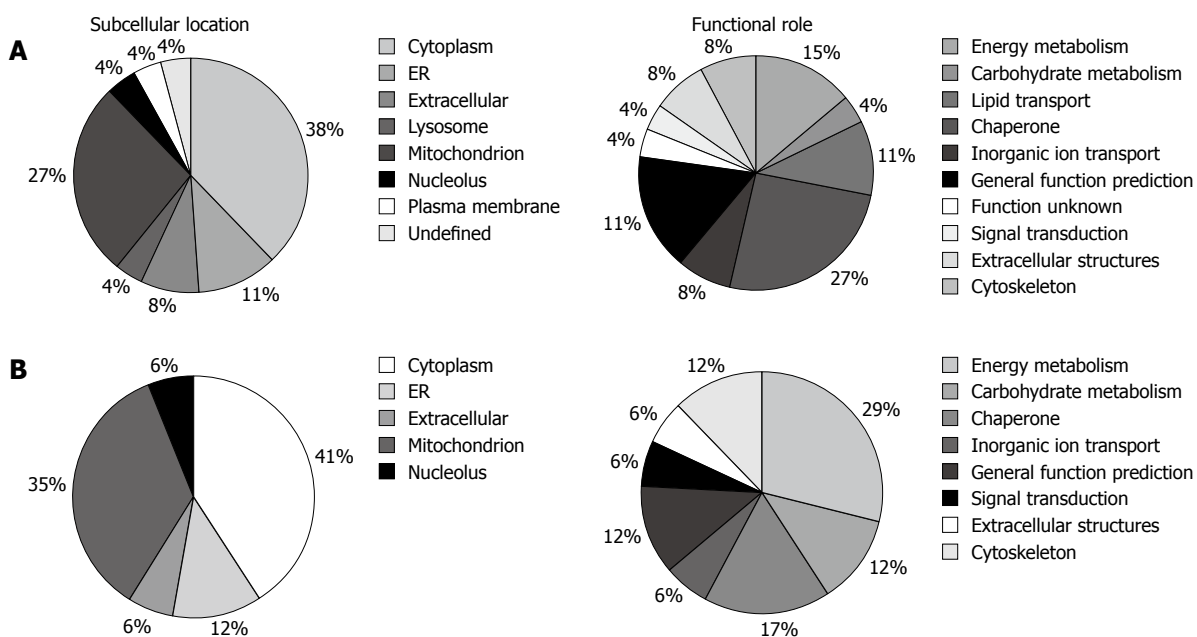
7	Q16822	PCK2	C	Mitochondrion	70.73/7.56	71.67/7.62	GM <sup>[19]</sup>	Up
8	Q71U36	Tubulin $\alpha$ -1A chain (TUBA1A)	Z	Cytoplasm	50.15/4.94	56.47/4.82	GM <sup>[17]</sup>	Down
9	Q8NBS9	TXNDC5	R	Endoplasmic reticulum	47.63/5.63	49.43/5.09	GM <sup>[20]</sup>	Down
10	P35900	KRT20	W	Cytoplasm	48.49/5.52	48.15/5.54	GM <sup>[19]</sup>	Down
11	P06733	ENO1	G	Cytoplasm	47.17/7.01	46.80/7.57	MALDI-TOF MS	Down
12	P12532	Creatine kinase, ubiquitous mitochondrial precursor (CKMT1B)	C	Mitochondrion	47.04/8.60	43.16/8.48	GM <sup>[20]</sup>	Down
13	P22695	Cytochrome b-c1 complex subunit 2, mitochondrial precursor (UQCRC2)	C	Mitochondrion	48.44/8.74	44.10/8.83	GM <sup>[21]</sup>	Up
14	P11177	PDHB	C	Mitochondrion	39.25/6.20	32.96/5.64	MALDI-TOF MS	Up
15	P17707	AMD1	T	Cytoplasm	38.34/5.71	31.91/5.74	MALDI-TOF MS	Up
16	P00918	Carbonic anhydrase II (CA2)	R	Cytoplasm	29.25/6.87	30.75/7.69	MALDI-TOF MS	Down
17	P60174	Triosephosphate isomerase (TPI1)	G	Cytoplasm	26.67/6.45	26.14/7.32	MALDI-TOF MS	Up

<sup>1</sup>Abbreviation of cellular role categories. Categories were taken from Cluster of Orthologous Groups (COG) (<http://www.ncbi.nlm.nih.gov/COG/>), and the abbreviation was used to mark the categories. C: Energy production and conversion; G: Carbohydrate transport and metabolism; I: Lipid transport and metabolism; O: Posttranslational modification, protein turnover, chaperones; P: Inorganic ion transport and metabolism; R: General function prediction only; S: Function unknown; T: Signal transduction mechanisms; W: Extracellular structures; Z: Cytoskeleton; <sup>2</sup>GM: Gel matching; <sup>3</sup>AB: Antibiotic; PB: Probiotic.

**Table 2** Summary of identification results of protein spots conserved in pouchitis/pouch remission and normal pouch/probiotic-treated pouch groups

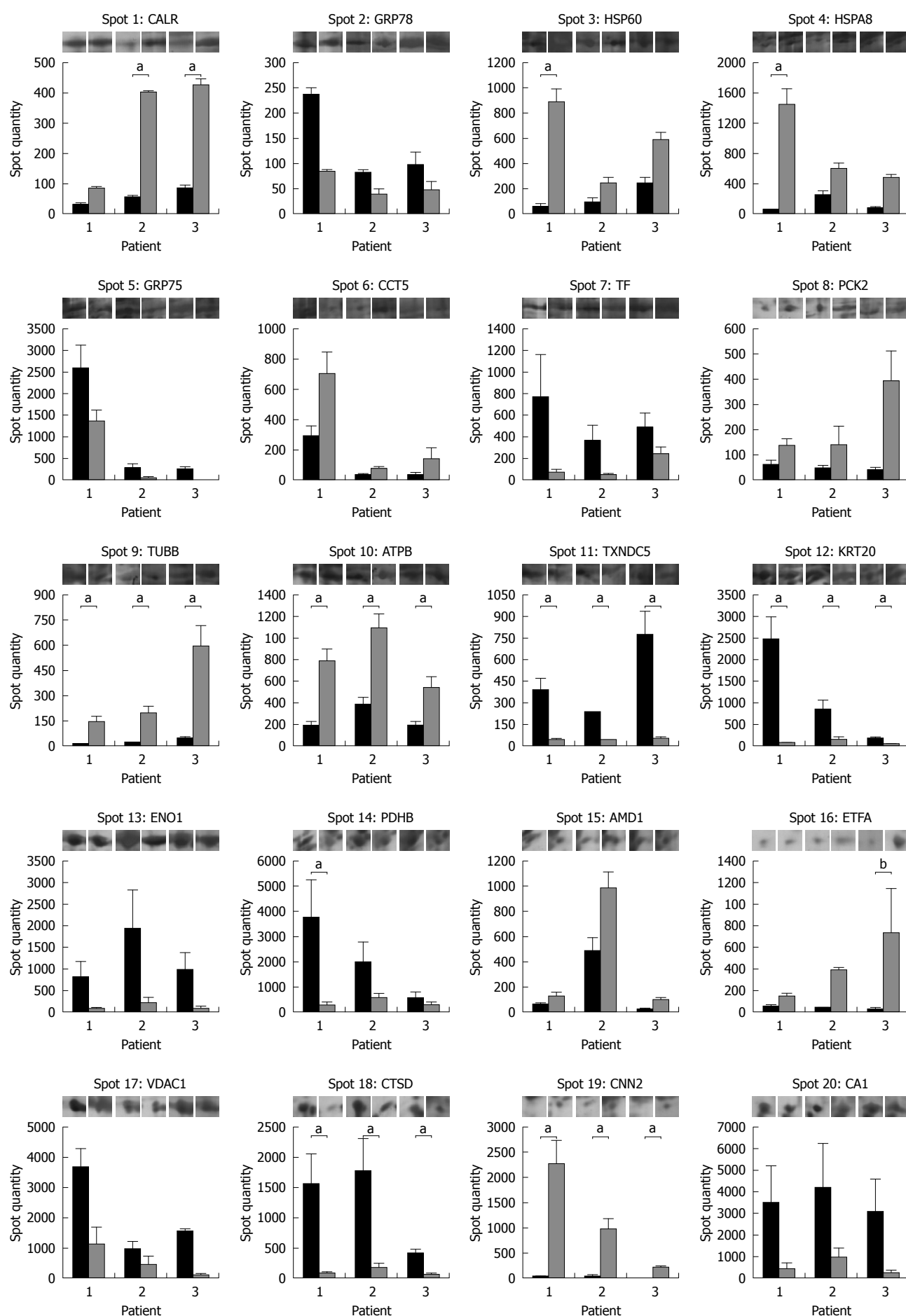
Spot ID	Swiss-Prot Acc. No.	Protein name	COG <sup>1</sup>	Subcellular location	Theoretical <i>M<sub>r</sub>/pI</i>	Experimental <i>M<sub>r</sub>/pI</i>	Method of identification <sup>2</sup>
A	Q15365	Poly(rC)-binding protein 1 (PCBP1)	A	Nucleus	37.53/6.66	35.99/7.17	MALDI-TOF MS
B	Q6IBM5	Rho GDP dissociation inhibitor (GDI) $\alpha$ , isoform CRA_a (ARHGDIa)	T	Cytoplasm	23.21/5.03	25.73/4.99	MALDI-TOF MS
C	Q5R8R5	Glutathione S-transferase P (GSTP1)	O	Cytoplasm	23.36/5.93	24.33/5.80	MALDI-TOF MS
D	P61088	Ubiquitin-conjugating enzyme E2 N (UBE2N)	O	Nucleus	17.14/6.13	19.00/6.10	MALDI-TOF MS
E	P68871	Hemoglobin subunit $\beta$ (HBB)	C	Extracellular	16.00/6.74	13.10/7.70	MALDI-TOF MS
F	Q1HDT5	Hemoglobin $\alpha$ 1-2 hybrid (HBA1)	C	Extracellular	15.27/9.04	12.50/9.55	MALDI-TOF MS

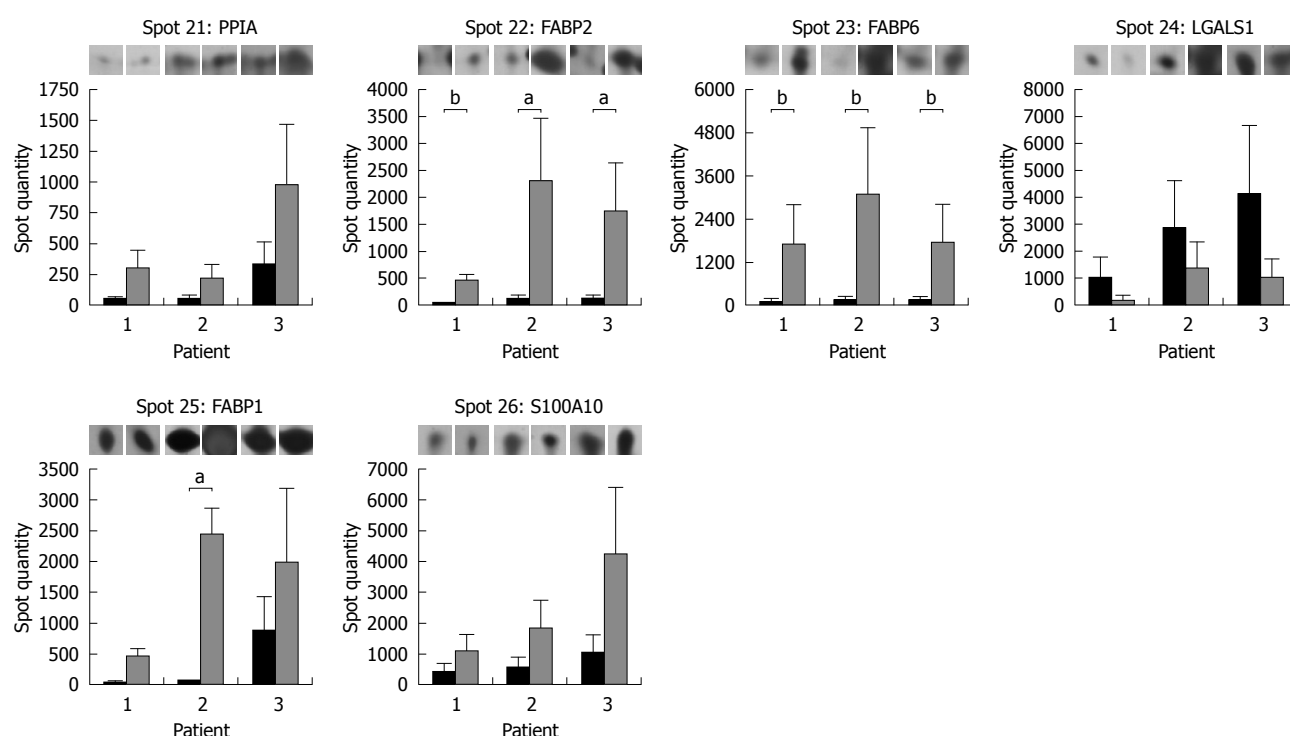
<sup>1</sup>A: RNA processing and modification. For other abbreviations see Table 1; <sup>2</sup>For each protein spot, the gel matching identification method was also employed. Spots A, B and D were identified by comparison with published 2D proteome patterns<sup>[17,18]</sup>; spots C, E and F by comparison with Swiss-2D PAGE.



**Figure 2** Pie charts representing the distribution of the differentially expressed proteins from pouchitis/pouch remission (A) and normal pouch/probiotic-treated pouch (B) group comparison, according to their subcellular location and biological function. ER: Endoplasmic reticulum.







**Figure 3** Protein expression histograms of the 26 differentially expressed protein spots between pouchitis (dark grey) and antibiotic-induced remission (light grey). Each bar represents the average spot quantity determined from 3 technical replicates for each patient condition by PDQuest. Representative gel images are displayed on top of each graph. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P = 0.06$  for ETFA and  $P = 0.07$  for FABP2 and FABP6.

expression after 3 mo of probiotic administration was detected for HSPA8 and PDHB in all the subjects enrolled.  $P$  values of 0.06, near the threshold of significance, were obtained for vinculin (VCL) and phosphoenolpyruvate carboxykinase (PCK2) in 3 and 2 patients, respectively. Among protein spots with downregulated expression levels after VSL#3 therapy, statistical significance was achieved in all patients for KRT20 and in only 1 for TXNDC5.

### Bibliometric analysis

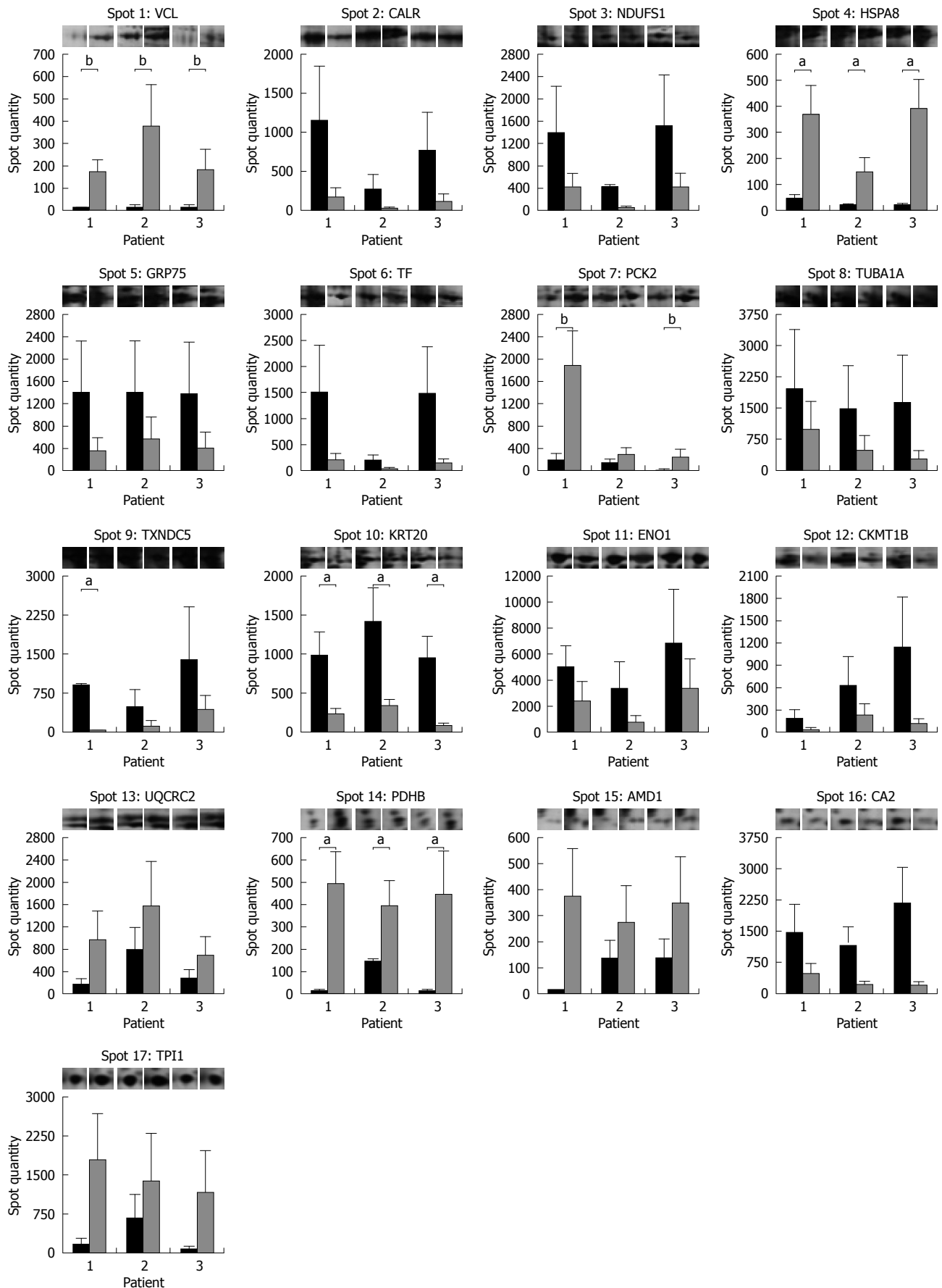
On the basis of literature co-citation from NCBI PubMed, a protein-protein network tree using the data-mining program Biblisphere software was generated. As shown in Figure 5, the network tree was compiled of 28 different proteins forming 2 network clusters. Group 1 consisted of 26 highly interrelated proteins including ATP5B, carbonic anhydrase I (CA1) and II (CA2), creatine kinase (CKMT1B),  $\alpha$ -enolase (ENO1), PCK2, PDHB and triosephosphate isomerase (TPI1), associated with energy, carbohydrate and amino acid metabolism, as well as glycolysis/gluconeogenesis, oxidative phosphorylation and electron transport chain. The second group was formed by 2 linear co-cited proteins, NADH-ubiquinone oxidoreductase 75 kDa subunit (NDUFS1) and cytochrome b-c1 complex subunit 2 (UQCRC2), related to energy production and conversion. The residual 5 detected proteins, S-adenosylmethionine decarboxylase (AMD1), CNN2, tubulin  $\alpha$ -1A chain (TUBA1A), TUBB and TXNDC5 were completely disconnected from the network tree.

### DISCUSSION

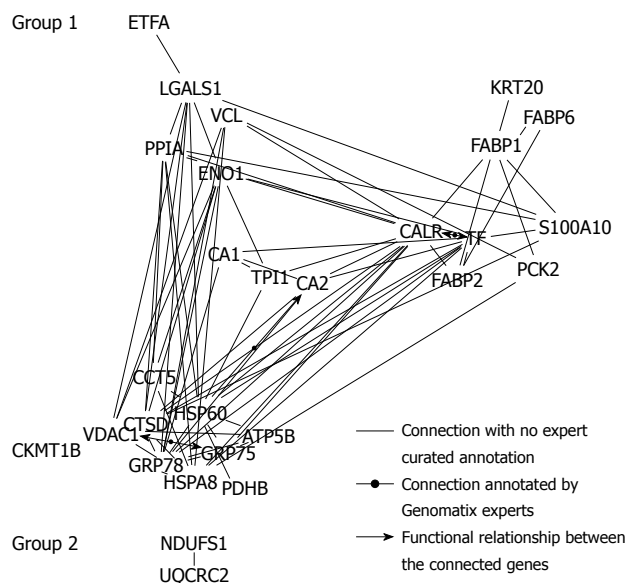
In this study, we provided for the first time 2D protein maps of mucosal biopsy samples collected during pouch endoscopy in patients who underwent IPAA.

The comparison between mucosal biopsy proteomes in pouchitis and in antibiotic-induced remission enabled the identification of 26 different proteins with at least 2-fold changes in their expression levels. Statistical significance was achieved for ATP5B, CNN2, CTSD, KRT20, TUBB and TXNDC5. In addition, a statistically significant altered expression pattern was obtained for CALR, HSP60, HSPA8, FABP1, FABP2 and PDHB in 1 or 2 of the 3 patients enrolled.

Among the identified mitochondrial proteins, ATP5B, ETFA and PCK2 directly participate in the process of energy production. The decrease of their expression levels in the inflamed pouch suggests the decline of mitochondrial function with pouchitis onset. This assumption is consistent with a previous hypothesis that chronic intestinal inflammation represents an energy-deficiency disease with alterations in the oxidative metabolism of the epithelial cells<sup>[23]</sup>. Moreover, the low expression of FABP1, FABP2 and FABP6, involved in enhancing the uptake of fatty acids into cells and facilitating their transport to intracellular organelles, could reinforce the speculation that pouchitis-diseased enterocytes do not perform  $\beta$ -oxidation/oxidative phosphorylation owing to a lack of normal supply of fatty acids<sup>[24]</sup>. Combined with these results, the overexpression of ENO1 found in the inflamed pouch may reflect a shift toward anaerobic



**Figure 4** Protein expression histograms of the 17 differentially expressed protein spots in non-inflamed pouch before (dark grey) and after (light grey) probiotic treatment. Each bar represents the average spot quantity determined from 3 technical replicates for each subject condition by PDQuest. Representative gel images are displayed on top of each graph. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P = 0.06$  for VCL and PCK2.



**Figure 5 Bibliometric data analysis.** Protein-protein network tree generation using the data-mining program Biblisphere software.

glycolysis to overcome the decreased ATP formation by a dysfunctional oxidative phosphorylation<sup>[21]</sup>.

The hypothesis of cellular stress and hypoxic conditions in chronically inflamed tissues is supported by the induction of several chaperone proteins, including 75 (GRP75) and 78 kDa glucose-related proteins (GRP78), TXNDC5, voltage-dependent anion-selective channel protein 1 (VDAC1), CTSD and peptidyl-prolyl cis-trans isomerase A (PPIA)<sup>[25-27]</sup>. In addition, we detected a statistically significant altered expression pattern for TUBB, KRT20 and CNN2, suggesting changes in cytoskeletal architecture with potential alterations in signal transduction and cellular transcription profiles<sup>[27,28]</sup>.

Next, we compared mucosal biopsy proteomes in the normal pouch before and after probiotic administration and we identified 17 different proteins with significant changes in their expression levels. Interestingly, 8 of the differentially expressed proteins exhibited the same pattern of deregulation as in the pouchitis/pouch remission group. Indeed, both antibiotic and probiotic therapy resulted in downregulation of GRP75, serotransferrin (TF), TXNDC5, KRT20, ENO1 and in upregulation of HSPA8, PCK2 and AMD1, suggesting profound structural and metabolic alterations in enterocytes. In particular, TXNDC5 is a newly identified member of the thio-redoxin family of endoplasmic reticulum proteins<sup>[29]</sup>, and it has been proposed as a promising biomarker for cancer diagnosis<sup>[30]</sup>. Because of its important role in redox regulation<sup>[31]</sup>, the altered expression profile of TXNDC5 in IPAA may be related to the increased oxidative stress with significantly lower plasma concentrations of lipophilic antioxidants and higher free radical activity measured in patients with restorative proctocolectomy compared to normal subjects<sup>[32]</sup>. Furthermore, for KRT20 and ENO1, widely applied as diagnostic markers for colon adenocarcinomas and many other tumors<sup>[33,34]</sup>,

as well as for PCK2 and AMD1 a differential protein profile in inflammatory bowel disease has been already reported<sup>[21,35,36]</sup>.

In addition to these results, in the VSL#3-treated pouch we found a statistically significant upregulation of VCL and an altered expression pattern for TUBA1A, supporting the assumption of a positive modulation exerted by probiotics at cytoskeleton level for cell morphology and integrity<sup>[37,38]</sup>. In addition, the dysregulated expression levels of NDUFS1, CKMT1B, UQCRC2, PDHB, CA2 and TPI1, directly involved in energy metabolism, strengthen the hypothesis of significant changes in the metabolic profiles of the host associated with probiotic administration<sup>[39,40]</sup>. Nonetheless, although the manipulation of the ubiquitin/proteasome pathway and the ability to intervene with the complex host system of detoxification of potentially harmful xenobiotics and endobiotic compounds may account for some of the cytoprotective effects of probiotics<sup>[37,41,42]</sup>, we did not find any significant change in glutathione S-transferase P (GSTP1) and ubiquitin-conjugating enzyme E2 N (UBE2N) protein expression levels.

The bibliometric data analysis including all the 33 differentially regulated proteins from the pouchitis/pouch remission and non-inflamed/probiotic-treated pouch group comparison generated a complex network with 26 highly interrelated proteins. As expected, the majority of clustered proteins were associated with glycolysis/gluconeogenesis, oxidative phosphorylation and electron transfer chain pathways.

In conclusion, the identified proteins, both upregulated and downregulated, may be involved in pouchitis pathophysiology and participate in disease onset or in maintenance of the non-inflamed pouch.

## COMMENTS

### Background

Restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA) is the procedure of choice for complicated ulcerative colitis. In the long-term, up to 50% of patients develop pouchitis, an idiopathic inflammatory disease of the ileal reservoir. The management of pouchitis is largely empirical and only few small placebo-controlled clinical trials have been conducted. Although antibiotics represent the mainstay of treatment, probiotics have recently gained more attention as an effective therapeutic option for pouchitis management.

### Research frontiers

The etiology and pathophysiology of pouchitis are still not entirely clear but the bulk of the evidence points towards an abnormal mucosal immune response to altered microbiota patterns. By investigating the dynamic nature of protein expression, cellular and subcellular distribution, posttranslational modifications and protein-protein interaction networks, proteomic technologies could play a major role in unraveling the mystery of immunopathogenic mechanisms of pouchitis and in discovering novel biomarkers for disease activity, diagnosis and prognosis.

### Innovations and breakthroughs

The current study is the first proteomic study to be reported in IPAA research. The authors provided the 2D protein maps of mucosal biopsy samples collected during pouch endoscopy in patients with chronic refractory pouchitis. The changes in the protein expression profiles following antibiotic or probiotic treatment were characterized.

### Applications

The identified proteins, upregulated or downregulated following antibiotic/



probiotic treatment, may be involved in pouchitis pathophysiology and participate in disease onset or in maintenance of the non-inflamed pouch. Future work will be focused in validating the list of proteins identified in larger patient cohorts.

# Peer review

The results are well described and interesting, although the number of patients is a bit on the small side. Even though this manuscript does not give a clear understanding to the mechanistic differences, the results may aid other scientists in making a follow-up study.

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## Is there an association between *Helicobacter pylori* in the inlet patch and globus sensation?

Hakan Alagozlu, Zahide Simsek, Selahattin Unal, Mehmet Cindoruk, Sukru Dumlu, Ayse Dursun

Hakan Alagozlu, Zahide Simsek, Selahattin Unal, Mehmet Cindoruk, Sukru Dumlu, Division of Gastroenterology, Department of Internal Medicine, Faculty of Medicine, Gazi University Hospital, Ankara 06500, Turkey

Ayşe Dursun, Department of Pathology, Faculty of Medicine, Gazi University Hospital, Ankara 06500, Turkey

Author contributions: Alagozlu H, Simsek Z designed and performed the research; Unal S, Cindoruk M, Dumlu S contributed research; Dursun A performed the pathology.

Correspondence to: Dr. Hakan Alagozlu, Division of Gastroenterology, Department of Internal Medicine, Faculty of Medicine, Gazi University Hospital, Ankara 06500, Turkey. [hakanalagozlu@gmail.com](mailto:hakanalagozlu@gmail.com)

Telephone: +90-346-2580938 Fax: +90-312-3186690

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**CONCLUSION:** Often patients with CHGM have a long history of troublesome throat symptoms. We speculate that disturbances in globus sensation are like non-ulcer dyspepsia.

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**Key words:** Barrett's esophagus; Cervical heterotopic gastric mucosa; Globus sensation; *Helicobacter pylori*; Inlet patch

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### Abstract

**AIM:** To determine the association between *Helicobacter pylori* (*H. pylori*) and globus sensation (GS) in the patients with cervical inlet patch.

**METHODS:** Sixty-eight patients with esophageal inlet patches were identified from 6760 consecutive patients undergoing upper gastrointestinal endoscopy prospectively. In these 68 patients with cervical inlet patches, symptoms of globus sensation (lump in the throat), hoarseness, sore throat, frequent clearing of the throat, cough, dysphagia, odynophagia of at least 3 mo duration was questioned prior to endoscopy.

**RESULTS:** Cervical heterotopic gastric mucosa (CHGM) was found in 68 of 6760 patients. The endoscopic prevalence of CHGM was determined to be 1%. *H. pylori* was identified in 16 (23.5%) of 68 patients with inlet patch. 53 patients were classified as CHGM II. This group included 48 patients with globus sensation, 4 patients with chronic cough and 1 patient with hoarseness. All the patients who were *H. pylori* (+) in cervical inlet patches had globus sensation.

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### INTRODUCTION

Islands of heterotopic gastric mucosa are found throughout the gastrointestinal tract, the most common site being the cervical esophagus. Cervical heterotopic gastric mucosa (CHGM) or cervical inlet patches are commonly seen during withdrawal of the gastroscope. Patients with CHGM have various laryngeal and oropharyngeal symptoms, ranging from asymptomatic to protracted symptoms such as globus sensation and chronic cough due to acid secretion from the inlet patch<sup>[1-4]</sup>.

Globus sensation (GS) is caused mainly by cervical disturbances. The usual complaint in patients with globus sensation or globus pharyngeus is the sensation of a ball or lump in the throat, generally not accompanied

by dysphagia. Globus sensation is felt medially deep in the throat during dry swallowing (empty swallow), and almost never while drinking or eating. It is not painful, and there is no obstruction of food<sup>[2,3]</sup>.

*Helicobacter pylori* (*H. pylori*) produces chronic inflammation in the CHGM (as in non-ulcer dyspepsia). *H. pylori* infection plays a role in altered gastric perception in non-ulcer dyspepsia. *H. pylori* in CHGM may cause altered cervical perception such as globus sensation. In this prospective study, we aimed to determine the association between *H. pylori* and globus sensation in patients with CHGM.

## MATERIALS AND METHODS

### Subjects

Over a one-year period, between 2005 and 2006, the number of patients with cervical inlet patches from a total of 6760 consecutive patients undergoing upper gastrointestinal endoscopy at the Hospital of the Gazi University in Ankara, Turkey were identified. Patients were referred for endoscopy for a variety of reasons, primarily for evaluation of dyspepsia. In these patients, symptoms of globus sensation (lump in the throat), hoarseness, sore throat, frequent clearing of the throat, cough, dysphagia and odynophagia of at least 3 mo duration were questioned prior to endoscopy.

The investigation conformed to the principles outlined in the Declaration of Helsinki. Informed consent was obtained from all subjects and the study was approved by the ethical review committee of Gazi University in Ankara, Turkey.

### Esophagogastroduodenoscopy

After an overnight fast, a routine esophagogastroduodenoscopy was performed with a standard endoscope using topical anesthesia with or without conscious sedation, depending on patient preference. Conscious sedation was performed with midazolam (2-5 mg). During all procedures the esophagus was carefully surveyed and special attention was paid to the area of the upper esophageal sphincter. This region was best examined when slowly withdrawing the endoscope, with repeated short inflations while rotating the instrument.

Heterotopic gastric mucosa was defined as patches covered with salmon-red mucosa distinguishable from surrounding greyish-pearly colored esophageal mucosa by well-defined margins (Figure 1). The size of the patches was determined by the top span of the fully open biopsy forceps. In all subjects, the distance between the patch and the frontal incisor was recorded and the patch size measured under the guidance of the open biopsy forceps.

### pH monitoring

pH monitoring was performed in our laboratory in the patients with inlet patch. A 2.1-mm diameter dual-electrode antimony pH catheter (pHersaflex ambulatory

catheter, MMS) was placed transnasally after an overnight fast. Recording sites were fixed on the catheter, with a distance of 15 cm to measure proximal and distal pH. The distal electrode was placed 5 cm above the manometrically defined lower esophageal sphincter. The proximal electrode was placed at 16-21 cm, which corresponded approximately with the endoscopic finding of inlet patches. The pH values from both intraesophageal electrodes were recorded continuously on an ambulatory Mark III Digitrapper (Synectics Medical Inc.). Abnormal distal esophageal reflux and proximal reflux were defined as the percentage of esophageal total acid exposure (pH < 4) of  $\geq 4.2\%$  and  $\geq 1\%$ , respectively<sup>[5]</sup>.

### Biopsies

A minimum of two biopsies were obtained from the CHGM (Figure 2) and antral gastric mucosa. The samples were taken using large cup and side-opening forceps. Pathology and/or the rapid urease test were performed to determine the presence of *H. pylori* in all patients (Figure 3). The presence of *H. pylori* was identified using hematoxylin-eosin, cresyl violet, giemsa and silver stain.

### Statistical analysis

All statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS) 13.0 software for Windows XP. Categorical variables were compared with the chi-squared test or Fisher's exact test, and continuous variables were compared using Student's *t*-test and univariate analysis. A *P* value of less than 0.05 was considered to be statistically significant.

## RESULTS

### Prevalence, demographic characteristics

CHGM patches were found in 68 of 6760 patients (3173 female, 3587 male). The endoscopic prevalence of CHGM was determined to be 1%. Demographic characteristics of the patients with and without patches are shown in Table 1. The female/male ratio was 1.12 in the 68 patients with patches and 0.88 in those without. There was no significant difference between the mean age of the patients with CHGM with and without *H. pylori* (*P* > 0.05). Female patients with inlet patches had higher colonization of *H. pylori* than male patients (*P* < 0.05).

### Size, number, symptoms, pH monitoring

The size of the inlet patches varied between 5 and 32 mm and occupied between 10% and 30% of the circumference. Five patients had "double" patches. Symptoms are shown in Tables 1 and 2. Five patients had distal reflux and 1 patient had both proximal and distal reflux. There were no patients with only proximal reflux. The other patients were normal.

### Histological characteristics

***H. pylori:*** *H. pylori* was identified in the CHGM in



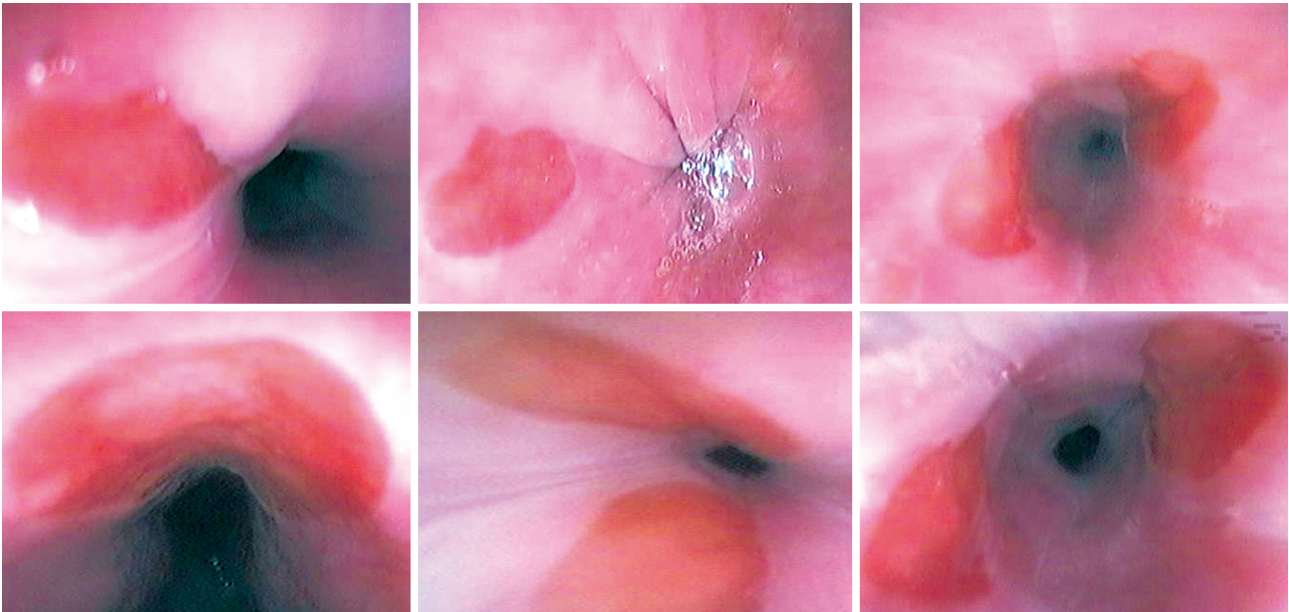


Figure 1 Various endoscopic images of inlet patches (single or double).

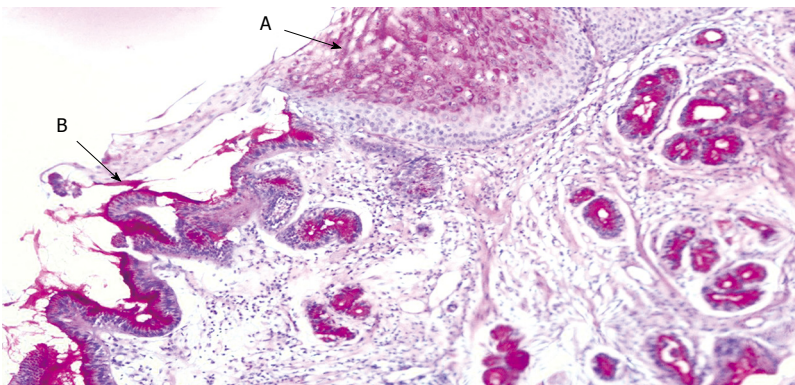


Figure 2 Biopsies. A shows heterotopic gastric mucosa, B shows squamous epithelium.

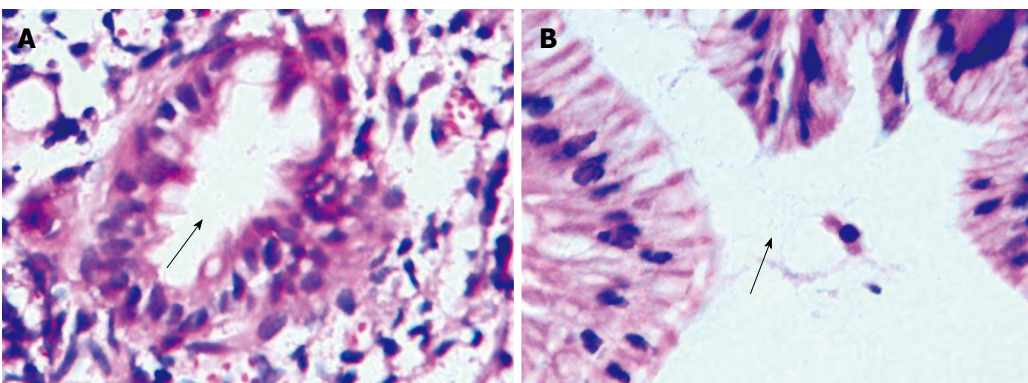


Figure 3 The presence of Hp bacilli in heterotopic gastric mucosa with HE stain ( $\times 1000$ ). A: Few Hp bacilli (arrow); B: Hp colonization (arrow).

23.5% of patients (16/68) (Table 3) and gastric *H. pylori* infection was positive in all (16/16) of these patients (Table 4, Figure 3). Colonization of *H. pylori* was most common in fundic-type mucosa (81.2%). All the patients who were *H. pylori* (+) in the cervical inlet patches had globus sensation ( $P < 0.05$ ).

**Mucosal type:** In the inlet patch, fundic-type mucosa was the most common histologic type (44/68), followed

by antral-type mucosa (15/68) (Table 3). Eight specimens of the inlet patch contained only foveolar epithelium and were therefore considered too superficial to be classified. In one patient, mucosal type was unremarkable because one specimen had complete replacement of the underlying mucosa with intestinal metaplasia.

**Intestinal metaplasia:** Intestinal metaplasia was identified in the inlet patch in seven patients (10.3%). One pa-

Table 1 Characteristics of the subjects

	HGM (+)	HGM (-)
<i>n</i>	68	6692
Sex		
Female (%)	36 (53.1)	3173 (47.4)
Male (%)	32 (46.9)	3519 (52.6)
Female/male	1.12	0.88
Age range (yr)	(17-56)	(16-90)
Mean age ( $\pm$ SE)	37.29 $\pm$ 1.85 <sup>a</sup>	47.5 $\pm$ 0.3
Endoscopic Barrett's esophagus	9 (13.2) <sup>a</sup>	166 (2.4)
Erosive esophagitis	7 (10.3)	636 (9.5)
Globus (%)	48 (70.6) <sup>a</sup>	0
Dyspepsia (%)	60 (88.2)	6550 (97.8)

HGM: Heterotopic gastric mucosa. <sup>a</sup>*P* < 0.05.

Table 2 Clinicopathologic classification and characteristics of CHGM patches

Clinicopathologic	Number (%)	Symptoms/findings
HGM I	12 (17.6)	Asymptomatic
HGM II	53 (77.9)	48 GS, 4 cough, 1 hoarseness
HGM III	2 (2.9)	1 ulcer, 1 polyp
HGM IV	0	
HGM V	1 (1.4)	Esophageal adenocarcinoma

CHGM: Cervical heterotopic gastric mucosa; GS: Globus sensation; Cough: Together with clearing of the throat.

tient had complete replacement of the underlying mucosa with intestinal metaplasia. Both *H. pylori* and intestinal metaplasia were observed in the inlet patch mucosa in one patient. In the remaining five patients, intestinal metaplasia was present, but no *H. pylori*. The type of mucosa was evaluated in these patients; three had antral-type and three fundic-type.

### Associated lesions

Endoscopic esophagitis (7 patients), duodenal ulcer (3 patients), hiatal hernia (9 patients) and endoscopic Barrett's esophagus (9 patients) accompanied CHGM.

### Clinicopathologic classification

Clinicopathologic classification was performed according to the classification reported by von Rahden *et al*<sup>[1]</sup> in patients with CHGM. (1) Asymptomatic carriers of esophageal CHGM were classified as CHGM I. Twelve patients were classified with CHGM I in our study. (2) Symptomatic individuals with esophageal CHGM complaining of globus sensation, cough, hoarseness or "extraesophageal manifestations" were classified as CHGM II without morphologic changes. Fifty-three patients were classified with CHGM II. This group included 48 patients with globus sensation, 4 patients with chronic cough and 1 patient with hoarseness. There were 48 (70.5%) patients with globus sensation out of the 68 patients with CHGM II. Symptoms of globus sensation were obvious attractive in these patients. (3) A smaller group of patients with additional morphologic changes (inlet patch complicati-

Table 3 Histological characteristics of heterotopic gastric mucosal patches

Histological characteristics	<i>n</i>
Type of patch	
Fundic	44
Antral	15
Foveolar epithelium	8
Complete IM	1
Chronic inflammation	68
IM (+), Hp (-)	6 (3 antral-type, 2 fundic-type, 1 complete IM)
IM (-), Hp (+)	15 (3 antral-type, 12 fundic-type)
IM (+), Hp (+)	1 (1 fundic-type)
IM (-), Hp (-)	46 (9 antral-type, 29 fundic-type, 8 foveolar epithelium)

IM: Intestinal metaplasia; Hp: *Helicobacter pylori*.

Table 4 Comparison of the HP (+) and Hp (-) subjects with CHGM

	Hp (+) CHGM ( <i>n</i> = 16)	Hp (-) CHGM ( <i>n</i> = 52)
Globus, <i>n</i> (%)	16 (100)	32 (61.5) <sup>a</sup>
Age (yr)	35.6 $\pm$ 2.3	38.6 $\pm$ 2.5
Female <i>n</i> (%)	11 (68.8) <sup>a</sup>	25 (48.1)
Male <i>n</i> (%)	5 (31.2)	27 (51.9) <sup>a</sup>
Fundic-type	13 (81.2)	31 (59.6) <sup>a</sup>
Antral-type	3 (18.8)	12 (23.1)
Intestinal metaplasia	-	1 (1.9)
Foveolar epithelium	-	8 (15.4)

<sup>a</sup>*P* < 0.05.

ons) were classified as CHGM III. This group included one patient with ulcer and polyp in the inlet patch. (4) If dysplasia was present, this was classified as CHGM IV. None of the patients belonged to this category. (5) If the diagnosis was invasive cancer and originated within the inlet patch, this was classified as CHGM V. One patient who had adenocarcinoma in the CHGM was classified as CHGM V.

### DISCUSSION

The usual endoscopic appearance of CHGM is a salmon-rose-colored mucosal patch with a sharp border or edge in the upper esophagus. The patches vary in diameter from 1 to 20 mm or more. Inlet patches are recognized endoscopically as 1 or 2 patches mostly in the lateral walls between the level of the cartilage and the fifth tracheal ring, and are seen as sharply demarcated, salmon-rose-colored oval or round patches.

The prevalence of CHGM varied between 0.29% and 2.27% in one prospective study<sup>[6]</sup>. Akbayir *et al*<sup>[7]</sup> reported a prevalence of 1.67% and Tang *et al*<sup>[8]</sup> reported a prevalence of 1.1%. In our prospective study, over a period of 1 year, 68 cases (1%) of CHGM were documented and confirmed by histology.

Microscopically, gastric mucosa containing either



cardiac, antral and potentially acid-secreting fundic mucosa can be found. In general, CHGM is uniformly of the fundic-type, containing both parietal and chief cells. Less frequently, histopathologic examination of CHGM shows an “antral pattern”, defined by the absence of chief cells and only a few parietal cells<sup>[1,8,9]</sup>. In our series, fundic-type mucosa was found in 44 of 68 patients examined histologically.

*H. pylori* is a well known pathogenic micro-organism responsible for chronic inflammation. Ectopic gastric mucosa of the inlet patch is an ideal location for *H. pylori* colonization<sup>[10]</sup>. Borhan-Manesh *et al*<sup>[11]</sup> found *H. pylori* in the inlet patch in 35% of patients in a subset with gastric *H. pylori*. Among our 68 patients with inflamed inlet patches, 16 were positive for *H. pylori* (23.5%), and all of these 16 patients also had *H. pylori* in the antrum. Coinfection of *H. pylori* in the inlet patch and gastric antrum has also been reported by others<sup>[10]</sup>. Since the infection by *H. pylori* is through the oral route, inlet patches may be important sites of *H. pylori* infection in the upper gastrointestinal tract because of its more proximal location. The inlet patches may function as reservoirs for *H. pylori*. Inlet patch colonization by *H. pylori* can occur during ingestion of food, and the presence of gastric *H. pylori* may play a role in the development of inlet patches. We searched but did not find any follow-up studies on antibiotic therapy for *H. pylori* and its impact on infection of inlet patches in the literature. We believe that the elimination of *H. pylori* in both the inlet patch and antrum is very important in the treatment of patients with coinfection.

In this study, the female/male ratio in the *H. pylori* (+) CHGM group was higher than that in the *H. pylori* (-) CHGM group. Females had higher inlet patch colonization with *H. pylori* than males ( $P < 0.05$ ). The mechanism of *H. pylori* colonization in the inlet patch is unclear.

A clinicopathological classification of CHGM as proposed by von Rahden *et al*<sup>[1]</sup> was carried out on all 68 patients, 53 of whom were classified as CHGM II; 48 had globus sensation, 4 had cough, one had hoarseness. Theoretically, laryngeal and oropharyngeal symptoms should be common due to acid secretion from the inlet patch. Several studies have reported cases of esophageal inlet patch presenting with various laryngeal and oropharyngeal symptoms, ranging from asymptomatic to protracted symptoms such as chronic cough and globus sensation<sup>[4,12-14]</sup>. In addition, CHGM can cause stricture, ulcer, perforation, web or polyp in the esophagus because of its capability to secrete acid<sup>[15,16]</sup>. In our two patients with CHGM III, one had a hyperplastic polyp and one had an ulcer.

There are also reports that CHGM is associated with an increased risk for Barrett's esophagus, suggesting a possible link. Traditionally, Barrett's esophagus is considered a distinct entity from esophageal inlet patch. Barrett's esophagus is an acquired precancerous lesion and the cell origin probably involves multipotential undifferentiated cells. Inlet patch is considered to be congenital. Up to half of all patients with cervical inlet patch have concurrent Bar-

rett's esophagus in some reports<sup>[17]</sup>. They have the same mucin core protein expression and cytokeratin pattern, suggesting a pathogenetic link between these two diseases<sup>[18]</sup>. Similarly, in the current study, the presence of CHGM in the upper esophagus was common and closely related to Barrett's esophagus (13.2%) compared with those without inlet patches ( $P < 0.05$ ). Gastro-esophageal diseases in patients with cervical inlet patch was not statistically significant when compared with those without patches ( $P > 0.05$ ). We speculated that the acid secretion from the inlet patches did not contribute to the pathogenesis of Barrett's esophagus. However, the patients with inlet patches were inherently predisposed to developing columnar metaplasia in the distal esophagus.

The usual complaint in GS is that of a ball or lump in the throat generally not accompanied by dysphagia. This sensation is often more pronounced when taking an “empty swallow”. In our study, all the patients with *H. pylori* in cervical inlet patches had globus sensation.

CHGM should be looked for, particularly in patients with GS. *H. pylori* positivity in the CHGM correlated with GS symptoms in our study. There were no reports on a link between *H. pylori* positivity in patients with CHGM and “globus sensation” or “globus pharyngeus” in our review of the English literature. Therefore, we were not able to compare our data.

*H. pylori* infection plays a role in causing symptoms in patients fulfilling the criteria for non-ulcer dyspepsia. There is agreement that *H. pylori* infection causes changes in gastric physiology. In addition, *H. pylori* infection plays a role in altered gastric perception in non-ulcer dyspepsia. We speculate that the disturbances in globus sensation are like non-ulcer dyspepsia. *H. pylori* produces chronic inflammation in the CHGM (as in non-ulcer dyspepsia). It could be speculated that globus sensation is a non-ulcer dyspepsia of CHGM. *H. pylori* is a potential cause of GS in patients with CHGM.

There are no follow-up studies on antibiotic therapy for *H. pylori* and its impact on infection of inlet patches in the literature. Additional studies are needed to understand the fundamental mechanisms leading to globus sensation in CHGM. These patients might benefit from *H. pylori* eradication therapy to alleviate this potentially aggravating factor. Based on these important findings, we expect to see more studies on inlet patches in the near future.

## COMMENTS

### Background

Cervical heterotopic gastric mucosa (CHGM) or cervical inlet patches are commonly seen on the cervical esophagus during withdrawal of the gastro-scope. Patients with inlet patches have various laryngeal and oropharyngeal symptoms, ranging from asymptomatic to protracted symptoms such as globus sensation and chronic cough due to acid secretion from the inlet patch. Globus sensation has a largely unknown etiology.

### Research frontiers

All the patients who were *Helicobacter pylori* (*H. pylori*) (+) in the cervical inlet patches had globus sensation. *H. pylori* produces chronic inflammation in the CHGM (as in non-ulcer dyspepsia). It could be speculated that globus sensa-

tion is a non-ulcer dyspepsia of CHGM. *H. pylori* is a potential cause of GS in patients with CHGM.

### Innovations and breakthroughs

The authors showed a causal association between *H. pylori* infection and the symptoms of globus sensation in these patients. Inlet patches may function as reservoirs for *H. pylori*. Inlet patch colonization by *H. pylori* can occur during ingestion of food and the presence of gastric *H. pylori* might play a role in the development of inlet patches.

### Applications

Patients might benefit from *H. pylori* eradication therapy to alleviate this potentially aggravating factor. Based on these important findings, we expect to see more studies on inlet patches in the near future.

### Terminology

CHGM in the cervical esophagus appears to result from incomplete replacement of the original columnar epithelium by stratified squamous epithelium in the embryonic period. Islands of heterotopic gastric mucosa are found throughout the gastrointestinal tract, the most common site being the cervical esophagus. Macroscopically, visible islands of CHGM, referred as "inlet patches" are often detected during endoscopic examination.

### Peer review

The manuscript presented interesting data on heterotopic gastric mucosa on the cervical esophagus. They proved the link between presence of inlet patch and globus sensation in these patients. Interestingly, they showed a causal association between *H. pylori* infection and the symptom of globus sensation in these patients.

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## Atrial natriuretic peptide signal pathway upregulated in stomach of streptozotocin-induced diabetic mice

Zhang-Xun Qiu, Bing Mei, Yi-Song Wu, Xu Huang, Zuo-Yu Wang, Yan-Fei Han, Hong-Li Lu, Young-Chul Kim, Wen-Xie Xu

Zhang-Xun Qiu, Yi-Song Wu, Xu Huang, Zuo-Yu Wang, Yan-Fei Han, Hong-Li Lu, Wen-Xie Xu, Department of Physiology, Shanghai Jiao Tong University, School of Medicine, Shanghai 200240, China

Bing Mei, Shanghai Institute of Brain Functional Genomics; Department of Environmental Science and Technology, East China Normal University, Shanghai 200240, China

Young-Chul Kim, Department of Physiology, Chungbuk National University College of Medicine, 12 Gaeshin-dong, Hungduk-gu, Cheongju, Chungbuk 361-763, South Korea

Author contributions: Qiu ZX, Mei B, Wu YS, Huang X, Wang ZY, Han YF, Lu HL, Kim YC performed the majority of experiments and data analysis; Xu WX designed the study and wrote the manuscript.

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Correspondence to: Wen-Xie Xu, MD, PhD, Department of Physiology, Shanghai Jiaotong University School of Medicine, 800 Dongchuan Road, 328 Wenxuan Medical Building, Shanghai 200240, China. [wexiexu@sjtu.edu.cn](mailto:wexiexu@sjtu.edu.cn)

Telephone: +86-21-34205639 Fax: +86-21-34201118

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### Abstract

**AIM:** To investigate atrial natriuretic peptide (ANP) secretion from gastric mucosa and the relationship between the ANP/natriuretic peptide receptor type A (NPR-A) pathway and diabetic gastroparesis.

**METHODS:** Male imprinting control region (ICR) mice (4 wk old) were divided into two groups: control mice, and streptozotocin-induced diabetic mice. Eight weeks after injection, spontaneous gastric contraction was recorded by using physiography in control and streptozotocin-induced diabetic mice. The ANP-positive cells in gastric mucosa and among dispersed gastric epithelial cells were detected by using immunohistochemistry and flow cytometry, respectively. ANP and natriuretic

peptide receptor type A (NPR-A) gene expression in gastric tissue was observed by using the reverse transcriptase polymerase chain reaction.

**RESULTS:** The frequency of spontaneous gastric contraction was reduced from  $12.9 \pm 0.8$  cycles/min in the control group to  $8.4 \pm 0.6$  cycles/min in the diabetic mice ( $n = 8$ ,  $P < 0.05$ ). However, the amplitude of contraction was not significantly affected in the diabetic group. The depletion of interstitial cells of Cajal in the gastric muscle layer was observed in the diabetic mice. ANP-positive cells were distributed in the gastric mucosal layer and the density index of ANP-positive cells was increased from  $20.9 \pm 2.2$  cells/field in control mice to  $51.8 \pm 2.9$  cells/field in diabetic mice ( $n = 8$ ,  $P < 0.05$ ). The percentage of ANP-positive cells among the dispersed gastric epithelial cells was increased from  $10.0\% \pm 0.9\%$  in the control mice to  $41.2\% \pm 1.0\%$  in the diabetic mice ( $n = 3$ ,  $P < 0.05$ ). ANP and NPR-A genes were both expressed in mouse stomach, and the expression was significantly increased in the diabetic mice.

**CONCLUSION:** These results suggest that the ANP/NPR-A signaling pathway is upregulated in streptozotocin-induced diabetic mice, and contributes to the development of diabetic gastroparesis.

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**Key words:** Diabetes mellitus; Atrial natriuretic peptide; Gastric mucosa; Gastroparesis

**Peer reviewer:** Giovanni Tarantino, MD, Professor, Department of Clinical and Experimental Medicine, Federico II University Medical School, VIA S. PANSINI, 5, Naples 80131, Italy

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## INTRODUCTION

Gastroparesis is a chronic complication of diabetes, also called delayed gastric emptying, occurs in > 50% of patients with long-standing diabetes<sup>[1]</sup>. Symptoms of gastroparesis include heartburn, pain in the upper abdomen, vomiting, nausea, and early feeling of fullness, but the worst effect of gastroparesis is that it can make diabetes worse by making blood glucose control more difficult<sup>[2]</sup>. Besides, there are deterioration in glycemic control and incapacitating symptoms such as malnutrition, water and electrolyte imbalance, and aspiration. However, the pathophysiology of diabetic gastropathy and gastroparesis, such as the mechanism of impaired fundic, pyloric relaxation and impaired electrical pacemaking, are still not established<sup>[3,4]</sup>. It is generally believed that diabetic gastropathy and gastroparesis may be caused by visceral autonomic neuropathy, hyperglycemia, and degeneration of smooth muscle. Hyperglycemia itself can cause antral hypomotility, gastric dysrhythmia, and delayed gastric emptying in some patients<sup>[5]</sup>. Several physiological studies have reported that dysfunction of gastric smooth muscle in diabetes is associated with neural factors and intracellular signaling pathways<sup>[6,7]</sup>.

Atrial natriuretic peptide (ANP) was isolated from the atrium by de Bold *et al.*<sup>[8]</sup> in 1981. From then on, brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), dendroapsis natriuretic peptide (DNP), micrurus natriuretic peptide (MNP), and ventricular natriuretic peptide (VNP) have been found in succession. Three types of single transmembrane natriuretic peptide receptors (NPRs) for ANP, BNP and CNP have been identified<sup>[9,10]</sup>, namely, NPR type A (NPR-A), type B (NPR-B), and type C (NPR-C). NPR-A and NPR-B have the membrane-bound particulate guanylate cyclase (pGC), which can catalyze the formation of cGMP from GTP<sup>[8-11]</sup>. NPR-A preferentially binds to ANP and BNP, but has a low affinity for CNP. NPR-B has a much higher affinity for CNP than either ANP or BNP<sup>[12]</sup>. Besides the heart, ANP is also distributed in other organs, for example, ANP can be secreted by gastric mucosa<sup>[13-15]</sup>. It is well known that ANP and other family members exert natriuretic-diuretic effects, vasorelaxation, and other functions including: decreasing blood pressure, and controlling electrolyte homeostasis. Some studies have demonstrated that the natriuretic peptide family plays an inhibitory role in regulating gastrointestinal motility, for example, in the chicken rectum<sup>[3]</sup>, rat tenia coli<sup>[16]</sup> and guinea-pig cecum<sup>[17]</sup>.

Our previous study also indicated that CNP relaxes gastric circular and longitudinal smooth muscles in human, rat and guinea-pig stomach, and NPRs are distributed in rat gastric smooth muscle layer<sup>[18-20]</sup>. Recently, we have also reported that the CNP-induced

relaxation and the production of cGMP of gastric smooth muscle are potentiated in streptozotocin (STZ)-induced diabetic rats. As well as the activity of pGC, the expression of NPR-B gene in gastric smooth muscle is upregulated in STZ-induced diabetic rats<sup>[21]</sup>. These results suggest that the CNP/(NPR-B)/pGC/cGMP signaling pathway is involved in the pathogenesis of diabetes. Our previous studies have confirmed that ANP-synthesizing cells exist in different regions of the gastric mucosa in rats<sup>[15]</sup>, therefore, ANP can be considered an endogenous natriuretic peptide of gastric mucosa. However, it is not clear whether the ANP/NPR-A signaling pathway is involved in the pathogenesis of diabetic gastroparesis.

In the present study, we investigated the relationship between the ANP signaling pathway and STZ-induced diabetic gastroparesis to confirm whether ANP contributes to the development of gastroparesis. The present study focused on whether the amount of ANP secretion from gastric mucosa was enhanced and the expression of the NPR-A gene in gastric smooth muscle was upregulated in a mouse model of STZ-induced diabetic gastroparesis.

## MATERIALS AND METHODS

### Drugs

STZ, TRIzol Reagent and chemicals were purchased from Sigma. C-kit antibody, ANP antibody and pronase were purchased from Santa Cruz Biotechnology and Roche. Other chemicals were purchased from Sangon Biological Company.

### STZ-induced diabetic mouse model

Male imprinting control region (ICR) mice (4 wk old) were purchased from the Experimental Animal Center of Shanghai Jiaotong University School of Medicine. A total of 80 mice were divided into two groups: control group and STZ-induced diabetic group. STZ-induced diabetes was created as follows: the mice were fasted overnight and intraperitoneally administered STZ solution (Sigma-Aldrich, St. Louis, MO, USA). STZ was diluted in 0.1 mol/L citrate buffer (pH = 4.0) and used at a dose of 200 mg/kg. Control mice were intraperitoneally administered with the same volume of 0.1 mol/L citrate buffer. The glucose concentration of blood was determined with One-touch Apparatus (Johnson & Johnson Medical Company). STZ-induced diabetic mice were confirmed by measuring glucose concentration from tail blood after fasting, and diabetes was defined when the blood glucose level was > 16 mmol/L. All experimental protocols included in this study were approved by the local Animal Care Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the Science and Technology Commission of P.R.C. (STCC Publication No. 2, revised 1988).

### Gastric motility in mouse intact stomach

Eight weeks after treatment with STZ, the animals

were euthanized by lethal dose of intraperitoneal pentobarbital sodium (50 mg/kg). The abdomen of each mouse was opened along the midline, and the intact stomach was removed and placed in pre-oxygenated Krebs solution (containing in 118.1 mmol/L NaCl, 4.7 mmol/L KCl, 1.0 mmol/L  $\text{KH}_2\text{PO}_4$ , 1.0 mmol/L  $\text{MgSO}_4$ , 25.0 mmol/L  $\text{NaHCO}_3$ , 2.5 mmol/L  $\text{CaCl}_2$ , and 11.1 mmol/L glucose), which was equilibrated with 95% oxygen and 5%  $\text{CO}_2$ . The connective tissue was removed and the pylorus was connected to a pressure transducer (Chengdu Equipment Factory, China) with a thin glass tubule, and the gastric cardia was tied with thin string. The stomach was incubated in a 15-mL organ bath filled with Krebs solution and gassed with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  at 37°C. The sensitivity of the pressure transducer was adjusted to an appropriate value and we recorded gastric motility by using the SMUP-E biological signal processing system (Chengdu Equipment Factory). The stomach was allowed to incubate for at least 60 min before the experiments were started, and we eliminated error by injecting an equal volume of Krebs solution into the stomach.

### Immunohistochemistry

The mice were euthanized by lethal dose of intraperitoneal pentobarbital sodium (50 mg/kg), the stomach was removed and washed with saline, and then fixed in 4% paraformaldehyde (4°C, 24 h). The fixed tissue was washed with running water (room temperature, 2 h), immersed with 95% and 100% alcohol (room temperature, 2 × 2 h), xylene (2 × 20 min) and paraffin (68.5°C, 30/40/50 min). Sections of 6  $\mu\text{m}$  thickness were cut and deparaffinized in xylene (4 × 30 min). The specimens were hydrated in graded concentrations of ethanol, and washed three times in PBS, and incubated in the blocking reagent for 30 min. The samples were incubated with polyclonal antibody against c-Kit protein (sc-5535; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:100 dilution). c-Kit protein was used as a marker of interstitial cells of Cajal (ICCs)<sup>[22,23]</sup> and with polyclonal antibody against ANP (sc-20158; Santa Cruz Biotechnology; 1:100 dilution) for 24 h at 4°C, followed by 3 h incubation in Rho-anti-rabbit and horseradish peroxidase-anti-rabbit second antibody, respectively. The negative control group was omitted by incubating with primary antibody against ANP. The slice was visualized and photographed under a fluorescence microscope (Olympus IX71, Tokyo, Japan).

### Flow cytometry analysis of gastric epithelial cell

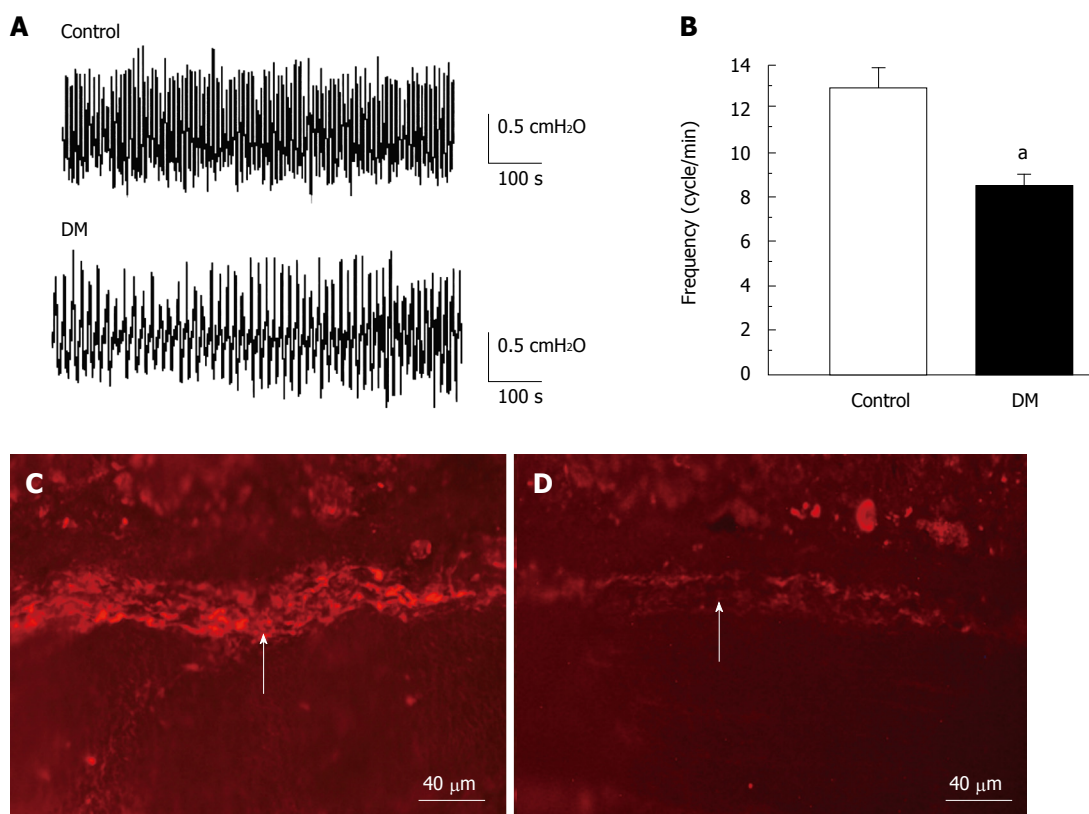
Eight weeks after injection with STZ, the mice were euthanized by lethal dose of intraperitoneal pentobarbital sodium (50 mg/kg). The stomach was removed and washed with saline. After the stomachs were inverted to make mucosal-side-out stomachs, the stomachs were rinsed with saline, and pronase solution was injected into the stomach. The pronase was diluted into 1 mg/mL with MA solution (0.5 mmol/L  $\text{NaH}_2\text{PO}_4$ , 1 mmol/L  $\text{Na}_2\text{HPO}_4$ , 20 mmol/L  $\text{NaHCO}_3$ , 80 mmol/L NaCl,

5 mmol/L KCl, 50 mmol/L HEPES, 11 mmol/L glucose, 20 g/L BSA, 2 mmol/L EDTA; pH = 7.4). The stomach sacks were incubated in the oxygenated MA solution for 3 × 30 min, at 37°C equilibrated with 95% oxygen and 5%  $\text{CO}_2$ , followed by gently stirring for 1 h in MB solution (0.5 mmol/L  $\text{NaH}_2\text{PO}_4$ , 1 mmol/L  $\text{Na}_2\text{HPO}_4$ , 20 mmol/L  $\text{NaHCO}_3$ , 80 mmol/L NaCl, 5 mmol/L KCl, 50 mmol/L HEPES, 11 mmol/L glucose, 10 g/L BSA, 1 mmol/L  $\text{CaCl}_2$ , 1.5 mmol/L  $\text{MgCl}_2$ ; pH = 7.4). MB solution that contained the gastric mucosal cells was collected, and filtered through a 200-mesh cellular sieve. The solution sample was centrifuged at  $1500 \times g$  for 5 min. The centrifuged sample was re-suspended with MC solution (0.5 mmol/L  $\text{NaH}_2\text{PO}_4$ , 1 mmol/L  $\text{Na}_2\text{HPO}_4$ , 20 mmol/L  $\text{NaHCO}_3$ , 80 mmol/L NaCl, 5 mmol/L KCl, 50 mmol/L HEPES, 11 mmol/L glucose, 1 mmol/L  $\text{CaCl}_2$ , 1.5 mmol/L  $\text{MgCl}_2$ , 1 mmol/L dithiothreitol; pH = 7.4) and centrifuged at  $1500 \times g$  for 5 min again. The density of the cell suspension was adjusted to  $3 \times 10^6/\text{mL}$ . The cells were fixed with 75% cold alcohol (-20°C, 24 h), and washed with PBS, followed by 0.2% Triton X-100 for 10 min. The antigen was blocked by 10% goat serum diluted in PBS, and incubated with antibody against ANP (sc-20158; Santa Cruz Biotechnology; 1:50 dilution) overnight at 4°C. The cells were stained with FITC-conjugated goat anti-rabbit IgG and examined by flow cytometry (Becton Dickinson). Using Cellquest software,  $10^4$  cells were analyzed per sample.

### Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of ANP, and NPR-A gene expression

The whole gastric tissue was quickly removed from the mouse. Total RNA was isolated from the tissue as recommended by the manufacturer of TRIzol Reagent (Sigma). RNA concentration was determined by absorbance reading at 260/280 nm. Reverse transcription was performed with a volume of 20  $\mu\text{L}$  mixture that contained 11  $\mu\text{L}$  mRNA, and 1  $\mu\text{L}$  oligo dT<sub>18</sub>, which was incubated at 70°C for 5 min, and 4  $\mu\text{L}$  5 × reaction buffer, 2  $\mu\text{L}$  dNTP, 1  $\mu\text{L}$  RNase inhibitor, 1  $\mu\text{L}$  M-MLV RT was added to the mixture, followed by incubation at 42°C for 1 h. The enzyme was inactivated by heating at 70°C for 10 min. cDNA samples were used for analyzing specific cDNA of ANP and NPR-A. One microliter of cDNA was added to 19  $\mu\text{L}$  PCR reaction mixture that contained: 7  $\mu\text{L}$  nuclease-free water, 10  $\mu\text{L}$  2 × reaction buffer, 1  $\mu\text{L}$  sense primer, and 1  $\mu\text{L}$  anti-sense primer. The following conditions were used for PCR amplification: for GAPDH, 95°C for 4 min; 95°C for 30 s; followed by 40 cycles at 52.9°C for 1 min; 72°C for 30 s; 72°C for 7 min; for ANP, 40 cycles at 54.6°C for 30 s; for NPR-A, 60°C for 30 s. RT-PCR was performed on an iCycler™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) using the Access RT-PCR System (Promega, Madison, WI, USA). Specific primers for murine GAPDH, ANP and NPR-A were synthesized by Sangon Biological Company (Shanghai, China). The primer sequences were as follows: GAPDH (sense) 5'TCAACGGCACAGTCAAGG3', GAPDH (antisense) 5'ACCAGTGGATGCAGGGAT3'; ANP





**Figure 1** Comparison of gastric contractility and expression of ICCs in normal and STZ-induced diabetic mice. A: Representative traces of spontaneous gastric contraction in control and STZ-induced diabetic mice; B: Summary of amplitudes and frequencies of gastric motility in control and STZ-induced diabetic groups ( $n = 8$ ;  $^aP < 0.05$  vs control group); C and D: Expression of c-Kit-positive cells in the muscle layer. Arrow indicates the positive staining area. Expression of c-Kit was more obvious in STZ-induced diabetic mice than in the normal controls. Scale bar = 40  $\mu\text{m}$ .

(sense) 5'TCCTTCTCCATCACCCTG3', ANP (antisense) 5'CCTAGAGCACTGCCGTCT3'; NPRA (sense) 5'AGACGATGGGCAGGATAG3', NPRA (antisense) 5'GGATGTCAGGAGGTGGGT3'. The PCR products were size-fractionated by 1% agarose gel electrophoresis, and visualized under ultraviolet light with 0.5% ethidium bromide staining. GAPDH, ANP and NPR-A cDNAs were quantified by Gel Doc XR System and Quantity One software (Bio-Rad). Gene expression for ANP and NPR-A was normalized to that for GAPDH.

### Statistical analysis

The data was expressed as the mean  $\pm$  SE. We evaluated differences between the treatment groups using Student's *t* test. Differences were considered to be significant at  $P < 0.05$ . The density index referred to the number of ANP-positive cell per field of vision.

## RESULTS

### Change in plasma glucose concentration

We detected changes in blood glucose concentration. Mice were used at 8 wk after injection of STZ. At the time of the study, most STZ-treated mice exhibited hyperglycemia; their average blood glucose concentration was  $26.7 \pm 1.3$  mmol/L ( $n = 32$ ), which was significantly higher than that of the non-diabetic control mice ( $6.9 \pm 0.6$  mmol/L,  $n = 32$ ;  $P < 0.01$ ).

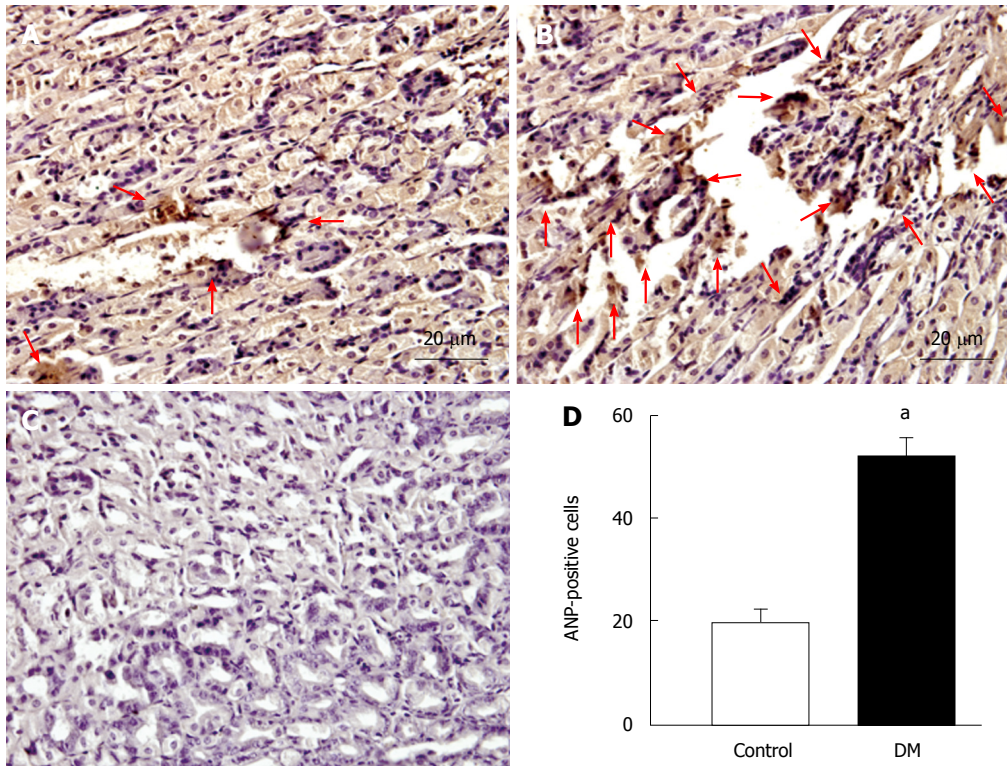
### Gastric motility and ICC expression in STZ-induced diabetic mice

To determine whether diabetic gastropathy occurred, the amplitude and frequency of spontaneous gastric contraction were observed in normal and STZ-induced diabetic mice. The frequency of spontaneous gastric contraction decreased significantly from  $12.9 \pm 0.8$  cycles/min in the control group to  $8.4 \pm 0.6$  cycles/min in diabetic mice ( $n = 8$ ,  $P < 0.05$ ; Figure 1A and B). However, the amplitude of contraction was not changed in the diabetic group. Spontaneous rhythmic contraction of gastrointestinal smooth muscle is triggered by ICCs, which are also mediators of neuromuscular transmission in the gastrointestinal tract. Depletion of ICCs contributes to dysfunction of gastrointestinal motility in patients and animal models<sup>[24]</sup>. ICCs distributed in the gastric smooth muscle layer were observed by immunochemistry. c-Kit protein, an ICC marker, was detected in the inter-muscular layer of normal and diabetic mice (Figure 1C and D), but the expression was significantly decreased in the diabetic group ( $n = 6$ ; Figure 1D). The results suggested that the STZ-induced diabetic mice exhibited gastric dysfunction, and the reduced frequency of gastric motility in diabetic mice might have been related to ICC depletion.

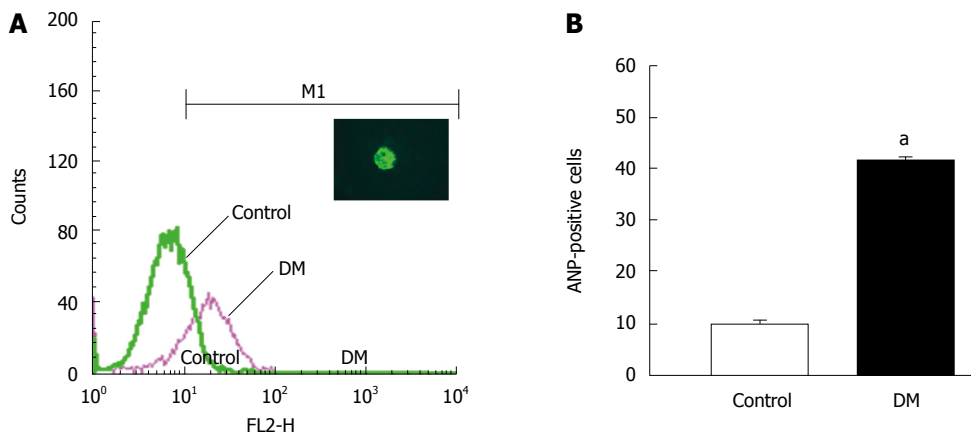
### ANP secretion from gastric mucosa

Besides the heart, the ANP family is also distributed in other organs, and some studies have found that ANP





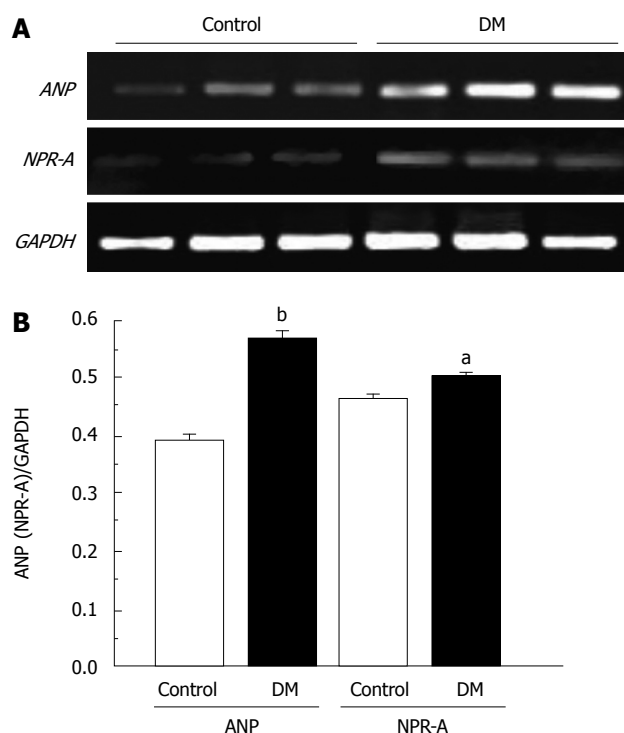
**Figure 2** Immunohistochemical analysis of atrial natriuretic peptide (ANP) in mouse gastric tissue. The photographs show ANP expression in gastric mucosa in the control group (A), STZ-induced diabetic group (B), and negative control group (C); The paranuclear cytoplasmic stained cells that display an intense dark brown color were ANP-positive-staining cells in the mucosa. The red arrows indicate ANP-positive cells; B shows that the number of ANP-positive cells increased in the STZ-induced diabetic group; D shows the density index that referred to the number of ANP-positive cells per field of vision ( $n = 8$ ,  $^aP < 0.05$  vs control group). Scale bar = 20  $\mu\text{m}$ .



**Figure 3** Flow cytometric analysis of ANP-positive cells among gastric mucosal cells. The percentage of ANP-positive cells was determined by flow cytometry with FITC-conjugated goat anti-rabbit IgG. A: Relative fluorescence intensity of gastric mucosal cells in the control and diabetic groups. M1 indicates the proportions of ANP-positive cells that displayed positive FITC labeling. The inserted fluorescent image is a single ANP-positive cell that had a positive fluorescent signal in its cytoplasm ( $\times 400$ ); B: ANP-positive cells among the gastric mucosal cells ( $10^4$  cells per sample) in control and STZ-induced diabetic mice ( $n = 3$ ,  $^aP < 0.05$  vs control).

can be secreted from gastric mucosa<sup>[13-15]</sup>. To determine whether ANP secretion from gastric mucosa is enhanced in STZ-induced diabetic mice, ANP-positive cells in the gastric mucosa were observed in the control (Figure 2A) and STZ-induced diabetic (Figure 2B) mice. The number of ANP-positive cells was increased significantly in the gastric mucosa of diabetic mice, and the density index was enhanced from  $20.9 \pm 2.2$  cells/field of vision in the controls to  $51.8 \pm 2.9$  cells/field of vision in the diabetic

group ( $n = 8$ ,  $P < 0.05$ ; Figure 2D). We further analyzed the number of ANP-positive cells in gastric epithelial cells by flow cytometry. The relative fluorescence intensity of the diabetic group was higher than that of the control group (Figure 3A). The number of ANP-positive cells was increased significantly in the diabetic group. The percentage of ANP-positive cells among dispersed epithelial cells was enhanced from  $10.0\% \pm 0.9\%$  in the control group to  $41.2\% \pm 1.0\%$  in the



**Figure 4** RT-PCR of gastric *ANP* and *NPR-A* genes in control and STZ-induced diabetic mice. A: Results of agarose gel electrophoresis, including *ANP*, *NPR-A* and *GAPDH* genes. The density signal was normalized to that of *GAPDH*. The mean value of the density was obtained from three separate experiments; B: Expression of *ANP* and *NPR-A* genes was increased in STZ-induced diabetic mice ( $n = 3$ ; <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs control).

diabetic group ( $n = 3$ ,  $P < 0.05$ ; Figure 3B). The results suggested that ANP secretion from gastric mucosa was potentiated in the stomach of STZ-induced diabetic mice.

### Expression of ANP and NPR-A gene

The number of ANP-positive cells was increased in the STZ-induced diabetic group, therefore, we investigated whether *ANP* and *NPR-A* gene expression was also increased. *ANP* and *NPR-A* gene expression was observed in gastric tissue by RT-PCR. *ANP* and *NPR-A* gene expression was increased in the diabetic group ( $n = 3$ ; Figure 4A). The signal was normalized to that of *GAPDH*. The ratio of *ANP*/*GAPDH* and *NPR-A*/*GAPDH* was  $0.54 \pm 0.02$  and  $0.5 \pm 0.01$  and  $0.39 \pm 0.01$  and  $0.46 \pm 0.02$  in the STZ-induced diabetic and control group, respectively ( $n = 3$ ,  $P < 0.05$  and  $P < 0.01$ ; Figure 4B).

## DISCUSSION

Diabetic gastroparesis is defined as slow gastric emptying in the absence of mechanical obstruction, and constipation is considered to be the most significant clinical manifestation. It occurs in 30%-60% of diabetic patients observed at tertiary referral centers<sup>[25]</sup>. Many investigators view these changes as the consequences of irreversible autonomic neuropathy, which affects the

vagus and sympathetic nerves primarily<sup>[2,5]</sup>. However, the cause of gastroparesis is multifactorial and also involves enteric neurons, mucosal endocrine cells, smooth muscle cells and ICCs<sup>[4]</sup>. The main conclusion drawn from the present study is that the ANP/NPR-A signaling pathway is upregulated and may contribute to the development of gastroparesis in STZ-induced diabetic mice, and then lead to gastric motility dysfunction.

In non-obese diabetic (NOD) mice, a well-established model of human type I diabetes mellitus, gastric emptying of solids is delayed after 6-8 wk of untreated diabetes<sup>[23]</sup>. In the present study, we established an STZ-induced diabetic model and gastric motility was observed *in vitro* after 8 wk of untreated diabetes. The frequency of spontaneous contraction in the STZ-induced diabetic group was decreased significantly, however, the amplitude of contraction was not significantly different between these two groups. The results suggest that diabetic gastropathy mostly exhibits slow rhythm and turbulence, however, the amplitude of spontaneous contraction is not affected significantly in the early stage of diabetes. ICCs play a key role in gastric motility, and damage to the ICC networks may contribute to the development of gastropathy and gastroparesis<sup>[26]</sup>. In the present study, we confirmed whether the number of ICCs in the gastric smooth muscle layer was changed, by using immunochemistry in the STZ-induced diabetic mice. c-Kit expression in the muscle layer was observed in normal and diabetic mice, however, c-Kit expression was decreased in diabetic mice. The results suggest that gastric dysfunction in STZ-induced diabetic mice may be related to ICC depletion.

Atrial myocytes are the main source of ANP, but ANP is also found in other tissues, such as the gastrointestinal tract<sup>[27]</sup>. ANP and its receptor NPR-A have been detected in the gastric antrum<sup>[28]</sup>. NPR-A has the same membrane-bound guanylate cyclase activity as NPR-B, which catalyzes the formation of cGMP from GTP<sup>[9,10]</sup>. Some studies have demonstrated that ANP has an inhibitory effect on the regulation of gastrointestinal motility; for example, in chicken rectum<sup>[16]</sup>, rat tenia coli<sup>[17]</sup> and guinea-pig cecum<sup>[3]</sup>. Our previous study also has indicated that NPs relax gastric circular and longitudinal smooth muscles in human, rat and guinea-pig stomach, and NPRs are distributed in the rat gastric smooth muscle layer<sup>[18,19]</sup>. Another previous study has indicated that ANP release is augmented in the atrium of STZ-induced diabetic rats<sup>[29]</sup>. Recently, we have found that the expression of NPR-B gene is increased and NP-dependent guanylate cyclase/cGMP signaling is upregulated in STZ-induced diabetic rats<sup>[20,21]</sup>. ANP is also secreted from the gastric mucosa<sup>[15]</sup>, therefore, ANP can be considered endogenous to the gastric mucosa. However, the relationship between the ANP/NPR-A signaling pathway and STZ-induced diabetic gastroparesis is not clear. In the present study, we confirmed that the expression of ANP in gastric mucosa was increased significantly in STZ-induced diabetic mice. We demonstrated that the percentage of ANP-positive

cells was also enhanced significantly among the dispersed gastric epithelial cells, and the expression of the *ANP* gene in gastric tissue was upregulated in STZ-induced diabetic mice. These results suggest that ANP secretion from gastric mucosa is increased significantly in STZ-induced diabetes. The amount of ANP secretion is increased in diabetic mice, therefore, we investigated whether expression of NPR-A in the stomach was upregulated in STZ-induced diabetic mice. We demonstrated that expression of the *NPR-A* gene was also increased significantly in gastric tissue of diabetic mice. The results suggest that the ANP/NPR-A signaling pathway is upregulated in the stomach of STZ-induced diabetic mice.

Our previous study has demonstrated that the CNP/NPR-B signaling pathway is upregulated in STZ-induced diabetic rats<sup>[20,21]</sup>, while the present study indicates that the ANP/NPR-A signaling pathway is also upregulated in STZ-induced diabetic mice. Previous studies have indicated that diabetes may also affect expression of the *ANP* gene; for example, *ANP* gene expression in heart and kidney are increased in STZ-induced diabetic rats<sup>[11,30]</sup> and plasma concentration of pro-ANP is increased in comparison with control rats<sup>[31]</sup>. Christoffersen *et al.*<sup>[32]</sup> also have reported that diabetic mice show an increase in *NPR-B* gene expression in the heart, and have suggested that increased NPR-B signaling affects development of diabetic cardiomyopathy. The NP/NPR signaling pathway has an inhibitory effect on gastrointestinal motility, therefore, upregulation of this signaling pathway may be involved in development of gastroparesis in STZ-induced diabetic mice.

In summary, gastroenteropathy causes considerable morbidity in patients with diabetes mellitus and it has become a major healthcare burden. Current treatments are mainly symptomatic and frequently ineffective. Development of new therapeutic options is hampered because of poor understanding of the underlying pathological mechanisms. Our study demonstrates that the ANP/NPR-A signaling pathway is upregulated. The results suggest that ANP/NPR-A signaling is involved in the development of gastroparesis in STZ-induced diabetic mice.

## COMMENTS

### Background

Gastroparesis is a gastrointestinal complication of diabetes, which is also called delayed gastric emptying. The mechanism of gastroparesis is not clear, but a recent study has reported that the C-type natriuretic peptide-natriuretic peptide receptor B (CNP/NPR-B) pathway is upregulated in the stomach of diabetic rats. Atrial natriuretic peptide (ANP) has an inhibitory effect on motility of the gastrointestinal tract, but whether secretion of ANP is increased in the stomach of diabetic mice has not been reported.

### Research frontiers

NPs are located in several organs besides the heart. Recently, the authors have found that ANP and its receptor (NPR-A) are also present in the mouse stomach, and the CNP/cGMP pathway in diabetic gastroparesis was investigated in STZ-induced diabetic rats. This study was designed to investigate whether ANP secretion is altered in the stomach of STZ-induced diabetic mice.

### Innovations and breakthroughs

ANP with an inhibitory effect on gastrointestinal motility is present in the

stomach, and previous studies have focused on the relationship between the CNP/NPR-B pathway and gastric motility. This is believed to be the first study to investigate ANP secretion and NPR-A in the stomach of STZ-induced diabetic mice. The results suggest that ANP secretion and expression of NPR-A mRNA are both increased in the stomach of STZ-induced diabetic mice. This may be involved in stomach motility dysfunction in STZ-induced diabetic mice.

### Applications

The dysfunction of stomach motility that occurs in diabetic gastroparesis may be related to ANP secretion and upregulation of NPR-A. This may contribute to the treatment and preventive intervention of diabetic gastroparesis in the future.

### Terminology

Gastroparesis also called delayed gastric emptying, and usually occurs in diabetes. Symptoms of gastroparesis include heartburn, pain in the upper abdomen, vomiting, nausea, and early feeling of fullness.

### Peer review

This study deals with questions about the mechanisms involved in gastroparesis in diabetes.

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## Double balloon enteroscopy in children: Diagnosis, treatment, and safety

Mike Thomson, Krishnappa Venkatesh, Khalid Elmalik, Willam van der Veer, Maartan Jacobs

Mike Thomson, Krishnappa Venkatesh, Khalid Elmalik, Centre for Paediatric Gastroenterology, Sheffield Children's NHS Foundation Trust, Sheffield, S10 2TH, United Kingdom  
 Willam van der Veer, Maartan Jacobs, Department of Gastroenterology, VU University Medical Center, Postbus 7057, 1007 MB, Amsterdam, The Netherlands

Author contributions: Thomson M and Jacobs M designed research; Thomson M, Venkatesh K, van der Veer W and Jacobs M performed research; Thomson M, Venkatesh K and Elmalik K wrote the paper.

Correspondence to: Dr. Mike Thomson, Centre for Paediatric Gastroenterology, Sheffield Children's Hospital, Sheffield, S10 2TH, United Kingdom. [mike.thomson@sch.nhs.uk](mailto:mike.thomson@sch.nhs.uk)

Telephone: +44-114-2717673 Fax: +44-114-2267659

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### Abstract

**AIM:** To assess the feasibility and utility of double balloon enteroscopy (DBE) in the management of small bowel diseases in children.

**METHODS:** Fourteen patients (10 males) with a median age of 12.9 years (range 8.1-16.7) underwent DBE; 5 for Peutz-Jeghers syndrome (PJ syndrome), 2 for chronic abdominal pain, 4 for obscure gastrointestinal (GI) bleeding, 2 with angiomatous malformations (1 blue rubber bleb nevus syndrome) having persistent GI bleeding, and 1 with Cowden's syndrome with multiple polyps and previous intussusception. Eleven procedures were performed under general anesthesia and 3 with deep sedation.

**RESULTS:** The entire small bowel was examined in 6 patients, and a length between 200 cm and 320 cm distal to pylorus in the remaining 8. Seven patients had both antegrade (trans-oral) and retrograde (trans-anal and *via* ileostomy) examinations. One patient underwent DBE with planned laparoscopic assistance.

The remaining 6 had trans-oral examination only. The median examination time was 118 min (range 95-195). No complications were encountered. Polyps were detected and successfully removed in all 5 patients with PJ syndrome, in a patient with tubulo-villous adenoma of the duodenum, in a patient with significant anemia and occult bleeding, and in a patient with Cowden's syndrome. A diagnosis was made in a patient with multiple angiomata not amenable to endotherapy, and in 1 with a discrete angioma which was treated with argon plasma coagulation. The source of bleeding was identified in a further patient with varices. DBE was normal or revealed minor mucosal friability in the remaining 3 patients. Hence a diagnostic yield of 11/14 with therapeutic success in 9/14 was achieved.

**CONCLUSION:** Double balloon enteroscopy can be a useful diagnostic and therapeutic tool for small bowel disease in children, allowing endo-therapeutic intervention beyond the reach of the conventional endoscope.

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**Key words:** Double balloon enteroscopy; Gastrointestinal; Peutz Jeghers syndrome; Wireless video capsule endoscopy; Children

**Peer reviewer:** William Dickey, Altnagelvin Hospital, Londonderry, BT47 6SB, Northern Ireland, United Kingdom

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### INTRODUCTION

The advent of flexible fiberoptic endoscopes transformed

the diagnosis and management of gastrointestinal (GI) disorders in adults and children, allowing direct visualization with targeted mucosal biopsies. Furthermore, endo-therapeutic procedures have now been possible throughout the upper GI tract and ileo-colon. However, the small bowel distal to the ligament of Trietz is inaccessible to conventional GI endoscopes. Recently, push enteroscopy, allowing the therapeutic endoscopist access up to 70-100 cm beyond the pylorus<sup>[1-4]</sup>, intra-operative enteroscopy techniques which are relatively invasive<sup>[5,6]</sup>, and wireless video capsule endoscopy (WCE) which affords excellent diagnostic yield combined with lack of morbidity but is non-therapeutic<sup>[7-9]</sup>, have been performed.

Double balloon enteroscopy (DBE) is a more recent modality which enables high resolution endoscopic imaging of the entire small bowel, allowing interventional endo-therapy (e.g. non-variceal hemostasis, snare polypectomy and pneumatic balloon stricture dilatation)<sup>[10-12]</sup>. It is clear that this technology could allow treatment of lesions, possibly identified by WCE or other less invasive investigations such as magnetic resonance imaging (MRI) enteroclysis, in parts of the small bowel inaccessible to standard endoscopy, and hence may preclude the need for formal surgical approaches in such children. We present the first pediatric-only experience of DBE, although 2 predominantly adult series have included a few children with an age range up to 20 years and no specification as to those under 16 years<sup>[13,14]</sup>.

## MATERIALS AND METHODS

We prospectively collected the following data on all consecutively enrolled children between January 2004 and December 2007. All had undergone upper endoscopy, ileo-colonoscopy, and most had had WCE. Various imaging techniques had been employed but none had undergone virtual CT. The double balloon enteroscopy system (Fujinon; Fujinon Inc., Japan) (Figure 1) consists of a high resolution video enteroscope (EN-450P5/20) with a flexible over-tube (TS-12140), as well described elsewhere. The enteroscope has a working length of 200 cm and an outer diameter of 8.5 mm. The flexible over-tube has a length of 140 cm and outer diameter of 12 mm. Two enteroscopes are available, currently with 2.2 mm and 2.8 mm working channels, allowing therapeutic intervention. The enteroscopes and over-tube have balloons fitted at the distal tip of each, which are sequentially inflated and deflated with air from a pressure controlled pump system with a maximum inflatable pressure of 45 mmHg.

Specifics of the procedure are not provided here as these have been well documented elsewhere and do not differ in pediatric practice compared to that in adults. Patients received bowel preparation as for colonoscopy in anticipation of a trans-anal approach. For bowel preparation, Senokot 1-2 mg/kg (max 30 mg) and sodium picosulphate 2.5-10 g (depending on age) were given on the evening prior to the day of procedure with sodium picosulphate repeated on the morning of the procedure.

The new double balloon enteroscopy (DBE) system features the following components  
An EN-450P5/20 video enteroscope  
A 400 (VP-402, XL-402) processor  
A TS-12140 overtube  
BS-1 balloons  
A PB-10 balloon controller



Figure 1 Double balloon enteroscope system configuration.

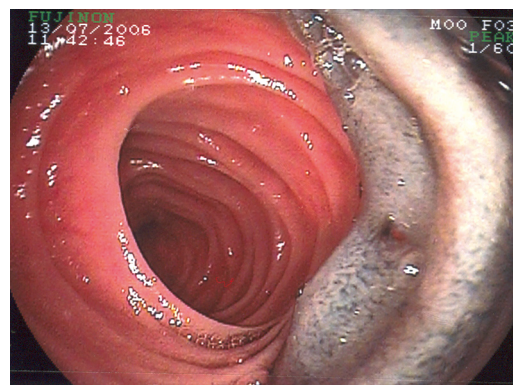


Figure 2 Double balloon tattoo.

The preference was for general anesthesia but moderate conscious sedation was employed in the older patients in one centre. If the terminal ileum (TI) was not reached then the most distal part of the small bowel negotiated was “tattooed” in the sub-mucosal plane with an endo-needle (Figure 2). The DBE could then be repeated *via* the trans-anal route and retrograde movement from the TI proximally was attempted to attain the marked area. No external compression, fluoroscopy, or other aides were necessary or useful in aiding intubation. No anti-spasmodics were employed, and air rather than carbon dioxide was insufflated. If lesions were encountered it was usual practice to treat as they were encountered rather than on withdrawal in case the lesions were not then found again. Potential adverse events such as pancreatitis, perforation, or bowel damage due to traction or torsion around the mesentery have been reported in the adult literature. Two of the authors (Thomson M and Jacobs M) performed all of the DBE having attained competence in DBE in adult patients first. Training and learning curves for this procedure are, it is estimated by the procedurists, similar to that encountered in ileo-colonoscopy, and clearly it is not yet apparent in pediatric practice how many DBE procedures are necessary in order to attain a high degree of competence.

## Patients

Fourteen patients (10 males), median age 12.9 years (range 8.1-16.7), median weight 39.6 kg (range 24.1-67.3)

**Table 1** Investigations performed on patients prior to DBE

1	WCE: ?small sessile polyp in mid-small bowel
2	Intra-operative enteroscopy with polypectomy MRI enteroclysis: normal
3	OGD: polyps in stomach and duodenum MRI: 3 big polyps in small bowel
4	WCE: multiple polyps in mid-small bowel
5	WCE: possible polyp in ileum
6	WCE: ?polyps seen in small bowel, ?intermittent intussusception Colonoscopy: polyp in rectum
7	WCE: normal, Abdominal Ultrasound: enlarged spleen
8	WCE: no source of bleeding
9	OGD, WCE: lymphonodular hyperplasia in duodenum of little clinical significance
10	OGD, WCE: no positive findings
11	OGD, ileo-colonoscopy, WCE: multiple blue rubber bleb nevus lesions throughout bowel
12	WCE: angioma in small bowel
13	WCE: polyp in mid-small bowel
14	WCE: multiple polyps seen throughout the small bowel including a lymphangitic polyp

DBE: Double balloon enteroscopy; WCE: Wireless video capsule endoscopy; OGD: Gastroduodenoscopy.

underwent DBE. Indications: patients 1-5 for Peutz-Jeghers syndrome (PJS); patients 6-7 for recurrent abdominal pain; patients 8, 9, 10 and 13 for obscure GI bleeding. Patients 11 and 12 had, respectively; blue rubber bleb nevus syndrome and an angioma identified by WCE, and had transfusion-dependent persistent GI bleeding. Patient 14 had Cowden's syndrome with previous episode of intussusceptions. Thirteen patients had undergone WCE and 1 MRI enteroclysis (Table 1).

Eleven patients received general anesthesia and 3 procedures were performed under sedation with fentanyl and midazolam. Thirteen patients had trans-oral DBE, of whom 6 patients also had trans-anal, and 1 patient had trans-stomal DBE through an ileostomy. One patient underwent intra-operative DBE.

## RESULTS

The results of this investigation suggest that DBE is both useful and feasible in children with small bowel disease. The entire small bowel was examined in 6 patients, either trans-oral alone or with both trans-oral and trans-anal DBE. When TI was not attained, trans-oral progression was assessed as approximately 200 to 320 cm beyond the pylorus (Table 2), based on the assumption that each set of maneuvers to advance the enteroscope traversed around 30 cm of bowel with diminishing distance the more attempts at advancement were made. No fluoroscopy was used hence these estimates of distance attained are presumptive. The median examination time was 118 min (range 95-195). No complications of DBE were encountered, and mild post-procedure abdominal discomfort, as occurs secondary to bowel insufflation in some ileo-colonoscopies, was temporary and controlled easily with simple analgesia.

Patients 1-5 were known to have PJS. Patient 1 had not undergone WCE, and both trans-oral and trans-anal DBE allowed the whole small bowel to be visualized. No polyps were found in the small bowel; however a rectal polyp was resected which confirmed PJS on histology. Patient 2 had undergone previous intra-operative enteroscopy with polypectomy, and DBE revealed a presumably new small polyp in the jejunum which was removed. Patient 3 underwent laparoscopic-assisted DBE and 7 polyps were removed. The postoperative period was complicated by pelvic abscess requiring surgical drainage, but no intestinal perforation had occurred, hence the reason for the laparoscopic complication was unclear. Patient 4 had multiple sessile polyps in the jejunum and patient 5 had one PJS polyp removed from the mid jejunum. It is therefore suggested that PJS patients undergo WCE prior to DBE and if no polyps are found, then no DBE should take place.

Patients 6 and 7 had recurrent abdominal pain as the main presenting complaint with multiple negative investigations. Patient 6 had a family history of PJS and WCE had suggested a polyp in the mid-ileum. However, trans-oral (200 cm post-pylorus) and trans-anal (35 cm proximal to ileo-cecal valve) DBE failed to identify a polyp. Patient 7 presented with recurrent abdominal pain over 3.5 years (repeated upper GI endoscopy and ileocolonoscopy were inconclusive), and WCE had suggested proximal jejunal polyps. Trans-oral DBE demonstrated thickened folds in the proximal jejunum, which on histology proved to be a tubulo-villous adenoma. Surveillance enteroscopy after 1 year identified progression to intra-mucosal carcinoma. Surgical excision of the affected bowel and pancreas has proved curative. Clearly, this finding is very uncommon and the literature does not suggest an incidence in this age group. Of course, it is not suggested that all patients with recurrent abdominal pain undergo DBE. Clear clinical indication and warning signs such as a family history of polyp syndromes, in spite of negative WCE, are reasonable pointers towards DBE.

Patients 8-13 were investigated for GI bleeding. Patient 8 was transfusion-dependent and DBE was non-contributory since esophago-gastric varices were identified which could also have been identified and treated by standard upper GI endoscopy, although prior to transfer to our unit this procedure had not identified the varices. Mid-small bowel varices were not found at DBE. Patient 9 was investigated for obscure GI bleeding and trans-oral DBE did not reveal a bleeding site. Subsequently a Meckel's scan, initially negative, was repeated, found to be positive and surgical resection occurred. Had trans-anal DBE been performed this may have identified the Meckel's diverticulum, but this was not attempted due to a technical failure of the system and remains conjectural. The technical failure was due to the distal balloon bursting and is not considered as a dangerous adverse event.

Patient 10 had intestinal aganglionosis, an ileostomy and a gastrostomy, and presented with a 3-year history



Table 2 Details of indications, approach and findings in patients undergoing DBE

Patient No.	Age/Sex	Indication	Approach	Complete /incomplete	Findings
1	13/M	PJS	Oral + anal	Complete	Rectal polyp
2	12/M	PJS	Oral	320 cm <sup>1</sup>	Small polyp in jejunum
3	16/M	PJS	Intra-operative	Complete	3 small polyps removed endoscopically and 3 large polyps removed surgically
4	11/M	PJS	Oral	250 cm <sup>1</sup>	Multiple polyps in mid-small bowel
5	9/M	PJS	Oral	Incomplete	Mid-small bowel polyp
6	10/F	Chronic abdominal pain	Oral + anal	Up to 200 cm <sup>1</sup> trans-orally, 35 cm TI proximal to ICV trans-anally	Normal
7	16/M	Chronic abdominal pain with family history of colorectal carcinoma	Oral + anal	Complete	Tubulo-villous adenoma in duodenum; Lymphoid aggregates in ileum
8	11/M	Upper GI bleeds/possible vascular malformation	Oral	300 cm <sup>1</sup>	Grade 1 esophageal varices; no source found in small bowel
9	16/M	Occult bleeding	Oral	200 cm <sup>1</sup>	No source found
10	8/M	Occult bleeding	Oral + <i>via</i> ileal stoma	Complete	Increased friable mucosa throughout the small bowel
11	12/F	Blue rubber bleb syndrome with persistent GI bleeding	Oral + anal	200 <sup>1</sup> cm trans-orally, 50 cm proximal to ICV trans-anally	Numerous angiomas throughout small bowel not amenable to therapy
12	9/F	Angioma	Oral	Incomplete	Angioma identified: APC applied
13	16/M	Occult bleeding with significant anemia	Oral + anal	Complete	Polyp 40 cm from TI: removed
14	12/F	Cowden's syndrome	Oral + anal	Complete	Multiple polyps: 2 snare polypectomies; Meckel's diverticulum found

<sup>1</sup>Post-pylorus distance achieved. PJS: Peutz-Jeghers syndrome; ICV: ileo-caecal valve; APC: Argon plasma coagulation.

of transfusion-dependent obscure GI bleeding. DBE identified very friable small bowel mucosa with contact bleeding, but no histological diagnosis was concluded with normal biopsies obtained. Patient 11 had transfusion-dependent recurrent GI bleeding due to multiple lesions consistent with blue rubber bleb nevus syndrome, identified in the colon at colonoscopy, and throughout the small bowel at DBE (Figure 3). Argon plasma coagulation (APC) was used in order to ablate some of the lesions and transfusion requirement diminished. The extensive nature of the lesions precluded definitive surgery and further DBE is planned, but has not occurred to date, therefore post-APC images are not available. However, transfusion dependency in both patients 10 and 11 had ceased.

Patient 12 had angioma detected in the mid-small bowel on WCE. This was identified with DBE, and APC was applied. Patient 13 presented with occult bleeding and significant anemia, and a polyp was detected in the small bowel on WCE. At DBE, a 4 mm polypoid structure was found (Figure 4A) and removed (Figure 4B). Patient 14 had Cowden's syndrome with a history of intussusception. DBE revealed presence of multiple sessile polyps and polypectomies were performed on 2 polyps. Incidentally, a Meckel's diverticulum was found (Figure 5) in this patient.

No patients referred and considered for DBE were rejected, i.e. there seems no reason not to consider this minimally invasive approach. No complications occurred in the 13 patients who had DBE alone without intra-operative assistance. Significant post-procedure abdominal pain was not encountered, and only paracetamol was needed to counter minor abdominal discomfort, except

in the individual who had undergone laparoscopy. All patients were in-patients although it is anticipated that day case procedures are viable. No evidence of pancreatitis, perforation or bowel damage was encountered, and the intra-abdominal abscess could have been the result of a micro-perforation rather than bowel damage due to the laparoscope, although the authors consider this unlikely. All patients were allowed home with no evidence of significant complications or discomfort within 24 h of the DBE being performed. All of those who did not undergo polypectomy were allowed home on the same day as the procedure. The longest duration at 195 min has to be considered as a long endoscopic procedure, but this has to be counter-balanced by the relative lack of invasiveness of the technique.

## DISCUSSION

Flexible GI endoscopy is sufficient for diagnostic and therapeutic procedures in the vast majority of pediatric cases, and in adult patients with obscure GI bleeding this procedure is known to determine the source in up to 90% of cases. However, in the small number of cases where the pathology is confined to the small bowel beyond the reach of conventional endoscopy, WCE and DBE have been recently employed. In our series the entire small bowel was examined in 6/14 patients in whom trans-oral and trans-anal approaches were combined. One cannot claim that DBE diagnosed the disease in this series of patients, but it can be considered that it had a very important role in treatment. In all patients with PJ syndrome polyps were detected. Prior to the advent of these technologies, modalities such



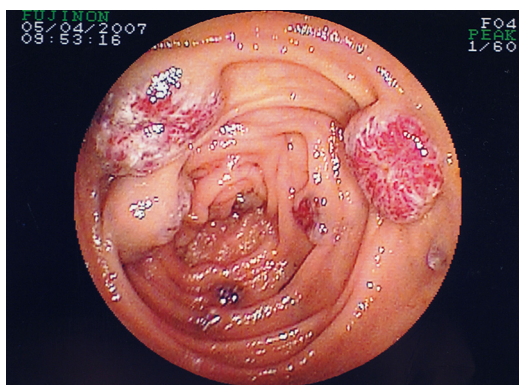


Figure 3 Multiple angiomas in small bowel.

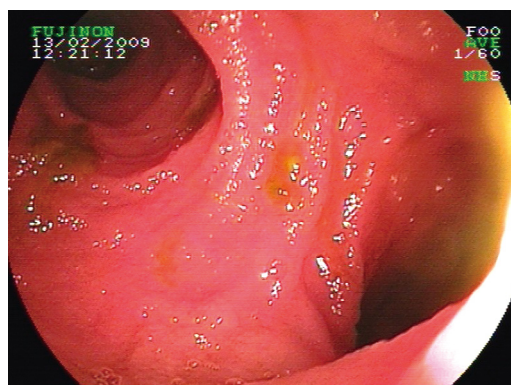


Figure 5 Meckel's diverticulum.

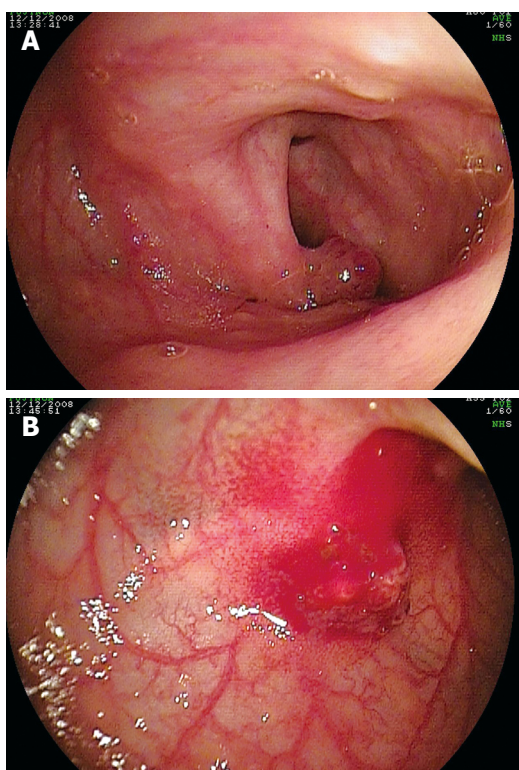


Figure 4 Polyp detected (A) and removed (B).

as push enteroscopy (PE) have had limited pediatric exposure due to safety concerns. In children, 80% of all mucosal lesions identified and biopsied by PE, and 20% of therapeutic procedures performed, were beyond the reach of a standard GI endoscope<sup>[13]</sup>. Small bowel series, angiography, scintigraphy and enteroclysis have been used with variable results in the evaluation of adult patients with obscure GI bleeding<sup>[15,16]</sup>. WCE has recently attained the position of investigation of first choice for such diagnoses, while intra-operative enteroscopy, despite its invasive quality, has been the mainstay in the subsequent treatment of obscure GI bleeding in children and adults<sup>[5,17,18]</sup>.

Wireless capsule endoscopy (WCE) has been compared favorably with intra-operative enteroscopy for

the diagnosis of obscure bleeding in adults, with 95% sensitivity and 75% specificity<sup>[19]</sup>. WCE has been found to be diagnostically superior to PE<sup>[20,21]</sup> and barium follow through/CT scan in obscure GI bleeding, and has been recently evaluated in children<sup>[22]</sup>. WCE is, however, non-therapeutic by its nature, and since the imperative in pediatric gastroenterology is the drive to diagnosis by mucosal histology, this is a shortcoming of WCE.

Trans-oral and, if necessary, subsequent trans-anal DBE allow therapeutic interventions such as polypectomy, hemostasis, balloon dilatation and placement of stents for the whole of the small bowel<sup>[23]</sup>. In a prospective comparative study between WCE and DBE in patients with obscure GI bleeding, the diagnostic rate was 80% for WCE and 60% for DBE; however, 51% of the patients had therapeutic intervention using argon plasma, underlining the therapeutic utility of DBE<sup>[24]</sup>. In a recent large retrospective analysis of 152 patients undergoing DBE for obscure GI bleeding in adults, 75% had the potential source of bleeding detected and, for 83% of patients, management was changed as a direct result of DBE<sup>[25]</sup>. Yamamoto has described full small intestinal examination in 86% of adults using DBE<sup>[23]</sup>. DBE in this series of children had a diagnostic yield of 11/14, and therapeutic success in 9/14 was achieved. Clearly if a regional or national small bowel diagnostic and therapeutic centre is contemplated then the duality of WCE and DBE is mandatory to achieve the goals of diagnosis and treatment without operative intervention, which should be the goal of a pediatric endoscopic centre of excellence. Hence, with the results of our prospective DBE study a case could be made for the discontinuation of push enteroscopy in the investigation and treatment of children with suspected small bowel pathology.

Complications have been reported in the literature with DBE, including intestinal perforation<sup>[26,27]</sup>, pancreatitis<sup>[28]</sup> and paralytic ileus<sup>[29]</sup>. However, the only complication in our group of children occurred secondary to surgical intervention in the child who underwent intra-operative DBE.

Training remains an issue with no clear resolution attempted by this small series which included only two

experienced endoscopists.

In conclusion, double balloon enteroscopy is a useful diagnostic and therapeutic tool for the investigation of small bowel disease. It is useful in conjunction with WCE for optimizing diagnostic potential in the small bowel and offering a therapeutic option. It is also of benefit in situations where diagnosis has not been reached by other investigative modalities, and particularly in those lesions amenable for therapeutic intervention endoscopically, but not reachable by the conventional endoscope.

## COMMENTS

### Background

The small bowel distal to the ligament of Trietz is inaccessible to conventional gastrointestinal (GI) endoscopes. Several techniques such as push enteroscopy, intra-operative enteroscopy techniques and wireless video capsule endoscopy have been developed. All these procedures have some limitations. Double balloon enteroscopy (DBE) is a recently developed tool which enables high resolution endoscopic imaging of the entire small bowel and allows interventional endo-therapy.

### Research frontiers

Small bowel is a particular area of the GI tract difficult to be imaged completely by conventional endoscope. DBE enables high resolution endoscopic imaging of the entire small bowel. In addition to diagnosis, DBE permits interventional endo-therapy.

### Innovations and breakthroughs

DBE has found application in the investigation of obscure GI bleeding in adults. This is the first study to assess the usefulness, safety and diagnostic potential of DBE in children.

### Applications

DBE is a valuable diagnostic and therapeutic tool for the investigation of small bowel disease both in adults and in children. DBE is useful in conjunction with other investigative modalities in optimizing diagnostic potential in the small bowel and offering a therapeutic option.

### Peer review

This is a good introductory study on DBE in children.

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## Z-line examination by the PillCam™ SB: Prospective comparison of three ingestion protocols

Ignacio Fernandez-Urien, Erika Borobio, Inmaculada Elizalde, Rebeca Irisarri, Juan Jose Vila, Jesus Maria Urman, Javier Jimenez

Ignacio Fernandez-Urien, Erika Borobio, Inmaculada Elizalde, Rebeca Irisarri, Juan Jose Vila, Jesus Maria Urman, Javier Jimenez, Department of Gastroenterology, Hospital of Navarra, Irunlarrea 3, 31080 Pamplona, Spain

**Author contributions:** Fernandez-Urien I designed the study, wrote the paper and reviewed capsule videos; Borobio E and Elizalde I reviewed capsule videos; Irisarri R and Urman JM collected the data; Vila JJ performed the statistical analysis; Jimenez J reviewed and corrected the paper.

**Correspondence to:** Ignacio Fernandez-Urien, MD, Department of Gastroenterology, Hospital of Navarra, Irunlarrea 3, 31080 Pamplona, Spain. [ifurien@yahoo.es](mailto:ifurien@yahoo.es)

Telephone: +34-948-647612350 Fax: +34-948-848422303

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detected was 71.3, 25.1 and 8.3, in groups A, B and C, respectively (both  $P < 0.001$ ). ETT times were longer in the supine group followed by the right supine and the standing groups (median of 237 s vs 64 s and 39 s, respectively;  $P < 0.001$ ).

**CONCLUSION:** Z-line visualization in patients undergoing SBCE can be accurately achieved in most cases when the capsule is swallowed in the right supine position.

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**Key words:** Barrett; Capsule endoscopy; Esophagus; Gastroesophageal reflux disease; Ingestion; Varices; Z-line

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Fernandez-Urien I, Borobio E, Elizalde I, Irisarri R, Vila JJ, Urman JM, Jimenez J. Z-line examination by the PillCam™ SB: Prospective comparison of three ingestion protocols. *World J Gastroenterol* 2010; 16(1): 63-68 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i1/63.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i1.63>

### Abstract

**AIM:** To evaluate the Z-line visualization by the PillCam™ SB2 using three different ingestion protocols.

**METHODS:** Ninety consecutive patients undergoing small bowel capsule endoscopy (SBCE) between January and May 2008 were included in the study. They swallowed the capsule in the standing (Group A = 30), supine (Group B = 30) and right supine positions (Group C = 30). Baseline patient characteristics, difficulties in capsule ingestion, esophageal transit times (ETT) and Z-line visualization were noted.

**RESULTS:** No significant differences were found between the groups with regard to baseline patient characteristics, ingestion difficulties and complete SB examinations ( $P > 0.05$ ). At least 1 frame of the Z-line was detected in 15.8%, 46.7% and 90% of patients in groups A, B and C, respectively ( $P < 0.001$ ). The average number of Z-line images was  $0.21 \pm 0.53$ ,  $3.23 \pm 6.59$  and  $5.53 \pm 7.55$  and the mean % of the Z-line

### INTRODUCTION

Small bowel examination has recently become possible because of emerging procedures such as capsule endoscopy. As demonstrated by previous studies, capsule endoscopy is an accurate, easy and safe method which allows examination of the entire small bowel in most cases<sup>[1-3]</sup>. Moreover, capsule endoscopy has been demonstrated to be more effective than small bowel follow-through and push-enteroscopy for small bowel exami-



nation<sup>[4-8]</sup>. Nevertheless, images and lesions outside the small bowel (i.e. esophagus, stomach and colon) can also be detected by the capsule<sup>[9-11]</sup>. These images or lesions are sometimes missed by conventional endoscopy<sup>[12,13]</sup> which means that non-small bowel segments of capsule videos should be carefully reviewed by physicians. As the small bowel capsule is usually swallowed in the standing position, the esophageal transit time becomes very short resulting in few images taken in the esophagus. Recent advances in capsule designs have demonstrated that an accurate examination of the esophagus is feasible<sup>[14-18]</sup>. In fact, the esophageal capsule is swallowed by the patient in the supine or right supine positions in order to increase esophageal transit time allowing the capsule to take more images in the esophagus. However, the esophageal capsule battery lasts 20 min on average, which means that only upper gastrointestinal segments, usually including the esophagus and stomach, can be examined. Since the small bowel capsule has longer battery time, the esophagus in addition to the stomach, small bowel and colon, could be explored. Whether esophageal mucosa can be accurately explored by the small bowel capsule in the supine and right supine positions has not been previously studied. The aims of this study were to evaluate and compare the Z-line visualization by the PillCam™ SB in patients undergoing small bowel capsule endoscopy using three different ingestion protocols: standing, supine and right supine positions.

## MATERIALS AND METHODS

### Patients

This study was conducted at a single hospital between January and May 2008. All patients who were not contraindicated to undergo capsule endoscopy, despite procedure indications, were suitable for inclusion in the study. Exclusion criteria were: age < 18 years, swallow and/or esophageal motility disorders and previous prokinetic drugs administration. Patients were randomized, by means of computer-generated random numbers, to swallow the capsule in one of the three different positions: standing (Group A), supine (Group B) and right supine position (Group C).

### Capsule endoscopy

All capsule procedures were performed with the PillCam™ SB2 (Given Imaging Ltd; Yoqneam, Israel). Two CE-experienced gastroenterologists (Fernandez-Urien I and Borobio E) reviewed the videos helped by the latest version of the program RAPID® 5.1.

### Ingestion protocols

All patients underwent capsule endoscopy after an 8-h fast. Prokinetics, laxatives or simethicone were not used, and all patients were asked to drink 100 mL of water before capsule ingestion in order to clear the esophagus of secretions. They were also kindly asked not to talk during the ingestion procedure.

**Standing position (Figure 1A):** Patients from Group A were asked to swallow the capsule in the standing position with a small amount of water if required (no more than 20 mL).

**Supine position (Figure 1B):** Patients from Group B were asked to swallow the capsule in the supine position with a small amount of water if required (no more than 20 mL). They remained in this position for two min and then they were raised to an inclination of 30 degrees for 2 min and 60 degrees for additional 1 min in order to facilitate the transit of the capsule through the esophagus. Then, all patients were asked to drink a small sip of water (10 mL), allowed to sit upright and then asked again to drink 10 mL of water (in order to ensure complete esophageal examinations).

**Right supine position (Figure 1C):** Patients from Group C were asked to swallow the capsule in the right supine position with a small amount of water if required (no more than 20 mL). They remained in this position for 7 min and then were asked to drink small sips of water (10 mL) every 30 s helped by a flexible straw in order to ensure that the capsule reached the distal part of the esophagus. After that, all patients were allowed to sit upright and asked to drink 10 mL of water (in order to ensure complete esophageal examinations).

### Variables analyzed

Baseline patient characteristics, difficulties in capsule ingestion, esophageal transit times (from mouth to stomach) and Z-line visualization were prospectively noted. Difficulties in capsule ingestion were classified as follows: easy when the capsule was swallowed before 1 min and without nausea, difficult when the capsule was swallowed after 1 min and/or with nausea, and impossible when the capsule was not swallowed by the patient. The Z-line visualization was measured on screen using a 4-quadrant scale (Figure 2).

### Sample size

Sample size estimation is not possible in the absence of data regarding the incidence of Z-line visualization with the PillCam™ SB2 ingested in the supine and right supine positions. However, assuming an incidence of Z-line visualization of 10% in the standing position, 35% in the supine position and 70% in the right supine position, 30 patients would be required in each group to detect significant differences (with  $\alpha$  level set at 0.05 and  $\beta$  at 95%).

### Statistical analysis

Data from quantitative variables which did not follow a Gaussian distribution are presented as median and interquartile range (IQR) and compared using the Kruskal-Wallis and the Mann-Whitney tests. Those data from quantitative variables which followed a Gaussian distribution are presented as mean and standard deviation

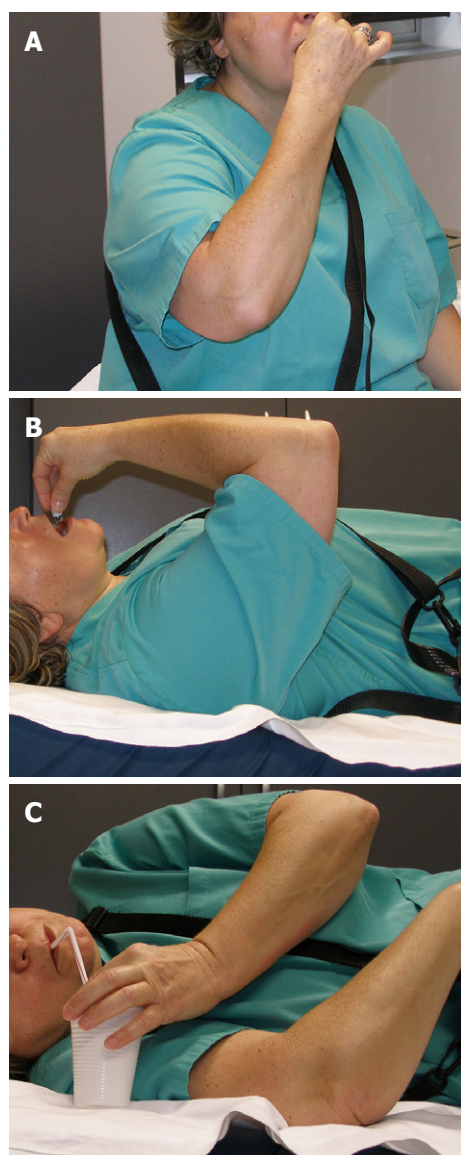


Figure 1 Capsule ingestion in the standing position (A: group A), supine position (B: group B) and right supine position (C: group C).

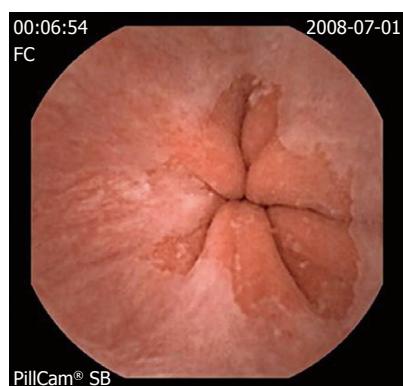


Figure 2 Z-line visualization by the PillCam™ SB.

and compared using ANOVA and Tamhane tests (for post-hoc comparisons, if needed). Qualitative variables (presented as simple proportions) and proportions are compared using the Pearson-Fischer  $\chi^2$  tests. Statistical

Table 1 Baseline patient characteristics

	Group A (standing)	Group B (supine)	Group C (right supine)	P value
n	30	30	30	(-)
Age <sup>1</sup> (yr)	58.6 ± 19.3	56.8 ± 19.1	51.8 ± 18.3	NS
Gender (M:F) <sup>2</sup>	14:16	16:14	17:13	NS
BMI <sup>1</sup> (kg/m <sup>2</sup> )	25.6 ± 2.8	25.2 ± 2.8	25.6 ± 5.8	NS
Indication (%) <sup>2</sup>	OGIB 73.1	OGIB 60	OGIB 66.7	NS
Outpatient (%) <sup>2</sup>	63.2	70	80	NS

<sup>1</sup>ANOVA test; <sup>2</sup>Pearson-Fischer  $\chi^2$  test. OGIB: Obscure gastrointestinal bleeding; NS: Not significant.

Table 2 Capsule ingestion n (%)

	Group A (standing)	Group B (supine)	Group C (right supine)	P value
Easy <sup>1</sup>	29 (96.6)	28 (93.3)	28 (93.3)	NS
Difficult <sup>1</sup>	1 (3.3)	2 (6.6)	2 (6.6)	NS
Impossible <sup>1</sup>	0 (0)	0 (0)	0 (0)	NS

<sup>1</sup>Pearson-Fischer  $\chi^2$  test.

analysis was performed with SPSS version 15.0 (SPSS Inc; Chicago, Ill, USA). Values of  $P < 0.05$  were considered to be statistically significant.

## RESULTS

### Baseline patient characteristics

Baseline patient characteristics are shown in Table 1. There were no statistically significant differences in age, gender, body mass index (BMI), procedure indication and outpatient setting between groups A, B and C ( $P > 0.05$ ).

### Capsule ingestion

Capsule ingestion was possible in all patients (results of capsule ingestion are summarized in Table 2). Capsule ingestion was easy in more than 90% of the patients in all positions. No significant differences were observed between groups A, B and C ( $P > 0.05$ ).

### Esophageal transit time

Esophageal transit times were significantly longer in the supine group followed by the right supine and the standing groups [median (IQR) of 237 s (80-474), 64 s (40-108) and 39 s (24-55), respectively;  $P < 0.001$ ]. Post-hoc comparisons showed that the differences group by group were also statistically significant ( $P < 0.05$ ) (Table 3).

### Z-line visualization

Table 4 shows the results concerning Z-line visualization in the standing, supine and right supine positions. At least one image of the Z-line was detected by the capsule in 15.8%, 37.3% and 90% of patients when the capsule was swallowed in the standing, supine and right supine positions, respectively ( $P < 0.001$ ). Post-hoc comparisons showed that the differences found group by group were also significant ( $P < 0.05$ ). The Z-line was detected by the

**Table 3** Capsule transit times (s)

	Group A (standing)	Group B (supine)	Group C (right supine)	P value
Median (IQR)	39 (24-55)	237 (80-474)	64 (40-108)	< 0.001 <sup>1</sup>
Standing <i>vs</i> supine		Standing <i>vs</i> right supine	Supine <i>vs</i> right supine	
Transit times	$P < 0.001^2$	$P = 0.024^2$	$P < 0.001^2$	(-)

<sup>1</sup>Kruskal-Wallis test; <sup>2</sup>Mann-Whitney test. IQR: Interquartile range.

**Table 4** Z-line visualization

	Group A (standing)	Group B (supine)	Group C (right supine)	P value
%	4/30 (15.8%)	11/30 (37.3%)	27/30 (90%)	< 0.001 <sup>1</sup>
Mean % Z-line	8.68 ± 22.96	25.16 ± 34.52	71.33 ± 33.47	< 0.001 <sup>2</sup>
Mean frames	0.21 ± 0.53	3.23 ± 6.59	5.53 ± 7.55	0.017 <sup>2</sup>
Standing <i>vs</i> supine		Standing <i>vs</i> right supine	Supine <i>vs</i> right supine	
% patients	$P = 0.027^3$	$P < 0.001^3$	$P < 0.001^3$	(-)
Mean % Z-line	NS <sup>4</sup>	$P < 0.001^4$	$P < 0.001^4$	(-)
Mean frames	NS <sup>4</sup>	$P = 0.002^4$	NS <sup>4</sup>	(-)

<sup>1</sup>Pearson-Fischer  $\chi^2$  test; <sup>2</sup>ANOVA test; <sup>3</sup>Pearson-Fischer  $\chi^2$  test; <sup>4</sup>Tamhane test (post-hoc comparisons).

capsule in  $5.5 \pm 7.5$ ,  $3.2 \pm 6.5$  and  $0.2 \pm 0.5$  frames per procedure in the right supine, supine and standing groups. Although these differences were significant ( $P < 0.05$ ), post-hoc comparisons showed that only the differences between the standing and the right supine groups were significant ( $P < 0.05$ ). The mean % of Z-line detected by the capsule was 71.3% in the right supine group, 25.1% in the supine group and 8.6% in the standing group. These differences were also significant ( $P < 0.001$ ) but post-hoc comparisons group by group demonstrated that the differences between the standing and the supine group were not significant ( $P > 0.05$ ).

### Complete small bowel examinations

The cecum was reached by the capsule in 89.5%, 86.2% and 96.7% of cases in the standing, supine and right supine positions, respectively. These differences were not statistically significant ( $P > 0.05$ ).

## DISCUSSION

Wireless capsule endoscopy has opened a new era for small bowel examination. In fact, more than 500 000 capsule procedures have been performed worldwide. Capsule endoscopy offers excellent images of the small bowel but also from the esophagus, stomach and colon in most cases. As demonstrated by some previous studies<sup>[12,13]</sup>, non-small bowel lesions detected by capsule endoscopy are sometimes missed by conventional endoscopy which means that non-small bowel segments of capsule videos should be carefully reviewed by physicians. However, esophageal examination with the PillCam™ SB has been demonstrated not to be feasible in the standing position<sup>[19]</sup> but possible in the supine and

right supine positions as shown with the PillCam™ ESO capsule<sup>[14-18]</sup>. Esophageal images taken by the capsule when it is swallowed in the standing position are not usually enough in terms of number and quality. Since the first small bowel examinations, capsule endoscopy has been performed in the standing position in most institutions and the reason for this seems to be simple, to reach the duodenum as soon as possible to ensure complete small bowel examinations. Currently, the rate of complete examinations is up to 80% in published series<sup>[20]</sup> and it depends on factors such as previous abdominal surgery, patient hospitalization and diabetes. Although there are no references in the literature, it seems that capsule ingestion in the standing position does not improve the rate of complete examinations. Moreover, there is a recent study which concludes that the right supine position after capsule ingestion improves the rate of complete examinations<sup>[21]</sup>. Thus, there are no specific reasons to perform small bowel capsule endoscopy in the standing position.

The main objective of our study which was to analyze the Z-line visualization with the PillCam™ SB in the supine and right supine positions has not been previously analyzed. This new modality of the small bowel capsule endoscopy procedure could optimize the capsule resources without affecting small bowel examinations and patients' tolerability. In fact, we did not find significant differences in the rate of complete small bowel examinations and patients' swallowing difficulties despite their positions during capsule ingestion. Capsule ingestion in the right supine position was significantly more effective for Z-line visualization than the standing and supine positions. On the one hand, our results showed that the Z-line was detected in most patients who swallowed the capsule in the right position. On the other hand, the frequency and the quality of Z-line images taken by the capsule were greater in the right supine position than in the standing and supine positions. Although in some patients it was not completely visualized by the capsule in the right supine position, the Z-line was detected more than 5 times per procedure on average. Therefore, it seems reasonable to affirm that the Z-line was almost completely visualized in most cases. These results are consistent with those previously obtained by esophageal capsule endoscopy<sup>[22,23]</sup>.

Surprisingly, esophageal transit times which were significantly longer in the supine group did not affect the Z-line visualization. More time in the esophagus did not mean more and better images from the Z-line. A reasonable explanation for this may be the position of the His angle at the gastroesophageal junction. While the capsule remains too long in the mid and distal esophagus but far away from the Z-line when is swallowed in the supine position, it rapidly reaches the distal esophagus but is kept by the His angle over the Z-line for several seconds in the right supine position. Therefore, the right supine position seems to be anatomically optimal for Z-line examination. Moreover, a previous study by Gralnek *et al*<sup>[22]</sup> in healthy volunteers using the PillCam™ ESO, tested



several ingestion procedures including standing, supine, right supine and left supine positions, concluding that the right supine position was the best approach to explore the distal esophagus.

Several studies have previously evaluated the feasibility of capsule endoscopy in the evaluation of the esophagus, however, the majority of them employed the PillCam™ ESO. The PillCam ESO and the ESO2 offer excellent images of more than 75% of the Z-line in most patients<sup>[22,23]</sup>. However, to our knowledge, there is only one study which has evaluated the role of the small bowel capsule for esophageal examinations<sup>[19]</sup>. In that study, an adequate assessment of the Z-line (50% and 100% of the circumference) was achieved in 10.4% and 0% of patients in the standing position and in 12.5% and 37.5% of patients in the supine position. Therefore, the authors concluded that esophageal examinations using small bowel capsule endoscopy was not feasible. Our results in patients who swallowed the capsule in the standing and supine positions are consistent with those obtained in that study, however, those authors did not include the right supine position as an additional comparative arm.

In this situation, the main question is: should all patients undergoing capsule endoscopy, despite indications, swallow the capsule in the right supine position? The answer is probably yes, because this alternative is easy to perform, is not uncomfortable for the patient, is not time consuming for physicians and the most importantly, it offers excellent images of the Z-line in most cases. However, the PillCam™ SB has to demonstrate that it is accurate in detecting esophageal lesions such as gastroesophageal reflux disease (GERD) lesions or varices. Other capsule prototypes such as the PillCam ESO capsule have demonstrated a high diagnostic accuracy for detecting GERD lesions, Barrett's esophagus and esophageal varices<sup>[14-16]</sup>. Nevertheless, it has to be taken into account that this capsule prototype takes 14 images per second and the PillCam SB, only 2 per second. Therefore, future studies in patients with suspected esophageal diseases should be performed. If favourable results are obtained, then this alternative should be used in all capsule procedures including small bowel, colon and of course, esophageal examinations.

## COMMENTS

### Background

Capsule endoscopy has become a very important tool for small bowel examination. However, images from other parts of the gastrointestinal (GI) tract, can also be detected by the capsule. These images or lesions are sometimes missed by conventional endoscopy, which means that images from the esophagus, stomach and colon should be carefully reviewed.

### Research frontiers

Esophageal examination is not feasible if the capsule is ingested in the standing position as shown by previous studies. With recent prototypes designed for esophageal examination, new ingestion protocols have been evaluated. The supine and right supine positions have been demonstrated to be good positions to achieve a good esophageal examination. Whether the small bowel capsule is capable of examining the esophagus in these positions has not been previously studied.

### Innovations and breakthroughs

This study demonstrates that the PillCam SB can accurately explore the Z-line when it is ingested in the supine and right supine positions.

### Applications

Esophageal examination could be of interest in those patients who undergo capsule endoscopy of the small bowel. Missed lesions in the esophagus by conventional endoscopy could be detected by the capsule if it is ingested in the right supine position.

### Peer review

This study demonstrate that Z-line examination is those patients undergoing small bowel capsule endoscopy is feasible if the capsule is ingested in the right supine position.

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## Systematic review of randomised controlled trials: Probiotics for functional constipation

Anna Chmielewska, Hania Szajewska

Anna Chmielewska, Hania Szajewska, Department of Paediatrics, The Medical University of Warsaw, 01-184 Warsaw, Dzialdowska 1, Poland

**Author contributions:** Szajewska H wrote the initial protocol of the review; Chmielewska A and Szajewska H were responsible for the literature search, study selection, methodological quality assessment, and data extraction; Szajewska H and Chmielewska A conducted the analyses and wrote the manuscript; Both authors contributed to the data interpretation; Szajewska H and Chmielewska A wrote the final report, which was approved by both authors; Szajewska H is the guarantor.

Supported by The Medical University of Warsaw, Poland  
Correspondence to: Hania Szajewska, MD, Department of Paediatrics, The Medical University of Warsaw, 01-184 Warsaw, Dzialdowska 1, Poland. [hania@ipgate.pl](mailto:hania@ipgate.pl)

Telephone: +48-22-4523309 Fax: +48-22-4523309  
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Lcr35, but not *L. rhamnosus* GG, showed a beneficial effect.

**CONCLUSION:** Until more data are available, we believe the use of probiotics for the treatment of constipation condition should be considered investigational.

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**Key words:** Randomised controlled trials; Constipation; Probiotics; Adults; Children

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### Abstract

**AIM:** To systematically evaluate and update evidence on the efficacy and safety of probiotic supplementation for the treatment of constipation.

**METHODS:** The MEDLINE, EMBASE, CINAHL, and Cochrane Library databases were searched in May 2009 for randomised controlled trials (RCTs) performed in paediatric or adult populations related to the study aim.

**RESULTS:** We included five RCTs with a total of 377 subjects (194 in the experimental group and 183 in the control group). The participants were adults (three RCTs,  $n = 266$ ) and children (two RCTs,  $n = 111$ ) with constipation. In adults, data suggests a favourable effect of treatment with *Bifidobacterium lactis* DN-173010, *Lactobacillus casei* Shirota, and *Escherichia coli* Nissle 1917 on defecation frequency and stool consistency. In children, *L. casei rhamnosus*

### INTRODUCTION

Constipation is a common condition affecting children and adults<sup>[1,2]</sup>. In the vast majority of cases, no underlying organic cause is found and functional constipation is diagnosed<sup>[3,4]</sup>. The standard treatment consists of disimpaction and the administration of laxatives to achieve a normal bowel habit of passing a soft stool without pain. Even though traditional treatment is well established and safe, for many patients it does not provide satisfying improvement, prompting interest in other therapeutic strategies<sup>[5]</sup>.

Currently, probiotics, defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host<sup>[6]</sup>, are increasingly being used in the management of constipation. Those most widely studied are organisms within the genera *Bifidobacterium*

and *Lactobacillus*. There are several reasons why probiotics might have therapeutic potential for the treatment of constipation. Firstly, there are data demonstrating differences in the intestinal microbiota between healthy individuals and patients with chronic constipation<sup>[7,8]</sup>. The key features are an increased number of clostridia and bifidobacteria, with different species of clostridia and enterobacteriaceae being frequently isolated. A number of key questions remain to be answered, principally, what is the origin of this dysbiosis? Is dysbiosis a secondary manifestation of constipation, or is it a factor contributing to constipation? Secondly, studies involving the administration of *B. lactis* DN-173 010 have shown improved colonic transit times, both in a healthy population<sup>[9]</sup> and in constipated patients<sup>[10]</sup>. Finally, probiotics lower the pH in the colon. This reduction in pH is due to the bacterial production of short-chain fatty acids (butyric acid, propionic acid, and lactic acid). A lower pH enhances peristalsis in the colon<sup>[8]</sup> and, subsequently, might decrease the colonic transit time.

In view of the uncertainty regarding the use of probiotics for the treatment of constipation, we decided to systematically review and update data from randomised controlled trials (RCTs) on the efficacy and safety of using probiotics for the treatment of constipation in both paediatric and adult populations. If the probiotics were effective, another aim was to determine what strain(s) of probiotic microorganisms is the most effective.

## MATERIALS AND METHODS

The guidelines from the Cochrane Collaboration for undertaking and reporting the results of this systematic review were followed<sup>[11]</sup>. Briefly, we searched three electronic bibliographic databases (MEDLINE, EMBASE, and CINAHL) and the Cochrane Library. Every database was searched from inception to May 2009. Additionally, the reference lists from identified studies and key review articles assessing the effects of probiotics on the treatment of constipation were searched. While no language restrictions were applied, in practice the search was restricted to English-language papers, papers written in languages known to the reviewers, or those with English-language abstracts. The review was restricted to RCTs only carried out in paediatric or adult populations. Participants in the experimental groups received any well-defined probiotic at any dosage regimen for at least several days; those in the control group received placebo or no intervention. The search strategy included the use of a validated filter for identifying RCTs, which was combined with a topic-specific search strategy. In brief, the search terms were: *constipation AND probiotic\**, *Lactobacillus*, *L. GG*, *L. acidophilus*, *L. rhamnosus*, *L. plantarum*, *L. casei*, *L. gasseri*, *L. reuteri*, *L. lactis*, *Bifidobacterium*, *B. breve*, *B. longum*, *B. infantis*, *B. adolescentis*, *B. lactis*, *Bacillus*, *Clostridium butyricum*, *Streptococcus thermophilus*, *Escherichia coli*, *Propionibacterium freundensisreichii*, *Enterococcus SF68*,

*Enterococcus faecalis*, *Saccharomyces boulardi*, and *VSL#3*. The primary clinical outcome measure was treatment success (as defined by the investigators). In addition, a priori it was decided to extract other data reported by the investigators if clinically relevant to the current review and/or adverse effects. All of the published studies that met our eligibility criteria were assessed for methodological quality, with the following strategies associated with good-quality studies: adequate generation of allocation sequences; concealment of allocation; blinding of investigators, participants, outcome assessors, and data analysts; intention-to-treat analysis (yes or no); and comprehensive follow-up ( $\geq 80\%$ ).

Data extraction was performed using standard data-extraction forms. For dichotomous outcomes, the total number of participants and the number of participants who experienced the event were extracted. For continuous outcomes, the total number of participants and the means and standard deviations were extracted. If feasible, the data were entered into Review Manager (RevMan) (Computer program. Version 5.0. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2007) for analysis.

## Statistical methods

As the studies we identified were not sufficiently similar and of sufficient quality we did not perform a meta-analysis. The binary measure for individual studies is reported as the risk ratio (RR) between the experimental and control groups with 95% confidence intervals (95% CI). The mean difference (MD) between the treatment and control groups was selected to represent the difference in continuous outcomes (with 95% CI).

A priori defined subgroup analyses were planned based on factors that could potentially influence the magnitude of the treatment effect, such as the probiotic strain or study population (children, adults); however, these analyses were not performed due to the limited data available.

## RESULTS

By means of our systematic search, we identified six trials<sup>[12-17]</sup>. One was a protocol of an ongoing study<sup>[15]</sup>, so it was not included. Thus, eventually five trials, including a total of 377 subjects (194 in the experimental group and 183 in the control group), met our predefined inclusion criteria. The characteristics of the included studies are presented in Table 1. The list of excluded trials ( $n = 22$ ) is available upon request. The most usual reason for exclusion of a study was that the study was not randomised, the study was carried out in healthy volunteers, or the intervention was treatment with a symbiotic, not a probiotic alone. In addition, some studies were published in Japanese with no English abstract, and thus, were not accessible to the reviewers, even for initial screening.

All of the trials were full peer-reviewed publications.

Table 1 Characteristics of included trials

Study ID	Probiotic	Design	Allocation concealment/ Blinding/Intention-to-treat analysis/Description of withdrawals or dropouts	Exp/cont (age, yr)	Definition of constipation	Duration of intervention	Intervention (daily dose)	Placebo
Studies in adults								
Mollenbrink <i>et al</i> <sup>[13]</sup> 1994 (Germany)	<i>E. coli</i> Nissle 1917	RCT, crossover	Unclear/Yes/No/Yes	35/35 (18-60)	< 2 BM per week	4 + 4 wk	25 × 10 <sup>9</sup> CFU	Placebo
Koebnick <i>et al</i> <sup>[12]</sup> 2003 (Germany)	<i>L. casei</i> Shirota	RCT, parallel	Unclear/Yes/Yes/Yes	35/35 (18-70)	Not provided	4 wk	6.5 × 10 <sup>9</sup> CFU, probiotic beverage	Placebo
Yang <i>et al</i> <sup>[14]</sup> 2008 (China)	<i>B. lactis</i> DN-173 010	RCT, parallel	Unclear/No/Yes/Yes	63/63 (25-65, only women)	< 3 BM per week; increased stool hardness; non-organic constipation and habitual constipation	2 wk	Fermented milk containing 1.25 × 10 <sup>10</sup> CFU of probiotic plus yoghurt strains	Placebo (acidified milk without any ferments or probiotics)
Studies in children								
Banaszkiewicz <i>et al</i> <sup>[17]</sup> 2005 (Poland)	<i>L. rhamnosus</i> GG	RCT, parallel (computer- generated randomisation list)	Yes/Yes/Yes/Yes	43/41 (2-16)	< 3 BM per week during 14 days for at least 12 wk	12 wk	Lactulose plus LGG 2 × 10 <sup>9</sup> CFU	Lactulose plus placebo
Bu <i>et al</i> <sup>[16]</sup> 2007 (Taiwan)	<i>L. casei</i> <i>rhamnosus</i> Lcr35	RCT, parallel (computer- generated randomisation list)	Yes/Yes/Yes/Yes	18/9 (< 10)	< 3 BM per week for > 2 mo plus at least one of the criteria: anal fissures with bleeding due to constipation, faecal soiling, or passage of large and hard stool	4 wk	8 × 10 <sup>8</sup> CFU	Placebo (starch)

RCT: Randomised controlled trials; CFU: Colony-forming units; BM: Bowel movements.

Four of the included studies were RCTs with a parallel design, and the remaining included RCT had a crossover design. All were placebo-controlled trials. The participants were adults (three RCTs,  $n = 266$ ) and children (two RCTs,  $n = 111$ ) with constipation defined as stated in Table 2. The following different probiotic strains were tested: *Bifidobacterium lactis* DN-173010, *E. coli* Nissle 1917, *Lactobacillus casei rhamnosus* Lcr35, *L. casei* Shirota, and *L. rhamnosus* GG. One RCT assessed the effectiveness of using *L. rhamnosus* GG as an adjunct to lactulose therapy compared with treatment with lactulose alone<sup>[17]</sup>. The durations of the interventions in the parallel-design studies were two weeks in one study, four weeks in two studies, and 12 wk in one study. The duration of the intervention in the crossover design study was eight weeks. The doses of the probiotic used ranged from  $8 \times 10^8$  to  $25 \times 10^9$  colony-forming units (CFU)/d.

The methodological quality of the trials varied. While all were randomised trials, the randomisation method was described and adequate in only two RCTs<sup>[16,17]</sup>. Except for one study<sup>[14]</sup>, double blinding was applied in the remaining RCTs. An adequate description of the intention-to-

treat analysis was provided in all but one study<sup>[13]</sup>. The withdrawals and dropouts were described adequately in all of the studies, and all included an adequate number (i.e.  $\geq 80\%$ ) of participants in the final analysis.

### Study description

**RCTs in adults:** The study by Mollenbrink and Bruck-schen<sup>[13]</sup> was a single-centre, randomised, double-blind, crossover trial that investigated the efficacy of treating 70 constipated patients with *E. coli* Nissle 1917 or placebo. After four weeks of treatment, there was a significant difference in the mean number of stools per week in the *E. coli* group compared with the placebo group ( $4.9 \pm 1.5$  vs  $2.6 \pm 1.0$ , respectively, MD 2.3 stools per week, 95% CI 1.7 to 2.9), which also remained significant at eight weeks ( $6 \pm 1.3$  vs  $1.9 \pm 1.5$ , respectively, MD 4.1, 95% CI 3.2 to 5). This study also revealed a significant difference between the probiotic and the control group in the incidence of hard stools (2/34 vs 16/30, respectively, RR 0.1, 95% CI 0.03 to 0.4). Both the effectiveness and tolerance of the treatment, as assessed both by a physician and the patients, were significantly better in



Table 2 The summary of study outcomes

Study ID	Probiotic	Outcomes
<b>Studies in adults</b>		
Mollenbrink <i>et al</i> <sup>[13]</sup> 1994 (Germany)	<i>E. coli</i> Nissle 1917 <sup>1</sup>	Number of stools per week [week 4: 4.9 ± 1.5 <i>vs</i> 2.6 ± 1.0, MD 2.3 (95% CI 1.7 to 2.9); week 8: MD 4.1 (95% CI 3.2 to 5)] Hard stools [2/34 <i>vs</i> 16/30, RR 0.1 (95% CI 0.03 to 0.4) ( <i>P</i> < 0.001)] Effectiveness of a probiotic compared to placebo assessed by physicians: 55.9% <i>vs</i> 6.7% Effectiveness of a probiotic compared to placebo assessed by patients: 52.9% <i>vs</i> 6.7% ( <i>P</i> < 0.001) Tolerance of a probiotic compared to placebo assessed by physicians: 58.85% <i>vs</i> 26.7% ( <i>P</i> = 0.01) Tolerance of a probiotic compared to placebo assessed by patients: 50% <i>vs</i> 26.7% ( <i>P</i> = 0.03)
Koebnick <i>et al</i> <sup>[12]</sup> 2003 (Germany)	<i>L. casei</i> Shirota <sup>2</sup>	Occurrence of moderate and severe constipation ( <i>P</i> < 0.001) Degree of constipation ( <i>P</i> = 0.003) Defecation frequency ( <i>P</i> = 0.004) Occurrence of hard stools ( <i>P</i> < 0.001) Degree of stool consistency ( <i>P</i> < 0.001) Occurrence of flatulence (NS) Degree of flatulence (NS) Occurrence of bloating (NS) Degree of bloating (NS)
Yang <i>et al</i> <sup>[14]</sup> 2008 (China)	<i>B. lactis</i> DN-173 010 <sup>1</sup>	Stool frequency ( <i>n</i> /wk) [week 1: 3.5 ± 1.5 <i>vs</i> 2.5 ± 0.9, MD 1 (95% CI 0.6 to 1.4); week 2: 4.1 ± 1.7 <i>vs</i> 2.6 ± 1.0; MD 1.5 (95% CI 0.7 to 1.6)] Defecation condition scores [week 1: 1.1 ± 0.9 <i>vs</i> 1.6 ± 1.1, MD -0.5 (95% CI -0.85 to -0.18); week 2: 0.8 ± 1.0 <i>vs</i> 1.6 ± 1.1; MD -0.8 (95% CI -1.14 to -0.44)] Grade I (0 points)-normal defecation Grade II (1 point)-only bearing down and uncomfortable sensation Grade III (2 points)-obvious bearing down and uncomfortable sensation, or frequent defecation with difficult and little defecation, seldom abdominal pain or anal burning sensation Grade IV (3 points)-often abdominal pain or anal burning sensation to influence defecation Stool consistency scores (according to classification method of Bristol) [week 1: 1.0 ± 0.8 <i>vs</i> 1.4 ± 1.0, MD -0.4 (95% CI -0.73 to -0.12); week 2: 0.6 ± 0.8 <i>vs</i> 1.3 ± 1.0, MD -0.7 (95% CI -1 to -0.4)] Grade I (0 points)-like sausage or snake, smooth and soft; like sausage, with fissure on the surface Grade II (1 point)-sausage-shaped, with lumps; noncohesive lumps, with coarse edges Grade III (2 points)-separating hard lumps, like fruit kernel (difficult discharge)
<b>Studies in children</b>		
Banaszkiewicz <i>et al</i> <sup>[17]</sup> 2005 (Poland)	<i>L. rhamnosus</i> GG <sup>2</sup>	Treatment success ( $\geq 3$ spontaneous BMs per week with no episodes of faecal soiling) (NS) Number of BMs per week (NS) Number of episodes of faecal soiling per week (NS) Straining at defecation frequency per week (NS)
Bu <i>et al</i> <sup>[16]</sup> 2007 (Taiwan)	<i>L. casei</i> rhamnosus Lcr35 <sup>1</sup>	Treatment success ( $\geq 3$ spontaneous BMs per week with no episodes of faecal soiling in the fourth week) (14/18 <i>vs</i> 1/9, RR 7, 95% CI 1.1 to 45; <i>P</i> = 0.01) Defecation frequency (times/d) (0.57 ± 0.17 <i>vs</i> 0.37 ± 0.1; MD 0.2, 95% CI 0.1 to 0.3) ( <i>P</i> = 0.03) Hard stool (%) (22.4 ± 14.7 <i>vs</i> 75.5 ± 6.1; MD -53% (95% CI -63 to -43) ( <i>P</i> = 0.01) Abdominal pain (times) (1.9 ± 1.6 <i>vs</i> 6.7 ± 3.3; MD -4.8, 95% CI -6.6 to -3) ( <i>P</i> = 0.03) Use of glycerin enema (times) (1.6 ± 1.9 <i>vs</i> 4.0 ± 2.1; MD -2.4, 95% CI -4 to -0.8) ( <i>P</i> = 0.04) Use of lactulose (times) (4.4 ± 3.6 <i>vs</i> 6.2 ± 3.8; MD -1.8, 95% CI -4.7 to 1.1) ( <i>P</i> = 0.66) Faecal soiling (times) (2.1 ± 3.8 <i>vs</i> 2.7 ± 1.4, MD -0.6 (95% CI -3.2 to 2) ( <i>P</i> = 0.95) Change of appetite (0.7 ± 0.8 <i>vs</i> 0.7 ± 0.6; MD 0, 95% CI -0.6 to 0.6) ( <i>P</i> = 0.81)

<sup>1</sup>Mean values are presented for the experimental group and control group, respectively; <sup>2</sup>Comparisons of experimental and control group. NS: Not significant.

those in the *E. coli* group. The authors concluded that *E. coli* Nissle 1917 is successful in the therapy of idiopathic chronic constipation.

The study by Koebnick *et al*<sup>[12]</sup> was a single-centre, double-blind, placebo-controlled, randomised trial involving 70 patients with symptoms of chronic constipation. All of the patients received either a probiotic beverage containing *L. casei* Shirota or placebo for four weeks. Patients completed a questionnaire related to their gastrointestinal symptoms, well-being, and stool habits, and underwent a medical examination weekly. The severity of constipation, flatulence, and bloating was divided into four categories (severe, moderately severe, mild, and no symptoms). Compared to the placebo group, those randomised to the *L. casei* Shirota group experienced a significant improvement

in the self-reported severity of constipation and stool consistency. That is, they experienced significant reductions in the occurrence of moderate and severe constipation (*P* < 0.001), the degree of constipation (*P* = 0.003), and the occurrence of hard stools (*P* < 0.001), and increased their defecation frequency (*P* = 0.004). However, the occurrence and degree of flatulence or bloating sensation did not significantly differ between the groups.

In the most recent study, Yang *et al*<sup>[14]</sup> administered a fermented milk product containing *B. lactis* DN-173 010 and some yoghurt strains (*S. thermophilus* and *L. bulgaricus* (1.2 × 10<sup>9</sup> CFU/pot 100 g) (experimental group) or an acidified milk containing non-living bacteria but no *B. lactis* DN-173 010 or yoghurt strains (control group) for two weeks to constipated women. Comparison of the experi-

mental group with the control group revealed a significantly higher stool frequency after one week of product administration ( $3.5 \pm 1.5$  *vs*  $2.5 \pm 0.9$ , respectively, MD 1.0 stool per week, 95% CI 0.6 to 1.4) and at two weeks ( $4.1 \pm 1.7$  *vs*  $2.6 \pm 1.0$ , respectively, MD 1.5 stool per week, 95% CI 0.7 to 1.6). The extent of defecation difficulty was assessed as 0-3 point defecation condition scores. In brief, 0 points indicates normal defecation, while 3 points indicates often abdominal pain or anal burning sensation to influence defecation. (Table 2 for complete categorisation of defecation condition scores). Both at one and two weeks after product consumption, there was a significant improvement in the defecation condition scores in the experimental group compared with the control group:  $1.1 \pm 0.9$  *vs*  $1.6 \pm 1.1$ , respectively (MD -0.5, 95% CI -0.85 to -0.18) at 1 wk and  $0.8 \pm 1.0$  *vs*  $1.6 \pm 1.1$ , respectively (MD -0.79, 95% CI -1.14 to -0.44) at 2 wk. The stool consistency score was determined according to the Bristol Stool Scale. In brief, 0 points indicates stools like a sausage or a snake, smooth and soft, while 2 points indicates separating hard lumps, like fruit kernel (difficult discharge) (Table 2). The stool consistency scores were significantly improved in the *B. lactis* DN-173010 group compared to the control group at 1 wk ( $1.0 \pm 0.8$  *vs*  $1.4 \pm 1.0$ , respectively, MD -0.4, 95% CI -0.73 to -0.12) and at 2 wk ( $0.6 \pm 0.8$  *vs*  $1.3 \pm 1.0$ , respectively, MD -0.7, 95% CI -1 to -0.4). There were no significant differences between groups in food intake and safety parameters. The researchers concluded that the administration of a fermented milk product containing *B. lactis* DN-173010 has a beneficial effect on stool frequency, defecation conditions, and stool consistency in adult women with constipation.

**RCTs in children:** Only two RCTs have addressed the use of probiotics in the treatment of constipation in children. In the study by Banaszekiewicz and Szujewska<sup>[17]</sup>, 84 children (aged: 2-16 years) with constipation ( $< 3$  spontaneous bowel movements per week for at least 12 wk) were enrolled in a double-blind, randomised, placebo-controlled trial in which they received 1 mL/kg per day of 70% lactulose plus  $10^9$  CFU of *L. rhamnosus* GG (experimental group,  $n = 43$ ) or a lactulose-containing placebo (control group,  $n = 41$ ) orally twice daily for 12 wk. The primary outcome measure was treatment success; all analyses were performed on an intention-to-treat basis. Treatment success was defined as  $\geq 3$  spontaneous bowel movements per week with no episodes of faecal soiling. Treatment success was similar in the control and experimental groups at 12 wk [28/41 (68%) *vs* 31/43 (72%), respectively;  $P = 0.7$ ] and at 24 wk [27/41 (65%) *vs* 27/43 (64%), respectively;  $P = 1.0$ ]. The groups also did not differ in their mean number of spontaneous bowel movements per week or episodes of faecal soiling per week at four, eight, and 12 wk. Adverse events and overall tolerance did not differ between groups. It was concluded that *L. rhamnosus* GG, as dosed in this study, was not an effective adjunct to lactulose in treating constipation in children.

The study by Bu *et al.*<sup>[16]</sup> evaluated the effect of treating children with chronic constipation with *L. casei rhamnosus* Lcr35 compared to magnesium oxide (MgO) or placebo; however, only the latter comparison is valid for this systematic review. For those treated with the probiotic ( $n = 18$ ) compared with placebo ( $n = 9$ ), the trial showed an increase in the treatment success defined as  $\geq 3$  spontaneous defecations per week with no episodes of faecal soiling (14/18 *vs* 1/9, respectively, RR 7, 95% CI 1.1 to 45), an increase in the defecation frequency (times/d) ( $0.57 \pm 0.17$  *vs*  $0.37 \pm 0.10$ , respectively, MD 0.2, 95% CI 0.1 to 0.3), a reduction in abdominal pain (frequency) ( $1.9 \pm 1.6$  *vs*  $6.7 \pm 3.3$ , respectively, MD -4.8, 95% CI -6.6 to -3), a reduction in the use of glycerin enemas during the four weeks of therapy (frequency) ( $1.6 \pm 1.9$  *vs*  $4.0 \pm 2.1$ , respectively, MD -2.4, 95% CI -4 to -0.8), and a decrease in the percentage of hard stools in the total number of defecations ( $22.4 \pm 14.7$  *vs*  $75.5 \pm 6.1$ , respectively, MD -53%, 95% CI -63 to -43). However, there was no difference between groups in the use of lactulose or the number of episodes of faecal soiling. No change in appetite was observed. However, the sample size was too small to draw any meaningful conclusion.

### Adverse events

The probiotics were well tolerated, and no adverse events associated with this supplementation were reported in any of the trials.

## DISCUSSION

### Principal findings

The objective of this review was to provide some resolution to the uncertainty regarding the use of probiotics for the treatment of functional constipation in paediatric and adult populations. The main finding of the review is that there is very limited evidence available from controlled trials to evaluate with certainty the effect of probiotic administration on constipation. Data published to date suggest that adults with constipation might benefit from ingestion of *B. lactis* DN-173010, *L. casei* Shirota, and *E. coli* Nissle 1917, which were shown to increase defecation frequency and improve stool consistency. However, in some cases, even if there was a significant difference in results, their clinical relevance is unclear. For example, compared with placebo, *B. lactis* DN-173010 increased only by one the number of stools per week. In children, the administration of *L. rhamnosus* GG was not effective, while the administration of *L. casei rhamnosus* Lcr35 augmented the number of stools and reduced the number of hard stools. Again, although the results were statistically significant, the overall effects were clinically modest. All of the conclusions are based on single studies, some of which had a very small number of participants and methodological limitations; thus, the conclusions should be interpreted with great caution. Repeat studies with the probiotic strains that have been proven effective are needed. A paucity of data did not allow us to con-

clude whether any particular probiotic is more effective than another.

### Previous reports

Previously, one systematic review<sup>[18]</sup>, co-authored by one of the authors of the current review, aimed at determining the effect of probiotics on constipation was performed. This systematic review, published in 2005 (search date: January 2004), identified two RCTs with a total of 140 adult participants. It was concluded that the administration of two probiotic strains (*E. coli* Nissle 1917, *L. casei* Shirota) significantly improved stool frequency and consistency, with no difference in the degree of bloating or flatulence; no adverse effects were reported. Our updated results include results from more RCTs, thus, more precisely define the effects of using probiotics for the treatment of constipation.

Evidence from non-RCTs suggests that at least some probiotics may be effective. For example, in children with constipation defined according to the Rome III criteria, the administration of *Bifidobacteria* (*B. bifidum*, *B. infantis*, and *B. longum*) and *Lactobacilli* (*L. casei*, *L. plantarum*, and *L. rhamnosus*) to 20 children aged 4-16 years resulted in an increased frequency of bowel movements, a decreased number of faecal incontinence episodes, and reduced abdominal pain, although there was no change in stool consistency<sup>[19]</sup>. In adults, preliminary data from a non-RCT revealed that the administration of *L. rhamnosus* and *Propionibacterium freudenreichii* resulted in a small, but significant, increase in defecation frequency<sup>[20]</sup>. However, this result was only true if the probiotics were administered together and not if only a single strain was given.

### Mechanism of action

Mechanisms by which probiotics might work in the treatment of constipation have been briefly discussed in the Introduction section. Clearly, they are not well understood. Perhaps the best mechanism documented is the mechanism by which *B. animalis* DN-173 010 exerts its effects. In healthy subjects, several RCTs have evaluated the effect of *B. animalis* DN-173 010 on colonic transit times. One double-blind RCT conducted in 72 healthy adults (aged 21-42 years) used radio-opaque pellets to measure colonic transit times. This study revealed a statistically significant reduction in the total colonic transit time of 21% (men:  $P < 0.03$ , women:  $P < 0.05$ ) and a reduction in the sigmoid transit time of 39% ( $P = 0.02$ ), particularly in women, with probiotic treatment. However, the beneficial effect was limited to the subjects who received living *B. animalis* DN-173 010 and was not observed in those who received heat-treated *B. animalis* DN-173 010<sup>[21]</sup>. Another double-blind RCT performed in 36 healthy women (aged 18-45 years) revealed significantly shorter total colonic and sigmoid colonic transit times ( $P < 0.05$ ) following ingestion of 375 g/d of a fermented milk containing yoghurt cultures plus *B. animalis* DN-173 010 for 10 d, compared with

the transit times observed with ingestion of the control probiotic-free product<sup>[22]</sup>. Two further non-blinded RCTs were carried out in healthy elderly subjects who were divided into groups according to their different baseline colonic transit times. Both studies demonstrated a reduction in transit times in all of the groups compared with baseline with consumption of fermented milk containing *B. animalis* DN-173 010<sup>[23,24]</sup>. Further studies are needed to confirm these findings.

### Strengths and limitations

The advantage of any systematic review is the low risk of subjective data selection. Study searches, assessment, and data synthesis were all based on predefined criteria and were performed with the use of well-established repetitive tools by two reviewers independently. Nevertheless, our analysis has some limitations. First, we cannot fully exclude publication bias, i.e. publication or non-publication of data depending on the results, with negative findings being less likely to be published irrespective of the methodological quality. As studies involving the administration of probiotics are often supported by the manufacturers, the possibility remains that negative results remain unpublished. No sufficiently effective strategy of identifying such studies has been developed. Second, even though no language limitation was imposed, in practice it was not feasible to assess data from reports written in Japanese. Third, any systematic review is only as good as the constituent studies. Only some of the trials included in our analysis seemed methodologically sound. Potential limitations included unclear or inadequate allocation concealment, no intention-to-treat analysis, and no blinding. Fourth, some trials included a small sample size. Finally, the effects of probiotics are strain specific as well as population specific. While a systematic review or a meta-analysis on probiotics does provide valid information, caution should be exercised in not over interpreting the results of a meta-analysis, particularly when all probiotics have been evaluated together.

### Safety issues

In general, the safety profile of probiotics seems to be good. In the included trials, no adverse effects were noted. The safety issue is important, as based on the available literature there is concern that the use of probiotics in at-risk populations may result in harmful events. Most complications have occurred in immunocompromised subjects or in patients with other life-threatening illnesses, who were managed in intensive care units and treated with probiotics.

In summary, this systematic review demonstrates that the data published to date do not yet provide sufficient scientific evidence to support a general recommendation about the use of probiotics in the treatment of functional constipation. Until such data are available, we believe that the use of probiotics for this condition should be considered investigational. Also, we believe that our demonstration of clinical uncertainty about this issue is



an important finding. As pointed out by Alderson and Roberts<sup>[25]</sup>, clinical uncertainty is a prerequisite for the large-scale RCTs needed to evaluate the influence of such interventions; it also helps to clarify available treatment options and stimulate new and better research.

## COMMENTS

### Background

Probiotics are increasingly being used in pediatric population. However, there is still uncertainty regarding the use of probiotics for the treatment of constipation.

### Research frontiers

Until more data are available, the use of probiotics for the treatment of constipation condition should be considered investigational. The large-scale RCTs are needed to evaluate the effect of specific probiotic strain(s) for the treatment of constipation.

### Innovations and breakthroughs

The updated results include results from more RCTs; thus, more precisely define the effects of using probiotics for the treatment of constipation.

### Applications

Until such data are available, the use of probiotics for this condition should be considered investigational.

### Peer review

This manuscript describes a systematic review of randomised controlled trials that evaluated the efficiency of probiotics in the treatment of functional constipation.

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## Incidental findings at MRI-enterography in patients with suspected or known Crohn's disease

Michael Dam Jensen, Torben Nathan, Jens Kjeldsen, Søren Rafael Rafaelsen

Michael Dam Jensen, Torben Nathan, Department of Internal Medicine, Section of Gastroenterology, Vejle Hospital part of Lillebaelt Hospital, Kabbeltøft 25, DK-7100 Vejle, Denmark  
Jens Kjeldsen, Department of Medical Gastroenterology, Odense University Hospital, Sdr. Boulevard 29, DK-5000 Odense, Denmark

Søren Rafael Rafaelsen, Department of Radiology, Vejle Hospital part of Lillebaelt Hospital, Kabbeltøft 25, DK-7100 Vejle, Denmark

**Author contributions:** Jensen MD, Nathan T, Kjeldsen J and Rafaelsen SR designed the research; Jensen MD performed the data collection and data analysis; Jensen MD drafted the article; Nathan T, Kjeldsen J and Rafaelsen SR critically revised the article; Jensen MD, Nathan T, Kjeldsen J and Rafaelsen SR approved the final version.

**Correspondence to:** Michael Dam Jensen, MD, Department of Internal Medicine, Section of Gastroenterology, Vejle Hospital part of Lillebaelt Hospital, Kabbeltøft 25, DK-7100 Vejle, Denmark. [michael.dam.jensen@slb.regionsyddanmark.dk](mailto:michael.dam.jensen@slb.regionsyddanmark.dk)  
Telephone: +45-79-406341 Fax: +45-79-406887

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### Abstract

**AIM:** To determine the frequency and clinical impact of incidental findings detected with magnetic resonance imaging (MRI)-enterography in patients with suspected or known Crohn's disease (CD).

**METHODS:** Incidental findings were defined as unexpected lesions outside the small intestine, not previously known or suspected at the time of referral, and not related to inflammatory bowel disease. Through a systematic review of medical charts we analyzed the clinical impact of incidental findings, and compared the MRI findings with subsequent diagnostic procedures.

**RESULTS:** A total of 283 patients were included in the analysis, and MRI detected active CD in 31%, fistula in 1.4% and abscess in 0.7%. Extra-intestinal findings not

related to CD were recorded in 72 patients (25%), of which 58 patients (20%) had 74 previously unknown lesions. Important or incompletely characterized findings were detected in 17 patients (6.0%). Incidental findings led to 12 further interventions in 9 patients (3.2%) revealing previously unknown pathological conditions in 5 (1.8%). One patient (0.4%) underwent surgery and one patient was diagnosed with a malignant disease. MRI detected incidental colonic lesions in 16 patients of which additional work-up in 4 revealed normal anatomy. Two patients (0.7%) benefitted from the additional examinations, whereas incidental findings led to unnecessary examinations in 9 (3.2%).

**CONCLUSION:** In a minority of patients with suspected or known CD, important incidental findings are diagnosed at MRI-enterography. However, a substantial number of patients experience unnecessary morbidity because of additional examinations of benign or normal conditions.

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**Key words:** Magnetic resonance imaging; Incidental findings; Crohn's disease; Small intestine

**Peer reviewer:** Marko Duvnjak, MD, Department of Gastroenterology and Hepatology, Sestre milosrdnice University Hospital, Vinogradska cesta 29, 10 000 Zagreb, Croatia

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### INTRODUCTION

In recent years magnetic resonance imaging (MRI) has been increasingly used for the assessment of small

bowel Crohn's disease (CD). MRI has a high diagnostic accuracy<sup>[1-12]</sup> and reproducibility<sup>[12]</sup>, both with enteroclysis and the oral contrast method (enterography), for evaluating CD. Unlike conventional enteroclysis, MRI enables visualization of disease extension beyond the intestinal wall, i.e. abscesses and fistulas. In comparison with enteroclysis, MRI detects additional extra-intestinal lesions in 24%-58% of patients<sup>[1,3]</sup>. However, some extra-intestinal findings are unexpected and not related to CD, and are often referred to as incidental findings. The ability to detect incidental findings presents a clinical dilemma. On one hand, modern imaging techniques may detect early extra-intestinal malignant disease or disease requiring clinical intervention, thereby reducing morbidity and mortality. On the other hand, incidental findings may lead to further diagnostic work-up or surgery of benign lesions causing increased morbidity.

Only one previous study has analyzed the frequency of incidental findings in MRI-enteroclysis. Herfarth *et al.*<sup>[13]</sup> found extra-intestinal lesions in 57% of 710 patients with suspected or known inflammatory bowel disease. Lesions of major clinical importance were detected in 12% of patients, of which the majority consisted of extra-intestinal manifestations of CD (abscesses). Findings were classified as tumor, metastasis or mass in 1.3% of patients. Ajaj *et al.*<sup>[14]</sup> performed MRI-colonography in 375 patients with suspected colonic diseases and detected extra-colonic lesions in 69%, with 12% requiring additional examinations. Approximately half of the extra-colonic lesions were previously unknown. These results emphasize that extra-intestinal findings are common when performing MRI of the abdomen. A significant proportion of incidental findings are clinically important and have an impact on clinical decision-making. However, these studies did not include the results of subsequent diagnostic work-up to reveal the benefit from detection of incidental findings.

The purpose of this study was to determine the frequency and clinical impact of incidental findings detected at MRI-enterography in patients with known or suspected CD.

## MATERIALS AND METHODS

This retrospective study was conducted in the Department of Radiology, Vejle Hospital part of Lillebaelt Hospital, Denmark. The Department introduced MRI-enterography in December 2003, and a study period from December 2003 to November 2007 was chosen, allowing a minimum of 1 year follow-up after MRI. All MRI-enterographies performed in the study period were identified in the hospital's computerized radiology information system, and radiology reports were printed out. Through a systematic review of medical charts we analyzed the clinical impact of incidental findings and compared the MRI findings with subsequent diagnostic procedures. All reports were reviewed independently by the first author.

### Criteria for inclusion and exclusion

MRI-enterographies performed in patients with suspected

or known CD having symptoms consistent with disease activity or complications were included in the study. The subsequent analysis focused on incidental findings defined as unexpected findings outside the small intestine not previously known or suspected at the time of referral and not related to inflammatory bowel disease. Hence, extra-intestinal manifestations of CD (abscesses and fistulas) were not regarded as incidental findings.

Examinations performed on indications other than CD, repeated MRI-enterographies, and examination failures because of technical malfunctions or patient discomfort were excluded. In order to minimize selection bias, the study population was restricted to patients with no previous MRI-enterographies. The likelihood of previously unknown findings outside the small intestine is substantially reduced in repeated scans during a short study period. Therefore, in cases of 2 or more examinations performed, only the first MRI scan was included.

A total 354 patients underwent MRI-enterography. Twenty-nine scans were performed on indications other than inflammatory bowel disease, and additionally 2 scans were excluded because of failure to perform the examination. Both patients were unwilling to ingest the enteral contrast. A total of 40 scans in 29 patients were excluded because of repeated MRI-enterographies in the study period. Hence, a total of 283 MRI-enterography examinations in 283 patients were included in the analysis.

A clinical impact was defined as one or more subsequent interventions, i.e. additional diagnostic work-up, medical and/or surgical treatment, solely caused by the incidental finding at MRI-enterography. The clinical impact was assessed by analyzing the number of patients with subsequent clinical interventions, and the number and type of interventions performed in each patient. Incidental findings were classified as true or false positive on the basis of the diagnostic work-up and as beneficial or unnecessary for the patient. Data were collected from radiological reports, medical charts, laboratory data and the results of subsequent diagnostic procedures. Information was collected from the hospital's computerized medical charts and radiology information system. In patients referred from other hospitals, referrals and medical charts were collected from the department in charge of treatment.

### Ethics

The study was approved by the local ethics committee of Southern Denmark and the Danish Data Protection Agency. In a few patients diagnostic work-up was performed at other hospitals, and prior to collecting these data, patients gave informed consent.

### Imaging technique

Scans were carried out with an Intera 1.5T MRI system with a 5 element Syn-body coil (Philips Medical Systems, Eindhoven, The Netherlands). The evening before the examination, patients were instructed to eat a light meal

**Table 1** Indications and results of 283 MRI investigations of the small intestine

Clinical indication for MRI		<i>n</i>	Total
Suspected CD	Diagnostic MRI in patients with suspected CD not confirmed at endoscopy	156	156
Known CD	Extension of newly diagnosed CD detected at endoscopy	17	127
	Evaluation of disease activity and extension or suspected complications of known CD	110	
Total			283
Results of MRI-enterographies	CD in the small intestine	87	31%
	Stenosis	38	13%
	Entero-enteric fistula	4	1.4%
	Intra-abdominal abscess	2	0.7%
	Suspected IBD in the colon	35	12%

MRI: Magnetic resonance imaging; CD: Crohn's disease; IBD: Inflammatory bowel disease.

and fast overnight. They were allowed to drink water prior to the examination. Patients received 1000 mL water mixed with psyllium husk fiber ingested gradually over one hour. Patients were examined in the supine position. The protocol contained the sequences Cor T1 (TR/TE, 7/3.4; flip angle 15; slice thickness 4 mm; 208 matrix; FOV 375), Cor T2 (B-FFE; TR/TE, 4.1/2.0 ms; flip angle, 60; slice thickness 5 mm; 224 matrix; FOV 400), Cor SPIR (TR/TE, 3000/125 ms; flip angle 90; slice thickness 7 mm; 256 matrix; FOV 400) and axial T1W (TR/TE, 7/3.4; flip angle 15; slice thickness 4 mm; 208 matrix; FOV 375) with discontinuous breath-hold before and after contrast. Gadodiamide 0.1 mmol/kg (GE Healthcare, Medical Diagnostics, Oslo, Norway) was given intravenously, and hyoscinebutylbromide 20 mg (Buscopan, Boehringer Ingelheim, Basel, Switzerland) was administered to reduce peristalsis during the procedure. All images were evaluated using an Impax PACS workstation (Agfa, Mortsel, Belgium) with 2 Coronis monitors (1600 × 1200 pixels) (Megapixels Diagnostic Display System, Barco, Kortrijk, Belgium). Radiologists performing the studies were all specialist doctors with experience in abdominal MRI techniques.

### Classification of scans

MRI-enterographies were classified according to the most important incidental finding. Lesions were assessed as proposed by Zalis *et al*<sup>[15]</sup> for computed tomography (CT) colonography. E0 is an examination in which technical factors severely limit evaluation, e.g. because of artifacts. E1 denotes a normal examination or variants in anatomy that are not expected to affect the patient's health status. E2 refers to examinations with clinically unimportant extra-intestinal findings. E3 denotes incompletely characterized findings that are likely to be benign and E4 refers to examinations with potentially important extra-intestinal findings. Classification of scans was performed by the first author on the basis of the radiological reports and prior to analyzing the clinical impact of incidental findings. The co-authors subsequently evaluated the classification of incidental findings, and agreement was attained. Incidental findings located in the colon were analyzed separately.

### Statistical analysis

Data were analyzed using descriptive statistics. Difference in means was calculated using the Wilcoxon rank-sum test and *P*-values less than 0.05 were considered significant.

## RESULTS

Of the 283 patients included in the study, 193 (68%) were female. The mean age of the study population was 38.7 years (range 9.9-84.9 years). The indication for MRI was suspected CD in 156, and newly diagnosed or known CD in 127. MRI examinations revealed active CD in 31%, fistula in 1.4%, and abscess in 0.7% of patients (Table 1). There was no difference in mean age between patients with known and suspected CD (*P* = 0.9).

### Extra-intestinal incidental findings

Extra-intestinal findings were recorded in 72 patients, of which 58 patients (20%) had previously unknown findings. Forty-one scans were classified E2, 11 were E3, and 5 were E4. In 225 scans no or previously known extra-intestinal lesions were recorded. In one examination the radiologist suspected multi-cystic ovaries, but evaluation of extra-intestinal organs was significantly compromised. The examination was classified E0, even though the finding led to further diagnostic work-up.

Seventy four incidental findings were detected in 58 patients (Table 2). In 43 patients only one finding was recorded, 14 patients had 2 findings, and 3 findings were recorded in one patient. The most frequent findings were benign cysts in the kidneys, ovaries and liver requiring no further work-up (*n* = 39). In 12 patients (4.2%) incompletely characterized extra-intestinal findings (E3) were detected. Of these, 2 patients had a large bladder suggesting previously unknown lower urinary tract disease, and one scan revealed a large hepatic cyst with a diameter of 15 cm displacing the right kidney. Potentially important findings (E4) were recorded in 5 patients (1.8%). Three patients had an undetermined mass or a cystic lesion in conjunction to the ovaries and pelvis wall, and further work-up was recommended. One scan revealed a focal hepatic lesion (atypical hemangioma), and one patient was diagnosed with an abdominal aortic

**Table 2** Previously unknown extra-intestinal findings in 58 patients

		Finding	n
E0	Female genitals	Suspected multi-cystic ovaries	1
E2	Liver	Hepatic cysts	3
		Gallstones	7
	Kidney	Renal cysts	19
		Renal anatomical variants	3
		Reduced kidney size	1
		Metallic artifact in the kidney	1
	Female genitals	Leiomyomas in the uterus	4
		Ovarian cysts	14
	Miscellaneous	Atrophy of the abdominal musculature after surgery	1
		Small amounts of free abdominal fluid	3
			56
E3	Liver	Large hepatic cyst with displacement of the right kidney	1
	Urinary tract	Bilateral nephropathy with reduced kidney size <sup>1</sup>	2
		Large bladder	2
	Female genitals	Free fluid in the pelvis and suspected leiomyoma of the uterus	1
		Two lobulated and cystic lesions in the pelvis	1
	Miscellaneous	Splenomegaly	1
		Ascites	1
		Bilateral hip joint effusion	1
		Lymphadenopathy in the mesentery	1
		Spondylosis and spinal stenosis	1
			12
E4	Focal hepatic lesion (atypical hepatic hemangioma)		1
	Unexplained mass in conjunction to the ovaries		3
	Abdominal aortic aneurysm		1
			5
Total			74

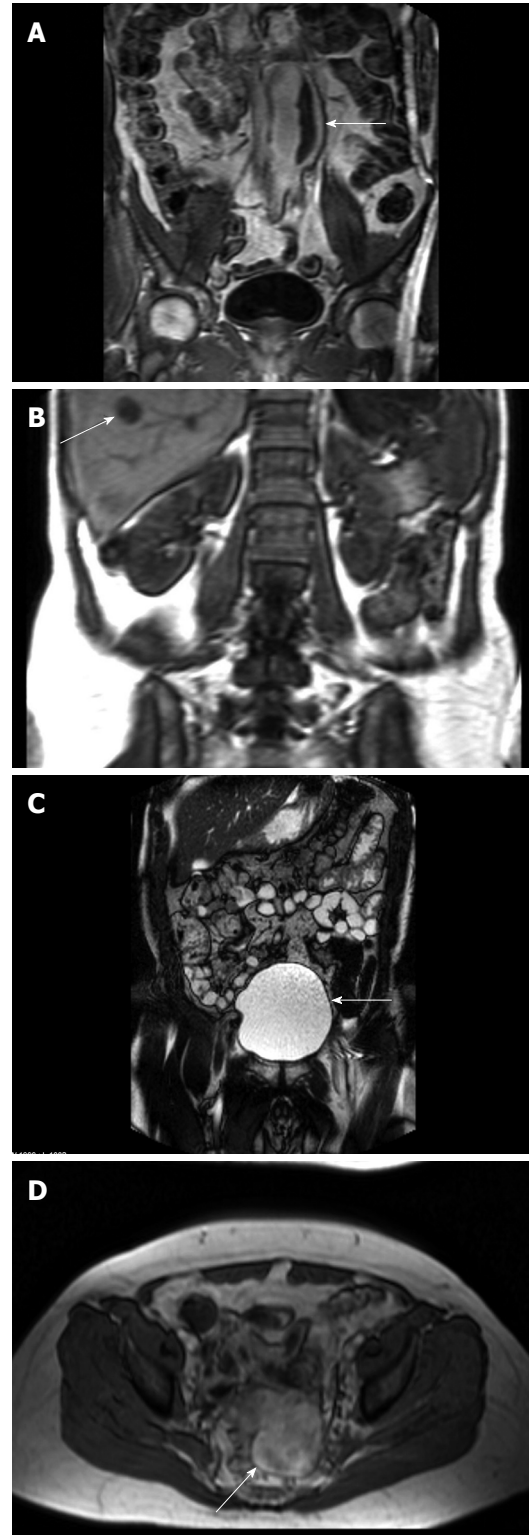
<sup>1</sup>Both patients had a normal S-creatinine at the time of MRI-enterography with intravenous contrast.

aneurysm (Figure 1).

Significantly more scans were classified E3 and E4 in patients with suspected CD (15 out of 156) than known CD (one out of 127) suggesting that incidental findings necessitating further diagnostic work-up are more common in this group of patients ( $P = 0.001$ ). Except for bilateral hip joint effusion, all E3 and E4 findings were detected in patients with suspected CD, and one patient had 2 E3 findings (Table 2).

### Clinical impact of extra-intestinal findings

Extra-intestinal findings resulted in 12 clinical interventions in 9 patients (3.2%). The interventions consisted of ultrasound examination in one, ultrasound-guided biopsy in one, contrast-enhanced ultrasound and biopsy in one, CT-scan in one, gynecological examination including transvaginal ultrasound in 5, surgery in one and biochemical tests in one (Table 3). Succeeding work-up resulted in 5 true positive extra-intestinal findings and 3 false positive findings. One patient with bilateral hip joint effusion failed to attend the follow-up ultrasound examination.



**Figure 1** Incidental findings at MRI-enterography. A: Abdominal aortic aneurysm (arrow). CT scan confirmed the aneurysm and ruled out rupture; B: Atypical hepatic hemangioma (arrow). The results of ultrasound-guided biopsy were benign; C: Large bladder leading to diagnostic work-up and diagnosis of prostate cancer (arrow); D: A lesion with a diameter of 6 cm in the small pelvis (arrow) was confirmed with transvaginal ultrasound. Surgery showed a torquated leiomyoma in the top of the uterus.

In a patient with suspected CD, MRI showed an enlarged bladder. The patient was referred for fur-



**Table 3** Previously unknown extra-intestinal findings leading to further examinations and the result of diagnostic work-up

	Extra-intestinal finding	Clinical intervention	Result of diagnostic work-up
5 true positive findings	Abdominal aortic aneurysm (E4)	CT-scan of the aorta	Abdominal aortic aneurysm without rupture
	Focal hepatic lesion (E4)	Contrast-enhanced US and biopsy (atypical hemangioma)	Hemangioma
	Two unexplained masses in conjunction to the ovaries (E4)	GE and transvaginal US	Leiomyomas
	Free fluid in the pelvis and suspected leiomyoma of the uterus (E3)	Surgery	Leiomyoma
	Large bladder (E3)	GE and transvaginal US	Prostate cancer
3 false positive findings	4.5 cm cystic lesion with an excrescens associated with the cyst wall (E4)	Transrectal US and biopsy	Normal
	2.9 cm solid lesion in the pelvis and displacement of the uterus (E4)	Abdominal CT scan	Normal
	Multicystic ovaries (E0)	Biochemistry (PSA)	Normal
Results not available	Bilateral hip joint effusion (E3)	GE and transvaginal US Referred for US examination	The patient did not attend the examination

US: Ultrasound; GE: Gynecological examination; PSA: Prostate-specific antigen; CT: Computed tomography.

**Table 4** Sixteen incidental findings located in the colon and their clinical impact

Finding	n	Clinical intervention	Result of diagnostic work-up
Suspected benign neoplasia (3 cm large polyp)	1	Colonoscopy and CT-colonography	Normal
Coprostasis	7	-	-
Indeterminate thickening of the cecum mucosa	2	Colonoscopy	Normal
Displacement of the cecum	3	-	-
Diverticulosis	2	-	-
Suspected malignant neoplasia	1	Colonoscopy and abdominal CT scan	Normal

ther urological examinations, and was subsequently diagnosed with a previously unknown prostate cancer. In another patient, MRI revealed a 6 cm wide and 9 cm long abdominal aortic aneurysm. CT scan of the aorta confirmed the aneurysm, and ruled out rupture. Five patients diagnosed with one or more lesions associated with the female genitals had further diagnostic work-up. In one patient, MRI showed a 6 cm large lesion in the small pelvis, and the finding was confirmed with transvaginal ultrasound. The patient underwent surgery, which showed a 5 cm × 4 cm × 5 cm torquated leiomyoma in the top of the uterus and 2 smaller leiomyomas in the anterior wall of the uterus. The surgeon performed a hysterectomy.

#### Incidental findings in the colon

MRI revealed incidental findings located in the colon and not related to inflammatory bowel disease in 16 patients (5.7%, Table 4), of whom 5 also had an extra-intestinal finding (E2 in all). In 12 patients, colonic findings were of minor or no clinical relevance. Four patients underwent additional examinations because of mucosal changes not characteristic of inflammatory bowel disease. The examinations revealed no pathological conditions.

## DISCUSSION

Few studies have dealt with incidental findings in abdominal MRI. In a recent retrospective study, Herfarth *et al*<sup>[13]</sup> analyzed extra-intestinal findings in MRI-enteroclysis. In 710 patients with suspected or known inflammatory bowel disease 57% had extra-intestinal lesions and 12% of the observed lesions were of major clinical importance. In 5 patients (0.7%) extra-intestinal findings were suspicious of previously unknown malignant disease. However, findings of major importance were mainly abscesses related to CD, and comparison with the present study is difficult because of different study designs.

Extensive work has been done on extra-intestinal findings in CT-colonography. Results are summarized in a comprehensive review from 2005 including 17 studies. In total 40% of patients were recorded to have extra-colonic abnormalities, 14% had further diagnostic work-up and extra-colonic cancers were detected in 2.7%<sup>[14]</sup>. The cancer detection rate was reported in 5 studies and varied from 0.4% to 4.6% with the highest rates in the elderly.

In the present study, MRI-enterography revealed incidental findings located outside the small intestine and not related to CD in 25% of patients resulting in additional examinations in 5%. Additional investigations

confirmed abnormal lesions in 1.8%, and one patient had a malignant disease. Two patients benefitted from the additional examinations (aortic aneurysm and prostate cancer), whereas incidental findings led to unnecessary examinations in 9 patients. Detection of extra-intestinal manifestations of CD was rare (1.8%).

Incompletely characterized or clinically important findings were more common in patients with suspected than known CD, suggesting that findings necessitating additional work-up are more frequent in this group of patients. Because of the retrospective nature of this study, and the small number of patients referred for additional examinations, it was not possible to elucidate further on this assumption or whether incidental findings could explain the patients' symptoms. A prospective study would clarify this issue.

Comparing studies can be troublesome because of differences in population characteristics, classification systems, examination protocols and study designs. In the present study we used an MRI technique with intravenous contrast in a young population with a low risk of malignant disease. Compared to the study by Ajaj *et al.*<sup>[14]</sup> we detected fewer extra-intestinal lesions, and the frequency of malignant disease was much higher when performing MRI-colonography. In an overall comparison with CT studies we also found a lower frequency of extra-intestinal findings and a lower rate of additional work-up. These discrepancies probably arise from differences in age, prior morbidity and the risk of malignant disease in the study populations.

MRI-enterography is a relatively new modality for evaluating CD in the small intestine. Ileo-colonoscopy, CT-enterography, capsule endoscopy, abdominal ultrasound and small bowel enteroscopy are alternative examinations. Choosing between modalities relies on several factors. Primarily a modality with a high sensitivity and specificity for luminal abnormalities as well as pathology in the bowel wall and extra-intestinal manifestations of CD is essential. Also other aspects of the investigations should be considered: risk of complications (aspiration, capsule retention, radiation exposure, *etc.*), patient discomfort, complexity of the examinations, accessibility, costs, and finally the impact of incidental findings. In the present study, the detection rate of clinically significant lesions outside the small intestine was low. In contrast, incidental findings led to unnecessary examinations in a substantial number of patients. Hence, in comparison with other modalities the detection rate of important incidental lesions was too low to be an argument in itself for performing MRI-enterography in patients with suspected or known CD.

Our study was limited by its retrospective design. Radiological reports were not performed with the focus on incidental findings, and underestimation of clinically unimportant findings are likely. The study population contained a preponderance of women (ratio 2:1), which is reflected by the frequency of incidental findings in the female genitalia. The second most common finding was ovarian cysts, and lesions in the female genitals were

common in all classification groups. It is well established that CD is more common in females (1.2-1.5:1) and in specialized centers for inflammatory bowel diseases the prevalence of women with irritable bowel syndrome is up to 4 times as high as that of men<sup>[17,18]</sup>.

In conclusion, incidental findings were common in patients with known and suspected CD having MRI for evaluation of small intestinal disease. Additional examinations revealed important disease in only a minority of patients. However, a substantial number of patients experienced unnecessary morbidity because of the additional examinations of benign or normal conditions. The detection rate of important incidental lesions not related to CD was too low to be an argument in itself for performing MRI-enterography in this group of patients.

## COMMENTS

### Background

Magnetic resonance imaging (MRI) is increasingly used in the assessment of small bowel Crohn's disease (CD). Unlike conventional radiology, MRI enables visualization of disease extension beyond the intestinal wall, i.e. abscesses and fistulas. However, some extra-intestinal findings are unexpected and without relation to CD (incidental findings).

### Research frontiers

Only a few studies have described the clinical impact of incidental findings in abdominal MRI. Lesions may represent important diseases and benefit patients, but may also cause unnecessary morbidity because of the diagnostic work-up of benign lesions.

### Innovations and breakthroughs

In 2 recent studies using abdominal MRI techniques, extra-intestinal lesions of major clinical importance were common. However, these studies did not include the results of subsequent diagnostic work-up to reveal the benefit from detection of these findings. In the present study, incidental findings were common in patients having MRI for evaluation of small bowel CD. Additional examinations revealed important disease in a minority of patients. However, a substantial number of patients experienced unnecessary morbidity arising from the additional examinations of benign or normal conditions.

### Applications

Several modalities for diagnosing small bowel CD are available. The present study emphasized that the detection rate of important incidental lesions was too low to be an argument in itself for performing MRI-enterography in this group of patients.

### Peer review

Jensen *et al* gave a nice and clear description of the research background, materials, methods, results and conclusions. Significant points have been presented and compared with data from prior research. Used methods are advanced, and detailed descriptions are provided allowing other investigators to reproduce or validate them. The statistical methods are appropriate. From the presented results, sufficient data can be drawn. In discussion, valuable conclusions are provided. References are appropriate and relevant. Tables and figures reflect the major findings of the study.

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## Association of autoimmune type atrophic corpus gastritis with *Helicobacter pylori* infection

Lea Irene Veijola, Aino Mirjam Oksanen, Pentti Ilmari Sipponen, Hilpi Iris Kaarina Rautelin

Lea Irene Veijola, Aino Mirjam Oksanen, Herttoniemi Hospital, City of Helsinki, Kettutie 8, 00800 Helsinki, Finland  
Lea Irene Veijola, Hilpi Iris Kaarina Rautelin, Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki, Haartmanink. 3, 00014 Helsinki, Finland  
Pentti Ilmari Sipponen, Repolar Oy, Box 26, 02101 Espoo, Finland

Hilpi Iris Kaarina Rautelin, HUSLAB, Helsinki University Central Hospital Laboratory, Haartmanink 3, 00014 Helsinki, Finland; Department of Medical Sciences, University of Uppsala, Dag Hammarskjölds väg 17, SE-75185 Uppsala, Sweden

**Author contributions:** Veijola LI, Rautelin HIK and Oksanen AM planned the study; Veijola LI and Oksanen AM interviewed and examined the patients, and performed the gastroscopies; Sipponen PI examined the histology of the biopsy materials; Veijola LI analyzed the data; Veijola LI and Rautelin HIK wrote the article; all authors participated in the revision of the manuscript.

**Correspondence to:** Lea Irene Veijola, MD, PhD, Herttoniemi Hospital, City of Helsinki, Kettutie 8, 00800 Helsinki, Finland. [lea.veijola@helsinki.fi](mailto:lea.veijola@helsinki.fi)

Telephone: +358-9-3105511 Fax: +358-9-19126382

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### Abstract

**AIM:** To study the association between *Helicobacter pylori* (*H. pylori*) infection and autoimmune type atrophic gastritis.

**METHODS:** Twenty-three patients with different grades of atrophic gastritis were analysed using enzyme immunoassay-based serology, immunoblot-based serology, and histology to reveal a past or a present *H. pylori* infection. In addition, serum markers for gastric atrophy (pepsinogen I, pepsinogen I/II and gastrin) and autoimmunity [parietal cell antibodies (PCA), and intrinsic factor (IF), antibodies] were determined.

**RESULTS:** Of the 14 patients with severe gastric

atrophy, as demonstrated by histology and serum markers, and no evidence for an ongoing *H. pylori* infection, eight showed *H. pylori* antibodies by immunoblotting. All eight had elevated PCA and 4/8 also had IF antibodies. Of the six immunoblot-negative patients with severe corpus atrophy, PCA and IF antibodies were detected in four. Among the patients with low to moderate grade atrophic gastritis (all except one with an ongoing *H. pylori* infection), serum markers for gastric atrophy and autoimmunity were seldom detected. However, one *H. pylori* negative patient with mild atrophic gastritis had PCA and IF antibodies suggestive of a pre-atrophic autoimmune gastritis.

**CONCLUSION:** Signs of *H. pylori* infection in autoimmune gastritis, and positive autoimmune serum markers in *H. pylori* gastritis suggest an etiological role for *H. pylori* in autoimmune gastritis.

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**Key words:** *Helicobacter pylori*; Autoimmune gastritis; Gastric atrophy; Vitamin B12 deficiency

**Peer reviewers:** Cuong D Tran, PhD, Research Fellow, Affiliate Lecturer, University of Adelaide, Gastroenterology Unit, Children, Youth and Women's Health Service, 72 King William Rd, North Adelaide, SA 5006, Australia; Dr. T Choli-Papadopoulou, Associate Professor, Department of Biochemistry, Aristotle University of Thessaloniki, School of Chemistry, Thessaloniki 55124, Greece

Veijola LI, Oksanen AM, Sipponen PI, Rautelin HIK. Association of autoimmune type atrophic corpus gastritis with *Helicobacter pylori* infection. *World J Gastroenterol* 2010; 16(1): 83-88 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i1/83.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i1.83>

### INTRODUCTION

Autoimmune type corpus gastritis, formerly named type



A gastritis, is severe atrophy of gastric corpus associated with hypochlorhydria<sup>[1]</sup>. Even without total gastric atrophy, many of these patients have an inability to absorb vitamin B12 from food<sup>[2]</sup>. Generally, 15%-20% of vitamin B12 malabsorption in elderly patients is due to pernicious anaemia, as defined as deficiency of intrinsic factor (IF)<sup>[3]</sup>. Over 90% of patients with pernicious anaemia have parietal cell antibodies (PCA) and 50%-70% have elevated IF antibodies<sup>[1]</sup>. The autoantigen for PCA is H+/K+-adenosine triphosphatase, the proton pump<sup>[4]</sup>.

In patients with *Helicobacter pylori* (*H. pylori*) infection, superficial gastritis proceeds to atrophic gastritis in about half of the patients<sup>[5]</sup>. Although this type of atrophic gastritis, which is associated with intestinal metaplasia, mainly involves the antrum, it can proceed to the corpus or affect the mucosa focally, *viz.* multifocal atrophic gastritis. Advanced atrophy develops over many years and *H. pylori* disappears from the gastric mucosa. In some patients, the antral intestinal metaplasia disappears and PCA appears; thus, the disease resembles classic autoimmune gastritis<sup>[6]</sup>. Gastric H+/K+-ATPase is also the major autoantigen in chronic *H. pylori* induced atrophic gastritis in corpus mucosa<sup>[7]</sup>.

In *H. pylori* induced atrophic gastritis, the activated CD4+ Th1 cells infiltrating the gastric mucosa cross-recognize the epitopes of the gastric parietal cell proton pump and various *H. pylori* proteins<sup>[8,9]</sup>. It is not known if *H. pylori* is the initiating factor in activating Th1 cells, which leads to inflammation and apoptosis, or is only a coincidental bystander<sup>[10]</sup>. If the classic autoimmune type gastric atrophy is an end-stage of *H. pylori* induced gastric autoimmunity with atrophic gastritis, the prevalence of pernicious anaemia should decrease with declining prevalence of *H. pylori*. It is not known if vitamin B12 malabsorption in the late stages of gastric atrophy could be restored or prevented if *H. pylori* were eradicated earlier<sup>[11-14]</sup>.

In the present study we investigated the signs of a previous *H. pylori* infection in patients with different grades of atrophic gastritis to assess the proportion of gastric atrophy not associated with *H. pylori* infection.

## MATERIALS AND METHODS

All patients with an earlier gastroscopy reprint available and who had undergone a gastroscopy for clinical indications at Herttoniemi Hospital during 2004 and 2005<sup>[15]</sup> were included in the present study if their follow-up histology indicated they had atrophic gastritis. Twenty-three of the 38 patients with different grades of atrophic gastritis had a blood sample available and were included in the study. The median age was 65 years and 18 were females.

The Ethics Committee of the Hospital District of Helsinki and Uusimaa approved the study and all the participants gave their written informed consent.

### Histology

Two biopsies from each of the antrum and the corpus

were taken during gastroscopy and stained with haematoxylin-eosin, Alcian blue (pH 2.5)-periodic acid Schiff, and modified Giemsa stains. All the samples were examined by one pathologist who was unaware of the identity of the samples. The samples were assessed according to the updated Sydney system<sup>[16]</sup>.

### Serum tests

*H. pylori* antibodies were detected by an enzyme immunoassay (EIA) and by immunoblotting. Serum samples were taken after gastroscopy and stored (-20°C) until analyzed for IgG antibodies to *H. pylori* using a locally validated in-house EIA with high sensitivity and specificity<sup>[17]</sup>. Immunoblotting was performed by MP Diagnostics Helico blot 2.1 (MP Biomedicals, Singapore). The interpretation criteria for an *H. pylori* seropositive sample, according to the manufacturer, were: (1) fulfilling the criteria for CagA positivity; (2) the presence of any bands at 89 kDa, 37 kDa, or 35 kDa; or (3) the presence of both the bands at 30 kDa and 19.5 kDa. The criteria for CagA positivity were the presence of 116 kDa CagA band (a) in combination with current infection marker CIM; (b) in combination of the 30 kDa (UreA) and 19.5 kDa bands; or (c) in combination of at least one of the following bands 89 kDa (VacA), 37 kDa, or 35 kDa.

PCA were measured by Varelisa (Pharmacia Diagnostics, Freiburg, Germany) using H+/K+-ATPase as an antigen. According to the manufacturer's instructions, values > 15 U/mL were interpreted as positive but equivocal values (10-15 U/mL) were interpreted negative as well as values < 10 U/mL.

Serum IF antibodies of the blocking type were measured routinely with the haemoglobin charcoal adsorption assay. The cut off value used was 2 U/L.

Serum pepsinogen I and II and gastrin-17 levels were investigated with Gastropanel (Biohit PLC Diagnostics, Helsinki, Finland). The reference ranges were 30-120 µg/L for pepsinogen I, 3-10 µg/L for pepsinogen II, 3-20 for pepsinogen I / II, and 2-10 pmol/L for gastrin-17.

### Statistical analysis

The differences between the groups were tested using two-tailed Fisher's exact test and the data were analysed using GraphPad software (QuickCalcs online calculators for scientists [www.graphpad.com](http://www.graphpad.com)). *P* values < 0.5 were considered significant.

## RESULTS

Of the 23 patients included in the study, 14 had severe gastric atrophy according both to histology and the serum markers, and the remaining nine patients had mild to moderate atrophic gastritis. The patients with severe atrophy were slightly younger (median age 64 years) than the other patients (median age 70 years). None of the patients with severe atrophy had either *H. pylori* in histology or elevated *H. pylori* antibodies in the EIA

**Table 1** Histological and serum findings in patients with mild to moderate ( $n = 9$ ) and severe ( $n = 14$ ) atrophic changes in the corpus

Findings	Number of patients with atrophic corpus gastritis						P-value
	Grade 1 or 2			Grade 3			
	IB+ ( <i>n</i> = 8)	IB- ( <i>n</i> = 1)	Total	IB+ ( <i>n</i> = 8)	IB- ( <i>n</i> = 6)	Total	
Chronic corpus gastritis	7	1	8	8	6	14	NS
Chronic antral gastritis	7	1	8	3	3	6	0.04
Antral intestinal metaplasia	3	0	3	0	0	0	0.05
<i>H. pylori</i> in histology	4	0	4	0	0	0	0.01
Elevated EIA <i>H. pylori</i> IgG <sup>1</sup>	7	0	7	0	0	0	0.0001
Vitamin B12 therapy	1	0	1	4	6	10	0.009
Elevated PCA <sup>2</sup>	2	1	3	8	4	12	0.02
Elevated IF antibodies <sup>3</sup>	0	1	1	4	4	8	0.04
Low pepsinogen I <sup>4</sup>	0	0	0	8	6	14	0.0001
Elevated pepsinogen II <sup>5</sup>	5	0	5	4	1	5	NS
Low pepsinogen I / II <sup>6</sup>	1	0	1	8	6	14	0.0001
Elevated gastrin-17 <sup>7</sup>	2	1	3	8	5	13	0.005

<sup>1</sup>In-house EIA positive  $\geq 700$ ; <sup>2</sup>Parietal cell antibodies PCA elevated  $> 15$ ; <sup>3</sup>Intrinsic factor IF elevated  $> 2$ ; <sup>4</sup>Pepsinogen I low  $< 30$ ; <sup>5</sup>Pepsinogen II elevated  $> 10$ ; <sup>6</sup>Pepsinogen I / II low  $< 3$ ; <sup>7</sup>Gastrin 17 elevated  $> 10$ . EIA: Enzyme immunoassay; *H. pylori*: *Helicobacter pylori*; PCA: Parietal cell antibodies; IF: Intrinsic factor; NS: Not significant; IB+: Immunoblot positive; IB-: Immunoblot negative. P-value: Total (grade 1 or 2) vs total (grade 3).

(Table 1); one patient had had a successful *H. pylori* eradication therapy 7 years earlier. Of the nine patients with mild to moderate atrophic gastritis, two had had a successful eradication therapy (4 mo and 6 mo earlier, respectively) and four had an ongoing infection shown in histology; seven had elevated *H. pylori* antibodies in the EIA (Table 1). All patients, except one with moderate atrophic gastritis, had chronic gastritis in the corpus, whereas the antrum was significantly less often affected in patients with severe atrophy compared to those with mild and moderate atrophic changes ( $P = 0.04$ , Fisher's exact test, Table 1). Antral intestinal metaplasia was not found in any of the patients with severe atrophy.

Serum markers for gastric autoimmunity were only rarely detected in patients with mild to moderate atrophic gastritis (PCA in three patients and IF antibodies in one patient, Table 1). In contrast, all 14 patients with severe atrophy had either elevated PCA or IF antibodies, six patients having both antibodies elevated. Furthermore, the levels of elevated PCA and IF antibodies were higher in patients with severe atrophy (eight of 12 patients with elevated PCA had a PCA titre over 100, and the mean IF antibody titre in eight patients with an elevated value was 8.7) compared to patients with only mild to moderate atrophic changes (only one of the three patients with elevated PCA had a PCA titre over 100 and the IF antibody titre in the only patient with an elevated value was 2.1).

*H. pylori* antibodies could be demonstrated by immunoblotting in 8/9 patients with mild to moderate atrophic gastritis and in 8/14 patients with severe gastric atrophy (Table 1). Patients with severe atrophy and a positive immunoblot result did not significantly differ from those with severe atrophy and negative immunoblot results as far as age, sex, histological findings, and serum results were concerned (Table 1). Although six patients with severe atrophy showed negative immunoblot results

(according to the criteria of the manufacturer) four of them had a positive CagA band in the immunoblot; thus, only two patients showed no evidence of previous *H. pylori* infection. In addition, one patient with mild atrophic gastritis had no evidence (not even a CagA band) for ongoing or previous *H. pylori* infection (Table 1). This particular patient showed clearly elevated PCA ( $> 100$  U/mL) and slightly elevated IF antibodies (2.1 U/L).

## DISCUSSION

In our study, of the 14 patients having autoimmune type atrophic gastritis (severe gastric atrophy with elevated PCA and/or IF antibodies) only two had no signs of previous *H. pylori* infection. In addition, all except one of the patients with mild to moderate atrophic corpus gastritis had an ongoing *H. pylori* infection or signs of previous infection. The *H. pylori* negative patient with minor atrophic changes in the gastric corpus had elevated PCA and IF antibodies; whether this particular patient goes on to develop severe gastric atrophy of autoimmune type remains to be shown. To the best of our knowledge, she is the first patient described in the literature as having preatrophic autoimmune gastritis with elevated serum markers of pernicious anaemia and no signs of *H. pylori* infection, despite being investigated with both invasive and non-invasive methods: antrum and corpus histology in two gastroscopies with a 5.4 years interval and negative EIA serology and immunoblotting, including CagA.

In severe gastric atrophy, the exclusion of previous *H. pylori* infection is controversial, as the sensitivity of histology is low<sup>[18]</sup>, and many of the EIA based serological tests are poorly validated<sup>[19]</sup>. In *H. pylori* gastritis, the antibodies in EIA serology decline below the cut-off values along with advanced atrophy<sup>[20]</sup>, as

well as after eradication therapy<sup>[21]</sup>; thus, the previous *H. pylori* infection cannot be deduced by negative EIA serology. Immunoblotting with CagA antibodies can give positive results for years after the disappearance of *H. pylori*<sup>[22,23]</sup>, but all *H. pylori* strains are not CagA positive. Discrepancies in CagA seropositivity yielded by immunoblotting in patients with severe gastric atrophy<sup>[24,25]</sup> may derive from the different sensitivities of the immunoblotting methods used<sup>[26]</sup>.

Studies of patients with preatrophic autoimmune type of corpus gastritis are rare. In a population-based study, all 12 patients with autoimmune type atrophic gastritis (diffuse lymphocytic infiltration of the entire lamina propria in the corpus mucosa) without severe gastric atrophy showed *H. pylori* in histology or serology<sup>[27]</sup>. In the same study, of the 28 individuals with severe autoimmune type gastric atrophy six were *H. pylori* positive in histology and another 13 were positive in serology (altogether 68% positive for *H. pylori*). Considering the moderately high prevalence (2.8% in the Kalixanda study<sup>[27]</sup>) of the autoimmune type of gastric atrophy in general, the description in the literature of patients with *H. pylori* negative autoimmune type gastritis in preatrophic stage is rare.

Uibo described a 17-year-old female with no signs of gastritis and *H. pylori* in histology developing atrophic gastritis during a 12-year follow-up<sup>[28]</sup>. However, the exclusion of *H. pylori* infection in this case was based only on histology, and the childhood infection rate in this population cohort was nearly 100%. Kuipers described two patients who were negative for *H. pylori* and without gastritis at first visit, who then developed atrophic gastritis (one developed also intestinal metaplasia and pernicious anaemia) during more than 10 years of follow-up<sup>[29]</sup>. However, although in this study the *H. pylori* infection was assessed with serology and histology at the first visit, in cases of discrepant results, histology was considered predominant over serology unless atrophic mucosa was observed. Whether these two patients had positive serology at the first visit was not mentioned. In the study of Segni *et al.*<sup>[30]</sup> of children with juvenile autoimmune thyroid disease, of the 18 children with elevated PCA who underwent gastroscopy, two children with hypergastrinaemia had *H. pylori* negative preatrophic gastritis, as shown by histology and EIA serology. Immunoblotting was not studied and follow-up has not been published. In the study of adult patients with Sjögren's syndrome, there was no difference in the prevalence of *H. pylori* infection, antigastric antibodies, or gastric histology between patients and controls, but after successful eradication therapy for *H. pylori*, the lymphocytic infiltration and atrophy in patients with Sjögren's syndrome, contrary to the controls, did not improve<sup>[31]</sup>. In addition, patients with Sjögren's syndrome who were positive for antigastric antibodies all had *H. pylori* infection and they more often had atrophic gastritis than the controls. In conclusion, from the previous studies, patients with autoimmune type atrophic gastritis without

*H. pylori* infection might rarely exist, but at the moment a study showing preatrophic gastritis proceeding to total gastric atrophy without *H. pylori* infection is lacking. This is in accordance with our results; as the patient having preatrophic gastritis without signs of *H. pylori* infection did not proceed to total gastric atrophy during 5 years of follow-up.

Several studies suggest that autoimmune atrophic corpus gastritis is associated with *H. pylori* infection in the majority of cases. In one study, two-thirds of patients with atrophic corpus gastritis had evidence of *H. pylori* infection, when assessed with histology and serology<sup>[32]</sup>. In another study, 62% of the patients with pernicious anaemia and severe atrophic corpus gastritis had positive *H. pylori* serology<sup>[33]</sup>. In one further study, patients with atrophic corpus gastritis were negative for *H. pylori* in histology and in EIA-serology, but positive when studied by immunoblotting<sup>[25]</sup>. In another study of atrophic corpus gastritis, among 111 patients with negative *H. pylori* EIA serology, 95.5% were positive in immunoblotting<sup>[34]</sup>. In a study of 10 patients with severe atrophic corpus gastritis, all were *H. pylori* negative in histology and in EIA-serology, and only one was positive in immunoblotting<sup>[24]</sup>. However, in this particular study, the immunoblotting method used to measure CagA antibodies was less sensitive than EIA-serology in detecting an ongoing *H. pylori* infection. In the present study, all except three patients had a positive CagA band on the immunoblot, including all EIA-serology positive patients. We have studied the sensitivity and specificity of this particular immunoblotting method previously, with good results<sup>[23]</sup>. However, not even the immunoblotting method used in our present study can rule out a previous *H. pylori* infection with 100% certainty, as all *H. pylori* strains are not CagA positive. On the other hand, the common occurrence of *H. pylori* antibodies in patients with autoimmune type of atrophic gastritis could be a random effect, as the *H. pylori* infection rate has been nearly 100% in populations now presenting as the peak age group of autoimmune gastritis. This could also be one explanation why *H. pylori* prevalence studied by immunoblotting in patients with serological markers of autoimmune type gastritis (PCA and IF antibodies) was no different from patients with no such markers in our study.

Thus, it still remains to be shown if *H. pylori* infection is crucial for the development of autoimmune type atrophic gastritis. However, bacterial infections might be important in autoimmune processes, as recently suggested by Torchinsky *et al.*<sup>[35]</sup>. In this *in vitro* study, phagocytosis of immune cells infected with bacteria and undergoing apoptosis promoted Th17 cell differentiation, the cell type having a potential role in autoimmunity. Thus, it is tempting to speculate that cells in the gastric mucosa infected with *H. pylori* could trigger an autoimmune response.

In conclusion, atrophic corpus gastritis, including

autoimmune type severe atrophy with vitamin B12 malabsorption, is associated with a longstanding *H. pylori* infection in most cases. There is an urgent need for population-based studies to assess the effect of *H. pylori* eradication on the development of vitamin B12 malabsorption.

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## COMMENTS

### Background

Autoimmune type atrophic gastritis is a severe gastric atrophy associated with pernicious anaemia with lifelong substitution therapy with vitamin B12. Longstanding *Helicobacter pylori* (*H. pylori*) infection proceeds in about 50% of patients to atrophic gastritis. *H. pylori* infection is much more prevalent than autoimmune type gastritis, and the association of these two conditions is possible without a causal relationship.

### Research frontiers

Previous studies have shown that *H. pylori* shares several epitopes with the proton pump, and the  $\beta$ -subunit of this pump is the causative antigen in autoimmune gastritis. In animal models, the passive transfer of these antibodies does not cause disease, but CD4<sup>+</sup> T-cells are responsible for the gastritis. Recently, it has been shown that bacterial infection can modify the immune response in the direction seen in autoimmune diseases, i.e. Th17 cell differentiation, thus linking infection and autoimmunity.

### Innovations and breakthroughs

It is difficult to differentiate severe end stage *H. pylori* atrophic gastritis and autoimmune type gastric atrophy, because the autoimmune serum markers appear in *H. pylori* gastritis with increasing grade of atrophy, as shown in previous studies and confirmed in our study. The preatrophic stage of autoimmune type gastritis without *H. pylori* infection is an unknown entity. Several patients with autoimmune type gastric atrophy have signs of a previous *H. pylori* infection when studied with sensitive methods and remain positive for years, as shown in this study.

### Applications

If *H. pylori* initiates the apoptosis that leads to gastric atrophy and vitamin B12 deficiency, eradication of the bacteria before the development of severe atrophic changes should abolish the development of pernicious anaemia and the need of lifelong vitamin B12 substitution therapy.

### Peer review

This is a very interesting paper and asks quite an important question as to whether there is an association between *H. pylori* infection and autoimmune type atrophic gastritis. This work could be accepted after revision.

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## On-treatment predictions of success in peg-interferon/ ribavirin treatment using a novel formula

Hidetsugu Saito, Hirotoishi Ebinuma, Keisuke Ojio, Kanji Wakabayashi, Mika Inoue, Shinichiro Tada,  
Toshifumi Hibi

Hidetsugu Saito, Hirotoishi Ebinuma, Keisuke Ojio, Kanji Wakabayashi, Mika Inoue, Shinichiro Tada, Toshifumi Hibi, Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 1608582, Japan

**Author contributions:** Saito H, Ebinuma H, Ojio K, Wakabayashi K and Tada S contributed to the analysis of the clinical data; Inoue M contributed as a clinical assistant to collect data from the affiliated hospitals; Hibi T provided financial support for this work; Saito H designed the study and wrote the manuscript.

**Correspondence to:** Hidetsugu Saito, MD, PhD, Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 1608582,

Japan. [hsaito@sc.itc.keio.ac.jp](mailto:hsaito@sc.itc.keio.ac.jp)

Telephone: +81-3-33531211 Fax: +81-3-33518705

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the formulae at weeks 4, 12 and 24 (the area under the curve of the receiver operating characteristic: 0.821, 0.802, and 0.891, respectively), but not at baseline (0.570). The formula at week 48 was also constructed and validation by test data achieved good prediction with 0.871 of the area under the curve of the receiver operating characteristic. Prediction by this formula was always superior to that by viral kinetics.

**CONCLUSION:** These results suggested that our formula combined with viral kinetics provides a clear direction of therapy for each patient and enables the best tailored treatment.

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**Key words:** Logistic regression analysis; Predictive formula; Prolongation of the therapy; Response-guided therapy; Viral kinetics

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### Abstract

**AIM:** To predict treatment success using only simple clinical data from peg-interferon plus ribavirin therapy for chronic hepatitis C.

**METHODS:** We analyzed the clinical data of 176 patients with chronic hepatitis and hepatitis C virus genotype 1 who received 48 wk standard therapy, derived a predictive formula to assess a sustained virological response of the individual patient using a logistic regression model and confirmed the validity of this formula. The formula was constructed using data from the first 100 patients enrolled and validated using data from the remaining 76 patients.

**RESULTS:** Sustained virological response was obtained in 83 (47.2%) of the patients and we derived formulae to predict sustained virological response at pretreatment and weeks 4, 12 and 24. The likelihood of sustained virological response could be predicted effectively by

### INTRODUCTION

Persistent hepatitis C virus (HCV) infection is the major chronic liver disease in Japan, and pegylated interferon- $\alpha$  (PEG-IFN) plus ribavirin (RBV) therapy is the current mainstay of treatment. The goal of treatment is a sustained virological response (SVR), which is defined as undetectable serum HCV RNA, according to a polymerase

chain reaction-based assay, 6 mo after the cessation of therapy<sup>[1]</sup>. Patients who achieve SVR gain the benefits of regression of fibrosis, decreased incidence of hepatocellular carcinoma, and reduced morbidity and mortality. The genotype of HCV affects treatment efficacy and HCV RNA levels (viral load) also may have an effect. Only a small percentage of patients infected with HCV genotype 1b and high viral load achieved SVR with conventional IFN therapy for 6 mo<sup>[2]</sup>. Patients infected with HCV genotype 1 should receive 48 wk of PEG-IFN plus RBV, while 24 wk of treatment is recommended for patients with HCV genotype 2<sup>[3,4]</sup>. When PEG-IFN plus RBV is administered in this manner, around 50% of patients infected with HCV genotype 1 achieve SVR<sup>[5]</sup>, which is a great improvement over the SVR associated with 24 wk conventional IFN- $\alpha$  therapy.

The likelihood of treatment success may also be predicted by viral kinetics on therapy as well as circulating core antigen and immune parameters<sup>[6,7]</sup>. In particular, recent studies have shown that SVR can be predicted by a rapid virological response (RVR), which is defined as an undetectable level of HCV RNA at 4 wk of treatment<sup>[8]</sup>, and an early virological response (EVR), which is defined as either an undetectable level of HCV RNA or a drop in HCV RNA levels of at least 2 log<sub>10</sub>IU/mL after 12 wk of treatment<sup>[9]</sup>.

Nevertheless, the current dosing regimens for PEG-IFN plus RBV could potentially under-treat some patients<sup>[10]</sup> and additional measurements of viral response are needed to facilitate individualization of therapy. Among predictive factors already reported<sup>[11-15]</sup>, many are not readily available from daily clinical assessment, because they require genomic analyses and/or advanced experimental methods. There is increasing evidence to support extending the duration of treatment beyond 48 wk for patients with an HCV genotype 1 infection who display a slow virological response, which is defined by HCV RNA levels > 50 IU/mL at week 12 but undetectable at week 24. Several trials suggested that 72 wk of treatment with PEG-IFN plus RBV results in a better SVR rate than the same treatment for 48 wk<sup>[16-18]</sup>. However, it is difficult to accurately determine whether the individual should have their therapy extended at week 48, because the predictive value of a slow virological response may be insufficient alone. It would be very valuable to have a more accurate predictive marker of SVR at week 48, derived from clinically available measurements.

In this study, to try to make better prediction of patients who would or would not respond to 48 wk of PEG-IFN plus RBV therapy, we analyzed the clinical data of patients with chronic hepatitis C who received 48 wk of therapy, derived a predictive formula to assess the likelihood of an SVR for each individual patient using a logistic regression model and confirmed the validity of this formula.

## MATERIALS AND METHODS

The study was approved by the Ethics Committee of the

Keio University, School of Medicine, and was performed in accordance with the internationally accepted ethical standards for human experimentation. The study was conducted by the Keio Association for the Study of Liver Diseases (KASLD). All patients received explanations of the purpose and protocol of the study and written informed consent was obtained from each patient.

## Patients

One hundred and seventy-six patients with chronic hepatitis C infected with HCV genotype 1b were enrolled prospectively and received PEG-IFN plus RBV therapy from December 2004 to May 2007. All patients had HCV RNA levels  $\geq 100$  KIU/mL, measured by a quantitative polymerase chain reaction (PCR) assay (COBAS HCV Amplicor MONITOR<sup>TM</sup>, sensitivity 500 IU/mL; Roche Diagnostic Systems, Inc., Tokyo, Japan). Pregnant women and women of childbearing potential, nursing mothers, male patients whose partner could have become pregnant, and those with anemia (hemoglobin concentration of 10 g/dL or less), leucopenia (1500 cells/ $\mu$ L or less), thrombocytopenia (80 000 cells/ $\mu$ L or less), severe dysfunction of organs other than the liver (these exclusion criteria are included in the instruction of the drug and provided by the manufacturer), infection with hepatitis B virus or human immunodeficiency virus, autoimmune hepatitis, primary biliary cirrhosis, and liver dysfunction caused by other etiologies were excluded. Some patients did not undergo a liver biopsy because not all of the centers could perform biopsies. All patients were treated for 48 wk and were followed for 24 wk after cessation of treatment. The formula was derived using data from the first 100 patients enrolled as selection data and validated using data from the additional 76 patients as test data. In this way it was possible to analyze the predictive accuracy and validity of the constructed formula.

## Treatment and data collection

PEG-IFN- $\alpha$ 2b (Schering-Plough K.K., Osaka, Japan) was administered weekly in doses adjusted for body weight according to the manufacturer's recommendations in Japan (45 kg or less, 60  $\mu$ g; 46-60 kg, 80  $\mu$ g; 61-75 kg, 100  $\mu$ g; 76-90 kg, 120  $\mu$ g; 91 kg or more, 150  $\mu$ g). Similarly, RBV (Schering-Plough K.K.) was given in daily doses adjusted to body weight according to manufacturer's instructions (60 kg or less, 600 mg/d; 61-80 kg, 800 mg/d; 81 kg or more, 1000 mg/d). Serum levels of HCV RNA were quantified and, when the level was below 500 IU/mL, HCV RNA was measured with the COBAS HCV Amplification and Detection version 2.0, sensitivity 50 IU/mL, Roche Diagnostic Systems). Blood cell counts and chemistry were analyzed at the beginning of treatment and every 4 wk thereafter. A questionnaire was used to review demographic data (gender, age, weight, height), previous treatment, histologic activity grade, and fibrosis stage, dose of PEG-IFN, dose of RBV, presence of diabetes, HCV RNA levels, SVR, white blood cell

counts (WBC), neutrophil counts (NC), red cell counts (RBC), hemoglobin levels (Hb), platelet counts (PLT), serum aspartate aminotransferase levels (AST), and serum alanine aminotransferase levels (ALT).

### Statistical analysis

The Mann-Whitney *U*-test was used to analyze continuous variables. Chi-squared and Fisher's exact tests were used for analysis of categorical data. One of our goals was to predict SVR using only simple clinical data, so a database was created containing the following basic information: for all patients, baseline age, sex, body weight (kg), height (cm), dose of PEG-IFN ( $\mu\text{g/kg}$ ), dose of RBV ( $\text{mg/kg}$ ), HCV RNA levels (KIU/mL), SVR (+/-), WBC ( $/\mu\text{L}$ ), NC ( $/\mu\text{L}$ ), RBC ( $/\mu\text{L}$ ), Hb (g/dL), PLT ( $/\mu\text{L}$ ), AST (IU/L), and ALT (IU/L). Statistical analyses were performed using the Statistical Package of Services Solutions (SPSS; SPSS Inc., Chicago, IL, USA) software, version 11.0. First, factors that differed significantly between SVR and non-SVR groups were identified at every time point by univariate analyses. The independent discriminative value of markers for predicting SVR was then assessed by logistic regression analysis. The third step was to construct a formula that combined independent factors. The best index for discrimination was the logistic regression function that combined the most discriminatory independent factors. The predictive formula was logically constructed by following basal formula:

$$1/p = 1 + \exp [-(\beta_0 + \beta_1 X_1 + \beta_2 X_2 + \dots + \beta_n X_n)]$$

Diagnostic values of indices and isolated factors were assessed by sensitivity, specificity, positive and negative predictive values (PPV and NPV), and receiver operating characteristic (ROC) curves.

## RESULTS

### Patient profile

Of the 176 patients, 101 (59.8%) were men and the median age was 56 years (18-77), which is greater than has been reported from Western countries. The median values of body weight and BMI were 61 kg (41.2-90.5) and 22.8 (15.7-32.0), respectively, which are lower than has been reported from Western countries. These conditions are characteristic of recent trends in Japanese patients; older and less obese patients. Ninety-four patients (66.2%) were treatment naïve and the median value of HCV RNA was 2165 KIU/mL (130 to > 5000). The pretreatment median values were as follows; RBC 464 cells/ $\mu\text{L}$ , Hb 14.4 g/dL, PLT  $165 \times 10^3$  cells/ $\mu\text{L}$ , WBC 4775 cells/ $\mu\text{L}$ , NC 2549 cells/ $\mu\text{L}$ , AST 51 IU/L, and ALT 63 IU/L.

### Response rate and factors associated with svr

SVR was obtained in 83 (47.2%) patients and in 54 (54%) of the first 100 patients (selection data) enrolled in this study (Table 1). Of the 83, 43 were male; 60.6% of the male patients achieved SVR and there was a statistically significant gender difference ( $P = 0.020$ ). The median

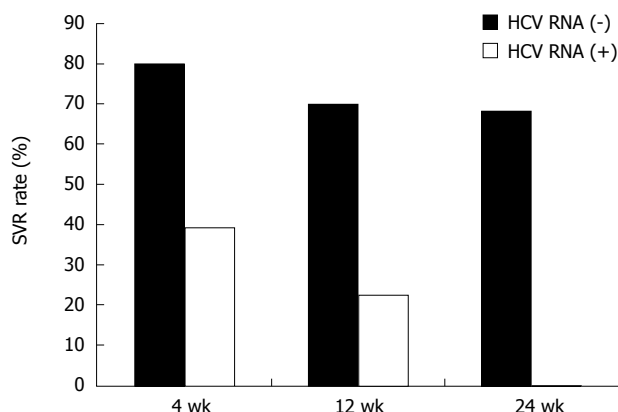
**Table 1** Basic demographic, virological, and clinical features of the 100 patients whose data were used as selection data

	SVR	non-SVR	P value
Number (%)	54 (54.0)	46	
Gender (%)			
Male	43 (60.6)	28	0.038
Female	9 (34.6)	18	
Age			
Median	53	57	0.0098
Range	18-72	37-77	
Weight (kg)			
Median	65.1	60	0.138
Range	42.5-90.5	43.9-86.0	
BMI ( $\text{kg/m}^2$ )			
Median	23.5	22.9	0.834
Range	17.5-31.8	18.2-31.2	
Previous + treatment	22	19	1.000
HCV RNA (KIU/mL)			
Median	1889	2263	0.554
Range	140 to < 5000	150 to < 5000	
RBC ( $\times 10^4/\text{mL}$ )			
Median	469	452	0.0041
Range	319-621	354-552	
Hb (g/dL)			
Median	15.0	14.1	0.0059
Range	10.9-17.7	11.3-17.2	
PLT ( $\times 10^3/\text{mL}$ )			
Median	172	159	0.039
Range	84-292	62-270	
WBC ( $/\text{mL}$ )			
Median	5000	4850	0.256
Range	3270-8900	2300-9200	
NC ( $/\text{mL}$ )			
Median	2550	2549	0.978
Range	1066-4231	1184-5626	
AST (IU/L)			
Median	54	50	0.898
Range	22-156	24-241	
ALT (IU/L)			
Median	76	55	0.027
Range	36-311	15-278	
PEG-IFN dose (48 wk) ( $\mu\text{g/kg}$ per day)			
Median	1.43	1.32	0.0043
Range	0.68-1.82	0.52-1.82	
RBV dose (48 wk) ( $\text{mg/kg}$ per day)			
Median	10.83	8.39	0.0002
Range	3.42-14.55	2.75-12.64	

SVR: Sustained virological response; BMI: Body mass index; RBC: Red blood cell count; Hb: Hemoglobin; PLT: Platelet count; WBC: White blood count; NC: Neutrophil count; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PEG-IFN dose (48 wk): Total dose of PEG-IFN at 48 wk ( $\mu\text{g/kg}$  per wk); RBV dose (48 wk): Total dose of RBV at 48 wk ( $\text{mg/kg}$  per day).

age was significantly lower in the SVR group. There was no difference in body weight and BMI between the SVR group and non-SVR group for the patients used for selection data. The pretreatment HCV RNA level did not differ significantly between the SVR and non-SVR groups, while pretreatment RBC, Hb, PLT and ALT levels differed between the groups. The average cumulative dose of RBV administered up to the time point indicated was always much greater in the SVR group than in





**Figure 1** Sustained virological response rates (%) of the patients during peg-interferon plus ribavirin therapy for chronic hepatitis C at week 4 (4 wk), week 12 (12 wk) and week 24 (24 wk). HCV RNA (-) means patients whose serum HCV RNA became undetectable at the time point indicated. HCV RNA (+) means patients whose serum HCV RNA was not cleared at the indicated time point.

the non-SVR group. The average cumulative dosage of PEG-IFN differed between the groups at week 48.

Among the first 100 patients, serum HCV RNA decreased 1 log or more at week 4 in 67 (67.0%) and became undetectable in 31 (RVR, 31.0%). SVR was attained in 25 of the 31 RVR patients (80.6%). Likewise, a complete EVR was attained in 66 patients, among whom 46 (69.7%) finally achieved SVR. SVR was achieved in 52 of 76 patients (68.4%) whose serum HCV RNA had disappeared by week 24. SVR also was achieved in patients whose serum HCV RNA had not disappeared until week 12. SVR was attained in 39.3%, 22.6% and 0% of patients who failed to achieve RVR, complete EVR and HCV RNA negativity at week 24, respectively (Figure 1). From these data, PPV and NPV determined by viral kinetics at week 4 were 80.6% and 60.7%, respectively. PPV at weeks 12 and 24 were 68.7% and 68.4% respectively, and NPV were 77.4% and 100%, respectively.

### Multivariate analysis for contributing factors to achieve SVR

Multivariate analysis was performed to determine the factors contributing to SVR. Analysis was made pretreatment and at weeks 4, 12 and 24. Factors available from pretreatment until at the time point were all included, and those calculated as  $P < 0.1$  by univariate analysis at each time point were analyzed using the logistic regression method. A statistical difference was found in gender, age, RBC, Hb, PLT and log (ALT 0 wk: ALT levels at week 0) at pretreatment by univariate analysis. The independent factor contributing to SVR was RBC ( $P = 0.024$ ) at pretreatment. Among significant factors found by univariate analysis at week 4, log (ALT 0 wk) ( $P = 0.015$ ), RVR (4 wk) ( $P = 0.0049$ ), and log (AST 4 wk) ( $P = 0.042$ ) were independent factors by multivariate analysis. Similarly, log (ALT 0 wk) ( $P = 0.0076$ ), EVR ( $P = 0.0083$ ), WBC (4 wk) ( $P = 0.035$ ), and average cumulative RBV dose ( $P = 0.045$ ) were significant factors at week 12. Independent

**Table 2** Logistic regression analysis of independent predictive factors for sustained virological response

Variables	Odds ratio	95% CI	P value
At pretreatment			
RBC ( $\times 10^4$ ) (0 wk)	1.011	1.002-1.021	0.024
PLT ( $\times 10^3$ ) (0 wk)	1.085	0.986-1.193	0.095
log (ALT 0 wk)	3.509	0.727-16.934	0.118
At week 4			
Age	0.941	0.885-1.000	0.051
log (ALT 0 wk)	27.090	1.891-388.001	0.015
RVR +/-	6.543	1.766-24.243	0.0049
log (AST 0 wk)	0.036	0.001-0.886	0.042
At week 12			
log (ALT 0 wk)	39.331	2.648-584.144	0.0076
RVR +/-	3.015	0.694-13.100	0.141
EVR +/-	8.340	1.728-40.265	0.0083
WBC (4 wk)	1.001	1.000-1.002	0.035
log (AST 12 wk)	0.049	0.002-1.037	0.053
RBV dose (12 wk)	1.519	1.010-2.284	0.045
At week 24			
log (ALT 0 wk)	68.688	3.669 to < 999.999	0.0047
RVR +/-	3.329	0.819-13.529	0.093
EVR +/-	31.775	2.840-355.460	0.0050
WBC (4 wk)	1.001	1.000-1.002	0.044
log (AST 12 wk)	0.036	0.001-0.918	0.044
RBV dose (12 wk)	1.607	1.021-2.528	0.040

contributing factors at week 24 were log (ALT 0 wk) ( $P = 0.0047$ ), HCV RNA (-/+) (24 wk) ( $P = 0.005$ ), WBC (4 wk) ( $P = 0.044$ ), log (AST 12 wk) ( $P = 0.044$ ) and average cumulative RBV dose (12 wk) ( $P = 0.040$ ) (Table 2). It was intriguing in addition to RVR and EVR that baseline ALT level (log) always affected SVR prediction from pretreatment until week 24.

### Predictive formulae of svr by logistic regression analysis

According to the method of logistic regression analysis, we derived four formulae to predict SVR of patients receiving 48-wk PEG-IFN plus RBV treatment in our cohort from significant factors selected by multivariate analysis at pretreatment and week 4, week 12 and week 24 as shown in Table 2. These formulae are as follows:

Pretreatment:  $1/p = 1 + \exp \{-[-8.8065 - 0.0114 \times \text{RBC} (\times 10^4) \text{ 0 wk} + 0.0812 \times \text{PLT} (\times 10^4) \text{ 0 wk} + 1.2552 \times \log (\text{ALT 0 wk})]\}$

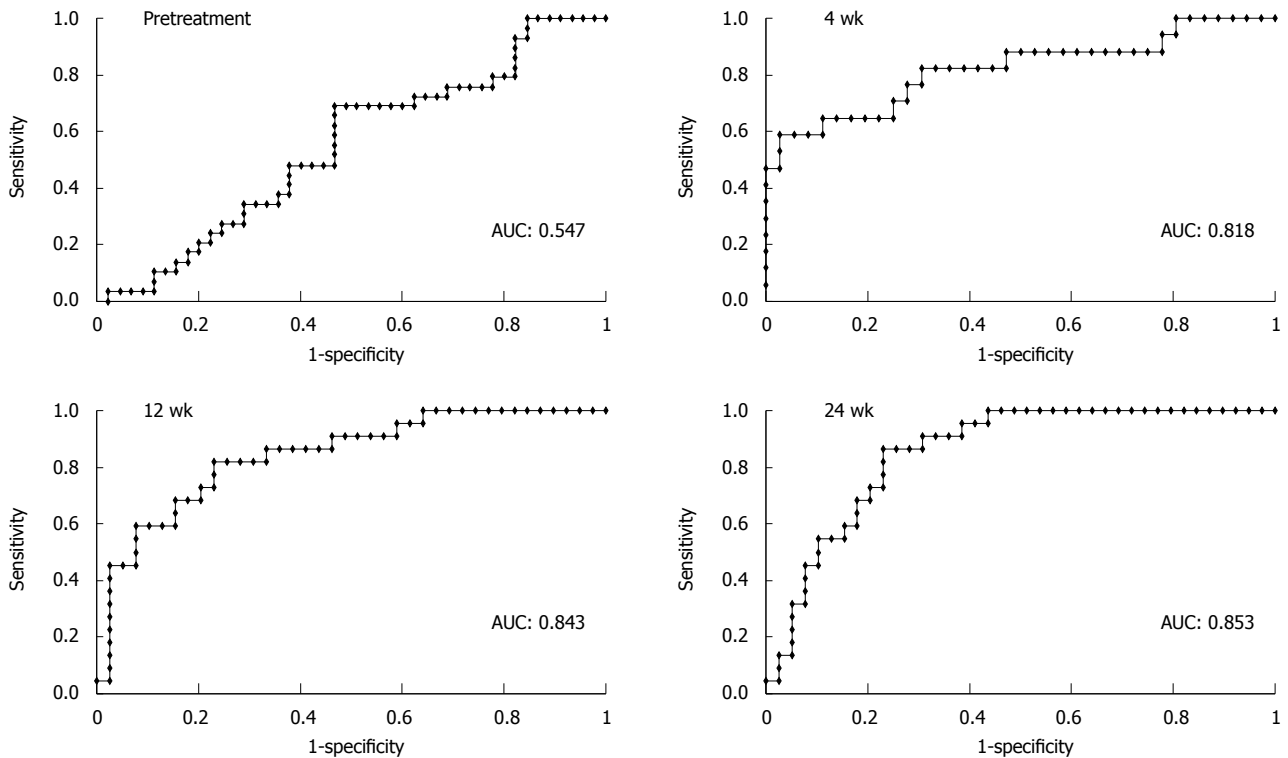
At week 4:  $1/p = 1 + \exp \{-[-1.8839 - 0.00607 \times \text{Age} + 3.2992 \times \log (\text{ALT 0 wk}) + 1.8784 \times \text{RVR} - 3.3364 \times \log (\text{AST 4 wk})]\}$

At week 12:  $1/p = 1 + \exp \{-[-11.5278 + 3.672 \times \log (\text{ALT 0 wk}) + 1.1036 \times \text{RVR} + 2.1211 \times \text{EVR} + 0.000837 \times \text{WBC 4 wk} - 3.0134 \times \log (\text{AST 12 wk}) + 0.418 \times \text{RBV dose 12 wk}]\}$

At week 24:  $1/p = 1 + \exp \{-[-14.5754 + 4.2296 \times \log (\text{ALT 0 wk}) + 1.2028 \times \text{RVR} + 3.4587 \times \text{HCV RNA 24 wk} + 0.0009 \times \text{WBC 4 wk} - 3.3224 \times \log (\text{AST 12 wk}) + 0.4741 \times \text{RBV dose 12 wk}]\}$

HCV RNA (-): 1, (+): 0.

ROC curve analysis was conducted to evaluate the accuracy of each prediction using both selection data and test data. The area under the curve of the ROCs



**Figure 2** Receiver operating characteristic (ROC) curves and the area under curve (AUC) of the predictive values made by the formulae during peg-interferon plus ribavirin therapy for chronic hepatitis C at week 4, 8, 12 and 24.

(AUCs) of multiple logistic regression analyses using selection data ( $n = 100$ ) at pretreatment and at weeks 4, 12 and 24 were 0.710, 0.828, 0.889, and 0.933, respectively. The predictive value at pretreatment was insufficient, but those after weeks 4, 12 and 24 were satisfactory.

The validity of the predictive formulae was evaluated further using test data ( $n = 76$ ). The AUCs of logistic regression analyses using test data were 0.547, 0.818, 0.843, and 0.853 at pretreatment and weeks 4, 12 and 24, respectively (Figure 2). Unlike prediction by viral kinetics, our formula is always applicable to all patients and the final predictive value is fairly high according to the ROC analysis, as described above. The median calculated values for patients who ultimately attained SVR were 0.545, 0.661, 0.589 and 0.656, at pretreatment and weeks 4, 12 and 24, respectively, and these values were always significantly higher than those of non-SVR cases (Table 3). The statistical difference of predictive values between SVR and non-SVR patients increased with the duration of therapy.

#### Prediction at week 48

It has been suggested that the prolongation of treatment is effective for patients who do not achieve HCV RNA negativity at week 12. There are also patients who achieve RVR and/or EVR but whose serum HCV RNA reappears after cessation of the treatment. We evaluated whether we can select such patients for whom treatment should be lengthened to 72 wk, using the same procedure as for on-treatment prediction of SVR with 48 wk of treatment. The predictive formula at week 48

**Table 3** The median predictive values for sustained virological response (SVR) rates calculated using our formulae at pretreatment and weeks 4, 12 and 24

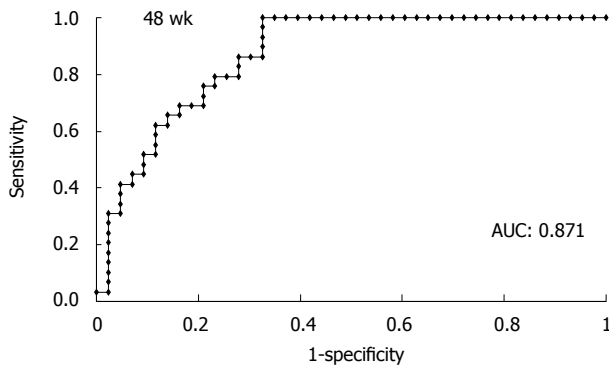
Median (range)	Patients who attained SVR	Patients with non-SVR	P value
Pretreatment	0.545 (0.286-0.926)	0.507 (0.108-0.945)	0.514
At week 4	0.661 (0.139-0.998)	0.251 (0.056-0.688)	0.000
At week 12	0.589 (0.079-0.994)	0.146 (0.001-0.952)	0.000
At week 24	0.656 (0.038-0.996)	0.026 (0.000-0.949)	0.000

The values were compared between patients who attained SVR and those who did not.

was constructed from the data of the 100 patients used for selection data. This formula included the parameter whether HCV RNA disappeared during therapy. When HCV RNA disappeared at week 4, 8, 12 and 24, each value, such as 4, 8, 12 and 24, was inserted into the formula. For non-SVR cases, 100 was inserted into the formula. The formula was determined as follows:

$$1/p = 1 + \exp \{ -[4.9107 - 0.0079 \times \text{Time of HCV RNA negative (wk)} + 0.1477 \times \text{PLT 0 wk} + 3.4941 \times \log(\text{ALT 0 wk}) - 1.7018 \times \log(\text{AST 12 wk})] \}$$

The ROC curve obtained from the test data of 76 patients is shown in Figure 3. The AUC derived from the test data ( $n = 76$ ) by logistic regression analysis was 0.871, suggesting that patients who can stop treatment at week 48 are predicted accurately by this formula. The median calculated value of patients who attained SVR was 0.775 (0.237-0.999) and that of patients who did not achieve



**Figure 3** Receiver operating characteristic (ROC) curves of the predictive values calculated by the formula from data up to 48 wk of peg-interferon plus ribavirin therapy for chronic hepatitis C.

SVR was 0.004 (0-0.966,  $P < 0.00001$ ). PPVs and NPVs calculated by the formula with various cut-off points are shown in Table 4. When we set the cut-off point at 0.2, NPV was 100% and 28 patients were included in this category.

There were 10 patients who relapsed after attaining complete EVR in 76 patients (4 cases achieved negativity of HCV RNA at week 8 and others achieved at week 12), and two of them could be pointed out as non-SVR by our calculation if the cut-off point was set at 0.5 (the value of one predicted person was 0.129 and another was 0.421).

## DISCUSSION

We propose here a method using formulae for the prediction of SVR in patients with chronic hepatitis C treated for 48 wk with PEG-IFN plus RBV. The predictive potential was very high when judged by AUC analysis, which was more than 0.8 from week 4. In particular, the validity at week 24 was more than 0.85 of AUC. The simplest method of prediction of SVR may be viral kinetics and a response-based on-treatment prediction, such as RVR and EVR, which are the outcomes of totally integrated viral and host factors. The PPV of the formulae were better at weeks 12 and 24 than the prediction with viral kinetics, and the NPV of the formulae were better at weeks 4 and 12. Evaluation of the formulae using data from the test patients revealed a very high AUC value of more than 0.85. These results suggest that formulae based on simple clinical data are superior to prediction by viral kinetics. These formulae, however, cannot be permanent, and may vary among different groups of patients, so should be re-evaluated or re-constructed, even for our series when the number of patients has increased. The most important outcome of this study is that we can predict SVR of our patients accurately with 48-wk PEG-IFN plus RBV therapy using only “simple” clinical data. Individual tailoring of treatment duration may be an option in the future to reduce relapse rates in HCV type 1-infected patients. The concept that

**Table 4** Positive and negative predictive values calculated by the formula constructed with data at week 48

Cut-off point	PPV (%)	NPV (%)	<i>P</i> value
0.2	65.9	100	< 0.00001
0.3	66.7	96.7	< 0.00001
0.4	66.7	86.1	< 0.00001
0.5	68.8	82.5	0.000013
0.6	71.4	79.5	0.000024
0.7	77.3	76.0	0.000048
0.8	80.0	70.2	0.00074

The calculated value was applied to the cut-off points and the numbers of patients with sustained virological response were evaluated.

extension of treatment duration can reduce relapse rates should be adopted only for a limited proportion of type 1-infected patients because the possibility of SVR may be very low in those whose serum HCV RNA remains positive at week 24. The patients who will benefit most from prolongation of therapy include those whose serum HCV RNA is positive at week 12 but negative at week 24, including even patients with RVR and EVR. The formulae we suggest might be helpful for such patients who are expected to achieve SVR but did not do so. For those individuals, our method based on logistic regression analysis will show a clear direction of therapy in each case and enable the best tailored treatment. Further prospective studies should be performed to determine whether this approach really increases the SVR rate by selection of patients and extension of treatment duration up to week 72.

RVR may be valuable for determining treatment duration but is not sufficient for predicting the response to treatment<sup>[8]</sup>. When SVR is predicted by RVR, the confirmed SVR rate within whole patients may be low. In the study of Yu *et al.*<sup>[19]</sup>, SVR was achieved in 42 of 100 patients, and all the patients who attained RVR achieved SVR. However, SVR was also attained in patients who did not attain RVR, and another 30 SVR patients who were not included in the RVR group. If 100% of patients with RVR attain SVR, the final prediction of SVR at this point (week 4) is 53.2%. In the study of Jensen *et al.*<sup>[20]</sup>, 95 of 374 (25.4%) genotype 1 patients attained RVR. The SVR rate of these 95 patients was 82% compared to 20.8% among the 374 treated subjects. The PPV of EVR for SVR is estimated to be less robust, less than 70%, and the validity may decrease more when we predict SVR by RVR only. We could predict SVR patients with 57.9% if the cut-off point was set at 0.5, and with 64.7% if that was set at 0.6 at week 4. The potent SVR of patients who did not achieve RVR could be predicted by our formula and the combination with viral kinetics may further improve predictive value.

Ferenci *et al.*<sup>[21]</sup> investigated response-guided treatment based on RVR and 78.8% of HCV genotype 1 patients with RVR attained SVR. Around 20% of patients with RVR failed to achieve SVR in their study. The SVR rate of patients with a complete EVR is around 80% and that

of patients without an EVR is around 15%. Therefore, there are several unfortunate patients with RVR and EVR but in whom serum HCV RNA reappears after the cessation of treatment. On the other hand, it may be difficult to attain SVR for patients whose serum HCV RNA does not disappear until after week 24 (late responders), even if they are treated for more than 48 wk with PEG-IFN plus RBV. Recent studies suggested that the extension of treatment to 72 wk would help to achieve SVR in such “unfortunate” patients, who should have responded well to the 48 wk therapy. It is not realistic that all patients who attain RVR and EVR should receive 72 wk therapy to ensure SVR. Our method of deriving a formula, predicting success or failure of response to 48 wk treatment, may serve as a good compass to identify patients who require extended treatment to achieve SVR. Of course, further prospective study is necessary and there has been no evidence that prolongation of therapy really decreases relapse rate. However, using the formula of week 48, we could predict patients who will benefit from an additional 24 wk of treatment and achieve an SVR. In fact, we could recognise 2 of 10 EVR patients as non-SVR by our formula and they would be rescued if they receive additional 24 wk therapy.

Perhaps the rule of stopping the treatment of patients with a decrease of less than  $2 \log_{10}$  in HCV RNA level within the initial 12 wk of therapy should be reconsidered because the high NPV of this rule (98%-100%) could be confirmed only for the 48 wk treatment group and not for the 72 wk group. As seen in our series, Japanese patients with chronic hepatitis C are older and have thinner physiques than those in Western countries. Because the time of infection was approximately 60 years ago<sup>[22]</sup>, and much earlier than elsewhere in the world, patients of an older age require treatment with PEG-IFN plus RBV<sup>[23]</sup>. However, these patients easily become anemic, probably because of their older age combined with their physical characteristics<sup>[24]</sup>, and the adherence to RBV, which may be critical for attaining SVR, is usually low. An RBV dosage of 1000-1200 mg/d is administered rarely in Japanese studies. The higher dose of PEG-IFN and RBV in 48 wk therapy suggested by Fried *et al.*<sup>[25]</sup>, who studied patients with a mean age of approximately 47, is almost impossible in Japan. When older patients opt for PEG-IFN plus RBV therapy, it is a “one-chance-treatment” and they always endure patiently the side effects of therapy but rarely agree to re-treatment after the cessation of therapy. Therefore, the on-treatment prediction of SVR is very important for older patients and, if the probability of success is reasonable, they would choose prolongation of the therapy. In this case, our formula is a very useful tool to decide whether the patient should receive additional therapy at week 48.

Many factors affecting the SVR rate have been reported, including viral- and host-related factors. Among the viral factors, amino acid substitutions in the interferon sensitivity determining region (ISDR) located in HCV nonstructural region 5A<sup>[11]</sup> and the core region (71st and 90th codons)<sup>[12]</sup> are well established. We also reported

amino acid substitutions in the RNA-dependent RNA polymerase (NS5B) region from our cohort study<sup>[26,27]</sup>. On the other hand, host factors, such as pretreatment intrahepatic CD8+ cell count<sup>[28]</sup>, the T-helper type 1 and 2 (Th1/Th2) ratio<sup>[13,29,30]</sup> and T-helper activity<sup>[31]</sup>, have also been demonstrated. Other factors, such as metabolic and diabetic factors, have been implicated in the efficacy of IFN therapy<sup>[32]</sup>. RBV plasma concentration at week 4<sup>[14]</sup> and a new index, named accordion index<sup>[33]</sup>, have also been proposed. These significant viral and host factors, except for the metabolic factors, are difficult to examine and daily clinical assessment is not practical. Shirakawa *et al.*<sup>[15]</sup> published an excellent report recently on the classification of patients according to their responsiveness to PEG-IFN plus RBV therapy. They predicted SVR successfully with pretreatment data, but the prediction included particular determinations, such as ISDR sequences and Th1/Th2 ratios, which are not easily available in clinics and are uneconomical. In contrast to their report, the formulae proposed in this study involve factors included among readily available data, and moreover, the validity was very high, especially at weeks 24 and 48.

In conclusion, our predictive formula, which is easily constructed in every institution with simple clinical data, would offer better prediction of SVR and non-SVR than the prediction by viral kinetics. Further study including extended protocol (72 wk treatment) and analysis with other measurement of HCV RNA, such as real-time PCR, should be evaluated in the future.

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## COMMENTS

### Background

The likelihood of treatment success of 48 wk peg-interferon (PEG-IFN) plus ribavirin (RBV) therapy for chronic hepatitis C may be predicted by viral kinetics on therapy. In particular, recent studies have shown that sustained virological response (SVR) can be predicted by a rapid virological response (RVR), and



an early virological response (EVR). Nevertheless, the current dosing regimens could potentially under-treat some patients and additional measurement of viral response is needed to facilitate individualization of therapy. Among predictive factors already reported, many are not readily available from daily clinical assessment, because they require genomic analyses and/or advanced experimental methods.

### Research frontiers

It is difficult to accurately determine whether the individual should have their therapy extended at week 48, because the predictive value of a slow virological response may be insufficient alone. It would be very valuable to have a more accurate predictive marker of SVR at week 48, derived from clinically available measurements. According to the method of logistic regression analysis, the authors of this study derived formulae to predict SVR of patients receiving 48 wk PEG-IFN plus RBV treatment in their cohort from significant factors selected by multivariate analysis at pretreatment and weeks 4, week 12 and week 24.

### Innovations and breakthroughs

The most important outcome of this study is that it is possible to predict SVR accurately with 48 wk PEG-IFN plus RBV therapy by formulae using only "simple" clinical data.

### Applications

This study may enable the best tailored treatment especially for patients with a high expectation of sustained virological response with 48 wk peg-interferon and ribavirin therapy for chronic hepatitis C but whose responses relapse. Prospective studies informed by this method will be of considerable value.

### Terminology

RVR is defined as an undetectable level of HCV RNA at 4 wk of treatment, and EVR is defined as either an undetectable level of HCV RNA or a drop in HCV RNA levels of at least 2 log<sub>10</sub> IU/mL after 12 wk of treatment. Recent studies reported that these on-treatment viral kinetics are useful for prediction of SVR.

### Peer review

Conclusively, establishing a new predictive formula to assess the likelihood of a SVR of the individual patient chronically infected with HCV is very important.

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## Reoperation for early postoperative complications after gastric cancer surgery in a Chinese hospital

Birendra Kumar Sah, Ming-Min Chen, Min Yan, Zheng-Gang Zhu

Birendra Kumar Sah, Ming-Min Chen, Min Yan, Zheng-Gang Zhu, Department of General Surgery, Ruijin Hospital, Shanghai Jiaotong University, School of Medicine, Shanghai Institute of Digestive Surgery, Shanghai 200025, China

Author contributions: Sah BK designed the study, collected the data, and drafted the manuscript; Chen MM and Yan M assisted in interpretation of data and assisted in drafting the manuscript; Zhu ZG participated in the design and final approval of the study and critical revision of the article.

Correspondence to: Zheng-Gang Zhu, Professor, Department of General Surgery, Ruijin Hospital, 197 Ruijin Er Road, Shanghai 200025, China. [rjzhuzhenggang@hotmail.com](mailto:rjzhuzhenggang@hotmail.com)

Telephone: +86-21-64370045 Fax: +86-21-53821171

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**CONCLUSION:** Reoperation significantly increases the mortality rate and raises the burden of the surgical unit. More prospective studies are required to explore the potential risk factors.

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**Key words:** Reoperation; Gastric cancer; Surgery; Postoperative complications

**Peer reviewers:** AM El-Tawil, PhD, Department of Surgery, University Hospital of Birmingham, Edgbaston, B15 2TH, United Kingdom; Michael Leitman, MD, FACS, Chief of General Surgery, Beth Israel Medical Center, 10 Union Square East, Suite 2M, New York, NY 10003, United States

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### Abstract

**AIM:** To investigate the occurrence of postoperative complications of gastric cancer surgery, and analyze the potential causes of reoperation for early postoperative complications.

**METHODS:** A total of 1639 patients who underwent radical or palliative gastrectomies for gastric cancer were included in the study. The study endpoint was the analysis of postoperative complications in inpatients.

**RESULTS:** About 31% of patients had early postoperative complications, and complications of infection occurred most frequently. Intra-abdominal hemorrhage and anastomotic leak were the main causes of reoperation, which accounted for about 2.2%. Mortality was 11.1% in the reoperation group, but was only 0.8% in other patients. The duration of postoperative stay in hospital was significantly longer and the total expenditure was markedly higher in the patients who underwent reoperation ( $P < 0.001$ ). There was no significant association of any available factors in this study with the high rate of reoperation.

### INTRODUCTION

Though the occurrence of postoperative complications and mortality rate after surgery for gastric cancer have significantly decreased over the past years, they are still considered high<sup>[1,2]</sup>. Radical gastrectomy with D2 lymph node dissection is widely accepted, but the extent of lymph node dissection is controversial among different centers<sup>[3-10]</sup>. It is well accepted that the extent of surgery (particularly aggressive dissection of the lymph nodes) does not extend the overall survival, and postoperative complications were significantly related to the extent of surgery, particularly the extent of lymph node dissection. This was proved by Japanese surgeons who conducted several clinical randomized controlled trials (RCTs)<sup>[3,9-11]</sup>. Sasako *et al*<sup>[9]</sup> conducted a RCT in 24 hospitals in Japan to compare D2 lymphadenectomy alone with D2 lymphadenectomy plus para-aortic nodal dissection

(PAND) in patients undergoing gastrectomy for curable gastric cancer. They concluded that compared with D2 lymphadenectomy alone, D2 lymphadenectomy plus PAND does not improve the survival in curable gastric cancer; extended D2 lymphadenectomy plus PAND should not be performed to treat curable stage T2b, T3, or T4 gastric cancer; and that D2 gastrectomy is associated with the low mortality and reasonable survival of the patients.

Many researchers at leading centers for gastric cancer, including those in Korea and China, have indicated that combined resection of other organs is not of long-term benefit and it significantly increased the prevalence of postoperative complications and mortality<sup>[3,4,11-13]</sup>. The occurrence rates of postoperative complications in the spleen-preservation group and splenectomy group were 11.6% and 29.3%, respectively. There was a higher frequency of pleural effusion, intra-abdominal abscess, and pancreatitis in splenectomized patients. A higher recurrence was observed in the splenectomy group (40.4%) compared with the spleen-preservation group (25.1%). The mean survival time was 72.0 mo in the spleen-preservation group compared with 56.7 mo in the splenectomy group<sup>[13]</sup>. Investigation of early postoperative complications would therefore be beneficial to optimize the extent of gastric cancer surgery.

Reoperation after routine surgery, particularly after gastric cancer surgery, increases the overall burden for both the surgical ward and the patients. We therefore investigated the factors causing reoperation and their effects on the recovery of patients who undergo surgery for gastric cancer.

## MATERIALS AND METHODS

A total of 1639 patients who underwent radical or palliative gastrectomies for gastric cancer in five consecutive years were included in the study (Table 1). Data were collected directly by comprehensive review of the original records of all patients. Sixty-seven patients with missing data and 13 patients who underwent emergency surgery were excluded from the analysis. Exclusion criteria were disease other than gastric cancer, and any type of palliative surgery (including exploratory laparotomy and gastrojejunal anastomosis) other than gastrectomy. The median age of the patients was 59 years (range, 17-93 years). The ratio of male and female patients was approximately 7:3.

Patients with early and resectable advanced gastric cancer underwent radical surgery (gastrectomies with D2 lymphadenectomy). Patients with late-stage gastric cancer underwent palliative gastrectomy. Most patients were diagnosed to be in stage III. The tumor invaded the serosa or adjacent structures in 38.1% of patients which was classified as pT3, and in 11.4% of patients classified as pT4 (Table 1). With respect to combined organ resection, 19 splenectomies, 14 partial pancreatectomies with splenectomy, 14 partial colectomies, two partial colectomies with splenectomy, seven partial hepatectomies (lobectomy),

Table 1 Demographic data of the patients

Items	Percentage (%)
Age group (yr)	
≤ 60	54.1
61-70	25.2
≥ 71	20.7
Sex	
Male	68.9
Female	31.1
Diagnosis	
Primary gastric cancer	97.4
Gastric stump cancer <sup>1</sup>	2.6
Site of tumor	
Proximal	17.6
Body	13.5
Distal	43.3
Large or multiple	25.6
No. of procedures	
Partial gastrectomy	76.0
Total gastrectomy	24.0
Type of resection	
Radical gastrectomy	91.6
Palliative gastrectomy	8.4
Combined resection	
Yes	4.4
No	95.6
Type of anastomosis	
Billroth I	47.6
Billroth II	13.9
Billroth reverse <sup>2</sup>	13.4
Roux-en-Y	21.6
Roux-en-Y (P-shape)	3.0
Others	0.5
Unknown	0.3
TNM stage	
I A	12.1
I B	8.5
II	18.1
III A	20.8
III B	16.2
IV	24.3

<sup>1</sup>Including recurrent gastric cancer; <sup>2</sup>Oesophago-gastric anastomosis.

one total hysterectomy and one partial pancreatectomy were carried out in 58 patients (Table 1). About 25% of the patients underwent total gastrectomy. Billroth I, typical Roux-en-Y, and Billroth reverse (esophagogastric anastomosis) were the preferred methods for anastomosis after distal gastrectomy, total gastrectomy and proximal partial gastrectomy. Above 85% patients underwent surgery by senior surgeons with experience of 20-30 years. The minimal working experience of the surgeons was > 15 years. No surgical fellow or surgeons-in-training was allowed to perform the surgery independently. All the patients were managed by senior attendants under direct supervision of the surgeons.

The endpoint was analysis of postoperative complications and postoperative mortality in inpatients. Complications were recorded according to the definitions stated in the Physiological and Operative Severity Score for the Enumeration of Mortality and Morbidity (POSSUM)<sup>[14]</sup>. As there are many complications that are not covered by its definitions, an undefined complication was therefore



recorded as “innominate” and the details were provided in separate tables. Severities of all complications were stratified according to Rui Jin Hospital Classification of Complications<sup>[15]</sup>.

We also audited the overall expenditure in US dollars (\$) of patients during their stay in hospital and compared it between the reoperation group and non-reoperation group.

### Statistical analysis

The statistical analysis was done using the Statistical Package for Social Science (SPSS) version 13.0 for Windows (SPSS, Incorporated, Chicago, IL, USA). Non-parametric methods were used to test the data without normal distribution.  $P < 0.05$  was considered significant.

## RESULTS

About 31% of patients had different types of complications according to POSSUM criteria. The prevalence of individual complications was not equal to the total number of complications. Multiple complications were possible in a single patient (Table 2). Postoperative infection was the most common complication. The occurrence of anastomotic leak was about 2%, and postoperative mortality was only 1%.

There were numerous innominate complications (Table 3), most of which were accompanied by complications described in POSSUM. Most patients had pleural effusion or/and seroperitoneum, most of which were accompanied by low fever but pathological diagnosis of infection could not be confirmed. A substantial number of patients had persistent fever or recurring fever of unknown origin. About 5% of patients had persistent nausea or vomiting caused by gastroplegia or enteroplegia, anastomosis edema, or ileus. Some patients were clinically suspected to have a minor leak but there was no sufficient objective evidence to support this finding. Although these patients were managed by conservative treatment (mainly NPO, intravenous antibiotics, and total parenteral nutrition), they still increased the burden on the surgical ward. Complications were rare such as pancreatic fistula, chyle leak, and bleeding at the anastomosis site.

Innominate complications were recorded empirically and merged to calculate different levels of complication type according to the Rui Jin Hospital Classification of Complications<sup>[15]</sup>. Most complications were minor (11.0%) or moderate (15.2%), only 8.3 % of patients had severe complications.

Patients were categorized into three levels according to the length of postoperative stay in hospital. About 75% of patients were discharged in good condition in less than 15 d after uneventful recovery and removal of sutures, 19.7% of patients discharged within 16-30 d and only 6% of patients stayed in hospital for more than a month.

There was a significant difference in the occurrence rate of overall complications between partial and total gastrectomy with radical lymphadenectomy, but no significant difference between partial and total gastrectomy with palliative lymphadenectomy was observed (Table 4).

Table 2 Details of complications

Complications	Frequency	Percentage (%)
Hemorrhage		
Wound	1	0.1
Deep	16	1.0
Wound dehiscence		
Superficial	9	0.5
Deep	5	0.3
Anastomotic leak	38	2.3
Infection		
Wound	15	0.9
Deep	80	4.9
Pyrexia of unknown origin	253	15.4
Septicemia	7	0.4
Chest	172	10.5
Urinary tract infection	20	1.2
System failure		
Renal	28	1.7
Respiratory	22	1.3
Cardiac	13	0.8
Hypotension	16	1.0
Deep venous thrombosis	4	0.2
Death	17	1.0
Overall	506	30.9

Table 3 Innominate complications

Complications	Frequency	Percentage (%)
Pleural effusion	213	13
Continuous or relapsing pyrexia of unknown origin	170	10.4
Seroperitoneum	118	7.2
Gastro or enteroplegia, anastomosis edema, ileus	75	4.6
Suspicious or sub-clinical anastomotic leak	57	3.5
Pancreatitis	22	1.3
Central vein catheter infection	14	0.9
Anastomosis site or upper GI bleeding	10	0.6
Chyle leak	8	0.5
Pancreatic fistula	6	0.4

The occurrence of complications of infection (including deep infection, pulmonary infection), system failure, and mortality was significantly higher in total gastrectomy with radical lymphadenectomy. After stratification of patients into partial and total gastrectomy groups, we noted no significant difference in complication occurrence between radical lymphadenectomy and palliative lymphadenectomy (Table 5).

Thirty-six patients underwent reoperation for different causes, with intra-abdominal hemorrhage and anastomotic leak as the main causes (Table 6). There was no significant difference in physiological score (PS;  $P = 0.382$ ) and operative severity score (OSS;  $P = 0.849$ ) between patients in the reoperation group and the non-reoperation group. Median values of PS and OSS in the reoperation group were 14.5 (range, 12-25) and 18 (range, 16-24) respectively, whereas they were 15 (range, 12-38) and 18 (range, 11-28) in the non-reoperation group. Mortality was significantly higher in patients who underwent reoperation ( $P < 0.001$ ), being 11.1% in the reoperation group but only 0.8% in

**Table 4** Difference of complication rate between partial and total gastrectomy

LN dissection Gastrectomy	Radical			Palliative		
	Partial	Total	Sig	Partial	Total	Sig
Overall	308 (26.7)	142 (40.9)	< 0.001	32 (34.8)	24 (52.2)	NS
Reoperation	23 (2.0)	12 (3.5)	NS	1 (1.1)	0	NS
Hemorrhage						
Wound	1 (0.1)	0	NS	0	0	
Deep	11 (1.0)	4 (1.2)	NS	1 (1.1)	0	NS
Wound dehiscence						
Superficial	7 (0.6)	1 (0.3)	NS	1 (1.1)	0	NS
Deep	4 (0.3)	1 (0.3)	NS	0	0	
Leak	22 (1.9)	12 (3.5)	NS	1 (1.1)	3 (6.5)	NS
Infection						
Wound	8 (0.7)	6 (1.7)	NS	1 (1.1)	0	NS
Deep	42 (3.6)	30 (8.6)	< 0.001	5 (5.4)	3 (6.5)	NS
PUO	165 (14.3)	65 (18.7)	0.044	13 (14.1)	10 (21.7)	NS
Septicemia	1 (0.1)	6 (1.7)	< 0.001	0	0	
Chest	86 (7.5)	61 (17.6)	< 0.001	14 (15.2)	11 (23.9)	NS
UTI	13 (1.1)	6 (1.7)	NS	1 (1.1)	0	NS
System failure						
Renal	15 (1.3)	12 (3.5)	0.008	1 (1.1)	0	NS
Respiratory	12 (1.0)	7 (2.0)	NS	3 (3.3)	0	NS
Cardiac	5 (0.4)	6 (1.7)	0.034	2 (2.2)	0	NS
Hypotension	8 (0.7)	6 (1.7)	NS	2 (2.2)	0	NS
DVT	1 (0.1)	3 (0.9)	NS	1 (1.1)	0	NS
Death	6 (0.5)	8 (2.3)	0.007	2 (2.2)	1 (2.2)	NS

Sig: Significance; LN: Lymph node; PUO: Pyrexia of unknown origin; UTI: Urinary tract infection; DVT: Deep vein thrombosis.

**Table 5** Difference of complications rate between radical and palliative LN dissection

LN dissection Gastrectomy	Partial			Total		
	Radical	Palliative	Sig	Radical	Palliative	Sig
Overall	308 (26.7)	32 (34.8)	NS	142 (40.9)	24 (52.2)	NS
Reoperation	23 (2.0)	1 (1.1)	NS	12 (3.5)	0	NS
Hemorrhage						
Wound	1 (0.1)	0	NS	0	0	NS
Deep	11 (1.0)	1 (1.1)	NS	4 (1.2)	0	NS
Wound dehiscence						
Superficial	7 (0.6)	1 (1.1)	NS	1 (0.3)	0	NS
Deep	4 (0.3)	0	NS	1 (0.3)	0	NS
Leak	22 (1.9)	1 (1.1)	NS	12 (3.5)	3 (6.5)	NS
Infection						
Wound	8 (0.7)	1 (1.1)	NS	6 (1.7)	0	NS
Deep	42 (3.6)	5 (5.4)	NS	30 (8.6)	3 (6.5)	NS
PUO	165 (14.3)	13 (14.1)	NS	65 (18.7)	10 (21.7)	NS
Septicemia	1 (0.1)	0	NS	6 (1.7)	0	NS
Chest	86 (7.5)	14 (15.2)	0.008	61 (17.6)	11 (23.9)	NS
UTI	13 (1.1)	1 (1.1)	NS	6 (1.7)	0	NS
System failure						
Renal	15 (1.3)	1 (1.1)	NS	12 (3.5)	0	NS
Respiratory	12 (1.0)	3 (3.3)	NS	7 (2.0)	0	NS
Cardiac	5 (0.4)	2 (2.2)	NS	6 (1.7)	0	NS
Hypotension	8 (0.7)	2 (2.2)	NS	6 (1.7)	0	NS
DVT	1 (0.1)	1 (1.1)	NS	3 (0.9)	0	NS
Death	6 (0.5)	2 (2.2)	NS	8 (2.3)	1 (2.2)	NS

other patients (Table 7). In the reoperation group, the mortality rate of patients with radical lymphadenectomy was higher than that of patients who underwent palliative lymphadenectomy. Mortality rate was higher in patients who underwent total gastrectomy than in those who

**Table 6** Causes of reoperation

Causes	Surgical management	Frequency
Intra-abdominal hemorrhage <sup>1</sup>	Simple hemostasis	16
Anastomotic leak	Repair and placement of drainage	10
Deep wound dehiscence	Closure of abdominal wall	4
Abdominal infection	Debridement and placement of drainage	3
Ileus	Adhesiolysis of small intestine	2
Anastomotic obstruction	Reconstruction	1

<sup>1</sup>Including 2 cases of anastomosis site bleeding.

**Table 7** Potential causes of death

Complications	Reoperation <i>n</i> (%)	
	Yes ( <i>n</i> = 36)	No ( <i>n</i> = 1603)
Death	4 (11.11)	13 (0.81)
Extent of surgery		
LN dissection		
Radical	4 (11.11)	10 (0.62)
Palliative	0	3 (0.18)
Gastrectomy		
Partial	1 (0.03)	7 (0.44)
Total	3 (8.33)	6 (0.37)
Complications		
Intra abdominal hemorrhage	1 (0.03)	0
Anastomotic leak	2 (5.55)	0
Infection		
Deep	1 (0.03)	2 (0.12)
Pyrexia of unknown origin	0	3 (0.18)
Septicemia	0	1 (0.06)
Chest	3 (8.33)	7 (0.44)
Urinary tract	2 (5.55)	0
System failure		
Renal	3 (8.33)	8 (0.49)
Respiratory	4 (11.11)	10 (0.62)
Cardiac	2 (5.55)	9 (0.56)
Hypotension	1 (0.03)	9 (0.56)
Deep venous thrombosis	0	3 (0.18)
Pancreatitis	0	1 (0.06)
Anastomosis site bleeding	0	1 (0.06)

underwent partial gastrectomy (Table 7).

Except for four patients with wound dehiscence who were discharged within one month, the other 32 patients were treated in hospital for more than one month. The length of postoperative stay was significantly longer in patients who underwent reoperation ( $P < 0.001$ ). The mean duration of postoperative stay was 44.6 d (standard deviation, SD = 29.41 d) in patients with reoperation, but was only 14.6 d (SD = 8.09 d) in other patients.

Reoperation caused a significant economic burden for patients. There was a significant difference in the total expenditure between groups of patients with or without reoperation ( $P < 0.001$ ). The median expenditure in patients with reoperation was 7946.36 \$ (SD = 8930.38 \$) but it was only 3238.32 \$ (SD = 4404.63 \$) in other patients.

Univariate analysis of the data revealed no significant association of any available factors in this study with the higher rate of reoperation, including age, hypertension,

anemia, hypoalbuminemia, hyperglycemia, type of gastrectomy, combined organ resection, type of anastomosis, surgeon's experience (number of operations performed), tumor stage.

## DISCUSSION

In the surgical approach for early and selective advanced gastric cancer, gastrectomy with D2 lymphadenectomy is justified<sup>[6,16-19]</sup>. The procedure of surgery, particularly the extent of lymphadenectomy for gastric cancer, varies among individual centers. The occurrence of postoperative complications was higher in inexperienced hands, and there was a considerable difference in early surgical outcomes among centers<sup>[3,20]</sup>. Postoperative complications were inversely correlated with the number of patients undergoing treatment in a surgical unit<sup>[21]</sup>; similar results were published for patients undergoing surgery for gastric cancer<sup>[15]</sup>. Overall survival rate was higher at specialized centers. It was therefore stressed in many articles that gastric cancer surgery was safe at specialized centers<sup>[3,6,22,23]</sup>.

The postoperative complications at our institution were in the acceptable range because most patients had a smooth recovery and postoperative mortality was not high. Overall surgical outcome was acceptable because of the occurrence rate of complications was below the moderate level. Postoperative infection was the commonest complication. There are several complications (e.g. gastroplegia or enteroplegia, suspicious anastomotic leak, pleural effusion) which are not covered by POSSUM. These complications cannot be ignored because they have a big impact on the overall burden (patient-related and economic) of our hospital. A substantial number of patients had persistent fever without a clear diagnosis; appropriate investigation was necessary to find the cause. Further investigation was required to classify or define the diagnosis of sub-clinical anastomotic leak. The Ruijin Hospital Classification of Complication, stratifies complications to different levels according to the severity of the disease, and is a validated classification<sup>[2,15]</sup>. We suggest that other hospitals use this classification for assessment of surgical outcome.

Reoperation was in the acceptable range as compared with a recent report from the Korean Institute, and the mortality caused by reoperation was low<sup>[24]</sup>. Most reoperations were carried out for intra-abdominal hemorrhage, which may be related to the experience of surgeons and necessitates additional efforts to examine the easily missed bleeding sites (particularly anastomosis sites). The four cases of rupture of the abdominal wall may be attributed to the poor surgical technique because these patients had their linea alba closed by an interrupted silk suture. We did not observe this complication in patients with linea alba closed by a continuous absorbable suture. Anastomotic leak was followed by intra-abdominal infection which often caused peripancreatic abscess, and eventually pancreatic fistulas in some cases. Improvement of surgical

technique is therefore crucial to lower the occurrence of intra-abdominal hemorrhage and anastomotic leak.

In conclusion, although the overall occurrence of postoperative complications was high after gastric cancer surgery, the occurrence rate of severe complications and mortality were low. Reoperation after gastric cancer surgery significantly increases the mortality and overall burden of the surgical unit. As the gastric cancer surgery is considered as a routine surgery, it is important to control the postoperative complications. Univariate analysis of the data revealed no significant association of any available factors in this study with the high rate of reoperation; however, more prospective studies are required to explore the potential risk factors for the higher rate of reoperation after gastric cancer surgery.

## COMMENTS

### Background

Though the occurrence of postoperative complications and mortality after surgery for gastric cancer have significantly decreased over the past years, they are still considered high. It was well accepted that the extent of surgery does not extend the overall survival and that postoperative complications were significantly related to the extent of surgery. Therefore, surgical extent should be seriously considered and postoperative complications should not be ignored.

### Innovations and breakthroughs

The postoperative complication is highly variable among different centers. However, surprisingly there are very few reports on this issue, especially from Chinese surgical centers. This study was conducted at a leading center for gastric cancer surgery in China, and analyzed a large cohort of patients for a long period. It provides the details on the occurrence of postoperative complications and analyzed its impact on patients and surgical ward. The finding of this study certainly provides very useful reference to the surgeons working in this field.

### Applications

The better understanding about the occurrence of different types of the postoperative complications and its underlying causes may help surgeons reduce the postoperative complications and upgrade the quality of surgical treatment.

### Terminology

"POSSUM" is an internationally accepted scoring system which is applied for the evaluation of surgical treatment. "Rui Jin Hospital Classification of the complications" is a novel system which stratifies all the complications in three different levels and provides objective idea about the severity of complications.

### Peer review

The article has some very good information and is worthy of publication.

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## Electro-acupuncture to prevent prolonged postoperative ileus: A randomized clinical trial

Zhi-Qiang Meng, M Kay Garcia, Joseph S Chiang, Hui-Ting Peng, Ying-Qiang Shi, Jie Fu, Lu-Ming Liu, Zhong-Xing Liao, Ying Zhang, Wen-Ying Bei, Bob Thornton, J Lynn Palmer, Jennifer McQuade, Lorenzo Cohen

Zhi-Qiang Meng, Hui-Ting Peng, Jie Fu, Lu-Ming Liu, Department of Integrative Oncology, Fudan University Cancer Hospital, Shanghai 200032, China

M Kay Garcia, Department of Anesthesiology and Pain Medicine/Integrative Medicine, M.D. Anderson Cancer Center, The University of Texas, Houston, TX 77030, United States

Joseph S Chiang, Department of Anesthesiology and Pain Medicine, M.D. Anderson Cancer Center, The University of Texas, Houston, TX 77030, United States

Zhi-Qiang Meng, Ying-Qiang Shi, Jie Fu, Lu-Ming Liu, Ying Zhang, Wen-Ying Bei, Department of Oncology, Shanghai Medical College, Shanghai 200032, China

Ying-Qiang Shi, Department of Abdominal Surgery, Fudan University Cancer Hospital, Shanghai 200032, China

Zhong-Xing Liao, Department of Radiation Oncology, M.D. Anderson Cancer Center, The University of Texas, Houston, TX 77030, United States

Ying Zhang, Wen-Ying Bei, International Center of Integrative Oncology, Fudan University Cancer Hospital, Shanghai 200032, China

Bob Thornton, Department of Palliative Care and Rehabilitation Medicine, M.D. Anderson Cancer Center, The University of Texas, Houston, TX 77030, United States

J Lynn Palmer, Lorenzo Cohen, Department of Behavioral Science, M.D. Anderson Cancer Center, The University of Texas, Houston, TX 77030, United States

Jennifer McQuade, Department of GI Medical Oncology, M.D. Anderson Cancer Center, The University of Texas, Houston, TX 77030, United States

**Author contributions:** Meng ZQ and Garcia MK are both first authors who oversaw all aspects of the study and wrote the manuscript; Peng HT and Fu J performed the acupuncture treatments; Zhang Y and Bei WY were research nurses responsible for recruiting and consenting patients and for data collection and management; Shi YQ oversaw all surgical aspects of the study and helped with patient recruitment; Palmer JL provided the statistical analysis; Thornton B, McQuade J and Liao ZX assisted with language translation throughout the study and writing of the manuscript; Chiang JS assisted with language translation, protocol development and oversaw all clinical aspects of the study; Cohen L and Liu LM were responsible for all administrative and financial aspects of protocol development, study implementation, and writing of the manuscript.

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Correspondence to: Lu-Ming Liu, MD, PhD, Department of Integrative Oncology, Fudan University Cancer Hospital, Shanghai 200032, China. [llm1010@163.com](mailto:llm1010@163.com)

Telephone: +86-21-64175590 Fax: +86-21-64437657

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### Abstract

**AIM:** To examine whether acupuncture can prevent prolonged postoperative ileus (PPOI) after intraperitoneal surgery for colon cancer.

**METHODS:** Ninety patients were recruited from the Fudan University Cancer Hospital, Shanghai, China. After surgery, patients were randomized to receive acupuncture (once daily, starting on postoperative day 1, for up to six consecutive days) or usual care. PPOI was defined as an inability to pass flatus or have a bowel movement by 96 h after surgery. The main outcomes were time to first flatus, time to first bowel movement, and electrogastroenterography. Secondary outcomes were quality of life (QOL) measures, including pain, nausea, insomnia, abdominal distension/fullness, and sense of well-being.

**RESULTS:** No significant differences in PPOI on day 4 ( $P = 0.71$ ) or QOL measures were found between the groups. There were also no group differences when the data were analyzed by examining those whose PPOI had resolved by day 5 ( $P = 0.69$ ) or day 6 ( $P = 0.88$ ). No adverse events related to acupuncture were reported.

**CONCLUSION:** Acupuncture did not prevent PPOI and

was not useful for treating PPOI once it had developed in this population.

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**Key words:** Acupuncture; Gastrointestinal motility; Gastrointestinal disorders; Gastrointestinal neoplasms

**Peer reviewers:** Dr. Kalpesh Jani, MS, DNB, FNB, MNAMS, FICS, FACS (USA), Consultant GI & Laparoscopic Surgeon, SIGMA Surgery, 390011 Baroda, Gujarat, India; Naoki Hiki, MD, PhD, Cancer Institute Hospital, 3-10-6, Ariake, Koto-ku, Tokyo 135-8550, Japan

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## INTRODUCTION

Bowel dysfunction following abdominal surgery is common and usually temporary, lasting no more than 3 d; however, if patients are unable to tolerate an oral diet, pass flatus, or have a bowel movement by postoperative day 4, they are considered to have prolonged postoperative ileus (PPOI). PPOI is uncomfortable for patients and potentially dangerous. The collection of gas and secretions related to PPOI causes pain and discomfort with bloating, distention, and often emesis<sup>[1]</sup>. Delayed gastric emptying also increases the risk of aspiration in patients during the early postoperative period. Patients with PPOI cannot be discharged from the hospital until the ileus has resolved. Few published studies have estimated the cost of PPOI, but expenses related to longer hospital stays, nursing care, laboratory/diagnostic testing, and interventional treatments are likely to be considerable.

Current treatment for postoperative ileus in China is primarily supportive and includes nasogastric suction, intravenous fluids, parenteral nutrition, and gradual ambulation with simple exercises. However, a variety of preventive interventions<sup>[2-6]</sup> for PPOI, such as preoperative carbohydrate loading, wrapping patients in warm blankets in the operating room, chewing gum, and rocking in a rocking chair postoperatively to stimulate gastrointestinal function, have been mentioned in the literature. Further research is needed, however, to evaluate the efficacy of these approaches. Two studies published by Sculati and colleagues have concluded that a preoperative bran-enriched diet (lasting 8-10 d) may help prevent PPOI<sup>[7,8]</sup>, but this is often not practical for patients undergoing gastrointestinal surgery.

Although prevention and treatment of PPOI with various pharmacologic agents has been explored for several years, success has been limited<sup>[9-17]</sup>. Alternatives

to systemic opioid analgesia, such as thoracic epidural analgesia<sup>[18]</sup> and non-opioid analgesics such as ketorolac tromethamine<sup>[10,11]</sup>, have been shown to shorten the duration of PPOI when compared with opioids, but non-opioid analgesia does not adequately control pain in all patients<sup>[10,11]</sup>. The combined use of a local anesthetic for chemical sympathectomy and sparing amounts of narcotic for improved pain control has been proposed, but there is no clear guidance as to which combination best promotes bowel motility while maintaining adequate pain control<sup>[17]</sup>.

A 2008 Cochrane review of the use of prokinetic agents in PPOI has concluded that there was no evidence to support the use of erythromycin, and insufficient evidence for cisapride, cholecystokinin-like drugs such as cerulein, and dopamine antagonists such as metoclopramide, propranolol and vasopressin<sup>[19]</sup>. Neostigmine rapidly decompresses the colon and has shown some potential in PPOI; however, side effects such as bradycardia, bronchospasm, and increased risk of anastomotic dehiscence are of major concern<sup>[17,20]</sup>. Lubiprostone, a bicyclic fatty acid that acts as a chloride channel opener and thereby increases intestinal water secretion, has been shown to be effective in constipation<sup>[21]</sup> and is currently being investigated in PPOI<sup>[16]</sup>.

Narcotic receptor antagonists represent another major class of drugs studied in the treatment of PPOI. Naloxone, for example, is limited by its central nervous system effects and potential to reverse analgesia<sup>[17]</sup>. Methylnaltrexone, a quaternary derivative of naltrexone that does not cross the blood-brain barrier, has shown some efficacy in opioid-induced constipation<sup>[22,23]</sup>, but preliminary results from two trials in PPOI showed no benefit over placebo<sup>[24]</sup>. In May 2008, alvimopan, a selective mu-receptor antagonist, was the first drug to receive United States Food and Drug Administration (FDA) approval specifically for the treatment of PPOI after showing benefit in several phase III trials<sup>[25,26]</sup>. However, there are concerns about the cost-benefit ratio of this drug, given that it shows only a modest reduction in hospital stay (7-15 h) and costs nearly \$1000/treatment cycle<sup>[27,28]</sup>.

Some research has suggested that traditional Chinese herbal medicines can also help bowel motility<sup>[29,30]</sup>. For example, *saussura cappa* and the formula *Liu Jun Zi Tang* have been associated with improved stomach and intestinal emptying time and increased plasma motilin levels<sup>[29,31]</sup>. Although herbal medicine shows some benefit for gastrointestinal motility, abdominal surgery patients generally cannot have anything by mouth during the perioperative period.

Acupuncture has been used in China for thousands of years to treat a variety of gastrointestinal problems<sup>[32]</sup>. The advantages of acupuncture are that it is a cost-effective, minimally invasive procedure with a very low incidence of side effects. Although prior studies have investigated the effects of acupuncture on gastrointestinal motility in humans<sup>[33]</sup>, few randomized clinical trials have been published. Controlled animal studies supported

by plausible physiological and laboratory evidence have, however, shown that acupuncture has positive effects on gastric and intestinal motility<sup>[34-38]</sup>. Although the exact mechanisms are not fully understood, one hypothesis is that acupuncture may help regulate the gastrointestinal tract *via* the autonomic nervous system. Several animal studies have revealed that the effect of acupuncture on gastrointestinal function is mediated through sympathetic and parasympathetic efferent pathways<sup>[39,40]</sup>.

To the best of our knowledge, only one study<sup>[41]</sup> has evaluated the efficacy of acupuncture in preventing PPOI after abdominal surgery. However, in that trial, the incidence of PPOI assessed at postoperative day 4 after ileostomy/colostomy closure was too small to show significance between the treatment and control groups. Therefore, in the current prospective, randomized study, we investigated whether acupuncture could prevent PPOI after invasive colon cancer surgery. Bowel motility was determined by time to first flatus, time to first bowel movement, and by electrogastroenterography (EGEG), a device that detects electrical signals from the abdomen. Secondary objectives were to compare postsurgical quality of life (QOL) between the treatment and control groups in terms of pain, nausea, insomnia, abdominal distension/fullness, and sense of well-being.

## MATERIALS AND METHODS

### Patient eligibility

Patients were recruited from the Fudan University Cancer Hospital between July 2004 and October 2006 and were enrolled in the trial after providing written informed consent. Regardless of sex or ethnicity, all patients 18-75 years old, who had colon cancer with a Duke A to D stage diagnosis (as long as metastatic disease did not affect bowel function) and were scheduled to undergo intraperitoneal surgery, were identified at the time of preoperative evaluation and screened for eligibility. Eligible patients had to meet the following criteria: physical status classification of category III or better according to criteria established by the American Society of Anesthesiologists<sup>[42]</sup>; planned use of epidural infusion for post-surgical pain management; no upper or lower extremity deformity or local skin infections that could interfere with accurate acupuncture point location; no active systemic infection; no chronic functional constipation as defined by Rome I criteria prior to the cancer diagnosis<sup>[43]</sup>; and no history of cerebrovascular accident or spinal cord injury. Patients were also excluded if they had chronic pain currently treated with any form of major opioid or with weak opioids at morphine equivalent doses > 30 mg/24 h; had a cardiac pacemaker; were mentally incapacitated or had a significant emotional or psychiatric disorder that precluded study participation; were pregnant; were using laxatives or other medicines known to affect bowel function, such as herbal preparations, high-dose vitamins, or iron sulfates; had known bleeding abnormalities or were on heparin

or warfarin; had any parasurgical complications needing intensive care; or were currently using acupuncture.

### Procedures

The study was designed collaboratively and conceived by faculty from Fudan University Cancer Hospital and M.D. Anderson Cancer Center, The University of Texas. An experienced statistician was involved in all stages of study development and analysis. The protocol was approved by both Institutional Review Boards. Two nurses from Fudan Cancer Hospital spent 3 mo at M.D. Anderson Cancer Center undergoing research nurse training, two physicians underwent 2 mo of faculty research training, and the acupuncturist from Fudan University Cancer Hospital spent 1 mo at M.D. Anderson Cancer Center. The acupuncturist was trained specifically in aspects of quality control and fidelity to study-related acupuncture procedures. During the course of the trial, faculty and staff from M.D. Anderson Cancer Center also visited Fudan Cancer Hospital four times to review the trial. Video conferences were conducted twice each month.

At the Fudan University Cancer Hospital, patients are generally admitted for preoperative evaluation 3-5 d prior to surgery. All patients were recruited during this time. The first 30 patients were randomized using simple randomization; however, group differences were noted for route of anesthesia administration. Therefore, from the 31st patient forward, in order to ensure group balance for anesthesia administration, patients were randomized into either treatment or control groups by a form of adaptive randomization, minimization<sup>[44]</sup>. Before a participant was assigned to a treatment group, the number of already randomized participants with similar covariate characteristics was totaled. The totals were computed based on marginal sums so that each covariate was considered separately. The treatment assignment for a participant was then based on which treatment group assignment would produce the best overall balance with respect to the covariate characteristics. The patient characteristic used for group assignment was the mode of anesthesia (iv, epidural, or iv plus epidural).

After surgery, if the patient continued to meet all eligibility criteria, she/he was randomized with equal probability into either the treatment or control group. Data were collected and recorded twice a day for 6 d or until the first bowel movement. Patients had to have a bowel movement prior to discharge; therefore, participation ended at the time of discharge for anyone leaving prior to the sixth postoperative day, regardless of whether they had received acupuncture treatment.

### Acupuncture treatment

The acupuncture treatments were performed in the patient's room by a hospital accredited physician who has over 30 years of acupuncture experience and is routinely involved in postoperative care to patients. Patients in the treatment group received acupuncture

once a day, starting on postoperative day 1, for six consecutive days or until the first bowel movement, whichever came first. After the skin was prepped with 70% alcohol, the needles were inserted and remained in place for approximately 20 min with each treatment. The treatment frequency was agreed upon by a small panel of experienced acupuncturists. Patients in the control group received standard postoperative care with no acupuncture.

During the acupuncture treatment, each patient lay in a supine position in his or her hospital bed. A tight abdominal dressing and binder was placed after surgery and not removed for 2-3 d, therefore, only points located on the extremities were selected. Electrical stimulation was applied concomitantly and continuously to two pairs of points [SJ-6 (positive) and GB-34 (negative)] bilaterally by placing lead wires on the needles connected to an electro-acupuncture stimulator (Model 980; Shanghai Medical Equipment Co. Ltd., Shanghai, China). This unit applied consistent stimulation throughout the treatment at a frequency of 2 Hz.

The acupuncture needles used (Huatuo, Suzhou, China) conformed to the requirements of the ISO 9002, EN46002 and CE certification, United States FDA International Good Manufacturing Practices, and the World Health Organization's standards for quality and safety. These stainless steel needles are 1.5 *cun* in length by 32-gauge diameter and are provided in individual sterile packages.

Standardized techniques for point location were used and were based on anatomical landmarks as well as proportional measurements of the patient's body. For example, finger breadth refers to the patient's middle finger, and the proportional unit of measure was the "*cun*", defined as the distance between the two medial ends of the creases of the interphalangeal joints when the patient's middle finger is flexed<sup>[32]</sup>. The following bilateral acupuncture points were selected specifically for the purpose of improving bowel motility. (1) SJ-6: located 3 *cun* proximal to the dorsal crease of the wrist, on the line connecting *Yangchi* (SJ-4) and the tip of olecranon, between the radius and ulna, on the radial side of the extensor digitorum communis muscle. The Chinese name for this point is *Zhigou*. According to the underlying theory of Traditional Chinese Medicine (TCM), this is a major point for stimulating the intestines<sup>[45]</sup> and is often paired with GB-34 to treat constipation. (2) GB-34: located in the depression anterior and inferior to the head of the fibula. The Chinese name for this point is *Yanglingquan*. This point is often paired with SJ-6 to treat constipation due to *qi* stagnation or heat<sup>[45]</sup>. (3) ST-36: on the lateral surface of the leg, 3 *cun* distal to the lower border of the patella, one finger's breadth from the tibia tuberosity between the tibia digitorum tibialis anterior muscle and the tendon of longus pedis. The Chinese name for this point is *Zusanli*. It lies just over the deep peroneal nerve and is commonly used by acupuncturists to harmonize (i.e. regulate) the gastrointestinal tract<sup>[45]</sup>. (4) ST-37: located 3 *cun* below *Zusanli* (St-36) and one finger's breadth (middle

finger) from the anterior border of the tibia. The Chinese name for this point is *Shangjuxu*. Based on TCM theory, this point also regulates the intestines<sup>[45]</sup>.

### Outcome measures

The main outcome measure of bowel motility was assessed by asking patients to record the exact date and time that they first passed flatus and the exact date and time of their first bowel movement after surgery. Time 0 was the time anesthesia ended according to the anesthetic record. The total numbers of hours between time 0 and the passage of flatus and between time 0 and the first bowel movement were then calculated. PPOI was defined as having no bowel movement for more than 96 h (4 d) after surgery.

Secondary measures included EGEG and QOL assessments. Electrical signals from the stomach and intestines associated with gastrointestinal motility were monitored *via* EGEG (Huake Electronic Technical Research Institute, Beijing, China). The surgeon placed the leads for EGEG monitoring at the time of wound closure and prior to placement of the abdominal dressing and binder. According to standard postoperative care at this hospital, the abdominal dressing and binder were not removed until postoperative day 2 or 3; therefore, assessment of bowel sounds was not performed. EGEG monitoring occurred twice per day. After attachment of the electrodes to the leads, the patient rested for 1 min, and then two consecutive 3-min recordings were obtained. Both frequency (per minute) and amplitude were measured using two channels.

Data related to postoperative QOL were obtained from the nursing and physician progress notes and the patient's self-evaluation using the Quality of Life Status (QOLS) assessment tool, which is based on the Edmonton Symptom Assessment System (ESAS)<sup>[46-48]</sup>. The QOLS used in this study was a slightly modified version of the ESAS and consisted of five items (pain, nausea, insomnia, abdominal distention, and general sense of well-being), which were rated using a 0-10 numeric rating scale.

### Statistical analysis

Our primary analysis was to determine the proportion of patients with ileus in each group at day 4 and to determine if the two groups differed in the proportions of ileus observed, using a binomial test. We powered our study to be able to estimate each proportion to within at most 16% by including 40 patients in each group. For example, if 50% of patients in one group (20 out of 40) developed ileus, the 95% confidence interval of this estimate would be 34%-66%. In addition, if we found that 40% of patients (16 out of 40) in one group developed ileus and 11% or fewer ( $\leq 4$ ) in the second group developed ileus, this difference would be considered statistically significant with 80% power and a two-sided significance level of 5%.

The time of occurrence of bowel motility indicators (i.e. first passage of flatus and first bowel movement)



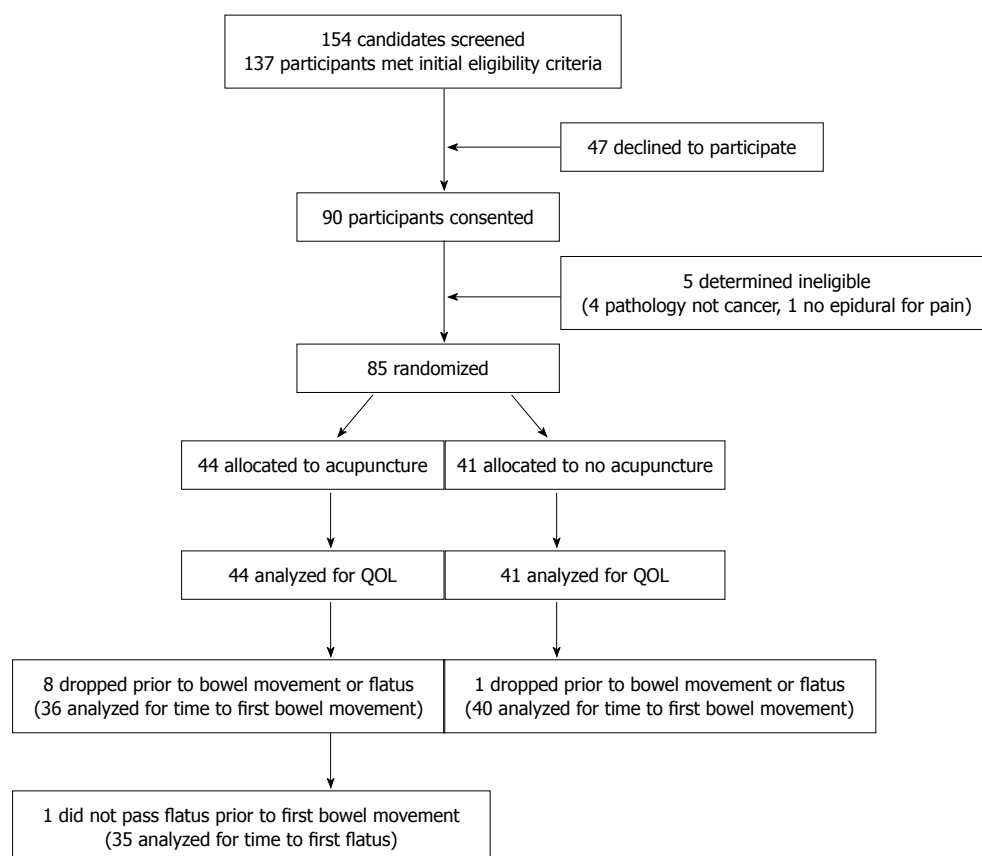


Figure 1 Flow of participants through the study.

was subtracted from the time anesthesia ended, and compared between groups using the Wilcoxon two-samples test. Unpaired *t* tests were used to compare EGEG indicators and QOL outcomes between groups.

## RESULTS

Of the 154 subjects screened, 137 met all the initial eligibility criteria. Forty-seven patients who were eligible declined to participate. Therefore, 65.7% of eligible patients (90 patients) agreed to participate. The overall mean age of the participants was 53.7 years (range, 29-73 years), and there were no group differences in mean age (acupuncture, 54.3 years; control, 53.1 years). There were 38 (45%) women and 47 (55%) men, with an even balance between groups (43% and 46% women in the acupuncture and control groups, respectively). All patients received intraperitoneal surgery, and a representative sample of the population that we wanted to assess was obtained as follows: 47 (52.2%) proctectomy, 21 (23.3%) right hemicolectomy, 11 (12.2%) sigmoidectomy, eight (8.9%) left hemicolectomy and three (3.3%) transverse colectomy. Of 90 patients, 70 (77.78%) received epidural anesthesia (37 acupuncture; 33 controls), 18 (20%) received both epidural and general anesthesia (7 acupuncture, 11 in controls), and two (2.2%) patients in the acupuncture treatment group received general anesthesia only. There were no differences between groups with regard to diagnosis,

operation methods, operation time, blood loss, and postoperative complications.

No participants were lost to follow-up. Five participants were dropped from the study after consent but prior to randomization either because a subsequent pathology report indicated that they did not have a cancer diagnosis, or because they did not receive epidural infusion for post-surgical pain management. Eighty-five patients were randomized (acupuncture = 44; no acupuncture = 41). Although some postoperative QOL data were obtained on all patients, eight in the acupuncture group and one in the no acupuncture group were dropped prior to first bowel movement or first passage of flatus, and before PPOI could be assessed on postoperative day 4, because the inconvenience of study participation (acupuncture-4 dropped on postoperative day 1, 2 on day 2, 1 on day 3, and 1 on day 4; no acupuncture-1 dropped on postoperative day 2). Figure 1 shows the flow of participants through enrollment, randomization, follow-up, and analysis.

Table 1 provides a summary of the study results. There were no significant differences between the groups in terms of bowel motility indicators, EGEG, or post-surgical QOL, thus, the null hypothesis was not rejected.

There were also no group differences when the data were analyzed on the basis of the subset of 46 patients who developed PPOI by day 4 (21 of 36 acupuncture patients *vs* 25 of 40 control patients, *P* = 0.71), and

Table 1 Summary of study results

Outcome	Acupuncture			Control			P value
	n	mean $\pm$ SD	Median (range)	n	mean $\pm$ SD	Median (range)	
Hours to first flatus	35	68.26 $\pm$ 23.38	72.25 (26.75-124.63)	40	65.24 $\pm$ 17.5	64.88 (30.25-105.17)	0.36
Hours to first bowel movement	36	119.04 $\pm$ 47.97	108.67 (34.00-241.17)	40	119.38 $\pm$ 60.21	104.25 (37.00-359.00)	0.77
EGEG at morning (Freq/min)	44	9.66 $\pm$ 1.34	9.32 (7.34-12.87)	41	9.59 $\pm$ 1.63	9.65 (6.80-14.98)	0.83
EGEG at afternoon (Freq/min)	44	9.76 $\pm$ 1.51	9.6 (6.11-15.93)	41	9.81 $\pm$ 1.46	9.94 (6.74-14.31)	0.65
EGEG at morning Amplitude (uv)	44	245 $\pm$ 61.1	243 (119-391)	41	239 $\pm$ 48.3	237 (131-343)	0.75
EGEG at afternoon Amplitude (uv)	44	250 $\pm$ 59.8	250 (131-380)	41	239 $\pm$ 51.7	222 (144-345)	0.39
Pain <sup>1</sup>	44	2.51 $\pm$ 1.74	2.22 (0-6)	41	2.37 $\pm$ 1.52	2.09 (0-5.73)	0.82
Nausea <sup>1</sup>	44	0.91 $\pm$ 1.67	0 (0-8)	41	0.45 $\pm$ 0.99	0 (0-5.25)	0.35
Insomnia <sup>1</sup>	44	5.11 $\pm$ 1.9	5 (1.83-9.00)	41	5.18 $\pm$ 2.06	5.17 (0.67-9.33)	0.76
Abdominal distention <sup>1</sup>	44	0.98 $\pm$ 1.35	0.21 (0-4.75)	41	0.76 $\pm$ 1.3	0.13 (0-6.50)	0.46
General well-being <sup>1</sup>	44	4.11 $\pm$ 1.57	4 (1.67-8.00)	41	3.73 $\pm$ 1.3	3.67 (1.17-6.29)	0.34

<sup>1</sup>Based on a 0-10 numeric rating scale.

whether their PPOI had resolved by day 5 (8 of 21 acupuncture patients and 11 of 25 controls,  $P = 0.69$ ) or day 6 (13 of 21 acupuncture *vs* 16 of 25 controls,  $P = 0.88$ ). The remaining 17 patients experienced a bowel movement by day 7. There were no adverse events greater than grade I (according to the Common Terminology Criteria for Adverse Events v3.0 (CTCAE)<sup>[49]</sup>) related to the acupuncture treatment reported during the study.

## DISCUSSION

PPOI developed in 46 patients, and there was no significant difference in the number of patients with PPOI in the acupuncture group ( $n = 21$ ) *vs* the control group ( $n = 25$ ). Similarly, there were no group differences in bowel activity as assessed by EGEG or in self-reported pain, nausea, insomnia, abdominal distention, or general well-being. In this patient population, PPOI occurred in up to 50% of participants in both groups, which suggested a lack of efficacy of acupuncture to prevent PPOI. Furthermore, analyses of the subset of patients who developed PPOI on day 4 and the resolution of PPOI on days 5 or 6 also revealed no group differences. Although this was a secondary analysis based on a small number of patients, it suggested that acupuncture was not effective in treating PPOI once it developed in this population.

Several important facts, however, were learned that will help with the development of future trials. First, standard postoperative care is different in China than in the United States. At Fudan University Cancer Hospital, patients undergoing this type of surgery often do not ambulate until after postoperative day 3, and in many cases, patients may not ambulate until postoperative day 5 or 6. In addition, patients have a nasogastric tube in place for several days and, thus, progression in diet is much slower than in the United States. Finally, a tight abdominal dressing and binder is placed after surgery and not removed for 2-3 d. Each of these factors could play an important role in gastrointestinal motility postoperatively and were not analyzed separately in the current study. As this was a randomized clinical trial, however, these

factors were likely balanced between both groups. No patients in either group were given enteral or nasogastric feeding before passage of first flatus, and all patients in both groups were given similar iv fluids that included fat emulsion, amino acids, glucose and equilibrium liquids.

One limitation of the current study is that the use of epidural anesthesia in the majority of patients may have diminished the possible effects of acupuncture because of the blockade of afferent and efferent pathways, as acupuncture may act on gastrointestinal motility through neural mechanisms. Moreover, opioid use was not tracked and analyzed formally; however, through the process of randomization and based on a brief review of patient records showing consistency in pre- and postoperative medication, the authors believe this was likely to have been similar in both groups. Nevertheless, it is important to note that the acupuncture treatment in this study was designed to stimulate gastrointestinal motility. The efficacy of acupuncture for pain control is well established<sup>[50]</sup>, and future trials should explore whether or not acupuncture designed to reduce pain can also reduce the amount of opioids used, and thus lessen the occurrence of PPOI. Although acupuncture alone may not be sufficient to reduce the risk of developing postoperative ileus, adding acupuncture to a regimen that includes other preventive measures such as less opioid use, increased ambulation, and progressive diet could have a synergistic effect and help prevent this debilitating complication following abdominal surgery. Future acupuncture trials should, therefore, be designed to include evaluation of these factors, as well as different point combinations, treatment schedules, and type of needle stimulation (i.e. manual *vs* electrical).

To the best of our knowledge, only one previous randomized trial has examined the use of acupuncture to prevent PPOI<sup>[41]</sup>. That study was conducted in the United States among a population of cancer patients who underwent ileostomy/colostomy reversal, a procedure which is not commonly performed in China. Unfortunately, the incidence of PPOI in that study was too small to determine any statistically significant

differences between the groups. It is important to note that no adverse events related to acupuncture were reported during either the previous or current trial.

Providing acupuncture treatment to post-surgical patients at the bedside was found to be feasible, and the logistics of so doing were determined in the current study. Future research should evaluate the efficacy of acupuncture in a different population and for other postoperative conditions, such as anxiety, pain, nausea, vomiting, and wound healing.

In conclusion, this study confirmed findings from previous research<sup>[42]</sup> that acupuncture can be safely administered in a postoperative setting; however, it was not found to be effective in preventing PPOI in this population. Future studies examining the use of acupuncture for PPOI should include assessment of activity, diet, and postoperative pain control, as well as different point combinations, treatment schedules, and type of needle stimulation. Finally, this was a prevention study, and the efficacy of acupuncture in treating PPOI once it has developed has not been evaluated in a prospective randomized trial. Since prior animal and human data<sup>[34-38]</sup> have shown that acupuncture can regulate gastrointestinal function, further investigation is warranted.

## COMMENTS

### Background

Postoperative ileus is a common problem in patients who have major abdominal surgery. The duration is usually short, but prolonged postoperative ileus (PPOI) may lead to increased hospital stay and costs. Acupuncture is often used to treat gastrointestinal disorders in China, but it is still not known whether it is effective for preventing or treating PPOI.

### Research frontiers

Information from this study may help surgeons choose appropriate therapy for PPOI after abdominal surgery.

### Innovations and breakthroughs

This study was conducted as part of a unique collaboration between researchers in the United States and China. Only one previous randomized trial, conducted in the United States, has examined the use of acupuncture to prevent PPOI in cancer patients. Standard postoperative care is very different in the United States than in China, and some of these treatment differences could play an important role in postoperative gastrointestinal motility.

### Applications

Future studies examining the use of acupuncture to prevent or treat PPOI should include assessment of activity, diet, and postoperative medication for pain control.

### Terminology

For the purposes of this study, PPOI was defined as an inability to pass flatus or have a bowel movement by 96 h after surgery.

### Peer review

In this study, the authors investigated whether acupuncture can prevent PPOI after intraperitoneal surgery for colon cancer. Their results show that acupuncture cannot prevent PPI in this population. Subset analyses also suggest that acupuncture is not useful for treating PPOI. This was a prospective, randomized study, with novelty and innovation. It will be helpful for the surgeons to choose appropriate therapy for PPOI after abdominal surgery. Presentation and readability of the manuscript is good for publication.

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## Silencing Fas-associated phosphatase 1 expression enhances efficiency of chemotherapy for colon carcinoma with oxaliplatin

Zhi-Yu Xiao, Wei Wu, Navada Eagleton, Huan-Qing Chen, Jing Shao, Hong Teng, Tian-Hao Liu, Zhi-Min Jiang, He-Rui Yao

Zhi-Yu Xiao, Wei Wu, Huan-Qing Chen, Jing Shao, Department of Surgery, Sun Yat-Sen Memorial Hospital of Sun Yat-Sen University, Guangzhou 510120, Guangdong Province, China  
Navada Eagleton, Department of Veterinary Physiology and Pharmacology, Texas A&M University, College Station, Texas 77843, United States

Hong Teng, Tian-Hao Liu, Zhi-Min Jiang, He-Rui Yao, Department of Oncology, Sun Yat-Sen Memorial Hospital of Sun Yat-Sen University, Guangzhou 510120, Guangdong Province, China

**Author contributions:** Xiao ZY and Wu W contributed equally and performed the majority of experiments; Eagleton N performed statistical analysis and was involved in editing the manuscript; Chen HQ, Shao J, Teng H, Liu TH and Jiang ZM performed part of experiments; Xiao ZY and Yao HR designed the study and wrote the manuscript; Yao HR also provided financial support for this work.

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**Correspondence to:** He-Rui Yao, Associate Professor, Department of Oncology, Sun Yat-Sen Memorial Hospital of Sun Yat-Sen University, 107 West Yanjiang Road, Guangzhou 510120, Guangdong Province, China. [yaoherui@163.com](mailto:yaoherui@163.com)  
Telephone: +86-20-81332107 Fax: +86-20-81332853

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**METHODS:** Expression of FAP-1 in mRNA and protein was detected by reverse transcription polymerase chain reaction (RT-PCR) and flow cytometry. Small interfering RNA (siRNA) was designed according to the FAP-1 mRNA sequence. Cell proliferation was evaluated by methyl thiazolyl tetrazolium (MTT) assay. Anenxin V- and propidine iodine (PI) were assayed by flow cytometry for the detection of apoptosis.

**RESULTS:** The expression of FAP-1 was increased in SW480 cells after chemotherapy with oxaliplatin. Transfection of FAP-1 siRNA into SW480 cells silenced the expression of FAP-1 and consequently abolished the inhibitory function of Fas/FasL-mediated apoptosis pathway, thus increasing the efficacy of chemotherapy for colon carcinoma with oxaliplatin.

**CONCLUSION:** RNA interference combined with conventional chemotherapy is more effective against colon cancer.

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**Key words:** Colon carcinoma; Fas-associated phosphatase 1; RNA interference; Oxaliplatin; Chemotherapy

**Peer reviewer:** Jamie Murphy, MRCS (Eng.), MA, Lecturer in Colorectal Surgery, Centre for Academic Surgery, Royal London Hospital, 3rd Floor Alexandra Wing, London, E1 1BB, United Kingdom

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### Abstract

**AIM:** To investigate whether silencing Fas-associated phosphatase 1 (FAP-1) expression enhances the efficiency of chemotherapy for colon carcinoma with oxaliplatin.

## INTRODUCTION

The incidence of colon carcinoma is increasing worldwide<sup>[1]</sup>. Although great achievements have been made in surgery, chemotherapy and even some novel molecule-targeted drugs, such as bevacizumab (Avastin) used in treatment of colon carcinoma, their efficacy is still limited<sup>[2]</sup>. Since tumorigenesis is a multiple step event involving multiple genes, a single treatment modality just targets a part of the pathogenesis of colon carcinoma. The mechanism underlying the limited efficacy of the above treatment modalities still needs to be further explored in order to prolong the survival time of such patients.

Fas/FasL system is the essential pathway of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells to induce cell apoptosis<sup>[3]</sup>. Fas, an apoptotic message receptor, is belonged to the tumor necrosis factor family and expressed in many normal tissues and malignancies. Its expression pattern is mainly located in activated T lymphocytes and NK cells. By binding to Fas antibody or FasL, Fas-induced apoptotic pathway can be activated, initiating tumor cell apoptosis<sup>[4-6]</sup>. However, it has been reported that Fas receptors are highly expressed in colon carcinoma cells at both mRNA and protein levels, and FasL levels are high in blood and tissues of colon carcinoma patients<sup>[7,8]</sup>, suggesting that colon carcinoma cells can escape from the immune clearance, resist to the cytotoxic activity of host immunocytes, and are, thus, insensitive to FasR-mediated apoptosis. Although the mechanism underlying the failure of immune system to protect humans against colon malignancies remains unclear, it has been recently shown that Fas-associated phosphatase 1 (FAP-1) may play an important role in the pathogenesis of colon malignancies<sup>[9]</sup>. FAP-1, a tyrosine phosphatase, inhibits FasR-mediated apoptosis. By interacting with the cytoplasmic death domain of Fas receptors, FAP-1 acts as a negative switch in the Fas pathway<sup>[10]</sup>. Transfection of FAP-1 into Fas-sensitive cells can block FasL-induced apoptosis<sup>[11]</sup>. Fas and FAP-1 are expressed in colon cancer tissue and the expression of FAP-1 is associated with resistance against Fas-mediated apoptosis and interrupting the correspondence between FAP-1 and Fas can reverse the anti-Fas inducing apoptosis function of FAP-1<sup>[12]</sup>. Down-regulating the expression of FAP-1 by interleukin 2 can promote the sensitivity of colon cancer cells to Fas-induced apoptosis<sup>[13]</sup> and interrupting FAP-1 also increases chemosensitivity to certain kinds of cancer<sup>[14]</sup>, suggesting that FAP-1 can be used as a target for treatment of malignancies. These findings lead to a question of whether interrupting FAP-1 sensitizes chemotherapy for colon carcinoma.

It has been recently shown that RNA interference (RNAi) plays an important role in the treatment of malignancies, virus infection, and other diseases<sup>[15-19]</sup>. Small interfering RNA (siRNA) is small in size, and can easily infiltrate cell membranes and other structures. Its efficiency and specificity are higher than those of anti-sense oligonucleotide<sup>[20-23]</sup>. RNAi may be used as

a novel gene therapeutic procedure combining with chemotherapy for colon carcinoma.

In this study, the expression of FAP-1 was up-regulated after treatment with oxaliplatin. Silencing FAP-1 by siRNA effectively reversed the apoptotic resistance and increased the efficacy of chemotherapy for colon carcinoma with oxaliplatin.

## MATERIALS AND METHODS

### Colon cancer cell line and culture

Human colon adenocarcinoma cell line SW480, obtained from Chinese Type Culture Collection Committee Cell Bank (Shanghai, China), was maintained in RPMI-1640 medium (GIBCO, Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal calf serum (FCS) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### FAP-1 siRNA design

Three different sequences of FAP-1 specific siRNA, including positive control glyceraldehyde phosphate dehydrogenase (GAPDH) siRNA and negative control siRNA, were designed and synthesized by Genepharm (Shanghai, China). The sequence of each siRNA is shown in Table 1.

### siRNA transfection

siRNA was transfected with a siPORT™ NeoFX™ transfection agent (AMBION) following its manufacturer's instructions.

### Detection of FAP-1 by reverse transcription polymerase chain reaction (RT-PCR)

RNA was harvested from colon cancer cells by extracting Trizol (Invitrogen) following its manufacture's instructions. cDNA was synthesized from 2 µg of total RNA in a 20 µL reaction system containing 0.5 µL of PrimeScript™ RTase, 4 µL of 5 × PrimeScript™ buffer, 0.5 µL of RNase inhibitor, 1 µL of Oligo dT, 2 µL of dNTP, 11 µL of RNase free H<sub>2</sub>O. The mixture was incubated for 60 min at 42°C and then for an additional 30 min at 53°C. The unhybridized RNA was digested with 10 units of RNase H at 37°C for 10 min.

PCR was performed on cDNA using the sense and anti-sense primers to amplify FAP-1 and a house keeping gene, GAPDH. All primers were designed according to the published sequences: FAP-1: (sense) 5'-AG-GTCTGCAGAGAAGCAAGAATAC-3' and (anti-sense) 5'-GAATACGAGTGTCTCAGACATGG-3'; GAPDH: (sense) 5'-AACGGATTGTGGTCGTATTG-3' and (anti-sense) 5'-GGAAGATGGTGATGGGATT-3'.

The PCR conditions were as follows: denaturation at 95°C for 5 min, followed by 30 cycles at 94°C for 30 s, at 50°C for 45 s, at 72°C for 1 min, and a final extension at 72°C for 7 min for FAP-1, and denaturation at 95°C for 5 min, followed by 30 cycles at 94°C for 30 s, at 58°C for 45 s, at 72°C for 1 min, and a final extension at 72°C for 7 min for GAPDH. Primers were used at a final concentration of 0.1 µmol/L each, dNTPs at 50 µmol/L,

Table 1 siRNA sequence

siRNA	From 5' to 3'
FAP-1 siRNA 1709	Sense: CGAAGGAAAGUAAACAUAATT Anti-sense: UUAUGUUUACUUUCCUUCGGT
FAP-1 siRNA 6267	Sense: CAGGUACAUAUAAAGAUAATT Anti-sense: UUCAUCUUUAAUGUACCUGGA
FAP-1 siRNA 3264	Sense: GGGAGAUACCCUAGUGAATT Anti-sense: UUCACUAAGGUGAUCUCCCTT
GAPDH positive control	Sense: GUAUGACAACAGCCUCAAGTT Anti-sense: CUUGAGGCUGUUGUCAUACTT
Negative control	Sense: UUCUCCGAACGUGACACGUTT Anti-sense: ACGUGACACGUUCGGAGAATT

siRNA: Small interfering RNA; FAP-1: Fas-associated phosphatase 1; GAPDH: Glyceraldehyde phosphate dehydrogenase.

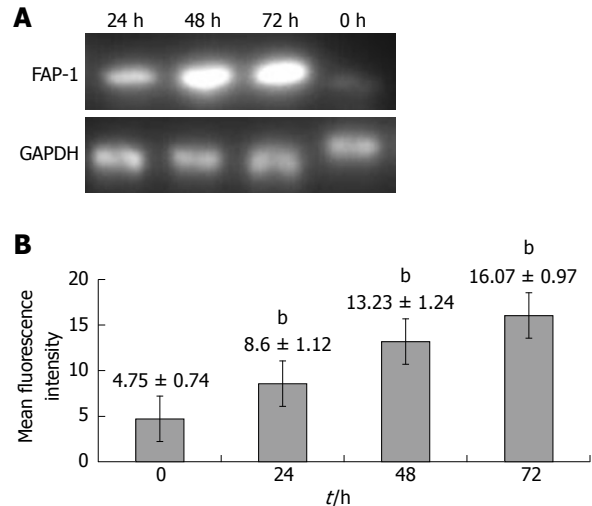
MgCl<sub>2</sub> at 1.5 mmol/L, Taq DNA polymerase at 1.0 µg per 50 µL reaction mixture. The 607 bp and 208 bp PCR products were the predicted FAP-1 and GAPDH, respectively, separated by electrophoresis on 2% agarose gel and stained with colloidal gold. The target bands were analyzed by densitometry. FAP-1 cDNA was semi-quantitated by densitometric comparison with GAPDH from the same sample.

#### Immunofluorescence analysis for FAP-1

Approximately 10<sup>6</sup> cells were incubated with 10 g/mL rabbit anti-human FAP-1 polyclone antibody (Santa Cruz) for 30 min at 4°C and washed with PBS containing 2% FCS. PE-conjugated secondary goat anti-rabbit antibody (Boster, Wuhan, China) was added to the cells for 30 min at 4°C. The cells were washed again with PBS containing 2% FCS and then the intensity of fluorescence was analyzed. Isotype-matched control antibody was used to determine the nonspecific binding. A total of 10000 cells were examined for each determination. Data were expressed as relative fluorescence intensity (RFI = mean fluorescence intensity of cells stained with anti-FAP-1 pAb/mean fluorescence intensity of cells stained with control pAb).

#### Cell proliferation assay

Cell proliferation was evaluated by methyl thiazolyl tetrazolium (MTT) assay. SW480 cells were seeded into a 96-well plate at the concentration of 3000 cells per well. Oxaliplatin (Henrui Co, LTD, Jiangsu Province, China) was administrated at a concentration of 5 µg/mL 24 h after the cells were plated. The proliferation status of SW480 cells was observed at 24, 48, 72, and 96 h, respectively, after treatment with oxaliplatin. Each group was quadruplicates and its mean OD value was used to represent the proliferation status of the group. MTT (Merck) was dissolved in RPMI 1640 and prepared at 1 mg/L for use. The medium was removed, the cells were washed three times with PBS, and 100 µL MTT solution was added into each well and incubated in dark at 37°C. Then, the MTT solution was removed and 100 µL DMSO (Sigma) was added into each well to dissolve the remaining formazan by gently shaking the plate



**Figure 1** Expression of Fas-associated phosphatase 1 (FAP-1) mRNA (A) and protein (B) after oxaliplatin administration. Oxaliplatin promotes FAP-1 expression of SW480 cells at 0, 24, 48 and 72 h after chemotherapy. <sup>b</sup>*P* < 0.01 vs 0 h group.

for 15 min. Finally, a 495 absorption value of each well was obtained with a spectrophotometer (Labsystems Dragon).

#### Detection of apoptosis by flow cytometry

Anexin V-FITC kit (Bender Medsystems) and propidium iodide (PI) were used to calculate the cells undergoing apoptosis with a flow cytometer (FacsCalibur, Becton Dickinson). Anexin V (+) PI (-) represents apoptotic cells, whereas Anexin V (+) PI (+) represents dead cells. The procedure was carried out according to the manufacturer's instructions for the Anexin V-FITC kit.

#### Statistical analysis

All experiments were performed in triplicate. The results were expressed as mean ± SE. Statistical analysis was performed by one-way analysis of variance (ANOVA) and comparisons among groups were performed by Bonferroni's multiple-comparison *t*-test. *P* < 0.05 was considered statistically significant.

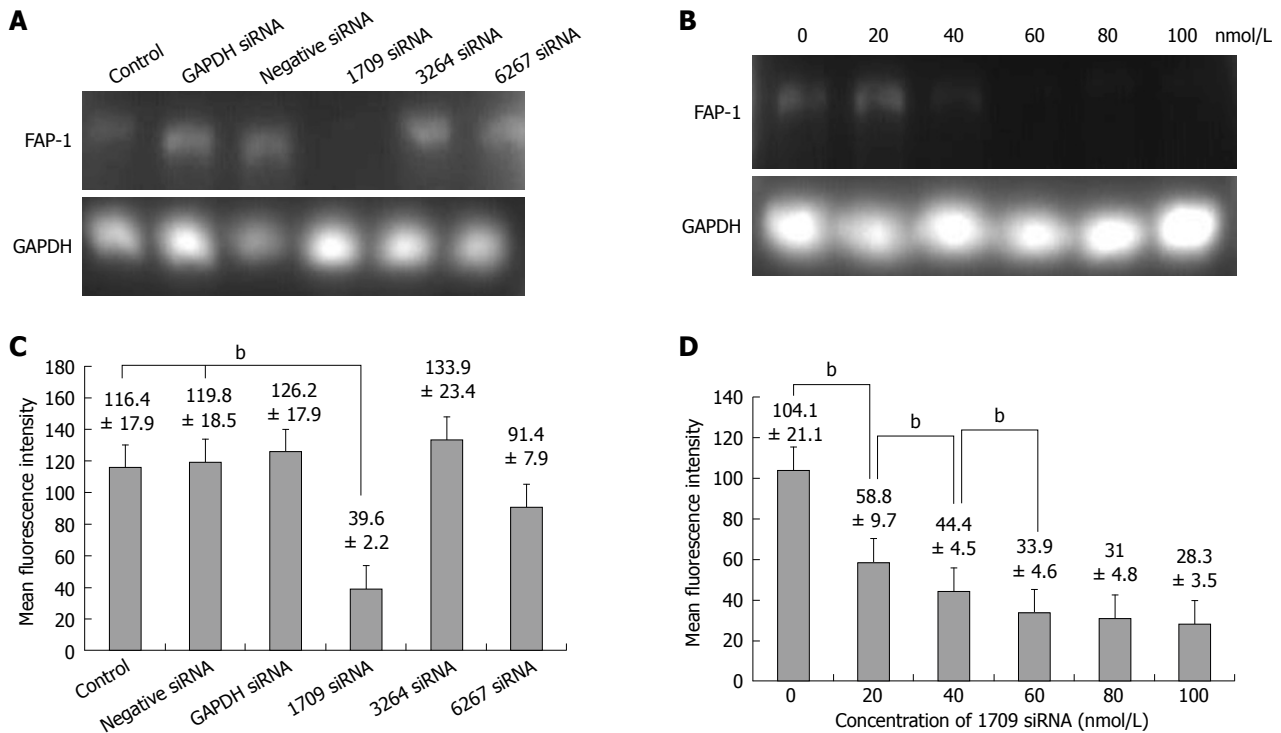
## RESULTS

#### Oxaliplatin promoted FAP-1 expression in SW480 cells

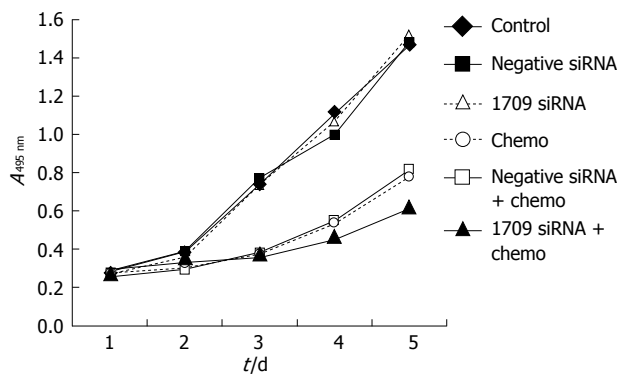
To investigate whether FAP-1 is resistant to chemotherapy for colon carcinoma with oxaliplatin, RT-PCR and flow cytometry were carried out to detect the FAP-1 expression in SW480 colon carcinoma cells at 0, 24, 48 and 72 h after chemotherapy for colon carcinoma with oxaliplatin. The FAP-1 expression was increased at both mRNA (Figure 1A) and protein levels (*P* < 0.01), and reached its peak at 48 h (Figure 1B).

#### siRNA silenced FAP-1 expression

siRNA was transfected into SW480 cells with a transfection agent, siPORT (AMBION). Three sequences of FAP-1 siRNA (1709, 6267 and 3264) were designed.



**Figure 2** Small interfering RNA (siRNA) silencing FAP-1 expression in siRNA 1709 group (A and C) and at the concentration of 60 nmol/L (B and D). <sup>b</sup> $P < 0.01$  vs 0 h group.



**Figure 3** FAP-1 siRNA increasing the inhibitory effect of oxaliplatin on proliferation of SW480 cells.

Forty-eight hours after transfection of siRNAs into SW480 cells, the FAP-1 expression at mRNA and protein levels was detected by RT-PCR and flow cytometry. The FAP-1 protein was expressed in siRNA 1709 group (Figure 2A and C) and at the concentration of 60 nmol/L (Figure 2B and D), respectively ( $P < 0.01$ ).

#### **FAP-1 siRNA combined with oxaliplatin inhibited proliferation of SW480 cells**

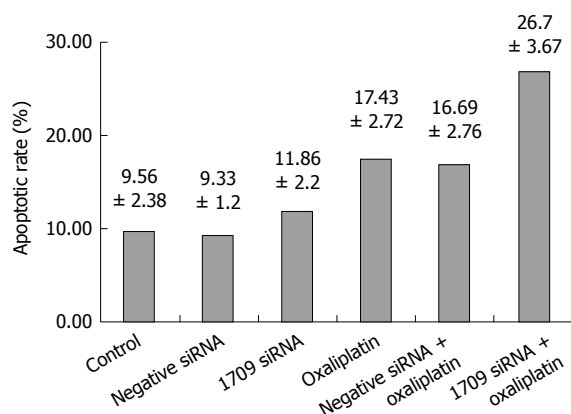
To investigate whether FAP-1 siRNA enhances the sensitivity of SW480 cells to oxaliplatin, cell proliferation was assayed in 6 groups including negative control group, negative siRNA group, siRNA 1709 group, oxaliplatin group, oxaliplatin+negative siRNA group, and oxaliplatin + siRNA 1709 group. Transfection was performed when

the cells were seeded. After 24 h, the culture medium was removed and washed three times with PBS and oxaliplatin dissolved in the culture medium (5  $\mu\text{g/mL}$ ) was added. The culture medium was replaced daily to keep the consistent concentration of oxaliplatin. Cell growth was observed daily for five days. Transfection of negative siRNA and siRNA into SW480 cells did not inhibit cell proliferation. Transfection of oxaliplatin combined with transfection of negative siRNA reduced cell proliferation. The greatest proliferation inhibition was found after treatment with oxaliplatin combined with transfection of siRNA 1709 (Figure 3).

#### **FAP-1 siRNA combined with oxaliplatin increased apoptosis of SW480 cells**

To investigate whether transfection of FAP-1 siRNA into SW480 cells combined with oxaliplatin increases apoptosis of colon carcinoma cells, the apoptotic rate of colon carcinoma cells was detected by flow cytometry and Annexin V and PI immunofluorescence. Transfection was performed when the cells were seeded. After 24 h, the culture medium was removed and washed three times with PBS and oxaliplatin dissolved in culture medium at the concentration 5  $\mu\text{g/mL}$  was added and the cells were harvested. The apoptotic rate of the negative control was  $9.56\% \pm 2.38\%$ , and similar in both siRNA transfection groups ( $P = 0.416$ ). The apoptotic rate of oxaliplatin combined with siRNA 1709 transfection group was  $26.7\% \pm 3.67\%$ , which was higher than that of the oxaliplatin treatment group ( $P < 0.01$ , Figure 4), suggesting that oxaliplatin promotes FAP-1 expression in SW480 cells.





**Figure 4** FAP-1 siRNA enhancing the apoptosis inducing effect of oxaliplatin.

## DISCUSSION

Colon cancer represents a major public health problem, resulting in more than 1 million new cases diagnosed each year and approximately a half million deaths worldwide. Colectomy is the only procedure that may cure colon carcinoma, but the 5-year survival rate mainly depends on the stage of tumor at the time of diagnosis. The majority of patients with colon carcinoma are at an advanced stage beyond surgical treatment when they visit a doctor<sup>[1]</sup>. For patients who cannot be cured by surgery, chemotherapy is another important and complementary treatment<sup>[24]</sup>. Among the chemotherapeutic drugs, oxaliplatin is commonly used in treatment of colon carcinoma. However, its efficacy, especially in patients at advanced stage, is still limited<sup>[2]</sup>.

The main mechanism of action of oxaliplatin is mediated through the formation of DNA adducts<sup>[25-27]</sup>. When the platinum compound enters the cells, one chloride ligand is dissociated to form a reactive mono-aqua monochloro complex, which reacts rapidly with the guanines on DNA to form monoadducts. The subsequent dissociation of the second chloro ligand allows conversion of the transiently formed monoadducts to a variety of stable diadducts<sup>[28,29]</sup>. The majority are intrastrand diadducts binding to a guanine residue<sup>[30,31]</sup>. Since intrastrand adducts are the most abundant adducts and capable of blocking both DNA replication and transcription, they are considered the major cytotoxic lesions. As a final result, oxaliplatin induces primary and secondary DNA lesions leading to apoptosis of human cancer cells<sup>[32]</sup>.

It has been shown that DNA lesion repair mechanism, over-expression of copper transporters, and enhanced drug detoxification result in an increased chemo-resistance to oxaliplatin<sup>[33,34]</sup>. However, the mechanism may be more complicated. Some researchers hold that the major process leading to chemotherapy resistance is the ability of cancer cells to evade cell death signals<sup>[35]</sup>. In our study, the expression of FAP-1, a negative switch in Fas-mediated apoptosis, was elevated in SW480 colon cancer

cells after treatment with oxaliplatin. We quantified the transcription level only by densitometry rather than by RT-qPCR. The role of MMP7 (matrix metalloproteinase 7) and its cross-talk with the Fas/FasL system during the acquisition of chemo-resistance to oxaliplatin have been reported<sup>[36]</sup>. Raymond D<sup>[37]</sup> also showed that oxaliplatin can activate the Notch-1 signaling pathway in colon cancer cells and enhance its chemo-resistance to SW480 colon cancer cells, indicating that the functional disorder of the Fas apoptosis pathway mediated by FAP-1 elevation may protect SW480 cells against apoptosis and is involved in chemo-resistance effect.

In our study, since FAP-1 was elevated after treatment with oxaliplatin and might account for chemo-resistance, the FAP-1 expression was inhibited by RNA interference to make clear whether it sensitizes chemotherapy. The apoptotic rate of oxaliplatin combined with siRNA transfection was higher than that of oxaliplatin only. The greatest proliferation inhibition was found in the group of oxaliplatin combined with siRNA transfection, suggesting that the elevated FAP-1 expression is involved in the mechanism enabling SW480 cells to be insensitive to oxaliplatin treatment. Based on the fact that siRNA used to silence the expression of FAP-1 and treatment with oxaliplatin increased the apoptosis of SW480 cells and reduced their proliferation, we can develop a novel therapeutic measure to enhance the efficacy of chemotherapy. The similar phenomenon was also observed in other malignances. Etodolac, a selective cyclo-oxygenase-2, can enhance carboplatin-induced apoptosis of human tongue carcinoma cells by down-regulating FAP-1 expression<sup>[38]</sup> and sphingosine kinase isoforms can regulate oxaliplatin sensitivity to human colon cancer cells through ceramide accumulation and Akt activation<sup>[39]</sup>. Secretase inhibitors have been recently used to abrogate oxaliplatin-induced activation of the Notch-1 signaling pathway in colon cancer cells, which can enhance chemo-sensitivity<sup>[37]</sup>. In the present study, FAP-1 siRNA combined with oxaliplatin reduced the proliferation of colon carcinoma SW480 cells compared with oxaliplatin alone. No study is available so far on the Fas/FasL system and FAP-1 interacting to influence cell proliferation. We hold that the higher reduction of proliferation is due to the enhanced apoptotic rate of FAP-1 siRNA combined with oxaliplatin treatment, decreasing the number of cells.

Since the pathogenesis of colon carcinoma remains largely unclear, a variety of chemotherapies have been designed to inhibit tumor growth. So far, no single strategy can solve all the complicated problems in the treatment of colon carcinoma. Our study is an attempt to integrate gene therapy targeting FAP-1 and conventional chemotherapy for colon cancer.

In conclusion, oxaliplatin increases the expression of FAP-1. RNAi can knockdown FAP-1 and sensitize chemosensitivity, and RNA interference combined with conventional chemotherapy is more effective against colon cancer.

## COMMENTS

### Background

Colon cancer represents a major public health problem, resulting in more than one million new cases diagnosed each year and approximately a half million deaths worldwide. Chemotherapy is an important and complementary treatment modality for colon carcinoma. Among the chemotherapeutic drugs, oxaliplatin is a commonly used in treatment of colon carcinoma, but its efficacy, especially in patients at advanced stage, is still limited.

### Research frontiers

The mechanism underlying oxaliplatin chemo-resistance is complicated. DNA lesion repair mechanism, over-expression of copper transporters, and enhanced drug detoxification cannot fully explain its mechanism. In this study, Fas-associated phosphatase-1 (FAP-1) was elevated in colon carcinoma cells after oxaliplatin treatment, implicating that the functional disorder of the Fas apoptosis pathway mediated by FAP-1 elevation may protect colon carcinoma cells against apoptosis and is involved in the chemo-resistance effect. Chemotherapy can be sensitized by inhibiting FAP-1 expression with RNA interference.

### Innovations and breakthroughs

Since the pathogenesis of colon carcinoma remains largely unclear, a variety of chemotherapeutic treatment modalities available have been designed. So far, no single treatment modality can solve all the complicated problems. This study is an attempt to integrate gene therapy targeting FAP-1 and conventional chemotherapy for colon cancer.

### Applications

Oxaliplatin can increase the expression of FAP-1. RNAi can knockdown FAP-1 and sensitize chemosensitivity. This in vitro study showed RNA interference combined with conventional chemotherapy is more effective against colon cancer.

### Terminology

FAP-1 is a tyrosine phosphatase, which inhibits FasR-mediated apoptosis. By interacting with the cytoplasmic death domain of Fas receptors, FAP-1 acts as a negative switch in the Fas pathway. Transfection of FAP-1 into Fas-sensitive cells can block FasL-induced apoptosis.

### Peer review

This study investigated the important factors that inhibit the apoptotic effect of oxaliplatin on colorectal cancer cells, which is of significance in the treatment of colon carcinoma.

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## Malignant schwannoma of the pancreas involving transversal colon treated with *en-bloc* resection

Miroslav P Stojanovic, Milan Radojkovic, Ljiljana M Jeremic, Aleksandar V Zlatic, Goran Z Stanojevic, Milan A Jovanovic, Milos S Kostov, Vuka P Katic

Miroslav P Stojanovic, Milan Radojkovic, Ljiljana M Jeremic, Aleksandar V Zlatic, Goran Z Stanojevic, Milan A Jovanovic, Milos S Kostov, Vuka P Katic, Department of Hepato-bilio-pancreatic, Surgical Clinic, Clinical Center Nis, Nis 18000, Serbia  
**Author contributions:** Stojanovic MP, Jeremic LM and Zlatic AV contributed to the literature review; Radojkovic M assisted in reporting the case; Stanojevic GZ contributed to revising the manuscript; Jovanovic MA, Kostov MS and Katic VP drafted and edited the manuscript and contributed to its final revision and approval.

Correspondence to: Miroslav P Stojanovic, MD, PhD, Professor, Chief, Department of Hepato-bilio-pancreatic, Surgical Clinic, Clinical Center Nis, Nis 18000, Serbia. [miskosparis@yahoo.com](mailto:miskosparis@yahoo.com)  
Telephone: +381-18-560925 Fax: +381-18-560925  
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Stojanovic MP, Radojkovic M, Jeremic LM, Zlatic AV, Stanojevic GZ, Jovanovic MA, Kostov MS, Katic VP. Malignant schwannoma of the pancreas involving transversal colon treated with *en-bloc* resection. *World J Gastroenterol* 2010; 16(1): 119-122 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i1/119.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i1.119>

### Abstract

Pancreatic schwannoma is a very uncommon tumor of the pancreas, with only 27 cases reported. Most pancreatic schwannomas are benign, with only four malignant tumors reported. We describe a case of giant malignant schwannoma of the pancreatic body and tail, which involved the transverse colon. The tumor was treated successfully with *en bloc* distal splenopancreatectomy and colon resection. This is believed to be the first reported radical operation for malignant schwannoma of the pancreatic body, with infiltration of the transverse colon, with excellent long-term results. The patient is alive and well 28 mo after the operation. The authors conclude that pancreatic schwannomas should be considered in the differential diagnosis of cystic neoplasms of the pancreas, although the diagnosis can only be confirmed by microscopic examination. In the case of the benign tumors, local excision is adequate, but in the case of malignant schwannoma, oncological standards must be fulfilled.

### INTRODUCTION

Mesenchymal tumors derived from Schwann cells that envelope the peripheral nerves (schwannoma/neurilemmoma) can be found throughout the body, with the most common localization in the extremities, trunk, head and neck, retroperitoneum, mediastinum and pelvis<sup>[1]</sup>. Visceral schwannomas, which arise from sympathetic and parasympathetic nerve fibers, are very rare<sup>[2]</sup>. Pancreatic schwannoma is notably uncommon, with only 27 cases reported in the English and Japanese literature to date<sup>[3-7]</sup>. Most pancreatic schwannomas are benign, with only four malignant tumors reported.

We describe a case of giant malignant schwannoma of the pancreatic body and tail, which involved the transverse colon. The tumor was treated successfully with *en bloc* distal splenopancreatectomy and colon resection.

### CASE REPORT

A 24-year-old woman was hospitalized because of unclear



abdominal symptoms, dyspepsia, weight loss and palpable tumor in the left hypochondrium. She had no anamnesis of pancreatitis or trauma, and there was no sign of von Recklinghausen's disease. The laboratory data (complete blood count, hepatic and pancreatic function tests) were within normal limits. Tumor marker levels also were within normal limits [carcinoembryonic antigen (CEA), 2.3 U/mL and carbohydrate antigen 19-9 (CA 19-9), 16.8 U/mL].

Ultrasonography of the abdomen revealed a well-demarcated, large, predominantly hyperechogenic mass, with hypoechogenic components in the body and tail of the pancreas, and compression of the posterior gastric wall. Computed tomography (CT) showed a well-circumscribed round hypodense mass (32 HU), 18 cm in diameter, which occupied the body and tail of the pancreas and displaced the splenic vein. There was suspect infiltration of the stomach and transverse colon (Figure 1).

Percutaneous needle aspiration was performed and a very small amount of the fluid was aspirated. Biochemical analysis showed normal amylase (48 U/L), CEA (1.9 ng/mL) and CA 19-9 (13 U/L) levels. Cytology revealed rare large cells with high nuclear-cytoplasmic ratios, prominent nucleoli, and cytoplasmic vacuoles, which were suggestive of malignancy. The cell block contained fragments of connective tissue and stroma with some spindle cells and hemosiderin-laden histiocytes.

We decided on operative treatment under the tentative diagnosis of a cystic neoplasm of the pancreas. Laparotomy revealed an encapsulated solid tumor in the pancreatic body and tail, which involved the transverse colon. There was no macroscopic regional lymphadenopathy. The patient underwent *en bloc* resection that consisted of hemipancreatectomy, splenectomy, omentectomy and transverse colon resection. Systematic lymphadenectomy was performed with removal of the 7, 9, 10 and 11 groups of lymph nodes, according to the Japanese Gastric Cancer Association Classification. Overall number of lymph nodes was 12, with micrometastases founded in two. The postoperative course was uneventful and the patient was discharged on postoperative day 11. No chemo or radiotherapy was added. She is free of symptoms 28 mo after surgery.

Surgical biopsies were fixed in 10% formaldehyde overnight, embedded in paraffin wax, and cut at a thickness of 4  $\mu$ m. The sections were stained with hematoxylin and eosin, Alcian blue/periodic acid-Schiff, van Gieson and immunohistochemical avidin biotin complex techniques, by using S-100 antibody for detection of schwannoma, and Ki 67 antibody to evaluate mitotic activity in tumor cells.

Microscopy showed that tumor cells infiltrated only the serosa of the transverse colon. The nuclei and cytoplasm of the spindle-shaped neoplastic cells were diffuse (Figure 2A) and strongly immunoreactive for S-100 protein (Figure 2B and C). Tumor cells showed increased proliferative activity, with numerous mitotic figures. In the hypercellular areas, we registered > 10 mitotic figures/



**Figure 1** Computed tomography (CT) of pancreatic schwannoma with involvement of the transverse colon.

high-power field. Intense nuclear staining for Ki67 (*MIB1*) in the neoplastic cells was observed (Figure 2D). There was no cystic component or secondary elements of mature cartilage or bone.

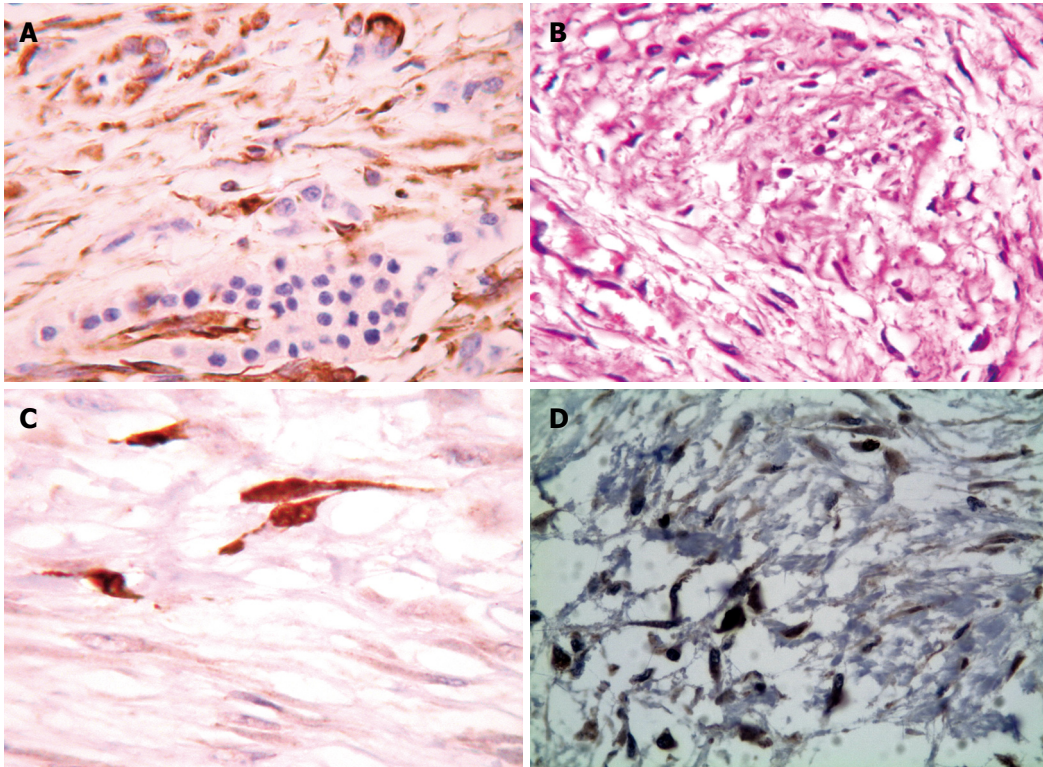
## DISCUSSION

Mesenchymal tumors derived from Schwann cells that envelope peripheral nerves (schwannoma/neurilemmoma) are uncommon. They can be found throughout the body, with the most common localization in the extremities, trunk, head and neck, retroperitoneum, mediastinum and pelvis<sup>[1]</sup>. Visceral schwannomas, which arise from sympathetic and parasympathetic nerve fibers are very rare<sup>[2]</sup>. Pancreatic schwannoma is notably uncommon, with only 27 cases reported in the English and Japanese literature to date<sup>[3-7]</sup>. The patients ranged in age from 41 to 87 years (mean 61 years), with a nearly equal sex distribution. The tumor size ranged from 1.5 to 20 cm (mean: 6.5 cm), with the pancreatic head involved in 44%, and the body and tail in 56% of cases<sup>[1]</sup>. Schwannomas usually occur as solitary lesions, but are occasionally multiple when associated with von Recklinghausen's disease<sup>[8]</sup>.

Although three cases of small solid pancreatic schwannomas have been reported<sup>[9-11]</sup>, typical presentation of schwannomas is in the form of cystic, thin-walled, and hemorrhagic masses<sup>[3]</sup>. In our case, we found a large, solid schwannoma with a well-defined capsule, along with infiltration of the transverse colon.

Typical microscopic features of schwannoma are two microscopic components: a highly ordered cellular component (Antoni A areas), and a loose myxoid component with degenerative changes (Antoni B areas)<sup>[12]</sup>. Tumor cells are invariably immunoreactive for S100 protein, vimentin and CD56, and negative for cytokeratin AE1/3, CD34, CD117 (c-kit), desmin, and smooth muscle myosin<sup>[13]</sup>.

A review of the literature has revealed only four cases of malignant schwannoma of the pancreas<sup>[2,12,14,15]</sup>. Malignant transformation of a benign schwannoma is extremely rare<sup>[8]</sup>. Also, malignant pancreatic schwannomas



**Figure 2** Microscopy of tumor cells infiltrating the serosa of the transverse colon. A: Schwannoma pattern of growth: Antoni B type. HE,  $\times 200$ ; B: Characteristic elongated cells with cytoplasmic processes in area with loose, myxoid background. Immunohistochemical ABC method,  $\times 400$ ; C: Pancreatic schwannoma with subtotal pancreatic tissue. Immunohistochemical ABC method,  $\times 400$ ; D: Intense nuclear staining for Ki67 (MIB1) in the neoplastic cells. Immunohistochemical ABC method,  $\times 400$ .

that were associated with von Recklinghausen's disease have been reported, but none of the benign pancreatic schwannomas were associated with von Recklinghausen's disease<sup>[2,15]</sup>.

Clinically, schwannoma is asymptomatic for a long time, or it is accompanied by nonspecific abdominal pain and discomfort. In the late clinical course, compression of the surrounding organs might be noticed.

To establish the diagnosis of pancreatic schwannoma, CT is the initial investigation of choice. CT findings usually show well-defined, round masses with multiple, low-attenuation, cystic necrotic areas. In tumors that are predominantly or exclusively composed of Antoni A areas (cellular component), CT shows inhomogeneous, hypodense, solid masses with contrast enhancement. When the tumor is predominantly composed of Antoni B areas (loose myxoid), CT shows homogeneous cystic masses without significant contrast enhancement<sup>[16]</sup>. On magnetic resonance imaging, schwannomas are present as masses of low signal intensity on T1-weighted images and of high signal intensity on T2-weighted images<sup>[17]</sup>.

Deep tumors tend to grow larger, therefore, they are more likely to show secondary degenerative changes such as cyst formation, calcification, hemorrhage, and hyalinization, and are known as ancient schwannomas<sup>[1]</sup>. As a result of these changes and the content of predominantly loose tissue, pancreatic schwannoma is often misdiagnosed as a pseudocyst or other cystic neoplasm of the pancreas.

Definitive preoperative diagnosis is proven using

fine-needle aspiration. However, this method correctly diagnoses only one in eight histologically proven schwannomas<sup>[18,19]</sup>. Definitive diagnosis requires histological examination and complex immunohistochemistry or ultrastructural examination<sup>[12]</sup>. For the definition of malignancy, which is often difficult in mesenchymal tumors, we use the following criteria: accentuated cell pleomorphism, high mitotic activity, rare stained necrosis, infiltration of the adjacent organs (colon), and locoregional lymph node micrometastases<sup>[2,12]</sup>.

The malignant transformation of pancreatic schwannoma is uncommon, therefore, simple enucleation of benign schwannoma is usually sufficient if the pathology is confirmed before surgery<sup>[4]</sup>.

In the case of the malignant schwannoma, oncological resection is indicated. A review of the literature has revealed one case of unresectable tumor of the pancreatic body, which was resolved by drainage<sup>[14]</sup>. In the case of pancreatic head localization, simple excision was performed in one case<sup>[2]</sup>, and radical (Whipple) operation was performed in two other cases. However, in the present literature, only short-term follow-up has been reported (maximum, 9 mo), with no data about maximum survival<sup>[12,15]</sup>.

As far as we are aware, this is the first report of radical surgery for malignant schwannoma of the pancreatic body, with infiltration of the transverse colon, with excellent long-term results. Our patient is alive and well 28 mo after the operation.

Radiotherapy has been shown to decrease tumor

growth and regression in neurogenic schwannoma, but there have been no previous reports of chemoradiation therapy<sup>[20]</sup>. However, the role of chemoradiation therapy in the management of pancreatic schwannoma has not been proven. Surgical excision with close follow-up and surveillance remain the mainstay of treatment<sup>[3]</sup>.

In conclusion, pancreatic schwannoma is very rare, but an important pathological condition. It should be considered in the differential diagnosis of cystic neoplasms of the pancreas, although the diagnosis can only be confirmed by microscopic examination. In the case of benign tumors, local excision is adequate, but in the case of malignant schwannoma, oncological standards must be fulfilled.

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## Successful endoscopic sclerotherapy for cholecystojejunostomy variceal bleeding in a patient with pancreatic head cancer

Yu-Chun Hsu, Hsu-Heng Yen, Yang-Yuan Chen, Maw-Soan Soon

Yu-Chun Hsu, Hsu-Heng Yen, Yang-Yuan Chen, Maw-Soan Soon, Department of Gastroenterology, Changhua Christian Hospital, Changhua City 500, Taiwan, China  
 Author contributions: Hsu YC and Yen HH contributed equally to this work; Chen YY and Soon MS approved the paper.  
 Correspondence to: Hsu-Heng Yen, MD, Department of Gastroenterology, Changhua Christian Hospital, Changhua City 500, Taiwan, China. 91646@cch.org.tw  
 Telephone: +886-4-7238592-5501 Fax: +886-4-7228289  
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### Abstract

Variceal bleeding outside the esophagus and stomach is rare but important because of its difficult diagnosis and treatment. Bleeding from cholecystojejunostomy varices has been reported to be a late complication of palliative biliary surgery for chronic pancreatitis. Such ectopic variceal bleeding has never been reported after palliative surgery for pancreatic cancer, probably because of the limited lifespan of these patients. Herein, we report our successful experience using endoscopic cyanoacrylate sclerotherapy to treat bleeding from cholecystojejunostomy varices in a 57-year-old man with pancreatic head cancer. To our knowledge, this is the first case report in the literature of this rare complication.

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**Key words:** Ectopic varix; Pancreatic cancer; Cholecystojejunostomy; Sclerotherapy

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### INTRODUCTION

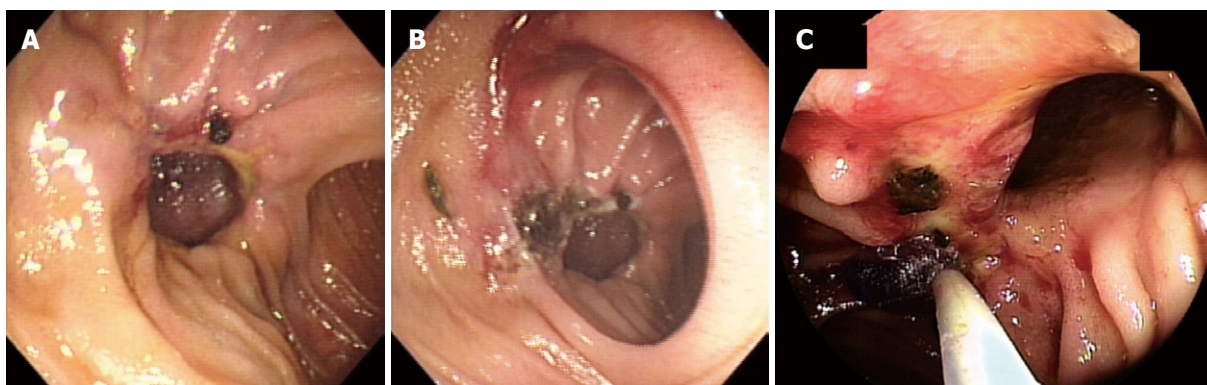
Variceal bleeding outside the esophagus and stomach is rare but important because of its difficult diagnosis and treatment. These ectopic varices are usually associated with cirrhosis and, less often, may result from portal vein thrombosis, chronic pancreatitis, mesenteric venous thrombosis, or adhesion caused by prior surgery<sup>[1]</sup>. Bleeding from cholecystojejunostomy varices has been reported to be a late complication of palliative biliary surgery for chronic pancreatitis<sup>[1-4]</sup>. To our knowledge, such bleeding has never been reported after surgery for pancreatic cancer, probably because of the limited lifespan of such patients<sup>[1]</sup>. Herein, we report our successful experience using endoscopic cyanoacrylate sclerotherapy to treat bleeding from cholecystojejunostomy varices in a 57-year-old man with pancreatic head cancer.

### CASE REPORT

A 57-year-old man was admitted to our hospital because of tarry stool passage. His surgical history included a Billroth-II operation for peptic ulcer 30 years ago and cholecystojejunostomy for biliary palliation due to pancreatic head cancer diagnosed 6 mo before this admission. The tumor progressed with portal vein invasion and multiple hepatic metastases during this period despite chemotherapy.

Hemoglobin count was 60 g/L on presentation and his coagulation profiles were within normal limits. An upper endoscopy disclosed no hemorrhagic lesion in





**Figure 1 Endoscopic view.** A: An anastomotic ulcer was observed in the cholecystojejunostomy; B: Anastomotic ulcer after argon plasma coagulation therapy; C: n-butyl-2-cyanoacrylate injection into the cholecystojejunostomy varices.



**Figure 2 Abdominal computed tomography.** A: Prominent vessels (arrow) formed around the gall bladder; B: Obliterated varicose vein (arrow).

the esophagus or stomach. The endoscope was then advanced to the cholecystojejunostomy area where shallow ulcers over the anastomosis were found (Figure 1A). The patient was administered intravenous proton pump inhibitor and blood component replacement therapy. The bleeding persisted and colonoscopy subsequently revealed a bleeding point above the terminal ileum. Therefore, a decision was made to perform local therapy for the anastomotic ulcer with argon plasma coagulation (Figure 1B).

The patient continued to bleed despite endoscopic therapy. abdominal computed tomography (CT) was performed, which revealed portal vein tumor invasion with collateral circulation formation. Prominent varices were found around the gall bladder and cholecystojejunostomy (Figure 2A). No tumor invasion to the bowel was noted. Retrospective review of the endoscopic images revealed that the folds were mildly engorged with mild blue color, which suggested underlying varices.

After discussion, the patient agreed to endoscopic therapy with n-butyl-2-cyanoacrylate for these varices on the 12th d of hospitalization. Endoscopic ultrasound with miniprobe was used to confirm the presence of varices beneath the anastomotic ulcer, and injection therapy with n-butyl-2-cyanoacrylate was carried out smoothly (Figure 1C). A follow-up CT revealed successful obliteration of the collateral circulation (Figure 2B). The patient died of his disease 4 mo later and had no recurrent gastrointestinal bleeding during the intervening period.

## DISCUSSION

Esophageal or gastric variceal bleeding is a common cause of severe gastrointestinal bleeding. Ectopic variceal bleeding from the duodenum<sup>[5-7]</sup>, jejunum<sup>[8]</sup>, ileum<sup>[9]</sup>, and colon<sup>[10,11]</sup> have been reported in the literature as a diagnostic and therapeutic challenge to clinicians. This ectopic variceal bleeding usually results from portal hypertension, portal vein thrombosis, mesenteric vein thrombosis, chronic pancreatitis, adhesion after surgery, or inflammatory bowel disease<sup>[1,12]</sup>. Our reported case, suffering from cholecystojejunostomy variceal bleeding, is even rarer and such bleeding has been reported to be associated only with chronic pancreatitis<sup>[1-4]</sup> in the literature. This is probably because these cancer patients have a limited lifespan, dying before such varices can develop<sup>[1]</sup>.

The diagnosis of ectopic varices is usually made after endoscopic examination: mesenteric venography<sup>[1,12]</sup>, abdominal ultrasound<sup>[13]</sup>, enteroclysis<sup>[3]</sup>, or CT<sup>[14]</sup>. In our case, the diagnosis was difficult to make during the initial endoscopic examination because the varicose vein was not prominent and was masked by an overlying anastomotic ulcer. A careful review of the CT and endoscopic images in this case suggested the presence of varices in the cholecystojejunostomy and led us to approach the case with different endoscopic measures. Consequently, we suggest that endoscopic ultrasound is mandatory, as in our case, to confirm the presence of varices prior to endoscopic therapy. Alternatively, needle aspiration or

injection of contrast may prove useful for diagnosis.

Unlike esophageal and gastric varices, the treatment options for ectopic varices have varied in the literature. Surgical resection<sup>[1]</sup> or radiological methods to decrease portal hypertension<sup>[15]</sup> have previously been reported. Both endoscopic band ligation<sup>[10]</sup> and sclerotherapy<sup>[15]</sup> have been successfully used to treat such ectopic varices. Endoscopic cyanoacrylate sclerotherapy was chosen in this patient with advanced cancer because it is minimally invasive and has previously been used to treat gastric varices endoscopically<sup>[16]</sup>. A follow-up CT demonstrated successful obliteration of the varices and thus, permanent hemostasis was achieved for our case.

In conclusion, we report a case of pancreatic cancer with bleeding from cholecystojejunal varices. The diagnosis was made by CT, endoscopy, and endoscopic ultrasound. Cyanoacrylate sclerotherapy was a successful method to achieve hemostasis in this case.

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## Ileum perforation due to delayed operation in obturator hernia: A case report and review of literatures

Hong Zhang, Jin-Chun Cong, Chun-Sheng Chen

Hong Zhang, Jin-Chun Cong, Chun-Sheng Chen, Department of Colorectal Tumor Surgery, Shengjing Hospital of China Medical University, Shenyang 110004, Liaoning Province, China  
Author contributions: Zhang H designed and wrote the paper; Cong JC managed the patient and examined radiologic findings; Chen CS performed the operation and supervised the research.  
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Correspondence to: Dr. Chun-Sheng Chen, Professor, Department of Colorectal Tumor Surgery, Shengjing Hospital of China Medical University, No. 36, San Hao Street, Heping District, Shenyang 110004, Liaoning Province, China. [zhanghong1203@yahoo.com.cn](mailto:zhanghong1203@yahoo.com.cn)  
Telephone: +86-24-96615-31411 Fax: +86-24-83955072  
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### Abstract

A 83-year-old woman was admitted to our hospital because of intermittent abdominal colicky pain and vomiting for 26 h. The pain localized over the periumbilical area with radiation along the medial side of the thigh. Computed tomography scan with three-dimensional reconstruction revealed a loop of small bowel protruding into the left obturator canal. Incarcerated obturator hernia was diagnosed and emergency laparotomy was arranged immediately. Unfortunately, her family refused surgery because of her worsening condition. On the third evening after admission, the patient developed peritonitis and sepsis. Perforation of small bowel due to the incarceration was noted during laparotomy. Bowel resection and an end-ileostomy were performed. She recovered well despite of the complication of multiple organ dysfunction syndrome. Literature is reviewed, and the pathogenesis, clinical manifestation, imaging features and treatment are discussed.

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### INTRODUCTION

Obturator hernia is considered to be rare and accounts for 0.05%-0.4% of all hernias<sup>[1]</sup>. The first case of obturator hernia was published in 1724 by Arnaud De Ronsil. Throughout the centuries, obturator hernia has been considered as an uncommon but important cause of mechanical intestinal obstruction, which usually occurs in elderly, emaciated women. Although some characteristics relevant to obturator hernia have been introduced and various imaging modalities have been applied, it remains a diagnostic and therapeutic challenge for surgeons. Here we report a case with correct diagnosis but delayed operation that directly results in bowel perforation, septic shock and multiple organ dysfunction syndrome (MODS).

### CASE REPORT

A 83-year-old woman was admitted to our hospital because of intermittent abdominal colicky pain and vomiting for 26 h. The pain localized over the periumbilical area with radiation along the medial side of the thigh. The patient had had several episodes of similar syndrome during the past 13 mo, but it relieved spontaneously or by treatment with intravenous fluids and nasogastric suction. A

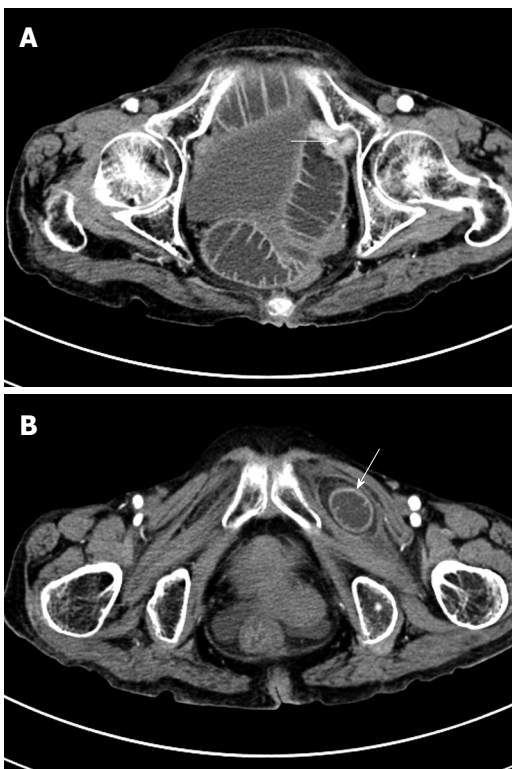




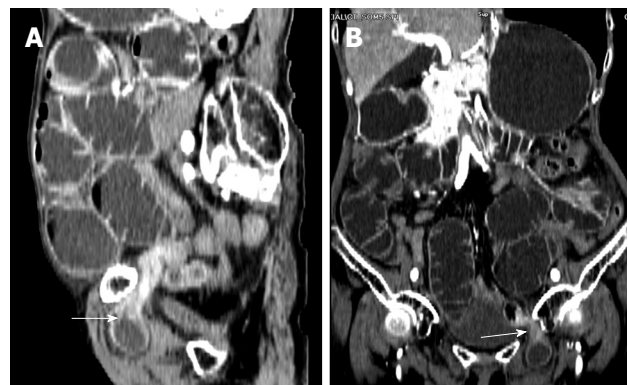
**Figure 1** Ultrasonographic image of the left groin demonstrating a fluid-filled loop of bowel with the neck of the hernia uncertainly identified (arrow).



**Figure 2** Plain abdominal film revealing multiple dilated loops of small bowel in an emaciated woman with scoliosis.



**Figure 3** Abdominal axial CT image. A: Severe dilated small bowel and abrupt stenosis at the terminal ileum in the pelvic cavity (arrow); B: A low-density mass with clear border located between the pectineus and the left external obturator muscles (arrow).

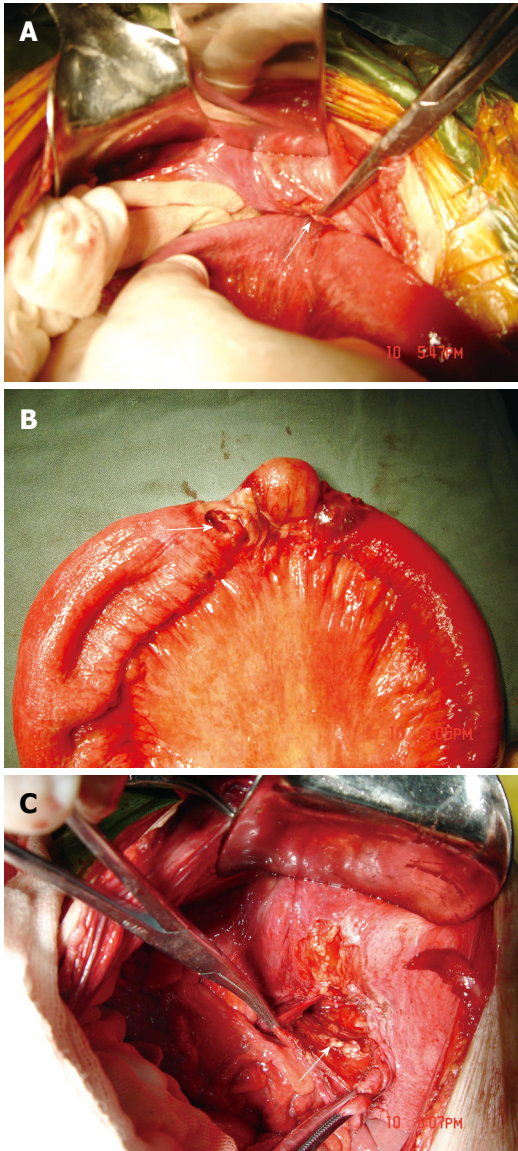


**Figure 4** Three-dimensional reconstructed CT (A: Sagittal section; B: Coronal section) finding extensive dilation of the small bowel loops and a loop of small bowel protruding into the left obturator canal with the transition zone from dilated to collapsed bowel (arrows).

diagnosis of intestinal obstruction was made 7 mo prior to current admission. But the cause of disease was unclear. She had a similar attack 5 mo earlier, was suspected of having incarcerated hernia by ultrasonography. A loop of small bowel at the left groin region was noted (Figure 1). But subsequent investigations and treatment were refused after the relief of pain. She also had a history of chronic constipation, ischemic heart disease and chronic bronchitis. She had no previous abdominal surgery. On her arrival, the body temperature was 36.8°C, blood pressure was 105/75 mmHg, heart rate was 92 beats per minute. Her body mass index was 18 (body weight 39 kg, height 147 cm). Physical examination revealed an

emaciated lady with a soft but distended abdomen, visible bowel coils, mild tenderness over the lower abdomen, and hyperactive bowel sounds. No mass or rebound pain of the abdomen was noted. There was no palpable hernia in the groin. No abnormal signs were found on rectal and vaginal examinations. The Howship-Romberg sign, which characterized by pain or paresthesia in the hip with radiation down the medial thigh to the knee on the affected side, was positive. The white blood cell count was  $11.2 \times 10^9/L$  and other serum analyses were within normal limits. The initial plain abdominal radiography showed dilated loops of small bowel (Figure 2). Computed tomography (CT) scan demonstrated an abrupt stenosis at the terminal ileum in addition to the dilation of small bowel (Figure 3A). A low-density mass was also noted between the pectineus and the left external obturator muscles (Figure 3B). Three-dimensional reconstructed CT revealed a loop of small bowel protruding into the left obturator canal. The stenosis of the lumen and distended small bowel loops in the abdomen were well visualized (Figure 4). Incarcerated obturator hernia was diagnosed and emergency laparotomy was arranged immediately. Unfortunately, her family refused surgery because of her worsening condition, general weakness and the high risk involved. The patient was managed conservatively with





**Figure 5** Intraoperative photography illustrating. A: Incarcerated small bowel at left obturator foramen (arrow); B: Perforation of ileum proximal to the site of incarceration (arrow); C: A defect in the left obturator canal (arrow).

intravenous fluids and nasogastric suction. On the third evening after admission, her abdominal pain suddenly became worse and constant. Subsequently, she developed restlessness and more unbearable generalized abdominal pain. On examination, the body temperature was 37.7°C, the blood pressure was 75/55 mmHg, and the heart rate was 128 beats per minute. There were obvious signs of peritonism and bowel sounds were absent. The diagnosis of perforation of small bowel and septic shock was made. The patient underwent emergency surgery after explanation to her guardian. Laparotomy revealed a loop of the terminal ileum about 80 cm from the ileocecal valve, herniating into the left obturator canal (Figure 5A). Perforation of small bowel was noted proximal to the port of incarceration (Figure 5B). The peritoneal contamination was severe. In view of the patient's poor conditions, we resected the perforated ileal segment and performed an

end-ileostomy with closure of the distal loop. The defect at the hernia site was 1.5 cm in diameter and was closed with a few interrupted non-absorbable sutures (Figure 5C). The operating time was 75 min. Postoperatively, the patient was complicated with MODS and received subsequent treatment in intensive care unit. She recovered well and discharged 21 d after surgery. The second operation for re-established intestinal continuity was suggested 3 mo after operation. But the patient declined and now she is still uncertain about it. No recurrence of the hernia was noted during the 5 mo follow-up.

## DISCUSSION

An obturator hernia is a herniated viscera through the peritoneal defect that is bounded superiorly by the pubic ramus and inferiorly by the free edge of the obturator membrane<sup>[2]</sup>. The incidence was approximately nine times higher in women than in men due to the wider pelvis and relatively greater diameter of the obturator foramen in females<sup>[3]</sup>. Recent published series show nearly exclusive incidence in women<sup>[4-7]</sup>. It occurs most commonly in emaciated elderly women between 70 and 90 years of age. The loss of protective preperitoneal fat from aging or malnutrition makes a larger space in the obturator canal and facilitates the formation of a hernia<sup>[8,9]</sup>. Thus, it is not surprising that most of the patients are markedly underweight. The majority of obturator hernias occur on the right side probably because the sigmoid colon may cover the left obturator foramen and prevents herniation<sup>[10]</sup>. About 6% of cases are bilateral and some may be associated with other types of hernia, such as the indirect inguinal hernia, the direct inguinal hernia, or the femoral hernia<sup>[11]</sup>. It is uncertain if concomitant conditions that lead to constant and increased intra-abdominal pressure such as constipation, ascites, chronic obstructive pulmonary disease, kyphoscoliosis, or multiparity are risk factors for obturator hernia. Our case presented most of these predisposing factors, including old age, emaciation, constipation and chronic bronchitis.

There are three anatomic stages in the development of obturator hernia<sup>[8]</sup>. Stage one: preperitoneal connective tissue and fat enter the pelvic orifice of the obturator canal. Stage two begins with a peritoneal dimple through the canal and progresses to the formation of a peritoneal sac. Stage three is characterized by clinically significant symptoms produced by the entrance of an organ, usually the ileum, sometimes the omentum or part of the bladder, into the sac. Rarely, it was reported that ovary or Meckel's diverticulum can also be incarcerated<sup>[12,13]</sup>. Our case appeared obviously to be in the third stage of development at the time of admission because of the presence of intestinal obstruction.

The most common presentation of obturator hernia is mechanical small bowel obstruction caused by incarceration of the bowel into the obturator canal. The symptoms may be acute or intermittent if the hernia content reduces into the peritoneal cavity spontaneously.

In some cases, the initial symptoms are mild nausea, vomiting and anorexia, probably due to incomplete herniation or Richter's type. Obturator neuralgia is also an important complaint that extends from the inguinal crease to the anteromedial aspect of the thigh. The Howship-Romberg sign is reported to be present in nearly 50% of cases which refers to pain along the medial thigh and sometimes in the hip caused by compression of the obturator nerve by the hernia sac<sup>[10,12]</sup>. Flexion of the thigh usually relieves the pain. Extension, abduction, or medial rotation of the hip may exacerbate the pain. The clinical diagnosis is often difficult to make when this sign is absent. However, the Howship-Romberg sign was generally masked by the severe abdominal symptoms, and it was always neglected before operation. Another possible sign on presentation is the Hannington-Kiff sign<sup>[14]</sup>. It is characterized by an absent adductor reflex in the thigh, resulting from obturator nerve compression. The obturator canal is easily identified by digital vaginal examination at either 2 o'clock or 10 o'clock position. Palpation of a tender mass in the obturator region is of great value in obtaining the correct diagnosis. However, it is evident in only a few cases because the incarcerated mass is usually small and deeply situated.

Various imaging modalities have been applied to establish accurate preoperative diagnosis of this rare disease, including plain abdominal radiography, herniography, ultrasonography, CT, and gastrointestinal imaging with contrast medium. Plain radiography provides no specific findings apart from a dilated bowel loop, and can not reveal any significant information as to the cause of intestinal obstruction<sup>[15]</sup>. It is almost useless in diagnosing obturator hernia. Herniography can directly demonstrate the hernial sac, but has no place in the emergency diagnosis of obturator hernia and is used only in elective cases. Ultrasonography has been considered as a reliable modality with the presence of a hypoechoic tubular structure or a cystic lesion in the region of obturator canal. However, it is not easily identified due to the deep location within the pelvic musculature and smaller hernia sac<sup>[1]</sup>. In 1983, CT was first reported to be useful for detecting obturator hernia by Cubillo, and now is regarded as the standard diagnostic modality for obturator hernia with a documented accuracy of 80%<sup>[10,16]</sup>. CT images may demonstrate an air-filled or fluid-filled bowel loop in the region of the obturator foramen. In a study of a series of patients, Kamori *et al.*<sup>[10]</sup> reported that 15 of 16 patients who were confirmed to have obturator hernia were diagnosed by CT alone. With the advent of multi-slice helical CT scan and the three-dimensional reconstruction technique, the accurate images are of great help in identifying obturator hernia and understanding the relationship between obturator canal and small bowel, as was seen in our study. In our case, the history taking, clinical manifestation and Howship-Romberg sign only gave the suspicion of obturator hernia. The definitive diagnosis was made by CT images.

There is a general agreement that obturator hernia

must be treated surgically. A variety of operative approaches to obturator hernias have been described including retropubic approach, obturator approach, inguinal extraperitoneal approach, transperitoneal approach and combined approach with either a laparotomy or laparoscopy<sup>[3,17-19]</sup>. However, because of the rarity of this condition, there is no consensus on the most proper approach. In patients with an established preoperative diagnosis, an extraperitoneal approach is the best surgical procedure. However, a transperitoneal approach will be necessary in those patients with intestinal obstruction of uncertain cause. We prefer transperitoneal approach because it can obtain adequate exposure, avoid vessel damage, facilitate the reduction of the incarcerated bowel, identify and allow resection of the strangulated bowel when necessary, and easy repair of the defect as well. It should be emphasized that careful dissection of the hernia sac is essential to avoid injury of the obturator nerve or vessels, and the contralateral side must be routinely explored because of the chance of underlying hernia<sup>[18]</sup>. Recently, with the advantage of minimal invasion, laparoscopic technique has been applied in management of obturator hernia. This minimally-invasive method may provide some benefits for these high-risk patients, such as less postoperative pain, fewer complications, earlier ambulatory and shorter hospital stay<sup>[17]</sup>. However, experience with laparoscopy is largely based on isolated case report. The laparoscopic operation for obturator hernia is infeasible in an emergency and is still limited to be performed widely due to the technical problems<sup>[20]</sup>. As in other hernias, after reduction of the contents, the defect of obturator canal should be repaired. Methods of repair vary from a simple suture or using autogenous adjacent tissue like broad ligament, ovary or uterus to permanent prosthetic mesh<sup>[21,22]</sup>. But the use of mesh is not advised in the presence of peritonitis or bowel resection because of the potential risk of infection.

Early surgical intervention is essential to the appropriate treatment of obturator hernia. However, surgeons are often reluctant to operate and family members always hesitate in proceeding with clinical management because of the age, the concomitant disease and the general condition of the patient. Conservative medical treatments were attempted in hopes that the obstruction would be reduced, which undoubtedly result in delayed surgical intervention and increased the morbidity and mortality rates. In our study, the duration from onset of symptoms to surgery was 3.7 d. Here we felt deeply regret that the delayed operation led to the gut resection with ileostomy and severe complications. Fortunately, the patient recovered well.

In conclusion, obturator hernia is relatively rare and is a significant cause of intestinal obstruction, particularly in emaciated elderly women without a history of abdominal surgery. It is important that physicians consider obturator hernia in mind when making a diagnosis in patients with small bowel obstruction. For the diagnosis of obturator hernia, the intermittent attacks of intestinal obstruction,

a positive Howship-Romberg sign, a palpable tender mass in the groin area by digital vaginal examination are helpful. Abdominal CT is useful for viewing a loop of small bowel herniated into the obturator canal. The point of early surgical intervention is highlighted because it is the only hope to lower the high morbidity and mortality associated with this condition.

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## Biliary cystadenocarcinoma diagnosed with real-time contrast-enhanced ultrasonography: Report of a case with diagnostic features

Xiao-Long Ren, Rui-Ling Yan, Xiao-Hui Yu, Ying Zheng, Jun-E Liu, Xiao-Bin Hou, Si-Yang Zuo, Xiao-Yan Fu, Hong Chang, Jian-Hong Lu

Xiao-Long Ren, Rui-Ling Yan, Xiao-Hui Yu, Ying Zheng, Jun-E Liu, Xiao-Bin Hou, Si-Yang Zuo, Xiao-Yan Fu, Hong Chang, Jian-Hong Lu, Department of Ultrasonography, General Hospital of Lanzhou Military Area Command, 333 Binhe South Road, Lanzhou 730050, Gansu Province, China  
 Author contributions: Ren XL and Yan RL performed the contrast enhanced ultrasound work and analysis; Yu XH, Zheng Y, Liu JE, Hou XB, Zuo SY, Fu XY, Chang H and Lu JH co-ordinated and provided the collection of all the human material and clinical materials; Ren XL designed the study and wrote the manuscript.

Correspondence to: Xiao-Long Ren, MD, PhD, Department of Ultrasonography, General Hospital of Lanzhou Military Area Command, 333 Binhe South Road, Lanzhou 730050, Gansu Province, China. [renxiaolong70@hotmail.com](mailto:renxiaolong70@hotmail.com)  
 Telephone: +86-931-8994574 Fax: +86-931-8994575  
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### Abstract

Biliary cystadenocarcinoma is a very rare malignant cystic tumor of the liver, which is often misdiagnosed due to a poor recognition of it. We report a case of a 60-year-old man with biliary cystadenocarcinoma with his real time contrast enhanced ultrasound (CEUS) characteristics compared to those of computed tomography (CT) and magnetic resonance imaging (MRI), which were correlated with the surgical and pathologic findings. Cystic wall enhancement, internal septations and intra-cystic solid portions in the arterial phase were observed on CEUS after contrast agent injection. The enhancement was washed out progressively and depicted as hypo-enhancement in the portal and late phases. CT revealed a large irregular cystic lesion in the left liver lobe with no clear septations and solid components. MRI showed an irregular cystic occupying lesion with septations.

### INTRODUCTION

Biliary cystadenocarcinoma is a very rare malignant cystic tumor of the liver. It is often misdiagnosed because of an insufficient recognition of it<sup>[1,2]</sup> and is hard to differentiate it from benign cystic lesions, such as simple cysts, hydatid cysts and its benign counterpart, cystadenoma. Although these cystic lesions of the liver are more frequently discovered because of the advances in abdominal imaging over the past several years, they are often incorrectly diagnosed, resulting in inadequate therapy<sup>[1-5]</sup>. In recent years, real time contrast-enhanced ultrasonography (CEUS) has gained substantial attention in liver imaging, and its role in differentiating benign from malignant focal liver lesions has been well established. The enhancement characteristics of common benign and malignant focal liver lesions on CEUS have been well described and analyzed, some of which are considered criteria for the differential diagnosis of focal liver lesions<sup>[6-8]</sup>. However, to



the best of our knowledge, no reports are available on the enhancement characteristics of biliary cystadenocarcinoma on real time CEUS, or on comparison between CEUS, computed tomography (CT) and magnetic resonance imaging (MRI) findings. We report a case of a 60-year-old man with a surgically proven biliary cystadenocarcinoma with its CEUS, CT, MRI and histopathologic findings compared.

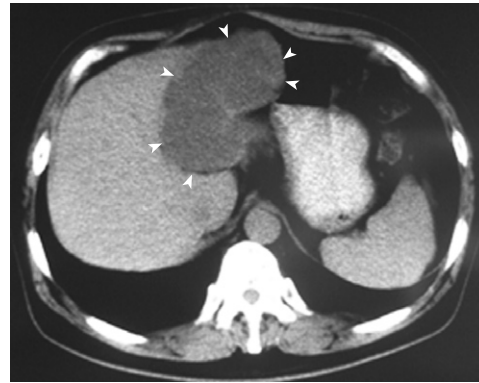
## CASE REPORT

A 60-year-old man was admitted to a local hospital due to an intermittent abdominal pain for 9 d, which was especially severe in the epigastrium. Physical examination revealed a palpable upper abdominal mass with tenderness in the epigastrium. Except for a 5-year history of diabetes mellitus, laboratory test showed normal carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), hepatitis B surface antigen (HBsAg), and anti-hepatitis B surface antigens (anti-HBs), but an elevated serum CA19-9 level of 1090 U/mL.

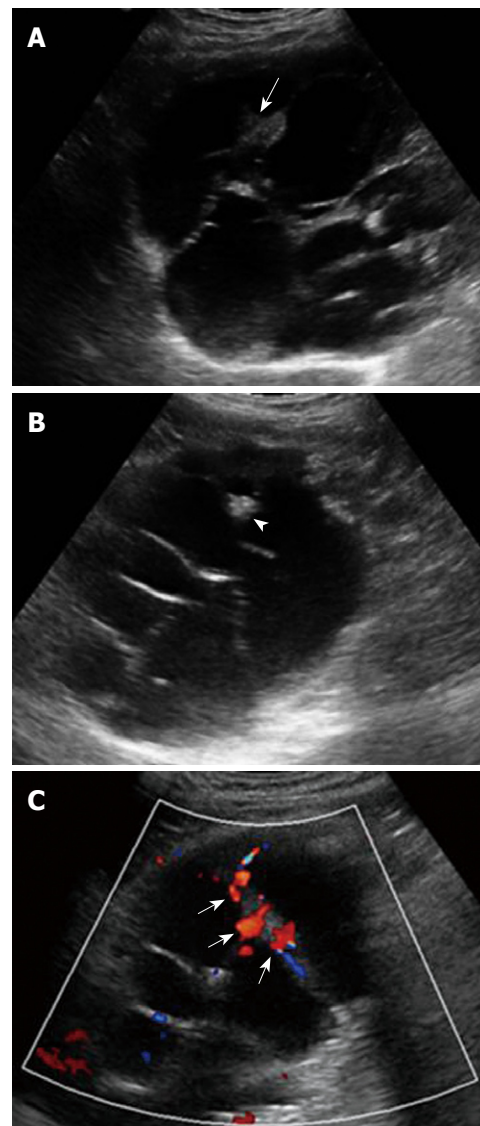
CT scan performed in the local hospital revealed a large irregular cystic lesion in the left liver lobe with no clear septations and solid components (Figure 1). After admitted to our hospital, two-dimensional ultrasonography and CEUS were performed with Philips iU22 (Philips Medical Systems, Bothell, WA) using a 1.0-5.0 MHz probe C5-1 transducer with a pure wave crystal technology to obtain B-mode and color Doppler images. The acoustic power output was adjusted to a low mechanical index of approximately 0.04 for CEUS.

B-mode ultrasonography showed a 12.5 cm × 10.6 cm × 8.2 cm anechoic cystic mass with a well-defined thick wall, mural nodules, and multiple internal septa (Figure 2A), as well as multiple thick and coarse mural and septal calcifications or stones within the septated cysts (Figure 2B). Color Doppler image demonstrated affluent blood flow in the internal septa (Figure 2C). After a conventional sonographic examination to depict the size, location, echogenicity, and internal color Doppler flow signals of the mass, pulse inversion harmonic imaging mode was initiated to examine the real-time CEUS using a sulfur hexafluoride microbubble contrast agent (SonoVue; Bracco SpA, Milan, Italy). A contrast agent (2.4 mL) was administrated intravenously in a bolus fashion *via* an antecubital vein, followed by a flush of 5 mL normal saline solution. CEUS displayed hyper-enhancement of the cystic wall, internal septations and intra-cystic solid portions in the arterial phase at 18 s after contrast agent injection. The intensity reached its peak at 31 s and the enhancement was washed out progressively and depicted as hypo-enhancement in the portal and late phases (Figure 3). MRI showed an irregular cystic occupying lesion with separations (Figure 4).

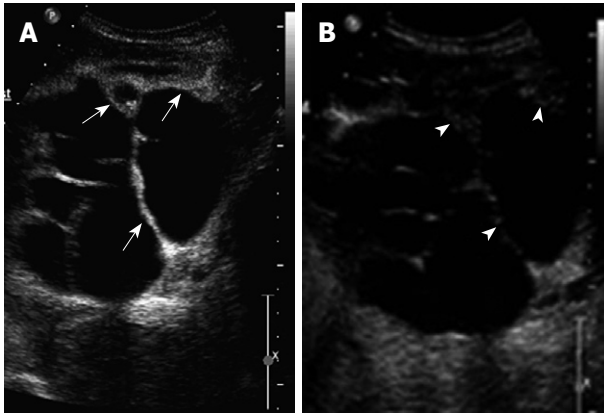
Finally, hepatic lobectomy was performed during which a large cystic mass was found with mucinous fluid present in some portions of the lesion. The mass was finally diagnosed as a biliary cystadenocarcinoma based on the pathological findings which confirmed the thick and coarse calcifications and stones on ultrasonography (Figure 5).



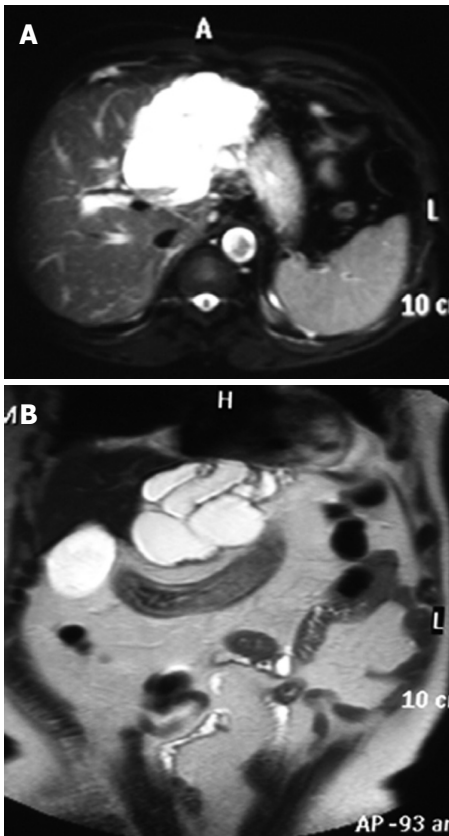
**Figure 1 Biliary cystadenocarcinoma.** Computed tomography shows a huge cystic tumor (arrowheads) in the left liver lobe, with no clear septations and solid components.



**Figure 2 Biliary cystadenocarcinoma.** A: Conventional sonography shows a multilocular cystic lesion in left lobe of the liver with nodular thickening of internal septa and mural nodules projecting into the cyst (arrow); B: Conventional sonography shows septal calcifications or stones (arrowhead); C: Color doppler flow imaging (CDFI) shows affluent vascularity in the internal septa (arrows).



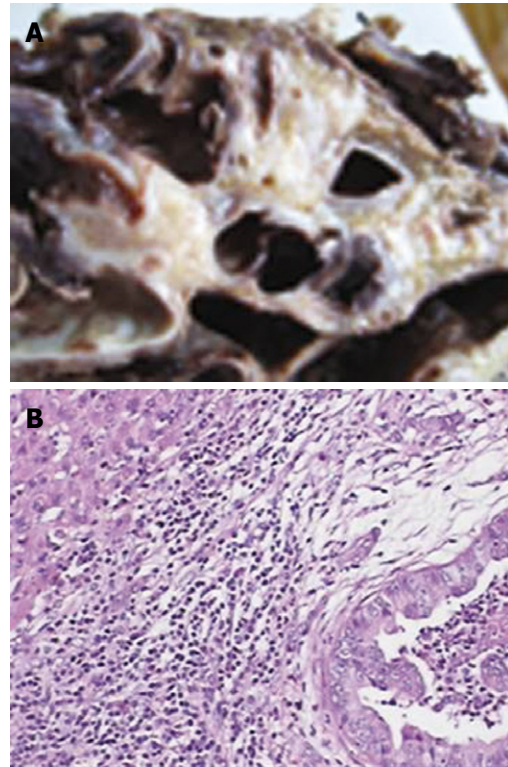
**Figure 3 Biliary cystadenocarcinoma.** A: Contrast enhanced ultrasound (CEUS) shows hyperenhancement of the cystic wall, internal septations and intracystic solid portions during the arterial phase (arrows); B: CEUS shows hypoenhancement of the cystic wall, internal septations and intracystic solid portions during the portal and late phases (arrowheads).



**Figure 4 Biliary cystadenocarcinoma.** A and B: Magnetic resonance imaging (MRI) scans showing a multilocular cyst in the left lobe of the liver.

## DISCUSSION

Biliary cystadenocarcinoma is a very rare malignant cystic tumor of the liver with an incidence of 0.41%<sup>[1]</sup>. Since it was first reported in 1943<sup>[9]</sup>, only a few cases of biliary cystadenocarcinoma have been reported in the literature<sup>[2,9]</sup>. Most biliary cystadenocarcinomas are primary malignant tumors originating from the intra-hepatic bile duct or from congenital intra-hepatic biliary



**Figure 5 Biliary cystadenocarcinoma.** A: Gross appearance of a cross-section of the formalin fixed specimen showing a multilocular mucinous cyst; B: Microscopically, tumor tissues showing moderately differentiated adenocarcinoma with a papillary growth pattern (HE, original magnification  $\times 100$ ).

malformation, or from benign cystadenoma<sup>[10-12]</sup>, or from very slowly growing bile ducts<sup>[2]</sup>.

Symptoms of biliary cystadenocarcinoma include abdominal pain, infection, dyspepsia, anorexia, nausea, vomiting, and occasionally, jaundice due to ductal compression. Most biliary cystadenocarcinoma patients are symptomatic with palpable upper abdominal masses<sup>[2]</sup>. Abdominal pain and a palpable upper abdominal mass were noted in our case with no jaundice. Because of insufficient knowledge about the biliary cystadenocarcinoma and its indistinctive clinical presentations, it is often misdiagnosed as a hepatic abscess, or a hydatid cyst, or a metastatic tumor with cystic degeneration, or even a simple cyst. It is most difficult to differentiate biliary cystadenocarcinoma from cystadenoma, due to their very similar clinical presentations and imaging features. Cystadenoma occurs predominately in women while 38%-44% of biliary cystadenocarcinomas occur in men<sup>[13-15]</sup>. Choi *et al*<sup>[16]</sup> reported that involvement of the left liver lobe is much more common. The majority of biliary cystadenocarcinomas are large in size, usually exceeding 10 cm in diameter, ranging 3.5-25 cm<sup>[16,17]</sup>. In our case, the lesion was found in the left liver lobe with a diameter of 12.5 cm.

Preoperative imaging studies are of key importance in differential diagnosis. The characteristic CT finding in these tumors is low-density intra-hepatic lesions with internal septa and mural nodules. Contrast enhancement can be seen along the internal septa and wall<sup>[2,3,5,17]</sup>. In our case, however, CT scan only revealed a large irregular



cystic lesion in the left liver lobe with no clear septations and solid components, while sonography showed internal septa and affluent blood signals. It was reported that CT scan fails to show any definite or probable septa while sonography reveals internal septa in the same cases. Because contrast enhanced CT was not performed for our case, CT enhancement characteristics of biliary cystadenocarcinoma could not be observed, suggesting that sonography is somewhat more sensitive than CT in detecting the septa of a cystic lesion<sup>[5,16]</sup> and is thus superior to CT in displaying the morphology of cystic hepatic lesions.

Although MRI can show small differences in tissue and provide further information concerning the nature of fluid in the cyst, and is therefore extraordinarily helpful in characterizing hepatic lesions. In our case, MRI showed an irregular cystic occupying lesion with separations. However, biliary cystadenocarcinoma could not be diagnosed in our hospital due to an insufficient recognition of it. In order to understand its enhancing characteristics and make a correct diagnosis, CEUS was performed with Philips iU22, which showed hyper-enhancement of the cystic wall with internal septations and intra-cystic solid portions in the arterial phase and reached its peak intensity in late arterial phase. However, the enhancement was washed out progressively and depicted as hypo-enhancement in the portal and late phases. An initial diagnosis of biliary adenocarcinoma was made by comparing the results in our study with published enhanced CT characteristics, which was confirmed at surgery. The hyper-enhancement in arterial phase and the hypo-enhancement in portal or late phase on CEUS in this case may indicate the malignant nature of the lesion. However, Xu *et al.*<sup>[18]</sup> have reported the hyper-enhancement of the cystic wall, internal septations, and intra-cystic solid portion in the arterial phase in 1 case with benign intrahepatic biliary cystadenoma on CEUS. The enhancement was washed out progressively and depicted as hypo-enhancement in the portal and late phases, suggesting that inadequate differences in enhancement characteristics can differentiate benign intrahepatic biliary cystadenoma from cystadenocarcinoma.

In addition to the features mentioned above, Li *et al.*<sup>[19]</sup> and Koffron *et al.*<sup>[20]</sup> have reported increased levels of CA125 and CA19-9. The CA19-9 level was also elevated in our case. Macroscopy can discover large monolocular or multilocular cystic lesions with mural nodules projecting into the cyst in most cases. Most of the cysts are filled with a great deal of clear, yellow mucous like fluid. The cystic fluid can be coffee-colored when complicated by intra-cystic hemorrhage and bile-like if the cyst communicates with intra-hepatic bile ducts<sup>[21]</sup>. Microscopy can display moderately-differentiated adenocarcinoma with a papillary growth pattern. The papillary structure is lined by monolayer columnar or pseudo-stratified epithelial cells. The tumor cells are characterized by loss of polarity, karyomegaly, and allotypic and pathologic mitotic figures. Although percutaneous liver biopsy contributes to a definite diagnosis, it is risky to induce

peritoneal implantation metastasis<sup>[22]</sup>.

Biliary cystadenocarcinoma should be suspected when CT or ultrasonography reveals an elevated mass or nodule in cystic wall or in folding. However, it is extremely difficult to differentiate cystadenoma from adenocarcinoma by imaging alone. In our case, benign hepatic cyst was considered at admission. It has been reported that tumor markers, carcinoembryonic antigen and carbohydrate antigen 19-9 are increased in serum or cystic fluid of biliary cystic tumor<sup>[23-25]</sup>. However, tumor markers cannot distinguish cystadenocarcinoma from cystadenoma or both from other cystic lesions of the liver. The increased level of serum CA125 and CA19-9 contributes to the differentiation of benign from malignant tumors and is a useful index for the prognosis of biliary cystadenocarcinoma patients<sup>[23-25]</sup>.

In conclusion, biliary cystadenocarcinoma is asymptomatic. When patients have a large cystic lesion in the liver, especially accompanying elevated serum CA125 and CA19-9 levels, a diagnosis of biliary adenocarcinoma can be established. Its definitive diagnosis is difficult to be made based on ultrasonography, CT and MRI findings. CEUS is useful in depicting the enhancing characteristics of cystic wall, internal septations and intra-cystic solid portions of biliary cystadenocarcinoma but cannot give a definite diagnosis. Further study is needed.

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## A dynamic model of once-daily 5-aminosalicylic acid predicts clinical efficacy

Deepak Parakkal, Eli D Ehrenpreis, Matthew P Thorpe, Karson S Putt, Bruce Hannon

Deepak Parakkal, Department of Gastroenterology, North-Shore University Hospital, 2650 Ridge Ave Evanston, IL 60201-1718, United States

Eli D Ehrenpreis, Chief of Gastroenterology and Endoscopy, Highland Park Hospital, NorthShore University Health System and Clinical Associate Professor of Medicine, University of Chicago, 777 Park Avenue West, Highland Park, IL 60035, United States

Matthew P Thorpe, Division of Nutritional Sciences, University of Illinois, Urbana, IL 61801, United States

Karson S Putt, Department of Biochemistry, University of Illinois, Urbana, IL 61801, United States

Bruce Hannon, Department of Geography/NCSA, University of Illinois, Urbana, IL 61801, United States

Author contributions: Parakkal D, Ehrenpreis ED, Thorpe MP, Putt KS and Hannon B contributed equally to this work; All authors designed and performed the research; Thorpe MP, Putt KS, Hannon B contributed to the new analytic tools; All authors analyzed the data; Parakkal D and Ehrenpreis ED wrote the paper.

Correspondence to: Eli D Ehrenpreis, Chief of Gastroenterology and Endoscopy, Highland Park Hospital, NorthShore University Health System and Clinical Associate Professor of Medicine, University of Chicago, 777 Park Avenue West, Highland Park, IL 60035, United States. [ehrenpreis@gipharm.net](mailto:ehrenpreis@gipharm.net)  
Telephone: +1-847-6305398 Fax: +1-847-9265350

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times a day, was compared to 2400 mg given once a day. Under ideal conditions, the predicted maximum drug in the total colon and individual colonic segments over 100 d differed by less than 3% between single and multiple doses. Despite changes in motility and defecation rates, the predicted maximum and average 5-ASA concentrations in the total colon and individual colonic segments differed by less than 10% between dosing regimens. Asymmetric distribution of 5-ASA in the colon was influenced by frequency of bowel movements and colonic transit rate. In active colitis, sigmoid 5-ASA concentration becomes negligible. Our model supports once daily administration of Asacol as standard treatment for ulcerative colitis.

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**Key words:** Ulcerative colitis; 5-aminosalicylate; Mesalazine; Asacol; Once-daily

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### Abstract

New once daily mesalamine formulations may improve adherence to medication usage. Response to Asacol and other forms of 5-aminosalicylic acid (5-ASA) is better correlated with tissue concentrations and best predicted by concentrations of the drug within the lumen of the colon. Our group used computer simulation to predict colonic 5-ASA levels after Asacol administration. In our study, the model simulated Asacol distribution in the healthy colon, and during quiescent and active ulcerative colitis. An Asacol dosage of 800 mg, three

### TO THE EDITOR

We read with a great interest the editorial by Lakatos<sup>[1]</sup> that summarizes the available literature on the short and medium term efficacy and safety of the new once-daily mesalazine formulations. Single dose regimens may improve adherence to medication usage. However, older forms of 5-aminosalicylic acid (5-ASA) may also be administered in a single daily dosage, apparently with adequate effects<sup>[2]</sup>. Most pharmacokinetic studies on

Asacol and other forms of 5-ASA are limited to data collected from serum, urine or fecal drug concentrations. However, response is better correlated with tissue than with plasma concentrations, and is best predicted by concentrations of the drug within the lumen of the colon<sup>[3,4]</sup>. A number of factors influence the concentrations of drugs in colon, such as 5-ASA. Our group<sup>[5]</sup> created a computer model to predict 5-ASA levels in colon after Asacol administration using STELLA software (Isee Systems, Inc., Lebanon NH, USA). This model divides the intestinal system into individual compartments-upper GI tract, right colon, transverse colon, descending colon and the recto-sigmoid colon, and predicts the movement of 5-ASA from one compartment to the other. Retrospective data for drug concentrations based on serum levels have been utilized<sup>[6,7]</sup>. In addition to local transfer of the drug, each colonic compartment loses a fraction of its drug concentration due to mass movements with defecation<sup>[8]</sup>. In our study, the model was run to simulate Asacol distribution in a healthy colon, and during quiescent and active ulcerative colitis. To achieve this, simulations were performed with increasing defecation rates up to 12 bowel movements daily along with variation of upper GI and colonic motility. One hundred 24 h cycles were studied. An Asacol dosage of 800 mg, three times a day, was compared to 2400 mg given once a day. Under ideal conditions, the predicted maximum drug in the total colon and individual colonic segments over 100 d differed by less than 3% between single and multiple doses. Despite changes in motility and defecation rates, the predicted maximum and average 5-ASA concentrations in the total colon and individual colonic segments differed by less than 10% between dosing regimens. The model could also predict almost no drug within the lumen of the recto-sigmoid colon during severe disease activities<sup>[5]</sup>.

Our model supports the once daily administration of Asacol, a concept catching on with new clinical trials.

Asymmetric distribution of 5-ASA in the colon is influenced by frequency of bowel movements and the rate of colonic transit is an important factor in determining 5-ASA dosing in active ulcerative colitis.

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**Ibrahim A Al Mofleh, Professor**, Department of Medicine, College of Medicine, King Saud University, PO Box 2925, Riyadh 11461, Saudi Arabia

**Jamie S Barkin, MD, Professor of Medicine, Chief**, Sinai Medical Center Division of Gastroenterology, Mt. Sinai Medical Center, University of Miami, School of Medicine, 4300 Alton Road, Miami Beach, FL 33140, United States

**Kevin E Behrns, MD, Professor & Chairman**, Department of Surgery, University of Florida, PO Box 100286, Room 6174, 1600 SW Archer Road, Gainesville, FL 32610-0286, United States

**Dr. Ronan A Cahill**, Department of General Surgery, Waterford Regional Hospital, Waterford, Cork, Ireland

**Jordi Camps, PhD**, Centre de Recerca Biomèdica, Hospital Universitari de Sant Joan, C. Sant Joan s/n, 43201 Reus, Catalunya, Spain

**Dr. Justin MM Cates, MD, PhD**, Department of Pathology, Vanderbilt University Medical Center, Medical Center North, C-3322, 1161 21st Avenue South, Nashville, TN 37232, United States

**Dr. Francesco Costa**, Dipartimento di Medicina Interna - U.O. di Gastroenterologia Università di Pisa - Via Roma, 67 - 56122 - Pisa, Italy

**Dr. Seyed Mohsen Dehghani, MD, Associate Professor**, Department of Pediatrics, Nemazee Hospital, Shiraz University of Medical Sciences, Shiraz, Iran

**Sharon DeMorrow, Assistant Professor**, Division of Research and Education, Scott and White Hospital and The

Texas A&M University System, Health Science Center College of Medicine, Temple, Texas 76504, United States

**Isabel Fabregat, PhD, Associate Professor**, Laboratori d'Oncologia Molecular, Institut d'Investigació Biomèdica de Bellvitge, Gran Via, Km 2,7, L'Hospitalet, 08907 Barcelona, Spain

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

**Dr. Mitsuhiro Fujishiro**, Department of Gastroenterology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan

**Yoshihide Fujiyama, Professor**, Internal Medicine, Division of Gastroenterology, Shiga University of Medical Science, Tsukinowa, Seta, Otsu 520-2192, Japan

**Jon C Gould, MD, FACS, Associate Professor** of Surgery, University of Wisconsin School of Medicine and Public Health, 600 Highland Avenue, H4/726, Madison, WI 53792, United States

**Ralph Graeser, PhD, Group Leader**, Molecular & Cellular Biology, ProQinase GmbH, Breisacher Str. 117, Freiburg, 79106, Germany

**Jin Gu, Professor**, Peking University School of Oncology, Beijing Cancer Hospital, Beijing 100036, China

**Invernizzi Pietro Invernizzi, MD, PhD**, Division of Internal Medicine and Hepatobiliary Immunopathology Unit, IRCCS Istituto Clinico Humanitas, via A. Manzoni 113, 20089 Rozzano, Milan, Italy

**Dr. Aydin Karabacakoglu, Assistant Professor**, Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey

**Dr. Cuneyt Kayaalp, MD, Professor**, Department of General Surgery, Staff Surgeon of Gastrointestinal Surgery, Turgut Ozal Medical Center, Inonu University, Malatya 44315, Turkey

**Ioannis E Koutroubakis, MD, PhD, Assistant Professor** of Medicine, University Hospital Heraklion, Department of Gastroenterology, PO Box 1352, 71110 Heraklion, Crete, Greece

**James YW Lau**, Department of Surgery, Prince of Wales Hospital, the Chinese University of Hong Kong, Hong Kong, China

**Kyu Taek Lee, MD, PhD, Professor**, Department of Medicine Samsung Medical Center, Sungkyunkwan, University School of Medicine, #50, Irwon-dong, Gangnam-gu, Seoul, 135-710, South Korea

**Dr. Sara Lindén, PhD, A.Professor**, Mucosal immunobiology and Vaccine Center, Gothenburg University, Box 435, Göteborg, 405 30, Sweden

**Lucia Malaguarnera, Associated Professor**, Department of Biomedical Sciences, University of Catania, Via Androne, 83, Catania, 95124, Italy

**Dr. Francesco Manguso, MD, PhD**, UOC di Gastroenterologia, AORN A. Cardarelli, Via A. Cardarelli 9, Napoli, 80122, Italy

**Roberto Mazzanti, MD, Professor, Chair** of Medical Oncology, Department of Internal Medicine, University of Florence, viale Morgagni, 85-50134 Florence, Italy

**Fanyin Meng, MD, PhD, Assistant Professor**, Department of Internal Medicine, Ohio State University, Room 514A Medical Research Facility, 420 West 12th Avenue, Columbus, Ohio 43210, United States

**Atsushi Nakajima, Professor**, Division of Gastroenterology, Yokohama City University Graduate School of Medicine, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236-0004, Japan

**Nakamura Hiroki Nakamura, MD**, Department of Gastroenterology and Hepatology, 1-1-1, Minami Kogushi, Ube, Yamaguchi 755-8505, Japan

**Dr. Robert Obermaier, Professor, MD**, Department of General- and Digestive Surgery, Albert-Luswigs University Freiburg, University Hospital, Hugstetter str. 55, Freiburg, 79106, Germany

**Keiji Ogura, MD, PhD**, Clinical Research Center, University of Tokyo Hospital, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan

**Maurizio Parola, Professor**, Department Medicina e Oncologia Sperimentale University of Torino Corso Raffaello 30, 10125 Torino, Italy

**Cesare Ruffolo, MD, PhD**, IV Unit of Surgery, Regional Hospital Cà Foncello, Piazza Ospedale 1, Treviso, 31100, Italy

**Francis Seow-Choen, MBBS, FRCSEd, FAMS, Professor**, Seow-Choen Colorectal Centre, Mt Elizabeth Medical Centre, Singapore, 3 Mt Elizabeth Medical Centre #09-10, 228510, Singapore

**Alain L Servin, PhD**, Faculty of Pharmacy, French National Institute of Health and Medical Research, Unit 756, Rue J.-B. Clément, F-92229 Châtenay-Malabry, France

**Mitsuo Shimada, Professor**, Department of Digestive and Pediatric Surgery, Tokushima University, Kuramoto 3-18-15, Tokushima 770-8503, Japan

**Ana Cristina Simões e Silva, MD, PhD, Professor**, Faculdade de Medicina UFMG, Departamento de Pediatria, sala 267, Avenida Professor Alfredo Balena, 190, Bairro Santa Efigênia, Belo Horizonte, Minas Gerais 30130-100, Brazil

**Stocchi Luca Stocchi, MD**, Desk A 30, Department of Colorectal Surgery, Digestive Disease Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, United States

**Yoshitaka Takuma, MD, PhD**, Department of Gastroenterology, Kurashiki Central Hospital, 1-1-1 Miwa, Kurashiki, Okayama, 710-8602 Japan

**Dr. Alberto Tommasini, MD, Professor**, Laboratory of Immunopathology, Institute for Maternal and Child Health, IRCCS Burlo Garofolo, Via dell'Istria 65/1, Trieste 34137, Italy

**Frank I Tovey, OBE, ChM, FRCS**, Honorary Research Fellow, Department of Surgery, University College London, London, United Kingdom

**Debbie Trinder, PhD**, School of Medicine and Pharmacology, University of Western Australia, Fremantle Hospital, PO Box 480, Fremantle 6959, Western Australia, Australia

**Dr. Dinesh Vyas**, Department of Minimally and Endoscopic Surgery, St John Mercy Hospital, 851 E Fifth Street, Washington 63090, United States

**Nathalie Wong, PhD, BSc(hons), Professor**, Department of Anatomical and Cellular Pathology, The Chinese University of Hong Kong, Shatin NT, Hong Kong, China

**Michael E Zenilman, MD**, Clarence and Mary Dennis Professor and Chairman, Department of Surgery, SUNY Downstate Medical Center, Box 40, 450 Clarkson Avenue, Brooklyn, NY 11202, United States

**Dr. Inti Zlobec, PhD**, Institute for Pathology, University Hospital Basel, Schoenbeinstrasse 40, Basel, CH-4031, Switzerland





## Meetings

### Events Calendar 2010

January 25-26  
Tamilnadu, India  
International Conference on Medical  
Negligence and Litigation in Medical  
Practice

January 25-29  
Waikoloa, HI, United States  
Selected Topics in Internal Medicine

January 26-27  
Dubai, United Arab Emirates  
2nd Middle East Gastroenterology  
Conference

January 28-30  
Hong Kong, China  
The 1st International Congress on  
Abdominal Obesity

February 11-13  
Fort Lauderdale, FL, United States  
21th Annual International Colorectal  
Disease Symposium

February 26-28  
Carolina, United States  
First Symposium of GI Oncology at  
The Caribbean

March 04-06  
Bethesda, MD, United States  
8th International Symposium on  
Targeted Anticancer Therapies

March 05-07  
Peshawar, Pakistan  
26th Pakistan Society of  
Gastroenterology & Endoscopy  
Meeting

March 09-12  
Brussels, Belgium  
30th International Symposium on  
Intensive Care and Emergency  
Medicine

March 12-14  
Bhubaneswar, India  
18th Annual Meeting of Indian  
National Association for Study of  
the Liver

March 23-26  
Cairo, Egypt  
14th Pan Arab Conference on  
Diabetes PACD14

March 25-28  
Beijing, China  
The 20th Conference of the Asian

Pacific Association for the Study of  
the Liver

March 27-28  
San Diego, California, United States  
25th Annual New Treatments in  
Chronic Liver Disease

April 07-09  
Dubai, United Arab Emirates  
The 6th Emirates Gastroenterology  
and Hepatology Conference, EGHG  
2010

April 14-17  
Landover, Maryland, United States  
12th World Congress of Endoscopic  
Surgery

April 14-18  
Vienna, Austria  
The International Liver Congress™  
2010

April 28-May 01  
Dubrovnik, Croatia  
3rd Central European Congress  
of surgery and the 5th Croatian  
Congress of Surgery

May 01-05  
New Orleans, LA, United States  
Digestive Disease Week Annual  
Meeting

May 06-08  
Munich, Germany  
The Power of Programming:  
International Conference on  
Developmental Origins of Health  
and Disease

May 15-19  
Minneapolis, MN, United States  
American Society of Colon and  
Rectal Surgeons Annual Meeting

June 04-06  
Chicago, IL, United States  
American Society of Clinical  
Oncologists Annual Meeting

June 09-12  
Singapore, Singapore  
13th International Conference on  
Emergency Medicine

June 14  
Kosice, Slovakia  
Gastro-intestinal Models in  
the Research of Probiotics and  
Prebiotics-Scientific Symposium

June 16-19  
Hong Kong, China  
ILTS: International Liver  
Transplantation Society ILTS Annual  
International Congress

June 20-23  
Mannheim, Germany  
16th World Congress for  
Bronchoesophagology-WCBE

June 25-29  
Orlando, FL, United States  
70th ADA Diabetes Scientific  
Sessions

August 28-31  
Boston, Massachusetts, United States  
10th OESO World Congress on  
Diseases of the Oesophagus 2010

September 10-12  
Montreal, Canada  
International Liver Association's  
Fourth Annual Conference

September 11-12  
La Jolla, CA, United States  
New Advances in Inflammatory  
Bowel Disease

September 12-15  
Boston, MA, United States  
ICAAC: Interscience Conference  
on Antimicrobial Agents and  
Chemotherapy Annual Meeting

September 16-18  
Prague, Czech Republic  
Prague Hepatology Meeting 2010

September 23-26  
Prague, Czech Republic  
The 1st World Congress on  
Controversies in Gastroenterology &  
Liver Diseases

October 07-09  
Belgrade, Serbia  
The 7th Biannual International  
Symposium of Society of  
Coloproctology

October 15-20  
San Antonio, TX, United States  
ACG 2010: American College of  
Gastroenterology Annual Scientific  
Meeting

October 23-27  
Barcelona, Spain  
18th United European  
Gastroenterology Week

October 29-November 02  
Boston, Massachusetts, United States  
The Liver Meeting® 2010--AASLD's  
61st Annual Meeting

November 13-14  
San Francisco, CA, United States  
Case-Based Approach to the  
Management of Inflammatory Bowel  
Disease

December 02-04  
San Francisco, CA, United States  
The Medical Management of HIV/  
AIDS



## Instructions to authors

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### Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

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### Format

#### Journals

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 Huaran Xiaohua 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

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- 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 and an organization as author

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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 *v* (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  $24.5 \mu\text{g/L}$ ; 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 23 243 641.

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Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

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Editorial Board of *World Journal of Gastroenterology*, Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China  
Telephone: +86-10-5908-0039  
Fax: +86-10-8538-1893  
E-mail: [wjg@wjgnet.com](mailto:wjg@wjgnet.com)  
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Telephone: +86-10-8538-1892  
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Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China  
Telephone: +86-10-8538-1892  
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## Tube feeding, the microbiota, and *Clostridium difficile* infection

Stephen JD O'Keefe

Stephen JD O'Keefe, Division of Gastroenterology, University of Pittsburgh, 570 Scaife Hall, 3550 Terrace St, Pittsburgh, PA 15134, United States

Author contributions: O'Keefe SJD wrote this paper.

Correspondence to: Stephen JD O'Keefe, MD, MSc, FRCP (UK), Professor, Division of Gastroenterology, University of Pittsburgh, 570 Scaife Hall, 3550 Terrace St, Pittsburgh, PA 15134, United States. [sjokeefe@pitt.edu](mailto:sjokeefe@pitt.edu)

Telephone: +1-412-6487217 Fax: +1-412-6487213

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### Abstract

*Clostridium difficile* (*C. difficile*) is now the leading cause of nosocomial diarrhea in the USA, accounting for 30% of patients with antibiotic-associated diarrhea, 70% of those with antibiotic-associated colitis, and most cases of pseudomembranous colitis. The organism has evolved over the last 8 years to become more virulent and resistant to antimicrobials (NAP1/027 strain) causing a more severe form of the disease that has increased mortality and healthcare costs. While it is generally accepted that the problem results from the overuse of antibiotics, and in particular second and third generation cephalosporins, fluoroquinolones and macrolides, recent studies suggest that acid suppression with proton pump inhibitors (PPIs) may be equally culpable. A further common, but less recognized, etiological factor is the prolonged use of elemental diets. Such diets are totally absorbed within the small intestine and therefore deprive the colonic microbiota of their source of nutrition, namely dietary fiber, fructose oligosaccharides, and resistant starch. The resultant suppression of colonic fermentation leads to suppression of the "good" bacteria, such as butyrate-producers (butyrate being essential for colonic mucosal health), and bifidobacteria and the creation of a "permissive" environment for *C. difficile* colonization and subsequent infection. Based on this analysis, the best chance of suppressing the emerging *C. difficile* epidemic is to

adopt a 3-pronged attack consisting of (1) avoidance of the use of prophylactic antibiotics, (2) the avoidance of prophylactic PPIs, and (3) the conversion of elemental diet feeding to a diet containing adequate indigestible carbohydrate after the first week of critical illness. In this review, we highlight the rising worldwide incidence of *C. difficile* associated diarrhea and the role played by non-residue diets in destabilizing the colonic microbiota.

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**Key words:** *Clostridium difficile*; Elemental diets; Enteral nutrition; Microbiota

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### INTRODUCTION

In under 10 years, *Clostridium difficile* (*C. difficile*) has risen from an obscure anaerobic bacterium to a notorious "superbug" responsible for epidemics of hospital-acquired infections worldwide, attracting major media concern (e.g. "Stomach Bug Crystallizes an Antibiotic Threat", New York Times, April 13, 2009). It is now the leading cause of nosocomial diarrhea in the USA, accounting for 30% of patients with antibiotic-associated diarrhea, 70% of those with antibiotic-associated colitis, and most cases of pseudomembranous colitis<sup>[1]</sup>. Furthermore, the organism has evolved over the last 8 years to become more virulent and resistant to antimicrobials

(NAP1/027 strain) causing a severe form of the disease that prolongs hospitalization and increases mortality, creating substantial increases in health service and economic burdens<sup>[2]</sup>. USA estimates report an additional annual cost to health care of \$1.1 billion<sup>[3]</sup>. Mortality rates have increased concomitantly with these epidemic outbreaks. For example, a report from Quebec, Canada, noted that mortality from infection associated with the use of prophylactic antibiotics for surgical procedures increased from 0.7 cases per 1000 procedures in 2002 to 14.9 in 2005<sup>[4]</sup>. Subsequent studies on bacterial isolates revealed strain changes that increased the production of toxins and made the organism resistant to a wider range of broad-spectrum antibiotics<sup>[5]</sup>. Second and third generation cephalosporins, fluoroquinolones and macrolides have been shown to increase the development of virulence and adherence enhancing colonization. It is therefore understandable that the observed recent exponential increases in incidence, morbidity, mortality and associated healthcare costs are of extreme national and international concern, as they threaten our current ability to support survival in septic critically ill hospitalized patients with the latest generation of potent antibiotics.

## HOSPITAL CONTROL

There is no consensus on how the problem can be solved. Despite increasing resistance, the organism is still generally sensitive to both oral metronidazole and vancomycin, and yet mortality continues to increase. Thus greater effort is being directed at prevention. Of the considered 3 most powerful risk factors, age, antimicrobials and exposure to healthcare facilities, the third should be the most controllable. The mode of transmission is thought to be chiefly due to contamination from the hospital environment and from the hands of healthcare workers. Most hospitals now impose strict isolation and hygiene control to detected cases, but the ability of the organism to form spores makes it difficult to control. Although the spores are easily washed down the drain with soap and water, *C. difficile* is not killed by most routinely used hospital germicides. Only 1:10 bleach used with a 10-min contact time serves as a *C. difficile* sporicide<sup>[6]</sup> and doing this routinely is logistically impossible.

## PROTON PUMP INHIBITORS

However, the overuse of broad-spectrum antibiotics cannot alone explain the *C. difficile* crisis. Recent studies have suggested that the increase in incidence is better correlated with the use of gastric acid suppressing proton pump inhibitors (PPIs) than with antibiotic usage<sup>[7]</sup>. While spores are resistant to acid, the vegetative form is killed by gastric acid but has been shown to survive passage through the stomachs of patients on PPIs<sup>[8]</sup>. As the vegetative form can theoretically survive on damp surfaces for short spaces of time, it is possible that

colonic infection may be propagated from patient-to-patient through the use of equipment such as bedpans. The use of PPIs may also promote the expansion and colonization of *C. difficile* by its recognized potential to induce small bowel bacterial overgrowth with anaerobic colonic organisms<sup>[9]</sup>.

## ELEMENTAL DIETS

In addition, there is the commonly overlooked question of enteral feeding. While there is irrefutable evidence that conventional enteral tube feeding reduces respiratory and bloodstream infectious morbidity<sup>[10]</sup>, the technique has been associated with increased risk of *C. difficile* infection<sup>[11]</sup>. Although this is usually explained by enteral feeding providing a high-frequency portal for inoculation of *C. difficile* spores deep into the gut by healthcare workers, it could simply be explained by the fact that patients requiring enteral feeding are usually sicker, at higher risk of any complication, and more often on antibiotics. Patients receiving percutaneous endoscopic gastrostomies (PEGs) may be at even higher risk due to their higher grades of chronic illness and the conventional use of prophylactic antibiotics to prevent placement infections<sup>[12]</sup>. Enteral feeding associated *C. difficile* infections may also be related to the fact that research has focused on the importance of luminal nutrition on the upper gastrointestinal (GI) tract - and not on the colon. Critically ill patients commonly have impaired upper GI function with poor motility and ileus. Studies have shown that feeding tolerance by such patients can be remarkably good if the feed is given in a residue-free predigested, or "elemental" form, and delivered beyond the stomach into the jejunum because it is totally absorbed within the upper small intestine<sup>[13]</sup>. Unfortunately, modification of normal eating in this way has some potentially deleterious effects. Firstly, the jejunal elemental diets suppress bacteriostatic gastric and pancreobiliary secretions<sup>[14,15]</sup> and also motility. These effects together promote colonization of the small intestine with colonic microbiota, leading to small bowel bacterial overgrowth. Secondly, experimental studies have shown that elemental diets are a perfect culture medium for *C. difficile* organisms<sup>[16]</sup>. Thirdly, elemental diets contain no complex carbohydrate residues, such as fiber or "resistant" starch that escape digestion in the small intestine and enter the colon to provide a fermentable food source for the colonic microbiota.

## THE CRITICAL IMPORTANCE OF COLONIC NUTRITION ON THE MICROBIOTA

The absence of fiber and resistant starches not only disturbs microbial balance further, but also deprives the colonic epithelium of its chief energy source and proliferation regulator, butyrate, a short chain fatty acid that is synthesized by the microbiota during the fermentation process<sup>[17]</sup>. A further twist to the story is that it

has been shown that butyrate deficiency in the colon potentiates the growth and toxin production of *C. difficile* organisms<sup>[18]</sup>. It is reasonable to thus speculate that the prolonged use of non-residue tube feeds will enhance *C. difficile* colonization and subsequent *C. difficile* associated colitis by reducing mucosal health and therefore resistance to pathogen adherence and subsequent cytotoxic injury. It has been shown experimentally that colony adherence results in mucosal inflammation induced by the transference of cytotoxic and enterotoxic factors, disrupting the epithelial barrier<sup>[19]</sup>. Toxins A and B enter the colonic cells and kill the cells by multiple mechanisms, including catalysis of the transfer of glucose to GTPases. This may be critically important as in the absence of butyrate, colonic cell survival will be totally dependent on efficient glucose utilization. The suggestion that pre-existing mucosal injury predisposes to *C. difficile* colonization and cytotoxicity is supported by the observation that patients with chronic ulcerative colitis are at dramatically increased risk of developing severe *C. difficile* associated colitis, and that many are resistant to antimicrobial treatment, with 20% requiring total colectomy and an overall mortality rate of 50%<sup>[20]</sup>. To date, fiber supplementation of enteral feeds has not been systematically tested in the critically-ill, but it is noteworthy that Lewis *et al*<sup>[21]</sup> found that oligosaccharide supplementation (12 g/d) increased bifidobacteria counts and decreased diarrhea in patients with chronic relapsing *C. difficile* infection.

## CONCLUSION

So what can we do to reduce the incidence of *C. difficile* infection and its progression to colitis? Survival in the ICU is commonly dependent on the protracted use of broad-spectrum antibiotics, and so withholding their use is not an option. However, prophylactic antibiotics post-operatively and in conditions, such as severe pancreatitis where they are of unproven benefit, should be avoided<sup>[22]</sup>. Secondly, PPIs are grossly overprescribed in the ICU usually with the intent of preventing stress ulceration, but there is poor correlation between this and gastric acidity. One study looking at the association between *C. difficile* associated colitis and PPIs, showed that 63% of patients had no valid indication for acid suppression<sup>[23]</sup>. Not only would restriction of PPI use likely decrease the incidence of *C. difficile* associated colitis but it would also have enormous health care savings worldwide. PPIs now account for 10% of the annual prescribing costs in the UK; more judicious use would save the National Health Service at least £100 million/year<sup>[24]</sup>. Finally, the use of non-residue tube feeds should be restricted to those critically-ill patients with ileus and borderline gut function and since, in practice, ileus usually resolves and function returns with the slow progression of tube feeding over three or 4 d<sup>[13]</sup>, even in these patients a change can often be made to a fiber or “prebiotic” containing formula after the first week.

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## Portal vein thrombosis: Insight into physiopathology, diagnosis, and treatment

Francesca R Ponziani, Maria A Zocco, Chiara Campanale, Emanuele Rinninella, Annalisa Tortora, Luca Di Maurizio, Giuseppe Bombardieri, Raimondo De Cristofaro, Anna M De Gaetano, Raffaele Landolfi, Antonio Gasbarrini

Francesca R Ponziani, Maria A Zocco, Chiara Campanale, Emanuele Rinninella, Annalisa Tortora, Luca Di Maurizio, Giuseppe Bombardieri, Raimondo De Cristofaro, Raffaele Landolfi, Antonio Gasbarrini, Department of Internal Medicine, Catholic University of Rome, 00168 Rome, Italy

Anna M De Gaetano, Department of Bioimaging and Radiological Sciences, Catholic University of Rome, 00168 Rome, Italy

**Author contributions:** Ponziani FR designed and wrote the review; De Gaetano AM and Zocco MA collected the images; Campanale C, Rinninella E, Tortora A and Di Maurizio L wrote the references and tables; Bombardieri G, De Cristofaro R, Landolfi R and Gasbarrini A revised the paper.

**Correspondence to:** Francesca R Ponziani, MD, Department of Internal Medicine, Catholic University of Rome, 00168 Rome, Italy. [francesca.ponziani@yahoo.it](mailto:francesca.ponziani@yahoo.it)

Telephone: +39-347-1227242 Fax: +39-6-30156018

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of liver transplantation and its possible influence on patients' future prognoses.

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**Peer reviewers:** Hiroshi Yoshida, MD, First Department of Surgery, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan; Diego Garcia-Compean, MD, Professor, Faculty of Medicine, University Hospital, Department of Gastroenterology, Autonomous University of Nuevo Leon, Ave Madero y Gonzalitos, 64700 Monterrey, NL, México

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### Abstract

Portal vein thrombosis (PVT) is a relatively common complication in patients with liver cirrhosis, but might also occur in absence of an overt liver disease. Several causes, either local or systemic, might play an important role in PVT pathogenesis. Frequently, more than one risk factor could be identified; however, occasionally no single factor is discernable. Clinical examination, laboratory investigations, and imaging are helpful to provide a quick diagnosis, as prompt treatment might greatly affect a patient's outcome. In this review, we analyze the physiopathological mechanisms of PVT development, together with the hemodynamic and functional alterations related to this condition. Moreover, we describe the principal factors most frequently involved in PVT development and the recent knowledge concerning diagnostic and therapeutic procedures. Finally, we analyze the implications of PVT in the setting

### INTRODUCTION

The term portal vein thrombosis (PVT) refers to the complete or partial obstruction of blood flow in the portal vein, due to the presence of a thrombus in the vascular lumen<sup>[1]</sup>. Although in the general population PVT is considered a rare event, its prevalence among cirrhotic patients ranges between 4.4%-15%, and is responsible for about 5%-10% of overall cases of portal hypertension<sup>[2]</sup>. The first case of PVT was reported in 1868 by Balfour and Stewart, describing a patient presenting splenomegaly, ascites, and variceal dilation<sup>[3]</sup>. Several etiological causes, either of local or systemic origin, might be responsible for PVT development, although more than one factor is often identified. Furthermore, PVT clinical presentation

is different in the context of acute or chronic onset and depends on the development and the extent of a collateral circulation. Intestinal congestion and ischemia, with abdominal pain, diarrhea, rectal bleeding, abdominal distention, nausea, vomiting, anorexia, fever, lactacidosis, sepsis, and splenomegaly are common features of acute PVT. In contrast, chronic PVT can be completely asymptomatic, or characterized by splenomegaly, pancytopenia, varices, and, rarely, ascites<sup>[3]</sup>. In the presence of portal hypertension, PVT must always be investigated, especially in cirrhotic patients, even if it is considered a rare event<sup>[2]</sup>. Indeed, an early diagnosis and appropriate management of secondary portal hypertension could be, in some cases, life-saving for the patient. Furthermore, in the diagnostic iter, the identification of possible local or systemic trigger factors is of primary importance however, occasionally no single factor is discernable.

Currently, several therapeutic options are available; however, their feasibility and efficacy are still being evaluated and the risks and benefits should be carefully considered for each patient.

In this review, we discuss the features of PVT, pointing out new insights into clinical, diagnostic, and therapeutic issues, making an overview of current beliefs regarding patient outcome and, finally, reporting controversies about the correct management of PVT in the setting of liver transplantation.

## **PATHOPHYSIOLOGY**

As a consequence of portal vein obstruction, systemic and splanchnic hemodynamics undergo specific and important modifications<sup>[4]</sup>. On the cessation of portal blood flow, the liver loses about two thirds of its blood supply. Interestingly, this condition is usually well tolerated and patients are often asymptomatic, while an acute arterial obstruction always leads to a severe hepatic dysfunction, which is sometimes fatal. It is probably that the immediate activation of two compensatory mechanisms might supplement the loss of portal vein's contribution to liver blood flow. The first mechanism is "arterial vasodilation" of the hepatic artery, similar to that observed in portal vein clamping during liver surgery<sup>[5]</sup>. This "arterial rescue" is a kind of vascular reflex present in every organ with both an arterial and a venous circulation and is capable of preserving liver function in the acute stages of PVT. The second compensatory mechanism is "venous rescue", consisting of the rapid development of collaterals to bypass the obstruction. This vascular neo-formation begins in a few days after portal vein obstruction, and finalizes within 3 to 5 wk<sup>[6,7]</sup>. As a result, the thrombosed portal vein is replaced by a network of collateral vessels, called "cavernoma", connecting the two patent portions proximally and distally to the thrombus. Usually, the original portal vein becomes a thin, fibrotic cord, which is difficult to visualize<sup>[8,9]</sup>. At this stage, the development of a hyperkinetic circulation, characterized by low systemic vascular resistance and a high cardiac output, is common<sup>[3]</sup>.

Despite the activation of this complex system of support, the impairment of portal flow has important consequences on liver tissue. It has been demonstrated in rats, that the progressive obliteration of the portal vein stimulates apoptosis of hepatocytes in the hypoperfused lobe<sup>[10]</sup>, while increasing the mitotic activity in the normal perfused one. The latter effect is well known, and is employed therapeutically in resective liver surgery. However, this process results in a progressive loss of tissue and might be responsible for the impairment of hepatic synthetic function observed in advanced stages of portal vein obstruction<sup>[11]</sup>.

## **EPIDEMIOLOGY**

The concept of PVT as a rare disease is mainly based on clinical series and case reports<sup>[2]</sup>. An epidemiological study performed in southern Sweden and based on autopsies, reported the incidental finding of a PVT in about 1% of the general population<sup>[12]</sup>. Cohen *et al*<sup>[13]</sup> confirmed these data and reported that most PVT patients were cirrhotics with a primary or metastatic liver cancer. Today, thanks to the availability of more sensitive and less invasive imaging, together with the existence of curative or palliative procedures, PVT is routinely investigated and recognized without any difficulty<sup>[14-16]</sup>. Thus, PVT seems more frequent than expected: it is estimated to be responsible for 5%-10% of the overall cases of portal hypertension, which can be 40% in developing countries<sup>[3]</sup>. The incidence among cirrhotic patients is still unknown, but recent data suggest a prevalence of about 0.6%-16%<sup>[17]</sup> (the highest) among orthotopic liver transplantation (OLT) candidates<sup>[2]</sup> and of about 6.5% in patients with a hepatocellular carcinoma at the time of diagnosis<sup>[16]</sup>.

## **ETIOLOGY**

Several causes can be involved in the pathogenesis of PVT and, frequently, more than one coexist. A simple classification distinguishes between local (70%) and systemic (30%) risk factors (Tables 1 and 2).

Inflammatory abdominal foci (such as appendicitis, diverticulitis, inflammatory bowel diseases, pancreatitis, cholecystitis, hepatic abscesses, and cholangitis), liver cirrhosis or tumors, represent the most common local thrombotic risk factors<sup>[8,12,18]</sup>.

Malignancies, frequently of hepatic or pancreatic origin, are responsible for 21%-24% of overall cases of PVT<sup>[13,19]</sup>. Direct vascular invasion, compression by tumor mass, or a hypercoagulable state are the mechanisms involved in neoplastic PVT development; hormonal factors might also play a role in this process, especially in men<sup>[16,20,21]</sup>.

PVT is common in patients affected by liver cirrhosis, with a risk related to the severity of the disease; the prevalence ranges from 1%, at the earlier stages, to 30% in candidates for liver transplantation<sup>[8,17]</sup>. Moreover, in patients with a hepatocellular carcinoma, the incidence of PVT rises to 10%-40%<sup>[9]</sup>.

**Table 1** Most frequent local risk factors for PVT<sup>[3,8,9,17,18,64,79]</sup>

Local risk factors for PVT (70%)
Cancer
Any abdominal organ
Focal inflammatory lesions
Neonatal omphalitis, umbilical vein catheterization
Diverticulitis, appendicitis
Pancreatitis
Duodenal ulcer
Cholecystitis
Tuberculous lymphadenitis
Crohn's disease, ulcerative colitis
Cytomegalovirus hepatitis
Injury to the portal venous system
Splenectomy
Colectomy, gastrectomy
Cholecystectomy
Liver transplantation
Abdominal trauma
Surgical portosystemic shunting, TIPS
Iatrogenic (fine needle aspiration of abdominal masses <i>etc.</i> )
Cirrhosis
Preserved liver function with precipitating factors (splenectomy, surgical portosystemic shunting, TIPS dysfunction, thrombophilia)
Advanced disease in the absence of obvious precipitating factors

PVT: Portal vein thrombosis; TIPS: Transjugular intrahepatic portosystemic shunt.

**Table 2** Most frequent systemic risk factors for PVT<sup>[3,8,9,17,18,64,79]</sup>

Systemic risk factors for PVT (30%)
Inherited
Factor V Leiden mutation
Factor II (prothrombin) mutation
Protein C deficiency
Protein S deficiency
Antithrombin deficiency
Acquired
Myeloproliferative disorder
Antiphospholipid syndrome
Paroxysmal nocturnal hemoglobinuria
Oral contraceptives
Pregnancy or puerperium
Hyperhomocysteinemia
Malignancy

Other less common PVT local causes are adenopathy, systemic inflammatory response syndrome, and surgical traumas to the portal venous system, such as portosystemic shunting, splenectomy, liver transplantation, ablative therapy for HCC, and fine needle aspiration of abdominal masses<sup>[1]</sup>.

On the other hand, myeloproliferative disorders and prothrombotic conditions belong to the group of systemic risk factors, with a prevalence of about 40% and 60%, respectively (Table 3)<sup>[8,22]</sup>.

Factor V Leiden mutation is the most common thrombophilia predisposing to PVT, followed by protein C (PC) deficiency<sup>[23-26]</sup>. The role of protein S (PS) and antithrombin III (AT) deficiency in PVT etiology has not yet been confirmed, and it is difficult to evaluate

**Table 3** Prevalence of thrombotic risk factors in series of routinely investigated, consecutive adult patients with non tumorous and non cirrhotic, acute or chronic, PVT<sup>[126]</sup>

Risk factor	PVT patients (%)
Myeloproliferative disorders	30-40
Atypical	14
Classical	17
Antithrombin deficiency	0-26
Protein C deficiency	0-26
Protein S deficiency	2-30
Factor V Leiden mutation	6-32
Prothrombin mutation	14-40
TT677 methylene tetrahydrofolate reductase (MTHFR) genotype	11-50
Antiphospholipid syndrome	6-19
Hyperhomocysteinemia	12-22
Recent pregnancy	6-40
Recent oral contraceptive use	12

the influence of anticoagulation therapy on the impairment in liver function. Indeed, in cirrhotic patients it is hard to distinguish between congenital and acquired deficiencies of natural coagulants and their role in PVT pathogenesis, because if liver function is impaired, levels of coagulation inhibitors, as well as those of pro-coagulation factors, are often decreased<sup>[27]</sup>. A clinical study conducted on eleven children with PVT<sup>[28]</sup>, reported a significant improvement in PC, PS, factors II, V, and VII levels and prothrombin time after surgical correction with a Rex Shunt (mesenteric-left portal vein bypass). In contrast, a distal spleno-renal shunt or an H-type meso-caval shunt, in the same condition, did not seem to be equally effective, probably due to insufficient residual portal vein flow and the consequent impairment in liver synthetic function<sup>[29]</sup>. However, the relatively low prevalence of genetic, in respect to acquired, thrombophilic disorders, might represent a potential diagnostic matter in PVT patients, and should be considered carefully in clinical practice<sup>[30]</sup>. To overcome this problem, an accurate genetic study of the patient and, eventually, his/her family (first degree relatives) might be useful in strongly suggestive cases. Unfortunately, in practice, this policy is not applicable without difficulty. A simple method to screen the deficiency of natural anticoagulants in patients with liver disease comprises the ratio of PS or PC or AT to [(factor II + factor X)/2]. If the result is less than 70%, a genetic deficiency has to be suspected and investigated<sup>[1]</sup>.

Among the other thrombophilic disorders, a prothrombin gene mutation seems to be frequent among cirrhotics with PVT<sup>[2,31-34]</sup>. However, in the general population, its role in PVT development seems less clear, as it is considered a weak prothrombotic risk factor. Moreover, a homozygous *methylene tetrahydrofolate reductase* (MTHFR) gene mutation might be associated with PVT development alone or, if heterozygote, in the presence of other cofactors<sup>[13,35-39]</sup>. Ammirato *et al.*<sup>[27]</sup> reported a strong correlation between the prothrombin A20210

mutation or the homozygous MTHFR C677-T genotype and PVT in cirrhotic patients without evidence of liver cancer.

Furthermore, the presence of anticardiolipin antibodies is quite frequent in patients with chronic liver disease; a transient positivity is often reported after infections, suggesting a relationship between microorganisms (i.e. *Bacteroides* species) and thrombotic events, such as PVT<sup>[40-43]</sup>. In contrast, other studies consider anticardiolipin antibodies simply as an epiphenomenon of liver damage<sup>[41,44]</sup>. Finally, the role of oral contraceptives, steroids, and pregnancy is still less clear<sup>[45-47]</sup>.

In about 22%-48% of patients, PVT is a manifestation of a myeloproliferative disease (MPD)<sup>[2,20,48]</sup>.

An intra-abdominal vascular thrombosis is often the sole presenting symptom and an overt MPD might successively develop in 51% of cases. In the Western Countries, 58% of idiopathic PVTs are associated with a latent MPD<sup>[49]</sup>. The principal diagnostic criteria are usually incompletely met in these patients, probably because of the atypical manifestation of the disease<sup>[50]</sup>. The 1849G→T point mutation in the gene encoding tyrosine-protein kinase JAK2, is a specific and easily detectable marker for MPDs, which can often be useful for a rapid diagnose in PVT patients<sup>[51-55]</sup>. Recent studies reported the presence of a JAK2 mutation in about 17%-35% of patients with PVT, but further studies are needed to confirm these data<sup>[56,57]</sup>.

Occasionally, it is not possible to recognize any overt cause of PVT; generally, the clinical course is favorable for these patients, with a low incidence of complications. However, at present, "idiopathic PVT" is less frequent, thanks to the amelioration in diagnostics and to a more scrupulous attention to patients' clinical history<sup>[12]</sup>.

In conclusion, it is reasonable to routinely investigate the most common prothrombotic disorders and exclude a local trigger, to provide a correct management of PVT and its original cause. However, the mechanism of PVT development is complex and multifactorial, and is not always attributable to a single risk factor. In the presence of sporadic local or systemic promoting events, an underlying intrinsic predisposition might be the access key to thrombosis development<sup>[1,13]</sup>.

## CLASSIFICATION

PVT onset can be acute or chronic. This is an arbitrary distinction, which is sometimes difficult to apply in clinical practice; patients who develop symptoms, such as abdominal pain, nausea, and fever, within sixty days prior to hospital admission, might have an acute PVT development<sup>[58,59]</sup>.

PVT can be classified into four categories, depending on the extension: (1) confined to the portal vein beyond the confluence of the splenic vein; (2) extended to the superior mesenteric vein, but with patent mesenteric vessels; (3) extended to the whole splanchnic venous system, but with large collaterals; or (4) with only fine collaterals<sup>[60]</sup>. This classification is useful to evaluate a

patient's operability and clinical outcome. In fact, when thrombosis is extended to both portal and mesenteric veins, the risk of bowel ischemia is considerable and mortality high, despite a lower risk of variceal bleeding<sup>[61]</sup>.

## CLINICAL PRESENTATION

PVT can occur either in childhood or in adulthood, with the same incidence<sup>[45]</sup>. Clinical presentation always depends on the onset and the extent of the thrombosis and the development of collateral circulation<sup>[62]</sup>.

### Acute PVT

Intestinal congestion and ischemia are typical manifestations of acute PVT; abdominal pain or distention, diarrhea, rectal bleeding, nausea, vomiting, anorexia, fever, lactacidosis, splenomegaly and sepsis might be variably present<sup>[63,64]</sup>. If the obstruction is not resolved quickly, intestinal perforation, peritonitis, shock, and death from multiorgan failure might occur<sup>[8]</sup>. On physical examination, the abdomen might be distended, but guarding is rare, except in case of intra-abdominal inflammation, intestinal infarction, and perforation<sup>[22]</sup>. The majority of patients exhibit splenomegaly, while ascites is rare or, eventually, present before the development of a collateral circulation. This mild, transient, ascites is due to intestinal venous congestion in the absence of the mechanisms activated in liver cirrhosis<sup>[65,64]</sup>.

### Chronic PVT

On the other hand, chronic PVT can be nearly asymptomatic, except for the presence of varices, cutaneous collaterals, or ascites<sup>[62]</sup>. Typically, patients with an advanced thrombosis do not always remember any previous trigger event or disease<sup>[22,63,64]</sup>. The majority of patients develop esophageal varices, in contrast to acute PVT; an episode of gastrointestinal bleeding is reported as the first presenting symptom in about 20%-40% of cases<sup>[9]</sup>. As this phenomenon is strictly time-dependent, it is advisable to screen all PVT patients endoscopically, at diagnosis<sup>[63]</sup>. In cirrhotics with PVT, the risk of variceal bleeding is nearly 80-120 times higher than in patients without liver disease, although the outcome seems better<sup>[65,66]</sup>.

Furthermore, hypersplenism and, consequently, pancytopenia, are commonly present in chronic PVT<sup>[1]</sup>; however, if one branch of the portal vein is preserved and the portal pressure is quite normal, they may even be absent. Ascites and encephalopathy are uncommon and only transient. They are more frequent after an episode of gastrointestinal bleeding or associated with renal failure or sepsis in older patients<sup>[8,64,67]</sup>. Abnormalities of the extrahepatic biliary tree have been reported in more than 80% of patients with chronic PVT; compression by choledochal or periportal varices or by the cavernoma, pericholedochal fibrosis, and ischemic structuring are the principal reasons<sup>[67-71]</sup>. Another finding is the "pseudocholangiocarcinoma sign"<sup>[1,11,17]</sup>, caused by the displacement, stricturing or thumbprinting of the biliary



Table 4 AASLD recommendations for diagnosis of acute and chronic PVT<sup>[126]</sup>

AASLD recommendations for diagnosis of acute PVT	AASLD recommendations for diagnosis of chronic PVT
Consider a diagnosis of acute PVT in any patient with abdominal pain of more than 24 h duration, whether or not there is also fever or ileus	Consider a diagnosis of chronic PVT in any patient with newly diagnosed portal hypertension
If acute PVT is suspected, computed tomography (CT) scan, before and after injection of vascular contrast agent, should be obtained for early confirmation of diagnosis. If CT scan is not rapidly available, obtain Doppler-sonography	Obtain Doppler-sonography, then either CT scan or MRI, before and after a vascular contrast agent, to make a diagnosis of chronic PVT
In patients with acute PVT and high fever, septic pylephlebitis should be considered, whether or not an abdominal source of infection has been identified, and blood cultures should be routinely obtained	Base the diagnosis on the absence of a visible normal portal vein and its replacement with serpiginous veins
In acute PVT, the possibility of intestinal infarction should be considered from presentation until resolution of pain. The presence of ascites, thinning of the intestinal wall, lack of mucosal enhancement of the thickened intestinal wall, or the development of multiorgan failure indicate that intestinal infarction is likely and surgical exploration should be considered	

ducts produced by contiguous neo-formed vessels; it is present in at least 80% of PVT patients at endoscopic retrograde cholangiopancreatography<sup>[67]</sup>, often mimicking a cholangiocellular cancer<sup>[72,73]</sup>. Physical examination and biochemical markers might be completely normal, but, sometimes, cholestasis, cholangitis, choledocholithiasis, cholecystitis and, at least, liver injury, might occur, configuring the so-called “portal biliopathy”<sup>[71,74]</sup>.

## DIAGNOSIS

### Imaging

The diagnosis of PVT can be quickly established by demonstrating the presence of solid material within the valsal lumen (Table 4)<sup>[22]</sup>. Nowadays, in developed countries, PVT is usually recognized at an early stage; cavernomatous transformation or the occurrence of gastrointestinal bleeding are rare. The clinical suspicion is often based on the incidental finding of hypersplenism, signs of portal hypertension or, less frequently, symptoms of portal cholangiopathy. Ultrasonography (US) is usually the investigation of choice, with a sensitivity and specificity ranging between 60% and 100%<sup>[17]</sup>; it can reveal the presence of solid, hyperechoic material into a distended portal vein or its tributaries, the presence of collateral vessels or a cavernoma (Figures 1-3)<sup>[18,22,75]</sup>. Doppler imaging can confirm the absence of flow in part or all the valsal lumen, and, if present, a cavernomatous transformation<sup>[22]</sup>. Recently, the endoscopic use of ultrasound (EUS) was demonstrated to be 81% sensitive and 93% specific in PVT diagnosis<sup>[76]</sup>, and to be capable of detecting small and non-occluding thrombi. It appears to be more accurate than US or computed tomography (CT) scans in discovering portal invasion by tumors<sup>[77,78]</sup>. However, the limit of EUS is the presence of a relatively blind area, which cannot be investigated, involving the distal superior mesenteric vein and the intrahepatic portion of the portal vein<sup>[76]</sup>.

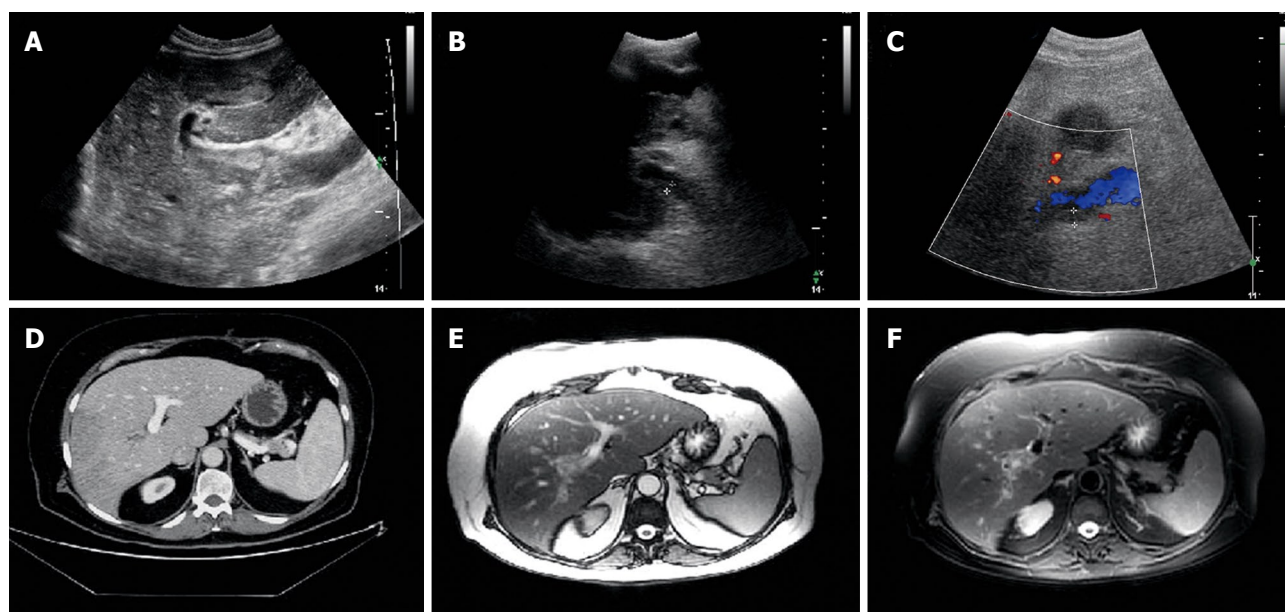
Incidentally, US is less reliable in determining the extension of the thrombus to the mesenteric circulation<sup>[79]</sup>. Instead, CT scanning and magnetic resonance imaging (MRI) can easily obtain this information, and, in addition,

can estimate the impairment of the bowel and other adjacent organs (Figures 1-3). CT scanning is able to demonstrate hyperattenuating material in the portal vein lumen and the absence of enhancement after contrast injection. In addition, in hypoperfused areas, hepatic enhancement appears increased during the arterial phase and decreased during the portal phase. CT is also useful for the identification of the possible cause of the thrombosis or potential complications, such as bowel ischemia and perforation<sup>[22]</sup>. MRI might also confirm the vascular occlusion; at spin-echo MR, the clot appears isointense on T1- weighted images, or hyperintense if recent, and usually has a more intense signal on T2 images. Gradient-echo MR might help to better evaluate any confusing spin-echo MR image<sup>[80]</sup>. Furthermore, contrast-enhanced MR angiography is useful to assess flow direction in the portal venous system and its patency, to identify a cavernomatous transformation, to determine the presence of varices, and to verify the correct function of surgical shunts<sup>[81,82]</sup>. In addition, MR angiography has a high accuracy in the follow-up of the portal venous system before and after liver transplantation<sup>[82-85]</sup>. Moreover, MRI-true fast imaging with steady state precession (true FISP), might overcome the difficulty of contrast injection in cases of poor venous access and the degradation of the images by respiratory motion<sup>[86]</sup>.

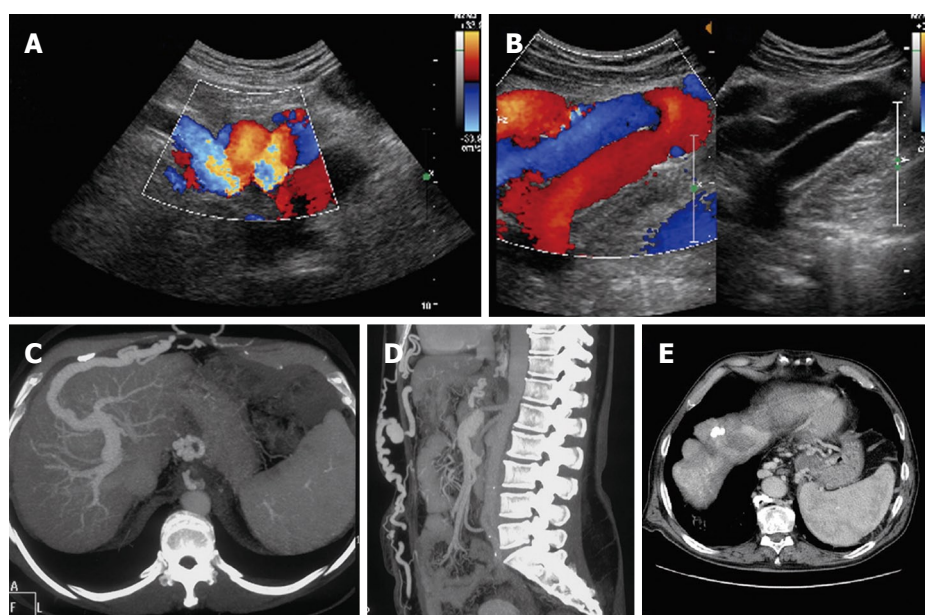
### laboratory investigations

In PVT patients, liver function is typically conserved. Laboratory investigations will be normal or quite normal, unless there is coexistence of a liver disease. However, levels of prothrombin and other coagulation factors could be moderately decreased, while D-dimer is usually increased<sup>[8,22]</sup>.

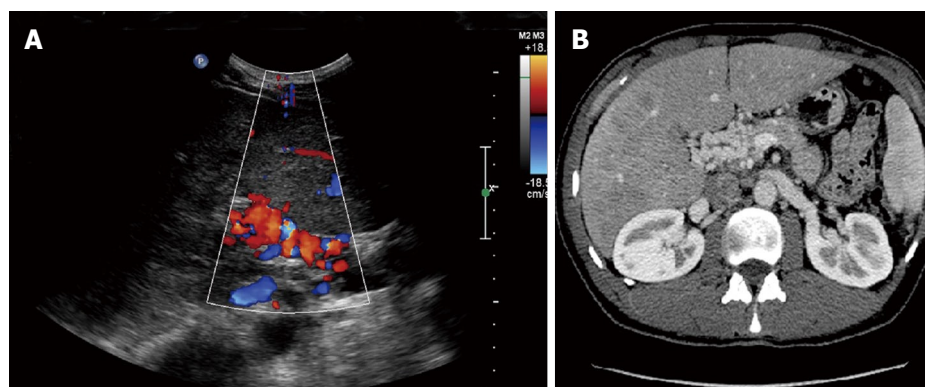
PVT is considered a milestone in the natural history of liver cirrhosis and it is related to serious complications, morbidity, and mortality, as previously discussed<sup>[87]</sup>. Thus, prevention is the first aim of PVT management in patients with an advanced liver disease. Recently, several studies tried to identify the strongest predictive factors for PVT development in these patients. In the past, male sex, previous surgery or interventional treatment for portal



**Figure 1 Portal vein thrombosis.** A: Complete thrombosis of the portal vein trunk (ultrasonography); B, C: Partial thrombosis of the right portal vein (ultrasonography and ultrasonography + doppler); D: Thrombosis of the right portal vein (CT scan); E, F: Thrombosis of the right portal vein (MRI).



**Figure 2 Collateral circulation.** A, B: Recanalization of paraumbilical vein (ultrasonography + doppler); C, D: Recanalization of paraumbilical vein (CT scan); E: Perigastric and paraesophageal varices (CT scan).



**Figure 3 Cavernomatous transformation of the portal vein.** A: Ultrasonography + doppler; B: CT scan.

hypertension, previous variceal bleeding, low platelet count, and advanced liver failure have been associated

with an increased risk of PVT development<sup>[2,31,85,88]</sup>. Interestingly, in a recent prospective study by Zocco *et al*<sup>[89]</sup>,

Table 5 Intraoperative grading of PVT extension<sup>[17]</sup>

Yerdel's grading
< 50% occlusion of the portal vein
> 50% occlusion of the portal vein (including total occlusion)
Complete thrombosis of both portal and proximal superior mesenteric vein
Complete thrombosis of portal vein and proximal and distal superior mesenteric vein

a portal flow velocity below 15 cm/s, at US-Doppler evaluation, was considered significantly predictive of PVT development, confirming the importance of Virchow's triad in the pathogenesis of vascular thrombosis.

### Original cause

As first, local causes such as cirrhosis, primary or metastatic malignancies, pylephlebitis, liver cysts, vascular abnormalities (webs or aneurysms), and pancreatitis have to be excluded. Imaging (US+Doppler, MRI or CT scan) or invasive procedures might be helpful<sup>[22]</sup>; needle biopsy of the obstructed portal vein might be specific, but also of relatively low sensitivity<sup>[90]</sup>. If no local risk factor is found, the presence of a thrombophilic disorder must be investigated. If no possible cause of the thrombosis is recognized, the PVT should be considered "idiopathic". Incidentally, a subclinical prothrombotic state has been reported in about 72% of idiopathic PVT, including an overt or occult MPD<sup>[91-93]</sup>.

### Complications

Once the diagnosis has been reached, the severity of liver and other organs' involvement should be assessed. Clinical and laboratory evaluation, as well as imaging, might be useful; the degree of the obstruction (complete or partial, limited or extensive) should be investigated. A partial thrombosis is often associated with few symptoms. Instead, a rapid and complete obstruction of the portal or mesenteric vein, without the involvement of the mesenteric venous arches, induces only intestinal congestion; the main feature is a diffuse thickening of the intestinal wall, visible even without alterations in contrast enhancement. Generally, there are no signs of other organ failures and liver function is usually preserved, probably because the increased hepatic arterial blood flow supplants portal obstruction. In addition, collateral circulation develops rapidly from pre-existing veins in the porta hepatis within 2 to 3 d after the onset of acute thrombosis, particularly in the gallbladder wall<sup>[61,94,95]</sup>. All these manifestations are completely reversible, even if a spontaneous recanalization or a cavernomatous transformation occurs. In contrast, when thrombosis spreads to mesenteric venous arches, the consequence is intestinal ischemia or infarction. Common radiological findings are the thinning of the intestinal wall and the presence of defects of enhancement after intravenous contrast injection<sup>[8]</sup>.

## PROGNOSIS

In non-cirrhotic and non-neoplastic patients, PVT has generally good outcome; exitus for gastrointestinal bleeding is uncommon<sup>[63,96,97]</sup>. Otherwise, prognosis depends on the underlying liver disease<sup>[1,12,22,79]</sup>. The overall mortality has been reported to be less than 10% in PVT chronic onset<sup>[18,98]</sup>, except for patients with malignancy or cirrhosis - about 26%<sup>[63]</sup>. Moreover, advanced age, malignancy, cirrhosis, mesenteric vein thrombosis, absence of abdominal inflammation, and serum levels of aminotransferase and albumin are associated with reduced survival<sup>[65]</sup>. Systemic risk factors, like MPD or other prothrombotic disorders, seem not to affect short-term survival<sup>[99]</sup>.

In addition, acute PVT, when recognized and treated before the occurrence of intestinal infarction, has good prognosis<sup>[61,100-103]</sup>. By contrast, in cases of bowel ischemia and multiorgan dysfunction or failure, patients in-hospital mortality rate is approximately 20%-50%<sup>[61]</sup>.

## PVT AND OLT

In the past, PVT was considered an absolute contraindication for liver transplantation. Currently, thanks to great innovations in medical care, surgical techniques, and radiological interventions, this belief has been confounded and PVT by itself can represent an indication for liver transplantation<sup>[11,64,104,105]</sup>. The first successful liver transplant in a patient with a thrombosed portal vein was reported by Shaw *et al.*<sup>[106]</sup>, in 1985. Several studies<sup>[107-111]</sup> showed that surgical thrombectomy, thromboendovenectomy with venous reconstruction, interposition of vein graft, porto-caval hemitransposition, and radiological endovascular interventions, can resolve venous obstruction in liver transplant recipients<sup>[112]</sup>. However, surgical options are various, and dependent on a correct intra-operative grading of the thrombosis (Table 5); terminal to terminal portal vein anastomosis with or without thrombectomy is the common choice in low grade PVT, while porto-caval hemitransposition is mandatory in grade 4<sup>[85,113]</sup>. Comparisons of technical difficulties, postoperative complications, survival, and mortality, in recipients with or without PVT are contrasting. Several studies reported a more complex surgical procedure, with a greater requirement of blood transfusions, an increased risk of complications (such as primary non function or dysfunction, thrombosis of the hepatic artery, relaparotomy, postoperative pancreatitis, sepsis, and renal failure), a poorer survival, and a higher mortality<sup>[113-115]</sup>. However, these data have not been confirmed and features of liver transplantation, comparing recipients with PVT and those without, are similar<sup>[74,113-116]</sup>. Interestingly, PVT patients' rates of survival at one and 5 years after OLT are equal, as if, once the peri-transplant period has been overcome, the future clinical destiny of recipients with or without a previous PVT could be overlapped. However, among patients with PVT, survival seems better in low grades of Yerdel classification; however, further studies are needed to confirm



this data. Transplantation at grade 1 PVT seems to carry results comparable to non-PVT patients<sup>[74,85,117]</sup>.

The rate of thrombosis recurrence has been estimated within 9% to 42% although some authors reported a lower incidence<sup>[74,107,108,116,118-120]</sup>. Male sex, previous treatment for PVT, Child-Pugh class C, and alcoholic liver disease might be associated with recurrence<sup>[85,114]</sup>. Furthermore, patients with an obstruction of more than half of the portal vein, extended or not to the superior mesenteric vein, seem to have increased risk of severe peri-operative complications, higher mortality, and decreased long-term survival<sup>[85,107,115]</sup>. In cirrhotics with PVT, surgical procedure may be more difficult, often complicated by rethrombosis and reintervention, but with the same morbidity and mortality of non-cirrhotic patients<sup>[113,115,121,122]</sup>.

After liver transplantation, PVT development is a rare but possible event, especially in the early postoperative period<sup>[60]</sup>. The incidence ranges between 1% and 2%<sup>[122-124]</sup>, with a preferential localization at the anastomotic site; technical complications, small diameter of the portal vein, pediatric recipient, presence of PVT pre-OLT, surgical shunting pre-OLT, or splenectomy are the principal predisposing risk factors<sup>[124]</sup>. The occlusion of the portal vein is always more scarring than the thrombosis of the hepatic artery and may be seriously threatening for both graft and patient survival<sup>[122]</sup>. Acute liver failure, bleeding from esophageal varices, and massive ascites could rapidly occur and immediate retransplantation must be quickly attempted in case of severe worsening of liver function<sup>[124]</sup>.

Thus, for all these reasons and the good results reported in literature, today PVT has no longer to be considered a contraindication but only a disadvantage and, in some cases, might present a possible indication to liver transplantation<sup>[114,116,124,125]</sup>.

## TREATMENT

Although spontaneous resolution of PVT has been reported in the literature<sup>[101,102]</sup>, a specific therapeutic management is mandatory to resolve portal vein obstruction and avoid serious complications. The goal of treatment is similar in acute and chronic PVT, and consists in correction of causal factors, prevention of thrombosis extension, and achievement of portal vein patency. However, in case of long standing thrombosis, the management of complications related to portal hypertension and portal cholangiopathy has to be concurrently considered<sup>[126]</sup>. Nowadays, anticoagulant therapy is the best way to obtain portal vein recanalization; however, there is no consensus on its application. Other modalities of treatment should be adopted only in case of partial or absent PVT resolution<sup>[12,126]</sup>. Furthermore, some conditions should be considered in the assessment of anticoagulant therapy, such as recent *vs* old thrombosis, the presence of a thrombophilic condition, or a liver disease.

### Anticoagulation in acute PVT

Although PVT might be compared to other cases of

deep vein thrombosis, there is no randomized controlled trial regarding the use of anticoagulants in acute PVT<sup>[126]</sup>. After 6 mo of therapy, a complete recanalization has been reported in about 50% of patients, with good results in the case of mesenteric vein involvement, and a low incidence of complications. In contrast, in about 10% of cases, PVT is resistant to anticoagulants<sup>[96,97,100-103]</sup>. In addition, when intestinal infarction occurs, anticoagulants administered prior to laparotomy seem to have a consistent benefit on survival<sup>[127-129]</sup>.

What is certain is that, in acute PVT onset, the sooner the treatment is given the better the outcome will be; the rate of recanalization is about 69%, if anticoagulation is instituted within the first week after diagnosis, while it falls to 25% when instituted at the second week<sup>[9,59,130]</sup>.

### Anticoagulation in chronic PVT

Opinions regarding therapeutic options in chronic PVT are more controversial and significantly variable. At present, anticoagulant treatment is administered to only 30% of patients with chronic PVT, reflecting concerns about the use of anticoagulation in the presence of gastroesophageal varices, low platelet counts, and coagulation dysfunctions<sup>[79]</sup>. However, the number of bleeding episodes in PVT patients receiving anticoagulant therapy did not increase, and in long-term follow-up studies, anticoagulants seem to be effective in preventing new thrombotic events with a low mortality<sup>[66,126]</sup>. Incidentally, a pragmatic approach, such as endoscopic eradication of varices prior to commencement of anticoagulation, should be reasonable<sup>[79]</sup>.

### Dose and duration of anticoagulants

If thrombosis is recent and there is no underlying thrombophilic condition, anticoagulation should be administered for 3-6 mo, as a complete portal vein recanalization can occasionally be delayed<sup>[79,100,101,131-133]</sup>. Recently, a panel of experts recommended the application of anticoagulant therapy only in PVT patients with a proven thrombophilic disorder or familial history of venous thrombosis<sup>[133,134]</sup>, thereby obtaining an improvement in survival and reduction in risk of gastrointestinal bleeding<sup>[135,136]</sup>.

### Anticoagulation in cirrhotic patients

The ubiquitous and long-term use of anticoagulants in cirrhotic patients with PVT should not be considered correct practice, until their safety and efficacy has been completely tested<sup>[62]</sup>. However, signs of intestinal ischemia or infarction, or an underlying prothrombotic disorder should be considered an indication for anticoagulants in cirrhotic patients, although only after an adequate prophylaxis for variceal bleeding<sup>[2,126]</sup>. In candidates for liver transplantation with a high risk PVT (obstruction of more than 50% of the portal vein), anticoagulation should be recommended, even if a scheduled prophylactic treatment has not yet been assessed<sup>[64,74,85,108,117]</sup>.



### Other treatments

Thrombolytic therapy, given either into the systemic venous circulation, the superior mesenteric artery, or the portal vein *via* a transjugular or transhepatic route, is also effective to provide recanalization in acute PVT<sup>[137-142]</sup>. However, efficacy is significantly lower and mortality increased in patients who undergo thrombolysis, if compared to conservative treatment<sup>[59,143,144]</sup>. Despite the high incidence of side effects, thrombolysis should be considered when initial anticoagulant therapy fails, even if there is no consistent evidence concerning in which conditions it should be preferred to anticoagulation<sup>[137]</sup>. Surgical thrombectomy is usually not recommended, as high morbidity and mortality have been reported; percutaneous transhepatic mechanical thrombectomy might also be effective in recent thrombosis, but vascular traumas are frequent and may stimulate rethrombosis<sup>[145]</sup>.

Other approaches, such as transjugular intrahepatic portosystemic shunt placement, should be reserved for patients developing acute PVT before or after liver transplantation, or in alternative to thrombolysis when anticoagulation fails<sup>[146,147]</sup>. It seems to be effective in resolving portal biliopathy, ascites, and portal hypertension, but it is not feasible if portal vein is not catheterizable or a cavernomatous vein cannot be dilated<sup>[148-150]</sup>.

Finally, shunt surgery (distal splenorenal shunt or Rex shunt, in children) might be applied as the last choice, and only in absence of splenic or superior mesenteric vein thrombosis<sup>[151]</sup>.

### CONCLUSION

PVT is relatively uncommon in the general population, but is more frequent among cirrhotic patients and represents a “milestone” in the natural evolution of liver disease. Local or systemic pro-thrombotic factors, alone or together, can play an important role in PVT pathogenesis, which is complex and different in each clinical context and in each patient. The consequent changes in hepatic and splanchnic hemodynamic are responsible for a mild impairment in liver function, in absence of an overt liver disease, or can precipitate a preexistent metastable clinical status in cirrhotic patients. Moreover, PVT might have indirect effects on other abdominal organs, causing intestinal ischemia and infarction, or predisposition to vascular neoformation and gastrointestinal bleeding. The identification of protean manifestations of PVT is essential to provide a prompt diagnosis, as the removal of the original trigger factor and an early therapeutic management is crucial to preserve patient health and, sometimes, life. The history of PVT has been characterized by difficulties in diagnosis and treatment, which, today, have almost been overcome. In the future, due to innovations in imaging and pharmaceuticals, clinical attention must be focused on the realization of a scheduled, preemptive, therapeutic approach to the patient, to better define the profile of toxicity and reduce side effects, especially in cirrhotic patients.

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## Treatment of biliary tract cancer with NVP-AEW541: Mechanisms of action and resistance

Samuel Wolf, Jana Lorenz, Joachim Mössner, Marcus Wiedmann

Samuel Wolf, Jana Lorenz, Joachim Mössner, Marcus Wiedmann, Department of Internal Medicine II, University of Leipzig, Liebigstr. 20, 04103 Leipzig, Germany

Marcus Wiedmann, Department of Internal Medicine I, St. Mary's Hospital, Gallwitzallee 123-143, 12249 Berlin, Germany

**Author contributions:** Wiedmann M designed the research and wrote the paper; Wolf S and Lorenz J performed the research; Mössner J analyzed the data and corrected the manuscript.

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Correspondence to: Dr. Marcus Wiedmann, PhD, Department of Internal Medicine I, St. Mary's Hospital, Gallwitzallee 123-143, 12249 Berlin,

Germany. [wiedmann@marienkrankenhaus-berlin.de](mailto:wiedmann@marienkrankenhaus-berlin.de)

Telephone: +49-30-76783251 Fax: +49-30-76783425

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### Abstract

**AIM:** To investigate *in vitro* treatment with NVP-AEW541, a small molecule inhibitor of insulin-like growth factor-1 receptor (IGF-1R), in biliary tract cancer (BTC), since this disease is associated with a poor prognosis due to wide resistance to chemotherapeutic agents and radiotherapy.

**METHODS:** Cell growth inhibition by NVP-AEW541 was studied *in vitro* in 7 human BTC cell lines by automated cell counting. In addition, the anti-tumoral mechanism of NVP-AEW541 was studied by Western blotting, cell cycle analysis and reverse transcription-polymerase chain reaction (RT-PCR). Anti-tumoral drug effect in combination with gemcitabine, 5-fluorouracil (5-FU) and Polo-like kinase 1 inhibitor BI2536 was also studied.

**RESULTS:** *In vitro* treatment with NVP-AEW541 suppressed growth in all human BTC cell lines, however response was lower in gallbladder cancer. Treatment with

NVP-AEW541 was associated with dephosphorylation of IGF-1R and AKT. In contrast, phosphorylation of p42/p44 and Stat3 and expression of Bcl-xL were inconsistently downregulated. In addition, treated cells showed cell cycle arrest at the G1/S-checkpoint and an increase in sub-G1 peak. Moreover, IGF-1R and its ligands IGF-1 and IGF-2 were co-expressed in RT-PCR, suggesting an autocrine loop of tumor cell activation. Combined with gemcitabine, NVP-AEW541 exerted synergistic effects, particularly at low concentrations, while effects of combination with 5-FU or BI 2536 were only additive.

**CONCLUSION:** Our findings suggest that NVP-AEW541 is active against BTC *in vitro* and potentiates the efficacy of gemcitabine.

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**Key words:** Tyrosine kinase inhibitor; Cholangiocarcinoma; Gemcitabine; NVP-AEW541

**Peer reviewer:** Shiu-Ming Kuo, MD, University at Buffalo, 15 Farber Hall, 3435 Main Street, Buffalo, NY 14214, United States

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### INTRODUCTION

Insulin-like growth factor-1 receptor (IGF-1R) is a tyrosine kinase receptor with a 70% homology to the insulin receptor<sup>[1]</sup>. When activated by its ligands IGF-1, IGF-2 or insulin at supraphysiological concentrations, the IGF-1R transmits a signal to its two major substrates, insulin receptor substrate-1 (IRS-1) and Shc. The signal is subsequently transduced *via* the common signal transduction

pathway, through ras, raf and p42/44 downstream of Shc and AKT downstream of IRS-1, all the way to the nucleus<sup>[2,3]</sup>.

The IGF-1R system has emerged as an interesting target for cancer therapy, as it represents an important promoter of tumor transformation and survival of malignant cells, but is only partially involved in normal cell growth<sup>[4-6]</sup>. This is in part attributed to interactions with oncogenes. Moreover, activation of IGF-1R may contribute to tumor angiogenesis by up-regulation of vascular endothelial growth factor (VEGF) expression in certain cancer entities<sup>[7-9]</sup>. In the past, different strategies were used to inhibit IGF-1R function, among them monoclonal antibodies and anti-sense RNA directed against the receptor or recombinant IGF binding proteins, and IGF-specific antibodies reducing levels of ligands<sup>[5]</sup>. Thus, targeting the IGF-1R system with small molecule tyrosine kinase inhibitors, such as NVP-AEW541, a novel compound which is 27-fold more selective for IGF-1R than the insulin receptor at the cellular level, may be a new strategy of cancer growth inhibition<sup>[10,11]</sup>. Anti-neoplastic efficacy of NVP-AEW541 has recently been shown in experimental models of acute myeloid leukemia<sup>[12]</sup>, multiple myeloma<sup>[13]</sup>, multiple myoblastoma<sup>[14]</sup>, neuroblastoma<sup>[15,16]</sup>, medulloblastoma<sup>[17,18]</sup>, malignant rhabdoid tumors<sup>[19]</sup>, Ewing's sarcoma<sup>[20,21]</sup>, ovarian<sup>[22]</sup> and breast cancer<sup>[23-25]</sup>, mesothelioma<sup>[26]</sup>, synovial sarcoma<sup>[27,28]</sup>, head and neck squamous cell carcinoma<sup>[29]</sup>, adrenocortical tumors<sup>[30]</sup>, hepatocellular carcinoma<sup>[31,32]</sup>, neuroendocrine gastrointestinal tumors<sup>[33]</sup>, gastrointestinal stromal tumors<sup>[34]</sup>, colorectal<sup>[32,35-38]</sup>, esophageal<sup>[32]</sup>, gastric<sup>[37]</sup>, and pancreatic cancer<sup>[32,37,39]</sup>. However, little is known about the situation for biliary tract cancer (BTC), a rare tumor with a grim prognosis and limited treatment options. Two recent studies showed expression of IGF-1R and its ligands in gallbladder carcinoma (GBC)<sup>[40]</sup> and cholangiocarcinoma (CC) specimens<sup>[41]</sup>. Therefore, the objectives of the current study were to investigate IGF-1R expression in BTC cell lines and to evaluate the efficacy of *in vitro* treatment with selective IGF-1R inhibitor NVP-AEW541 alone or in combination with gemcitabine, 5-fluorouracil (5-FU) or Polo-like kinase 1 inhibitor BI 2536, which is currently being investigated in phase II studies including our hospital for the treatment of solid tumors<sup>[42]</sup>.

## MATERIALS AND METHODS

### Drugs and cells

Seven BTC cell lines; five extrahepatic CC cell lines (EGI-1, TFK-1, CC-SW-1, CC-LP-1, and SK-ChA-1)<sup>[43-47]</sup> and two GBC cell lines (Mz-ChA-1, Mz-ChA-2)<sup>[46]</sup>, were examined. All cell lines were cultured in a 37°C incubator with 5%-10% CO<sub>2</sub> in appropriate media, which were changed every 3 d. NVP-AEW541 (targeting IGF-1R) was obtained from Novartis (Basel, Switzerland), dissolved in dimethyl sulfoxide (DMSO) (as 10 mmol/L stock) and stored at -20°C according to manufacturer's

instructions. BI 2536 (targeting Plk-1) was kindly provided by Boehringer (Ingelheim, Germany). Gemcitabine and 5-FU (diluted in 0.9% NaCl) were provided by our hospital pharmacy.

### Inhibition of cell growth

Cytotoxic effects of drugs alone and in combination were determined by automated cell counting (Casy Cell Counter Model TT; Innovatis AG, Reutlingen, Germany) according to manufacturer's instructions. Briefly,  $2 \times 10^5$  cells were seeded in duplicates in T25 flasks with media containing the designated drugs or vehicle control followed by incubation for 3 or 6 d. For the 6 d experiment, medium was changed after 3 d and treatment repeated. At the end of incubation, cells were trypsinized, washed, and analyzed in triplicates by automated cell counting.

### Immunoblotting

Cell culture monolayers were washed with ice-cold PBS and lysed in flask with a buffer containing Tris-HCl (50 mmol/L, pH 7.4), NP-40 (10 g/L), NaCl (200 mmol/L), sodium-orthovanadate (200 mmol/L), 2-glycerophosphate (1 mmol/L), sodium fluoride (20 mmol/L), DTT (10 mmol/L), PMSF (200 mmol/L) and 0.2% proteinase inhibitor cocktail (Sigma-Aldrich, Munich, Germany) on ice for 30 min. The lysate was then centrifuged at 13000 r/min for 15 min and proteins in supernatant were quantified by Bradford protein assay (Bio-Rad, Munich, Germany) and stored at -80°C. Next, 50 or 60 µg of cell lysates were separated on SDS-polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Freiburg, Germany). Membranes were then incubated in blocking solution [25 g/L dry milk or BSA in TBS-T (10 mmol/L Tris-HCl, 140 mmol/L NaCl, 1 g/L Tween-20)], followed by incubation with the primary antibody at 4°C overnight (50 g/L BSA in TBS-T). The membranes were then washed in TBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Antibody detection was performed with an enhanced chemoluminescence reaction (SuperSignal West Dura or SuperSignal West Femto, Pierce, Rockford, USA). Monoclonal (mc) β-actin antibody was purchased from Sigma (Sigma-Aldrich Chemie GmbH Munich, Germany), polyclonal IGF-1Rβ antibody from Santa Cruz (Santa Cruz Biotechnology Inc., Santa Cruz, USA), mc p-IGF-1R, mc p-p42/44 (p-Erk1/2, p-MAPK), mc p42/44, mc p-AKT, mc AKT, mc p-Stat3, mc Stat3 and mc Bcl-xL antibodies were all from Cell Signaling (Cell Signaling Technology, Beverly, USA). Recombinant human IGF-1 was purchased from Biomol (Biomol, Hamburg, Germany).

### Cell cycle analysis

Cells were seeded in T-25 flasks ( $3.5 \times 10^5$ ), treated with various concentrations of NVP-AEW541 or vehicle control for 36 h, washed with PBS, trypsinized,

centrifuged, and fixed in ice-cold ethanol with phosphate-buffered saline containing 1 mmol/L EDTA. DNA was labelled with 1:100 diluted propidium iodide after digestion of RNA by RNase A. Cells were analysed by flow cytometry with a FACSCalibur system (Becton Dickinson, San Diego, USA) and cell cycle profiles were determined using ModFitLT 2.0 for Macintosh (Verity Software House, Topsham, ME, USA). Doublets were excluded by gating for width of fluorescence signal (FL2-W). Each experiment was performed at least in triplicate.

### Reverse transcription-polymerase chain reaction (RT-PCR) for ligands IGF-1 and IGF-2

Total cell RNA was extracted using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) after homogenization with the QIAshredder (Qiagen) according to manufacturer's instructions. RNA was dissolved in water and quantified at 260 nm with a biophotometer (Eppendorf, Hamburg, Germany); purity was verified by optical density ( $A_{260\text{ nm}}/A_{280\text{ nm}}$  between 1.93 and 2.06. Single step quantitative RT-PCR analysis was carried out in the LightCycler system (Roche), primers and fluorochromes were obtained from Qiagen (QuantiTect Primer Assays Hs\_IGF1\_1\_SG and Hs\_IGF2\_1\_SG and SYBR-Green RT-PCR kit) and used according to manufacturer's instructions. Products of RT-PCR were separated by gel electrophoresis to confirm correct amplification and size. RNA samples extracted from hepatocellular carcinoma tissue and from HepG2 cell line served as positive controls. Water was used to detect primer interactions and GAPDH as housekeeping gene to assure equal loading. Relative gene expression was calculated with REST software tool as used by Pfaffl and Horgan<sup>[48]</sup>.

### Caspase-3 assay

Using the caspase-3 colorimetric assay kit (Sigma, Missouri, USA) according to manufacturer's instructions, caspase-3 activity was measured. Cells were grown, treated, lysed and centrifuged, and supernatants were used. Based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide by caspase-3, resulting pNA was measured photometrically at 405 nm after incubation at 37°C and 5% CO<sub>2</sub>. A pNA calibration curve was used to calculate results.

### Statistical analysis

Statistical calculations were performed using SPSS, version 10.0 (SPSS Inc., Chicago, USA). Numeric data were presented as mean value with SD. Inter-group comparisons were performed with the Student *t* test. *P* values less than 0.05 were considered significant.

## RESULTS

### Inhibition of cell growth

After 3 d of incubation, tested cell lines were sensitive to NVP-AEW541 (mean IC<sub>50</sub> = 0.51 ± 0.44 μmol/L) with

**Table 1** Inhibition of cell growth by *in vitro* treatment with NVP-AEW541

Cell line	IC <sub>50</sub> (μmol/L)	
	3 d	6 d
TFK-1	0.26	0.28
EGI-1	0.28	0.14
CC-LP-1	0.15	0.12
CC-SW-1	0.54	0.20
Sk-ChA-1	0.20	0.18
Mz-ChA-1	1.39	0.52
Mz-ChA-2	0.73	0.07

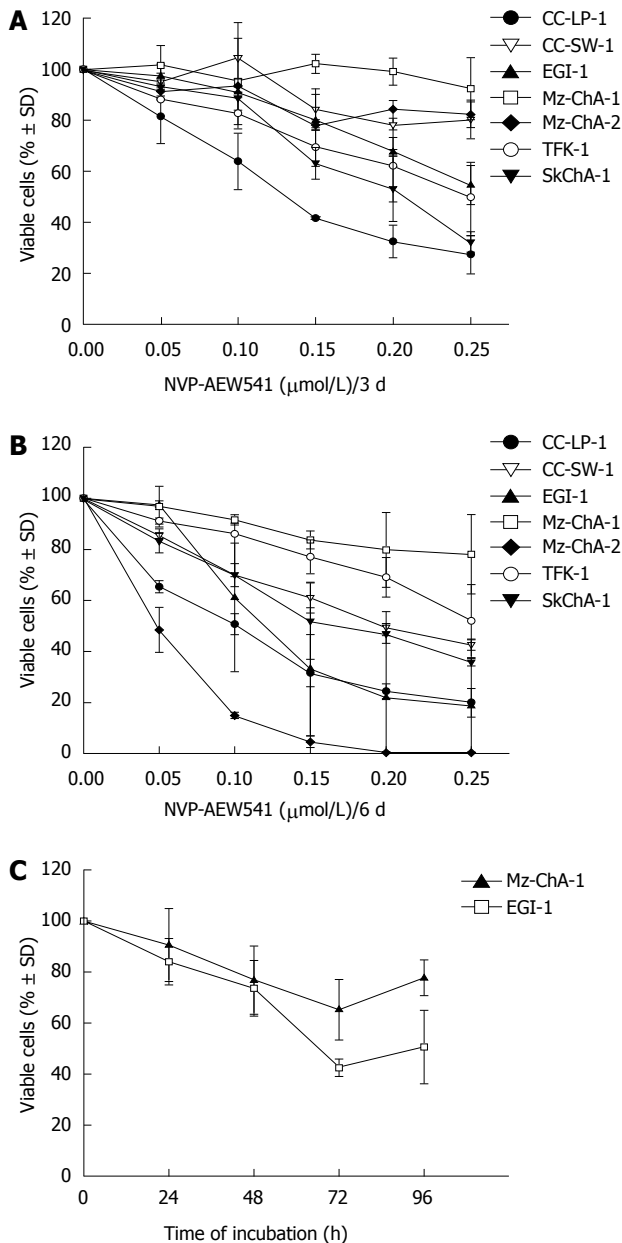
CC-LP-1 being the most sensitive and Mz-ChA-1 the least sensitive cell line (IC<sub>50</sub> values were calculated with linear regression model). Response differed markedly between the group of extrahepatic CC cell lines (mean IC<sub>50</sub> = 0.29 ± 0.15 μmol/L) and the group of the two GBC cell lines (mean IC<sub>50</sub> = 1.06 ± 0.47 μmol/L) (*P* < 0.05). Inhibition of cell growth was more pronounced if incubation time was extended to 6 d (treated twice, on days 0 and 4) with a mean IC<sub>50</sub> value of 0.22 ± 0.15 μmol/L. Although not statistically significant, there was again a difference in response for the 6 d experiment between the group of extrahepatic CC cell lines (mean IC<sub>50</sub> = 0.18 ± 0.06 μmol/L) and the group of the two GBC cell lines (mean IC<sub>50</sub> = 0.3 ± 0.32 μmol/L) (Figure 1A and B, Table 1).

Cell lines Mz-ChA-1, showing a weak, and EGI-1, showing an intermediate response to NVP-AEW541, were selected for further studies of drug mechanism, because each of these lines represented one entity of BTC (GBC and extrahepatic CC). Firstly, the *in vitro* cell growth inhibition experiment was repeated with a broader spectrum of drug doses to determine the correct 3 d IC<sub>50</sub> concentration. To verify calculated 3 d IC<sub>50</sub> concentrations and to determine the most effective length of incubation, additional experiments with drug incubation times ranging from 1 to 4 d, with 3 d IC<sub>50</sub> concentration, were carried out (Figure 1C), which showed best response after 3 d of incubation. Using data from this experiment, replication times for Mz-ChA-1 (68.16 h) and EGI-1 (30.7 h) were calculated. This difference could be one factor influencing response to NVP-AEW541. DMSO, the solvent of NVP-AEW541, had no influence on cell growth when administered alone (data not shown).

### Mechanism of drug action

IGF1-R protein was detectable in all seven cell lines by immunoblot, with expression level variation between the different cell lines (Figure 2A). However, there was no correlation between level of IGF1-R protein expression and inhibition of cell growth by NVP-AEW541. For further analysis of drug mechanism, EGI-1 and Mz-ChA-1 cells were used, representing cells with good and little response to the inhibitor, but intermediate IGF1-R protein expression, respectively. Stimulation of cell





**Figure 1** *In vitro* cell growth inhibition. A: Treatment of seven human biliary tract cancer cell lines with NVP-AEW541 for 3 d ( $n = 3$ ); B: 6 d incubation ( $n = 3$ ); C: Incubation of selected cell lines EGI-1 and Mz-ChA-1 with calculated  $IC_{50}$  for 24–96 h ( $n = 3$ ). Error bars represent SD.

lines EGI-1 and Mz-ChA-1 with recombinant human IGF-1 30 min prior to lysis resulted in phosphorylation of IGF-1R, which was not present in cells that were serum-starved for 24 h (Figure 2B, lanes 1 and 2). Twelve hours of pre-incubation with NVP-AEW541 prior to stimulation and lysis inhibited phosphorylation of IGF1-R (Figure 2B, lanes 3–5), while levels of whole IGF1-R protein remained unchanged during treatment. Assessment of phosphorylated intracellular signal transduction proteins AKT, p42/44, and Stat3, all located downstream of the IGF-1R pathway, showed variable results. Level of p-AKT was already increased in unstimulated Mz-ChA-1 cells, but not in EGI-1 cells. Treatment with NVP-AEW541 resulted in dephosphorylation in both cell lines.

Level of p-p42/44 was already increased in unstimulated EGI-1 cells, but not in Mz-ChA-1 cells. Treatment with NVP-AEW541 resulted in dephosphorylation in Mz-ChA-1 cells, but not in EGI-1 cells. This could be explained by a k-ras mutation (G12D) in the EGI-1 cell line, as already described in the COSMIC catalogue<sup>[49]</sup>, and would result in a consecutively active state of downstream target p42/44, as found by our group. Level of p-Stat-3 was already increased in both unstimulated cell lines. While dephosphorylation of Stat-3 occurred in EGI-1, phosphorylation of Stat-3 remained unchanged during treatment with NVP-AEW541 in Mz-ChA-1 cells. Furthermore, repetition with higher doses of NVP-AEW541 (up to 5  $\mu\text{mol/L}$ ) did not show any effect (blot not shown).

Anti-apoptotic protein Bcl-xL was significantly reduced after treatment with NVP-AEW541 in Mz-ChA-1 cells, but we could not determine any significant change of expression in EGI-1 cells after treatment. Treatment with higher doses of NVP-AEW541 did not reveal greater effects (blots not shown). Staining with  $\beta$ -actin antibody confirmed equal protein loading in all immunoblots.

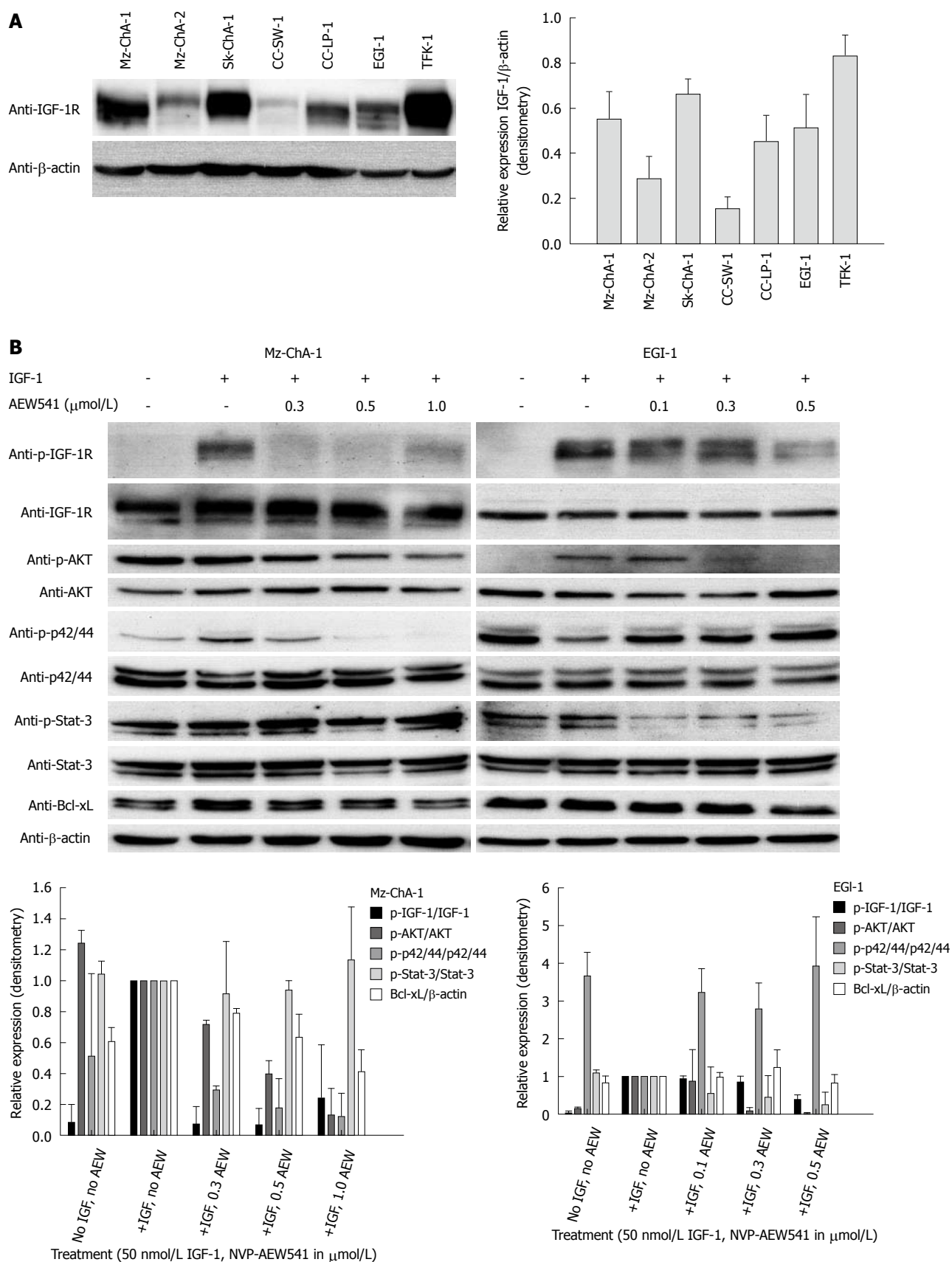
Cell cycle analysis of cell lines EGI-1 and Mz-ChA-1, treated with NVP-AEW541 for 36 h, showed an increase of G0/G1 fraction, possibly caused by G1/S-checkpoint arrest. This arrest was more pronounced when the dose was increased (Figure 3A and B). Analysis of flow cytometry sub-G1 fraction showed no significant apoptosis for NVP-AEW541 concentrations below 1  $\mu\text{mol/L}$ , but marked apoptosis if NVP-AEW541 concentration was increased above 1  $\mu\text{mol/L}$  (Figure 4C). Treatment with 5  $\mu\text{mol/L}$  resulted in marked apoptosis, influencing cell cycle proportions. There was no significant change in caspase-3 activity induced by treatment with NVP-AEW541 (data not shown).

#### RT-PCR analysis of IGF-1R ligands IGF-1 and IGF-2

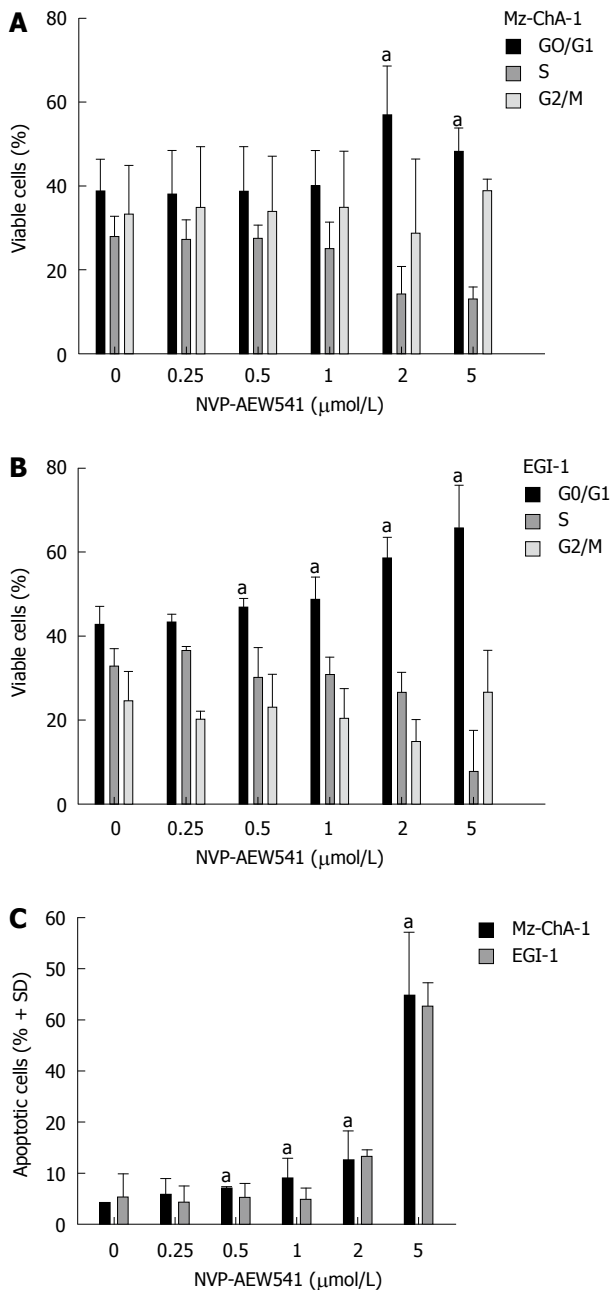
Semiquantitative RT-PCR revealed expression of ligands IGF-1 and IGF-2 in selected MzChA-1 and EGI-1 cells, suggesting an autocrine loop of IGF-1R activation. Samples of human hepatocellular carcinoma tissue served as a positive control. There was no significant change in expression levels of either IGF-1R ligand induced by treatment with  $IC_{50}$  doses of NVP-AEW541 for 3 d (Figure 4).

#### *In vitro* combination of NVP-AEW541 with gemcitabine, 5-FU or BI 2536

To evaluate efficacy of NVP-AEW541 in combination with commonly used chemotherapeutics for the treatment of BTC, further *in vitro* experiments were performed. NVP-AEW541 at  $IC_{20}$  concentration (0.8  $\mu\text{mol/L}$  for Mz-ChA-1 and 0.20  $\mu\text{mol/L}$  for EGI-1) was combined with increasing concentrations of gemcitabine or 5-FU over 3 d (Figure 5). Assuming that the drugs do not directly interact and using the model of effect multiplication postulated by Berenbaum<sup>[50]</sup>,

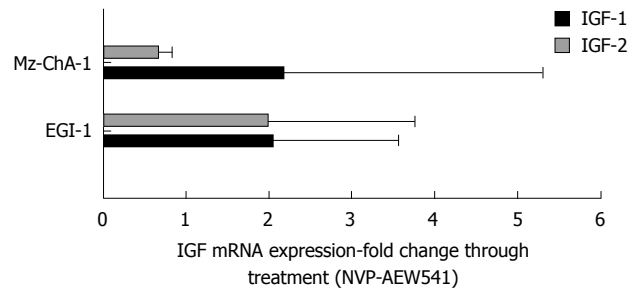


**Figure 2 Mechanism of NVP-AEW541 drug action.** A: Protein expression of IGF-1R in tested human biliary tract cancer cell lines was determined by immunoblot; B: p-IGF-1R, IGF-1R, p-AKT, AKT, p-p42/44, p42/44, p-Stat-3, Stat-3, and Bcl-xL protein levels were examined by immunoblot for selected cell lines EGI-1 and Mz-ChA-1. After 24 h of starvation, cell lines EGI-1 and Mz-ChA-1 were treated with NVP-AEW541 (12 h prior to lysis) and stimulated with ligand IGF-1 (30 min prior to lysis). β-actin served as loading control in all experiments. Densitometry was performed to analyze results which are shown relative to lane 2 as standard.



**Figure 3 Cell cycle analysis.** A: *In vitro* treatment of selected cell line EGI-1 with NVP-AEW541 for 36 h ( $n = 3$ ); B: *In vitro* treatment of selected cell line Mz-ChA-1 ( $n = 3$ ). Cells were stained with propidium iodide and analyzed by flow cytometry. ModFitLT 2.0 software was used to determine cell cycle proportions according to cellular DNA content; doublets were excluded by gating for width of fluorescence signal (FL2-W) ( $^aP < 0.05$  for G0/G1 fraction, treated cells *vs* control); C: Cells having less than single DNA content (sub-G1 fraction) were presumed to be apoptotic ( $n = 3$ ) ( $^aP < 0.05$ , treated cells *vs* control; error bars represent SD).

calculated values representing synergistic effects were compared to measured values. Even though  $IC_{50}$  doses of NVP-AEW541 for both cell lines are different, gemcitabine showed synergistic effects at low concentrations for both tested cell lines (Figure 5A and B), whereas combinations of NVP-AEW541 with 5-FU showed only additive effects (Figure 5C and D). In addition, new small-molecule Plk-1 inhibitor BI 2536 was tested



**Figure 4 Influence of NVP-AEW541 (3 d treatment with calculated  $IC_{50}$ ) on mRNA expression of IGF-1R ligands IGF-1 and IGF-2 in two of the tested cell lines.** Ratio of IGF mRNA expression in treated *vs* untreated cell lines is shown ( $n = 3$ ).

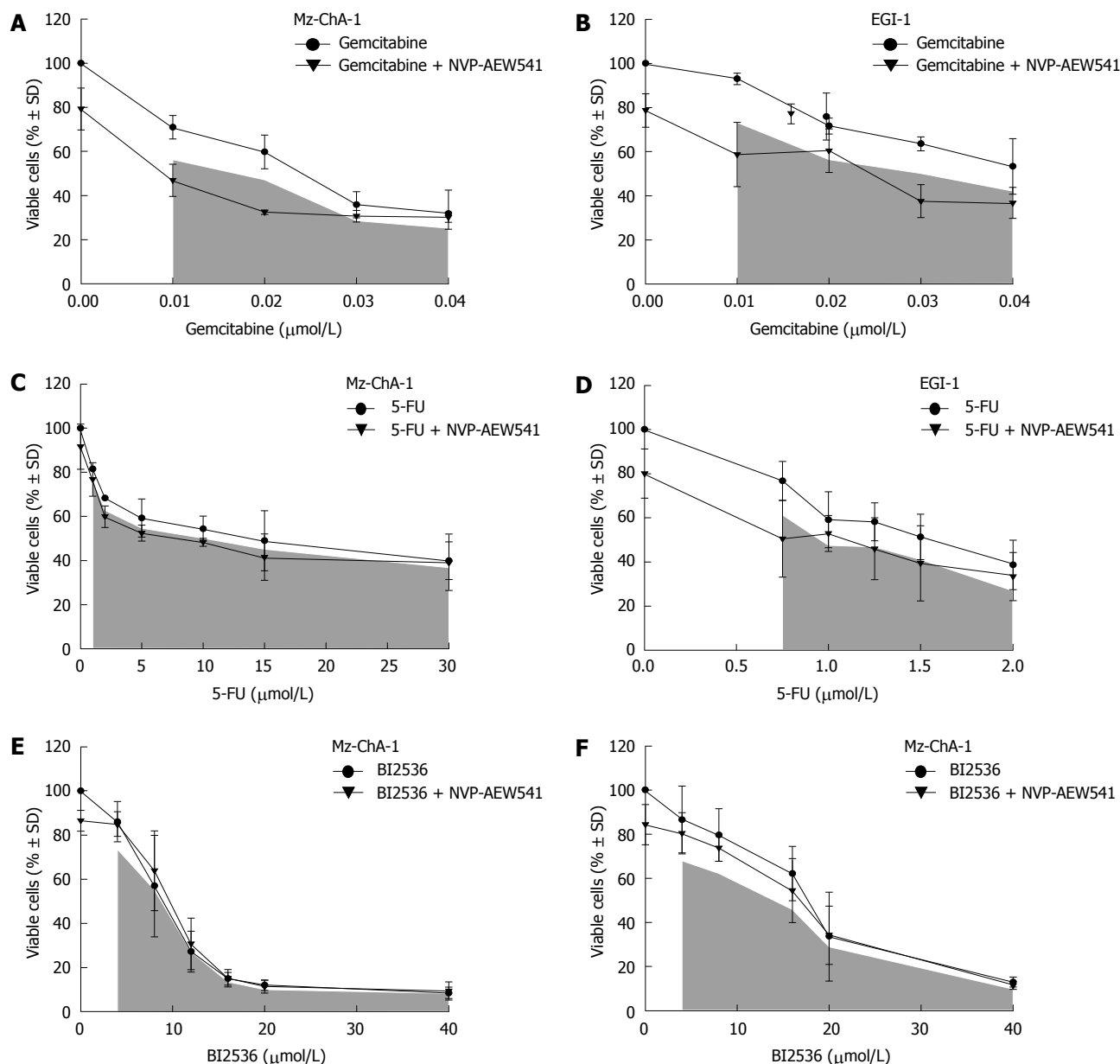
in combination with NVP-AEW541. This compound was available to us due to our participation in a phase II study for the treatment of solid tumors. Similar to the combination with 5-FU, the combination with BI 2536 resulted only in additive effects (Figure 5E and F).

## DISCUSSION

Non-resectable BTC is associated with a poor prognosis due to wide resistance to chemotherapeutic agents and radiotherapy. It is therefore essential to search for new therapeutical approaches<sup>[51]</sup>. In a recent study, analyzing tumor specimens from 57 patients with GBC, a strong immunoreactivity for IGF-1R was shown in 95%. IGF-1 and IGF-2 expression was detected in 45% and 25% of specimens, respectively<sup>[40]</sup>. Another study found IGF-1 and IGF-1R expression in all 18 examined biopsy samples of intrahepatic CC, whereas cholangiocytes of intrahepatic bile ducts of normal human liver were all negative<sup>[41]</sup>. Results of our own *in vitro* experiments support the co-expression of IGF receptor and its ligands IGF-1 and IGF-2. This co-expression, which is not a physiological state, leads to the assumption of an autocrine loop, rendering the IGF system a possible target for specific anti-cancer therapy of BTC.

Different approaches to inhibit IGF-1/-2 effects, at receptor or downstream levels, have already shown promising results in other studies. While IGF-1 neutralizing antibodies or IGF-binding proteins reduce ligand levels, strategies directed against IGF-1R employ receptor-specific antibodies which are mainly effective through degradation and downregulation of the receptor after binding, i.e. siRNAs, and small molecule tyrosine kinase inhibitors<sup>[5]</sup>. Several IGF-1R specific fully humanized monoclonal antibodies are currently already in phase II clinical trials (MK-0646 by Merck Oncology for solid tumors; AMG 479 by Amgen Oncology for Ewing's sarcoma, breast and pancreatic cancer; CP-751,871 by Pfizer Oncology for prostate cancer and NSCLC).

Our results for the small molecule tyrosine kinase inhibitor NVP-AEW541 showed a potent growth inhibition of BTC cell lines, supported by the low 3 d  $IC_{50}$



**Figure 5** *In vitro* treatment with drug combinations of NVP-AEW541 and gemcitabine, 5-fluorouracil (5-FU) or BI 2536. Selected cell lines EGI-1 and Mz-ChA-1 were incubated with increasing concentrations of gemcitabine (A, B), 5-FU (C, D) or BI 2536 (E, F) alone (black circles) and in combination with fixed IC<sub>20</sub> concentration of NVP-AEW541 (black triangles). The shaded area represents a possible drug synergism calculated according to the model of Berenbaum<sup>[50]</sup>; error bars represent SD (*n* = 3).

doses we determined. Although our results show different responses toward treatment between the groups of CC and GBC cell lines, comparable results have only been reported for acute myeloid leukemia<sup>[12]</sup>, medulloblastoma<sup>[17]</sup>, neuroblastoma<sup>[15,16]</sup>, Ewing's sarcoma<sup>[21]</sup>, fibrosarcoma cells<sup>[11]</sup>, and breast cancer cell line MCF-7<sup>[25]</sup> whereas IC<sub>50</sub> concentrations were much higher for ovarian cancer<sup>[22]</sup>, mesothelioma<sup>[26]</sup>, osteosarcoma<sup>[21]</sup>, synovial sarcoma<sup>[27]</sup>, neuroendocrine gastrointestinal tumor<sup>[33]</sup>, gastrointestinal stromal tumor<sup>[34]</sup>, pancreatic cancer<sup>[39]</sup>, hepatocellular carcinoma<sup>[31]</sup>, and colorectal cancer cells<sup>[35]</sup> (up to 50 μmol/L). However, the exact molecular mechanisms for these differences in response are still unclear. One factor might be the level of IRS-1<sup>[25]</sup>; other factors

might be mutations (e.g. of k-ras), and differences in replication time of cells.

Small molecule inhibitors act only by reducing IGF-1R activation and seem not to influence receptor expression. We were able to show the suppressive effect of NVP-AEW541 on phosphorylation of IGF-1R, while whole IGF-1R protein expression remained stable. At the same time, treatment with NVP-AEW541 did not result in significant upregulation of IGF-1 and IGF-2 levels. These results support treatment efficacy of NVP-AEW541.

Dephosphorylating effects on downstream targets p42/44 (thus reducing rate of proliferation) and AKT (thus increasing rate of apoptosis) were consistent with



previous studies (with the exception of consecutively activated p42/44 in cell line EGI-1)<sup>[11,12,15,21,26,28,34,35,39]</sup>.

The JAK/STAT (signal transducer and activator of transcription) signal cascade is another important pathway in oncogenesis in general<sup>[52]</sup>, and in the pathogenesis of CC in particular, since it has already been shown that upregulation of anti-apoptotic Mcl-1 (myeloid cell leukemia-1) by interleukin-6 depends on Stat-3 activation<sup>[53,54]</sup>. Moreover, RACK1 (receptor for activated C kinase 1) may be the adaptor for IGF-1R-mediated Stat-3 activation<sup>[55]</sup>. Moser *et al.*<sup>[39]</sup> recently reported a decrease of Stat-3 phosphorylation after blockade of IGF-1R in pancreatic cell line HPAF-II. In our own *in vitro* experiments, this effect was only seen in cell line EGI-1, but not in cell line MzChA-1.

Further results of our experiments showed that effects of NVP-AEW541 seem to be based mainly on an inhibition of cell growth, at least at low concentrations, while only high doses seem to trigger apoptosis. Although we could not detect any apoptosis by caspase-3 assay, levels of anti-apoptotic protein Bcl-xL decreased after treatment of MzChA-1 cells. In flow cytometry, the rate of apoptotic cells (sub-G1 peak) increased markedly at drug concentrations above 1  $\mu\text{mol/L}$ .

Another possible anti-cancer mechanism of NVP-AEW541, which was not examined in our experiments, might be the inhibition of angiogenesis, since it has been demonstrated that IGF-1 can induce expression of VEGF<sup>[9]</sup> and that NVP-AEW541 significantly reduces vascularization and VEGF expression in an *in vivo* mouse model for pancreatic cancer<sup>[39]</sup>. In addition, it has recently been shown that IGF-1R blockade reduces the invasiveness of gastrointestinal cancers *via* blocking production of matrilysin<sup>[37]</sup>.

Regarding the obviously different response rates of extrahepatic CC cell line EGI-1 and GBC cell line Mz-ChA-1, it is not easy to find a simple explanation. While a longer replication time and resistance to Stat-3 dephosphorylation would predict the lower response of Mz-ChA-1 cells to NVP-AEW541, it is unclear why resistance to dephosphorylation of p-p42/44 *via* k-ras mutation and resistance to Bcl-xL downregulation did not induce a lower response of EGI-1 cells to NVP-AEW541. Perhaps these factors have a different impact of contribution to resistance.

A possible side effect of NVP-AEW541 could be induction of diabetes due to the high homology of the kinase region of IGF-1R and the insulin receptor. While *in vitro* kinase assays showed a 27-fold selectivity of NVP-AEW541 towards IGF-1R<sup>[11]</sup>, a recent *in vivo* study found neither an increase of blood glucose level nor other side effects in treated animals<sup>[20]</sup>.

Since resistance against anti-cancer drugs evolves rapidly, a combination of different approaches seems necessary. The combined drugs should ideally tackle the cancer cells in different cell phases and use different modes of action. Gemcitabine and 5-FU are currently used as chemotherapy for BTC<sup>[56]</sup>. Gemcitabine is a nucleoside

analogue that is utilized instead of cytidine during DNA replication, leading to premature chain termination and subsequent apoptosis. While 5-FU is principally also a nucleoside analogue, its main effects are exerted through inhibition of thymidylate synthase and hence reduction of thymidine necessary for DNA replication. Both drugs affect cells mainly during S phase, while our flow cytometry experiments showed that treatment with NVP-AEW541 led to G1 phase arrest, leading to synergistic effects in combination with gemcitabine. In contrast, the combination with 5-FU was less effective, possibly derived from the facts that 5-FU in general seems to be less effective than gemcitabine in the treatment of BTC<sup>[56]</sup> and that 5-FU is more effective at reduced doses for extended periods of exposure<sup>[57]</sup>. Additionally, we tested the combination of NVP-AEW541 with BI 2536. Polo-like kinases are increasingly recognized as key regulators of mitosis, meiosis and cytokinesis<sup>[58]</sup> and have been implicated in the transformation of human cells<sup>[59]</sup>. BI 2536, a novel selective inhibitor of Plk-1, inhibits tumor growth *in vivo*<sup>[60]</sup>. The lack of synergistic effects of BI 2536 in combination with NVP-AEW541 may be attributed to the assumption that BI 2536 affects the M phase of the cell cycle, which occurs less frequently because of NVP-AEW541-mediated G1 arrest. Another promising combination therapy may be dual inhibition of IGF-1R and FAK (focal adhesion kinase) as has already been shown for the novel single small molecule inhibitor TAE226, which targets specifically both FAK and IGFR-1<sup>[61]</sup>. Further suggested combination partners are trastuzumab (HER-2 inhibitor) in HER-2 positive breast cancer<sup>[23]</sup>, erlotinib (EGF-R inhibitor)<sup>[38]</sup>, ICR62 (anti-EGF-R monoclonal antibody)<sup>[36]</sup>, and mammalian target of rapamycin inhibitor RAD001<sup>[13]</sup>.

In summary, our findings suggest that NVP-AEW541 is active against BTC *in vitro*. In addition, the compound potentiated the efficacy of gemcitabine. Based on this data, further preclinical and clinical evaluation of this new drug for the treatment of BTC is recommended.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Carcinomas of the biliary tree are rare tumors of the gastrointestinal tract with a rising incidence worldwide for intrahepatic cholangiocarcinoma (CC) in recent years. At present, complete resection is the only potentially curative therapy, but most patients present with already advanced disease. In the palliative setting, non-resectable biliary tract cancer (BTC) is associated with a poor prognosis due to wide resistance to chemotherapeutic agents and radiotherapy.

### Research frontiers

Receptor tyrosine kinase inhibitors are currently under preclinical and clinical evaluation as new treatment options.

# Innovations and breakthroughs

After several years of preclinical research, the first clinical study data are now available for this tumor entity. Inhibitors of the epidermal growth factor receptor family, such as erlotinib, cetuximab, and lapatinib were recently investigated. Bortezomib, an inhibitor of the proteasome; imatinib mesylate, an inhibitor of c-kit; bevacizumab, an inhibitor of vascular endothelial growth factor (VEGF); and Sorafenib (BAY 43-9006), a multiple kinase inhibitor that blocks not only receptor tyrosine kinases but also serine/threonine kinases along the RAS/RAF/MEK/ERK pathway, have also been studied. Although early evidence of antitumor activity was seen, the results are still preliminary and require further investigations.

# Applications

The aim of the authors' study was to investigate the *in vitro* treatment with NVP-AEW541, a small molecule inhibitor of insulin-like growth factor-1 receptor (IGF-1R), in BTC. Their findings suggested that cell growth suppression was successful in seven human BTC cell lines. Combined with gemcitabine, NVP-AEW541 exerted synergistic effects, particularly at low concentrations, while effects of combinations with 5-fluorouracil or Polo-like kinase 1 inhibitor BI 2536 were only additive.

# Terminology

The IGF-1R system has emerged as an interesting target for cancer therapy, as it represents an important promoter of tumor transformation and survival of malignant cells, but is only partially involved in normal cell growth. This is in part attributed to interactions with oncogenes. Moreover, activation of IGF-1R may contribute to tumor angiogenesis by up-regulation of VEGF expression in certain cancer entities. In the past, different strategies were used to inhibit IGF-1R function, among them monoclonal antibodies and anti-sense RNA directed against the receptor, or recombinant IGF binding proteins and IGF-specific antibodies to reduce levels of ligands.

# Peer review

The strength of this manuscript is that it characterized a highly-resistant BTC cell line in comparison with an extrahepatic CC cell line. Also, to some extent, the mechanism leading to the resistance was examined.

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## A randomized double-blind trial on perioperative administration of probiotics in colorectal cancer patients

Luca Gianotti, Lorenzo Morelli, Francesca Galbiati, Simona Rocchetti, Sara Coppola, Aldo Beneduce, Cristina Gilardini, Daniela Zonenschain, Angelo Nespoli, Marco Braga

Luca Gianotti, Francesca Galbiati, Sara Coppola, Angelo Nespoli, Department of Surgery, Milano-Bicocca University, San Gerardo Hospital, Via Pergolesi 33, 20052 Monza, Italy  
 Lorenzo Morelli, Daniela Zonenschain, Department of Microbiology, Università Cattolica Sacro Cuore, 29121 Piacenza, Italy

Simona Rocchetti, Aldo Beneduce, Cristina Gilardini, Marco Braga, Department of Surgery, San Raffaele University, 20132 Milan, Italy

**Author contributions:** Gianotti L and Braga M were the study supervisors; Gianotti L, Morelli L, Nespoli A and Braga M conceived and designed the study and analysed and interpreted the data; Gianotti L drafted the manuscript; Rocchetti S, Beneduce A and Gilardini C were responsible for acquisition of the data and quality analysis; Galbiati F, Coppola S and Zonenschain D were responsible for acquisition and analysis of the data and technical support; all authors critically reviewed the manuscript and gave important intellectual contributions to the final version of the manuscript.

Supported by A grant from Nestec Ltd, Vevey, Switzerland  
 Correspondence to: Luca Gianotti, MD, PhD, Department of Surgery, Milano-Bicocca University, San Gerardo Hospital, Via Pergolesi 33, 20052 Monza, Italy. [luca.gianotti@unimib.it](mailto:luca.gianotti@unimib.it)  
 Telephone: +39-39-2332391 Fax: +39-39-2333652

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### Abstract

**AIM:** To investigate whether probiotic bacteria, given perioperatively, might adhere to the colonic mucosa, reduce concentration of pathogens in stools, and modulate the local immune function.

**METHODS:** A randomized, double-blind clinical trial was carried out in 31 subjects undergoing elective colorectal resection for cancer. Patients were allocated to receive either a placebo (group A,  $n = 10$ ), or a dose of  $10^7$  of a mixture of *Bifidobacterium longum* (BB536) and *Lactobacillus johnsonii* (La1) (group B,  $n = 11$ ),

or the same mixture at a concentration of  $10^9$  (group C,  $n = 10$ ). Probiotics, or a placebo, were given orally 2 doses/d for 3 d before operation. The same treatment continued postoperatively from day two to day four. Stools were collected before treatment, during surgery (day 0) and 5 d after operation. During the operation, colonic mucosa samples were harvested to evaluate bacterial adherence and to assess the phenotype of dendritic cells (DCs) and lymphocyte subsets by surface antigen expression (flow cytometry). The presence of BB536 and La1 was evaluated by the random amplified polymorphism DNA method with specific polymerase chain reaction probes.

**RESULTS:** The three groups were balanced for baseline and surgical parameters. BB536 was never found at any time-points studied. At day 0, La1 was present in 6/10 (60%) patients in either stools or by biopsy in group C, in 3/11 (27.2%) in group B, and none in the placebo group ( $P = 0.02$ , C vs A). There was a linear correlation between dose given and number of adherent La1 ( $P = 0.01$ ). The rate of mucosal colonization by enterobacteriaceae was 30% (3/10) in C, 81.8% (9/11) in B and 70% (7/10) in A ( $P = 0.03$ , C vs B). The *Enterobacteriaceae* count in stools was 2.4 (log10 scale) in C, 4.6 in B, and 4.5 in A ( $P = 0.07$ , C vs A and B). The same trend was observed for colonizing enterococci. La1 was not found at day +5. We observed greater expression of CD3, CD4, CD8, and naive and memory lymphocyte subsets in group C than in group A with a dose response trend (C > B > A). Treatment did not affect DC phenotype or activation, but after *ex vivo* stimulation with lipopolysaccharides, groups C and B had a lower proliferation rate compared to group A ( $P = 0.04$ ). Moreover, dendritic phenotypes CD83-123, CD83-HLADR, and CD83-11c (markers of activation) were significantly less expressed in patients colonized with La1 ( $P = 0.03$  vs not colonized).

**CONCLUSION:** La1, but not BB536, adheres to the colonic mucosa, and affects intestinal microbiota by

reducing the concentration of pathogens and modulates local immunity.

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**Key words:** Probiotic; Dendritic cell; Microbiota; Colon cancer; Lymphocyte; Surgery; Intestinal immunity

**Peer reviewers:** Dimitrios V Aygerinos, MD, Department of Surgery, Beth Israel Medical Center, 1st Ave. and 16th Str, 16 Baird Hall, New York, NY 10010, United States; Dr. Terence C Chua, Department of Surgery, St George Hospital, Level 3 Pitney Building, Sydney 2217, Australia

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## INTRODUCTION

Probiotics are mono or mixed cultures of live micro-organisms that might beneficially affect the host by improving the characteristics of indigenous microflora<sup>[1]</sup>. The composition and the equilibrium of microbiota are known to influence important host activities, including the local immune response and several intestinal metabolic traits<sup>[2-5]</sup>.

For therapeutic purposes, probiotics should have certain features: to be of human origin, safe for the host, and genetically stable<sup>[1,6]</sup>. Furthermore, it is important that probiotics, in order to be active, survive passage through the gastrointestinal (GI) tract irrespective of gastric acids, pancreatic enzymes, and bile acids so that they can reach the ileum and colon and colonize the intestinal mucosa and stools<sup>[6,7]</sup>.

Although the effects of probiotic administration has been intensively investigated *in vitro*, in animal models, in healthy volunteers, and in some human GI diseases (i.e. inflammatory bowel diseases, alimentary allergy, infectious diarrhea, and pouchitis)<sup>[1-6]</sup> very little is known on the possible cross-interactions among probiotic administration, changes of intestinal flora, and local immune response in surgical patients. Probiotic administration in patients undergoing GI operations has been attempted to obtain a competitive action on microbiota that contain bacteria responsible for postoperative infections. The results of randomized clinical trials (RCTs) are inconsistent with significant reduction of infection rate in upper GI surgery<sup>[8-10]</sup> and show a lack of clinical benefits in other types of operations and clinical settings<sup>[11-13]</sup>. This might be due to the substantial differences in study design, probiotic dose and strain, duration, period and combination of treatments, and particularly, the paucity of preliminary phase II studies investigating in detail

the relationship between probiotics and changes in intestinal pathophysiology.

The objectives of the study were to investigate whether peri-operative probiotic bacteria could adhere to the colonic mucosa, to assess whether this treatment could change the gut microflora by reducing potentially pathogenic bacteria, and whether the gut immune response could be modulated in a selected cohort of patients undergoing colorectal resection for cancer.

## MATERIALS AND METHODS

This was a prospective, randomized, controlled, double-blind trial of three groups in parallel. The study was carried out in two university hospitals (Department of Surgery, San Gerardo Hospital, Milano-Bicocca University, Monza, Italy and Department of Surgery, San Raffaele Hospital, Vita e Salute University, Milan, Italy).

### Patient enrolment

Eligible patients were those with histological documentation of cancer of the colon or rectum. Inclusion criteria were: both genders, age between 18 and 80 years and candidate for elective colorectal resection. The exclusion criteria were: denied written informed consent, no collection of a stool sample 4 d before the operation, unresectable tumor, neoplastic ascitis, clinically relevant pulmonary, cardiovascular, hepatic and kidney dysfunction or failure, ongoing total parenteral nutrition, immunological disorders, ongoing or recent infections (within last 30 d), pregnancy, and participation to another clinical trial. After applying these criteria, patients were allocated by an individual random number into three study arms.

Concealment assignment was by central randomization by computer. Both probiotic and placebo preparations were in foil sealed sachets that were stored in identical numbered containers. The study products and the placebo were both white powders, identical in weight, smell, and taste. Thus, the identity of the specific product was blind to participants, support staff and investigators for the entire duration of the study period.

Individually numbered treatment packs were allocated to the subjects as per the randomization schedule. Randomization was done by a program trial balance. Balancing variables were sex (male and female) and age (18-54 years and 55-80 years). All data captured by the investigator were recorded directly on the case report forms (CRF). Data entering, from CRFs into a computer database, was blinded. The blind codes were broken after all the collected data were analyzed. All data were analyzed by intention-to-treat.

The Ethical Committees of both hospitals approved the protocol.

The trial was registered at ClinicalTrial.gov of the National Institute of Health with the identifier number NCT00936572.

### Procedures and treatment

Four days before the scheduled surgery, subjects were randomly assigned to one of three groups: Placebo ( $n = 10$ ); oral treatment with low dose of probiotics every 12 h [total dose:  $2 \times 10^7$  colony forming units (CFU)/d,  $n = 11$ ]; oral treatment with high dose of probiotics ( $2 \times 10^9$  CFU/d,  $n = 10$ ). Treatments were composed of a mixture 1:1 of *Lactobacillus johnsonii* (*L. johnsonii*) (La1) and *Bifidobacterium longum* (*B. longum*) (BB536) in spray-dried form and blended with maltodextrin. The placebo was maltodextrin only. Both probiotics and placebo were mixed in 100 mL of a nutritional supplement (Clinutren 1.5, Nestle Nutrition, Milan, Italy) before drinking. All groups received treatments or placebo for three consecutive days before surgery (from day -3 to day -1 included). Treatments or placebo resumed postoperatively on day +2 until day +4 for a total of 6 d of treatment (12 doses).

Two mucosal samples were collected during surgery for probiotic adherence testing, microbiological evaluation of microbiota, and immune parameters. Stool samples were collected before treatment initiation (day -4), during surgery (day 0) and postoperatively at day +5 and analyzed as described below.

All patients received a single dose of prophylactic antibiotic (Cefoxitin, single dose, 30 min before incision).

Bowel preparation was done by an isosmotic solution (Macrogol; 3L) the evening before operation after the last preoperative dose of probiotics.

### Microbiological analysis of feces and mucosa

Weighed feces samples and mucosa (1 g) were homogenized for 1.5 min in a stomacher (PBI, Milan, Italy) before dilution in saline solution (9 g/L NaCl). Appropriate dilutions were plated using Rogosa Acetate agar (Difco) to enumerate *Lactobacillus* spp. *Bifidobacteria* isolates were enumerated using BSM media (MRS Broth, Bacto agar, Difco), 0.5 g/L of L-Cysteine Hydrochloride (Merck), while *Enterobacteriaceae* were counted on MacConkey Agar (Oxoid), enterococci on Bile Esculin Azide agar (Oxoid), and *Clostridium perfringens* (*C. perfringens*) was counted on Neomycin Nagler Agar (Eiken Chemical Co., Tokyo). Plates for *Enterobacteriaceae* and enterococci were incubated at 37°C for 24 h aerobically while *lactobacilli*, *bifidobacteria* and *C. perfringens* were incubated at 37°C for 48 h in anaerobic jars (GasPak, BBL, Coskeysville, MD, USA).

Counts of the CFU were performed for all countable plates (containing 30-300 CFU). Randomly selected CFU of *lactobacilli* and *bifidobacteria* (about 15% of colonies counted on readable plates) were isolated and cultivated in MRS broth and MRS broth with 0.5 g/L of L-Cysteine Hydrochloride, respectively to identify the *L. johnsonii* and *B. longum* species, and subsequent the identification of La1 and BB536 strains.

### DNA extraction

An overnight culture was collected by centrifugation at 5000 r/min for 10 min, the pellet was dissolve in 1 mL 0.9%

NaCl and transferred to a tube containing 0.5 g of glass beads (Sigma, St. Louis, Mo.). Cell lysis was performed with the Mini-Beadbeater (Biospec product) for three min at maximum speed. Subsequently, the suspension was centrifuged for five min at 10000 r/min and 1 µL of the supernatant was used directly for polymerase chain reaction (PCR).

### Species identification

Detection of *L. johnsonii* isolates was performed with primers LJ1 (GATGATTTTAGTTCTTGCACTAA) and P6 (CTACGGCTACCTTGTTACGA) using conditions described by Ventura *et al.*<sup>[14]</sup>, while *B. longum* isolates were detected with primers Blon1 (5'-TTCCAGTTGATCGCATGGTC-3') and Blon2 (5'-GGGAAGCCGTATCTCTACGA-3') with conditions described by Mullié *et al.*<sup>[15]</sup>. All amplification reactions were performed in a total volume of 25 µL containing 200 µmol/L of each deoxynucleoside triphosphate, 2.5 U of *Taq* (Gold), 10 pmol of each primer, and 1 µL of the respective template DNA (which equaled about 20 ng of DNA). The PCR reactions were carried out in a Gene Amp 9700 thermal cycler (Applied Biosystem, Foster City, USA). The resulting amplicons were visualized under UV light in 1% and 1.5% (w/v) agarose (Bio-Rad Laboratories, Milan, Italy) electrophoresis gels, respectively for *L. johnsonii* and *B. longum*, followed by subsequent 0.5 µg/mL ethidium bromide staining.

### Genetic identification of La1 and BB536 strains

Detection of *L. johnsonii* La1 strains was performed using the primers NCCE722 (GCATCATGCCCTTGAGTAGC) and NCCE723 (AATGCCCACTTTTGGCCTC) while the *B. longum* BB536 strains detection was carried out using the primers NCC3001-A (5'-GAA-CAGGGTGTGCTGAGTGA-3') and NCC3001-B (5'-CAAGCGAGAAGATCATCGAA-3'), both of them provided by Nestec Ltd. All amplification reactions were carried out in a total volume of 25 µL containing 200 µmol/L of each deoxynucleoside triphosphate, 2.5 U of *Taq* (Gold), 10 pmol of each primer, and 1 µL of the respective template DNA. The PCR reactions were carried out in a Gene Amp 9700 thermal cycler. Amplification cycle for La1 was as follows: initial denaturation was performed at 94°C for five min, followed by 35 cycles of: 94°C for 30 s, 53°C for 30 s and 68°C for 1 min, and a final extension at 68°C for 7 min. The conditions for BB536 were: 94°C for five min, followed by 30 cycles of: 94°C for 30 s, 60 for 30 s and 72°C for 30 s, and a final extension at 72°C for 5 min. The 540 bp La1 PCR products were analyzed on 1.5% (w/v) agarose gels, while the 461 bp BB536 PCR products were analyzed on 1.5% (w/v) agarose gels, both at 80 V, using a 200 bp ladder (Promega Corporation, Madison, USA) for molecular weight standards.

### Extraction and identification of dendritic cells (DCs) and lymphocytes

Colon DCs and lymphocytes were isolated from specimens

of healthy mucosa (> 10 cm from neoplasm) as previously described<sup>[16]</sup>. Briefly, after surgical excision of the colon, samples of mucosa and submucosa were separated mechanically from the muscular tunicae. Filtration through nylon mesh was used to isolate enterocytes. The enterocytes were discarded, and the remaining tissue was resuspended in medium with enzymes (liberase, DNAase, and hyaluronidase). The solution was centrifuged and the cells (containing lamina propria DCs and lymphocytes) were resuspended in HBSS before separation on a Percoll gradient. The Percoll gradient allowed separation of the lamina propria DCs from lymphocytes. Cells were washed with phosphate buffered saline and their phenotype was analysed by fluorescence activated cell sorter analysis.

DC and lymphocyte phenotypes were analyzed using antibodies to surface markers (CD11c: dendritic myeloid; CD123: dendritic plasmacytoid; CD HLA-DR: dendritic activated; CD83: dendritic mature; CD3: T cells; CD4: T helper; CD8: T suppressor; CD19: B cells; CD45RA: T naive; and CD45RO: T memory).

### Statistical analysis

Discrete parameters, such as rate of colonization and adherence, were analyzed by a  $\chi^2$  test or Fisher's exact test. The Sidak procedure was used to correct for multiple testing.

The count of bacteria and the other continuous variables were compared and analyzed using non-parametric tests (Kruskal-Wallis and Wilcoxon tests) or using ANOVA after log10 transformed of the data.

The dose effect on *La1* colonization was tested by the Cochran-Mantel-Hanzel test for a linear trend.

We also adopted another approach on data grouping according to *La1* colonization by dividing colonized patients (*La1*+) from non-colonized (*La1*-), regardless of dose or treatment.

Categorical variables are described by frequency, while continuous variables by mean  $\pm$  SD.

## RESULTS

A total of 49 patients were eligible for the study. Due to exclusion criteria, 31 patients were randomized, completed the study, and were analyzed on an intention-to-treat basis (Figure 1). The three groups were well balanced for baseline characteristics and surgical procedures, as shown in Table 1.

### Microbiology

Before treatment (day -4) none of the patients was colonized by *La1* or *BB536*. The effect of treatments on microbiota is shown in Table 2.

At day 0, the group receiving the high dose had a rate of adherence of 60% (6/10) *vs* 27.2% (3/11) of the low dose groups and none in the placebo group ( $P = 0.02$  high dose *vs* placebo). Moreover, there was a significant linear positive correlation ( $P = 0.01$ ) between the number

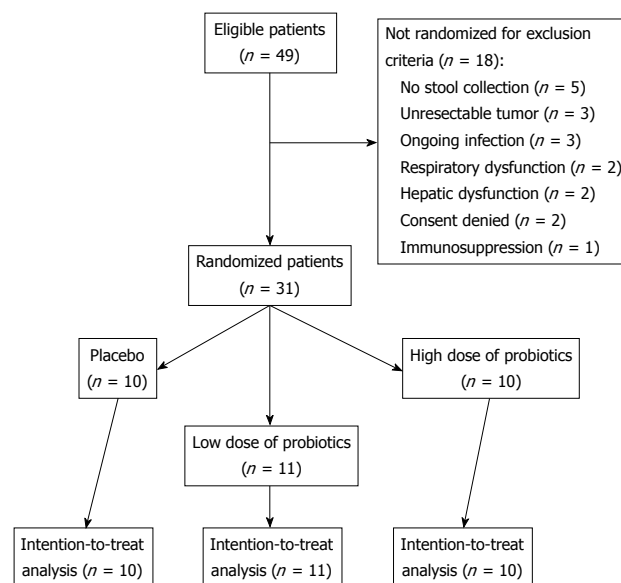


Figure 1 Study diagram according to CONSORT statement.

Table 1 Patient characteristics (mean  $\pm$  SD)

	Placebo (n = 10)	Low dose (n = 11)	High dose (n = 10)	P
Age (yr)	63.3 $\pm$ 10.2	64.7 $\pm$ 4.8	62.7 $\pm$ 7.8	0.49
Male/female	7/3	8/3	7/3	0.86
BMI (kg/m <sup>2</sup> )	25.6 $\pm$ 2.6	26.5 $\pm$ 4.1	24.4 $\pm$ 3.7	0.24
Hemoglobin (g/L)	121 $\pm$ 21	131 $\pm$ 21	128 $\pm$ 17	0.30
Leukocytes (cells/mm <sup>3</sup> )	6.5 $\pm$ 1.9	7.5 $\pm$ 1.5	7.9 $\pm$ 2.3	0.15
Blood glucose (mg/dL)	109.2 $\pm$ 48.3	100.7 $\pm$ 19.5	103.4 $\pm$ 30.1	0.63
Creatinine (mg/dL)	0.8 $\pm$ 0.1	0.9 $\pm$ 0.1	0.7 $\pm$ 0.1	0.12
Total protein (g/dL)	7.1 $\pm$ 0.7	7.3 $\pm$ 0.7	7.6 $\pm$ 0.5	0.13
Bilirubin (mg/dL)	0.5 $\pm$ 0.4	0.7 $\pm$ 0.2	0.7 $\pm$ 0.3	0.17
ALT (IU/L)	18.7 $\pm$ 5.5	26.3 $\pm$ 12.2	16.7 $\pm$ 5.7	0.08
Type of operation				
Left colectomy	4	6	5	0.72
Right colectomy	3	2	2	
Rectal resection	3	3	3	

BMI: Body mass index; ALT: Alanine aminotransferase

of adherent *La1* and the dose given (data not shown). *BB536* was never found in mucosa and feces samples. We also evaluated the changes in count of enterobacteriaceae and enterococci. We observed that the group of patients receiving the high dose of probiotics had a lower count of enterobacteriaceae in stool samples than the groups treated with the low dose or placebo ( $P = 0.07$ ). The same trend was observed for the enterococci count. The percent of patients with enterobacteriaceae adherent to colonic mucosa was 30% (3/10) in the high dose group, 81.8% (9/11) in the low dose group, and 70% (7/10) in the placebo group ( $P = 0.03$  high dose *vs* low dose). Similar results were observed for enterococci adherence rate, but without reaching statistical significance among the groups ( $P = 0.372$ ).

On postoperative day five, we did not observe



**Table 2** Variation of microbiota at day 0 (mean  $\pm$  SD) *n* (%)

	Placebo ( <i>n</i> = 10)	Low dose ( <i>n</i> = 11)	High dose ( <i>n</i> = 10)
Patients colonized with <i>La1</i>	0	3 (27.2)	6 (60) <sup>a</sup>
Patients colonized with <i>BB536</i>	0	0	0
Lactobacillus count in stools, Log10	3.1 $\pm$ 1.1	4.2 $\pm$ 0.4	5.3 $\pm$ 0.9 <sup>b</sup>
Enterobacteriaceae count in stools, Log10	4.5 $\pm$ 0.2	4.6 $\pm$ 0.6	2.4 $\pm$ 0.3 <sup>c</sup>
Enterococci count in stools, Log10	4.3 $\pm$ 0.5	4.1 $\pm$ 0.4	3.4 $\pm$ 0.7
Rate of enterobacteriaceae adherence to colonic mucosa	7 (70)	9 (81.8)	3 (30) <sup>d</sup>
Rate of enterococci adherence to colonic mucosa	6 (60)	5 (45.5)	3 (30)

<sup>a</sup>*P* = 0.02 *vs* placebo; <sup>b</sup>*P* = 0.04 *vs* placebo; <sup>c</sup>*P* = 0.07 *vs* placebo; <sup>d</sup>*P* = 0.03 *vs* low dose.

**Table 3** Percent of patients with variation of enterococci and enterobacteriaceae colonization of feces (day 0 *vs* day -4) *n* (%)

	Patients colonized with <i>La1</i> ( <i>n</i> = 9)	Patients not colonized with <i>La1</i> ( <i>n</i> = 22)
Enterobacteriaceae		
Unchanged/increased	2 (22.2)	9 (40.9)
Decreased	7 (77.8)	13 (59.1)
Enterococci		
Unchanged/increased	1 (11.1)	11 (50)
Decreased	8 (88.9) <sup>a</sup>	11 (50)

<sup>a</sup>*P* = 0.004 *vs* unchanged/increased.

colonization/adherence in any of the three groups, for both *La1* and *BB536*.

Specific stool cultures for *Clostridium perfringens* were always negative for both mucosa and stools in all three groups.

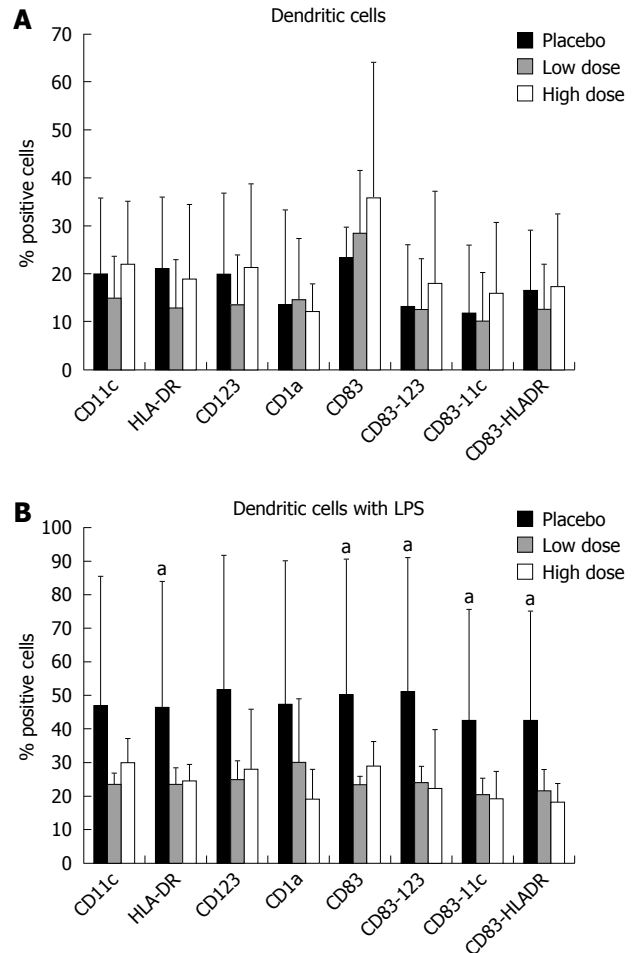
We also compared, regardless of treatment, patients who had colonization/adherence with *La1* (*La1*+) with those who did not have any *La1* adherence (*La1*-) (Table 3).

By comparing day -4 to day 0, we found that only *La1*+/ patients had a decrease (at least 1 log) of enterococci (*P* = 0.004) and of enterobacteriaceae colonization (*P* = 0.06).

### Immunology

On day 0, the *ex-vivo* analysis of intestinal DC phenotypes did not show any significant variation with respect to the type of treatment (Figure 2A). When DC were stimulated *in vitro* with lipopolysaccharide (LPS), there was a significant increase in proliferation of HLA-DR, CD83, CD83-123, CD83-11c, and CD83-HLA-DR subsets in the placebo group compared to the high dose and low dose groups. The same trend was observed for the other subsets, but without reaching a statistical difference (Figure 2B).

The Log number of dendritic subsets for CD83-123, CD83-11c, and CD83-HLA-DR was plotted in a linear correlation analysis with the total Log number of enteric bacteria in the stools, which includes also probiotics



**Figure 2** Percent of positive dendritic cell subsets of the three groups. A: *Ex vivo* analysis; B: *In vitro* analysis after lipopolysaccharide (LPS) stimulation. <sup>a</sup>Minimum *P* = 0.04 *vs* low and high dose.

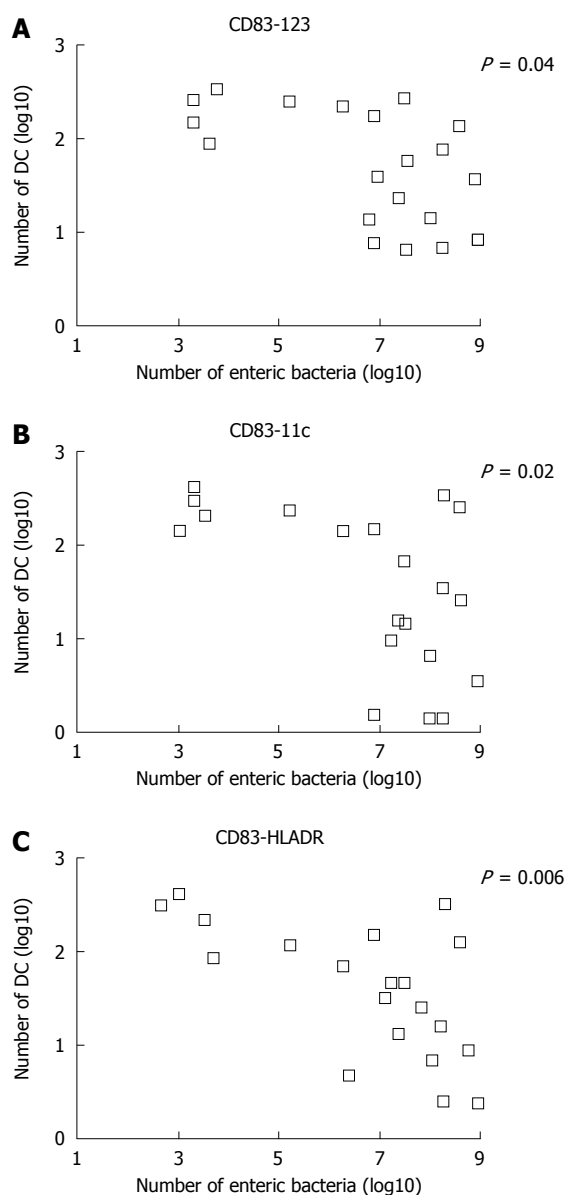
given. The analysis showed a significant inverse correlation between these two parameters (Figure 3).

By grouping patients according to colonisation (*La1*+/ *vs* *La1*-), we observed that those subjects with *La1* adherence to colonic mucosa (*La1*+) had a significant blunted proliferation of CD83-123, CD83-11c, and CD83-HLA-DR subsets compared to *La1*- (Figure 4).

The *ex-vivo* analysis of lymphocytes suggested the ability of probiotics to stimulate a non-specific proliferation of all subsets, with the exception of CD19 (B cells). The increased percent of positive cells also seemed dependent on the dose of probiotics given (Figure 5A). However, these variations did not reach statistical significance. LPS stimulation of lymphocytes did not affect T cell proliferation (Figure 5B).

### DISCUSSION

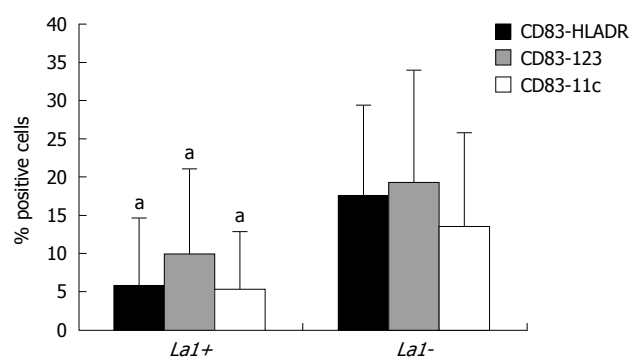
To our knowledge, this is the first clinical trial investigating the *in vivo* interaction among probiotic administration, variation of microbiota, and modulation of intestinal immune function in patients undergoing colorectal resection for cancer. This type of study might be important to better understand the potential mechanisms



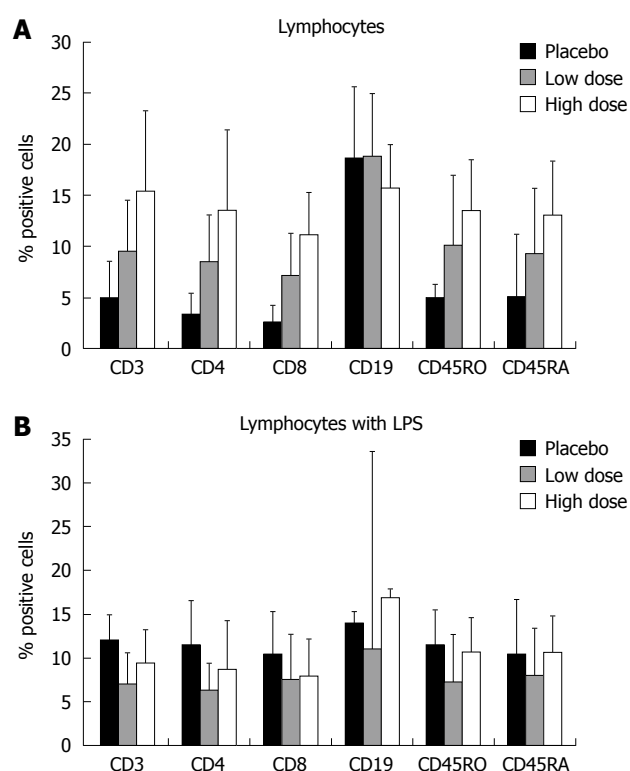
**Figure 3** Correlation between number of colonic dendritic cells (DCs) and number of bacteria in feces. A: Phenotype CD83-123; B: Phenotype CD83-11c; C: Phenotype CD83-HLADR.

of action of probiotics and subsequently to design appropriate and safe phase III trials with relevant clinical endpoints. Our data suggest that of *La1* (but not *BB536*) administered pre-operatively, is somehow able to adhere to colonic mucosa and colonize feces. This event seems to be correlated with a reduction of potentially pathogenic bacteria and modulation of the intestinal immune response. The dose given and the time of administration with respect to the operation appear to be key factors in obtaining these results.

Probiotic is a generic term that includes several species of bacteria. However, probiotic strains differ greatly in their mechanisms of action, survival during GI transit, modulation of intestinal metabolism, ability to affect the type of immune response, and competition with microbiota and pathogens<sup>[2,6]</sup>. Most of the data available are from animal or *in vitro* studies. Furthermore,



**Figure 4** Percent of positive subsets of DCs in *Lactobacillus johnsonii* (*La1*) colonized patients (*La1+*) vs non-colonized patients (*La1-*). <sup>a</sup>Minimum  $P = 0.03$  *La1+* vs *La1-*.



**Figure 5** Percent of positive lymphocyte populations of the three groups. A: *Ex vivo* analysis; B: *In vitro* analysis after LPS stimulation.

the same probiotic strain might have dissimilar clinical effects and efficacy in different GI illnesses, and a specific disease might not be successfully treated by different probiotics<sup>[2,6,17]</sup>.

We decided to test a mixture of two probiotics (*La1* and *BB536*) because they have been previously and repeatedly shown to have positive effects, such as safety for clinical use, non-pathogenicity, genetic stability, survival during GI transit<sup>[18]</sup>, antimicrobial properties, competitive antagonism with pathogens<sup>[19,20]</sup>, and the ability to modulate the intestinal immune system<sup>[21-24]</sup>. In particular, *BB536* has been shown, in single arm studies, to survive during intestinal passage and reach the colon intact, even though the experimental setting was different from ours<sup>[25]</sup>. These characteristics should make

these probiotics suitable for testing in a trial with patients who are candidates for colorectal resection, because infectious complications are usually sustained by the subject's own intestinal microbiota, more frequently than in other types of operation. *In vivo*, potential synergistic or antagonistic effects between these two probiotics are unknown in this kind of patients.

We believe that it is essential to have a comprehensive knowledge of the potential positive and negative interactions between defined probiotic strains and the host before designing a trial with a therapeutic strategy, such as reduction of surgical morbidity. This might avoid negative results or worse outcomes in specific clinical conditions, such as severe pancreatitis where the deleterious effect of probiotics on oxygen intestinal metabolism was not fully investigated *a priori*<sup>[13]</sup>.

Several RCTs tested such therapeutic strategies in surgery. They have been recently reviewed by van Santvoort *et al.*<sup>[26]</sup>. In most of these trials, synbiotics rather probiotics were used; the majority of the enrolled patients were candidates for hepato-biliary-pancreatic surgery, liver transplantation, or mixed cases. Most of the protocols were designed for exclusively postoperative treatment. Moreover, among these studies there were large variations of probiotic strain and dose, timing, duration, and route (oral *vs* enteral) of administration. Only one trial by Reddy *et al.*<sup>[27]</sup> was selective for colorectal patients. They reported a synergistic positive effect of synbiotics, neomycin, and bowel preparation on the prevalence of enterobacteriaceae colonization and bacterial translocation, but these events were not associated with a significant reduction of septic morbidity. The different trial characteristics make it quite difficult to compare them and draw any firm conclusion on efficacy.

Our trial suggest that the rate of preoperative colonic adherence and colonization by *La1* was suboptimal, reaching 60% with the higher dose tested, while *BB536* was never recovered. Several factors might explain these results: all patients underwent bowel preparation, which might affect transit time and peristalsis, thereby reducing the capability of probiotics to adhere. In fact, it has been shown that preoperative intestinal washout might cause loss of superficial mucus and epithelial cells<sup>[28]</sup>. Other factors might have been the dose ( $10^9$  not being enough), the preoperative timing of administration being too short, and the strains tested (particularly *BB536*) not being ideal in this type of patient. In any case, we did observe a reduction of pathogens and a modulation of the intestinal immune system in *La1* colonized patients. Moreover, we did not observe colonization and survival of both probiotics in the post-operative period. Our explanation is based on two hypotheses. First, the tolerance of probiotic administration in the postoperative period was quite low (less than 50%), in contrast to the tolerance of the pre-operative period (100%). This might explain why we could not recover live probiotics at day five postoperatively. Second, it might be due to the presence of postoperative ileus. This condition slows down

the GI transit time and increased residence in the lumen might have killed the probiotics.

Animal and *in vitro* data strongly suggest that probiotics possess the ability to modulate the intestinal and systemic immune response<sup>[1-5]</sup>. DCs are bone-marrow-derived "professional" antigen-presenting cells. They can acquire antigens and then interact with lymphocyte populations. The different response after DCs - T cell cross talk is influenced by several factors, including the phenotype of DC and signals received in the local environment. Specific intestinal DCs acquire, on activation, signals that might drive the development of Th1, Th2, T regulatory, or Th0 cell responses<sup>[29]</sup>. The nature of T cell polarization is largely dependent on the type of microbial products. In particular, specific strains of lactobacilli, including *La1*, can modulate DC and T-cell specific responses *via* the release of anti-inflammatory cytokines<sup>[21]</sup>. This might explain the beneficial effect of probiotic treatment of a number of inflammatory bowel diseases, such as alimentary intolerance or allergy, infectious diarrhea, and pouchitis in Crohn's disease<sup>[30-34]</sup>. Our data agree with the above findings and suggest that administration of probiotics can partially affect intestinal DC phenotype and activation. In fact, we observed an *in vivo* inverse correlation between number of enteric bacteria in feces (including probiotics administered) and number of mature and activated intestinal DCs. Thus, increases in microbiota appear to be correlated with a significant decrease of specific DC subsets. When DCs were stimulated "*ex vivo*" with LPS, only in placebo patients did we observe an enhanced ability of DCs to proliferate. Moreover, DCs isolated from patients colonized with *La1* had a significantly blunted ability to proliferate. Although speculative, there results suggest that increasing the probiotic concentration in stools might modulate the immune activity of DCs. In particular, it is possible that probiotics might avoid an excessive activation of DCs with a possible Th1 driven pro-inflammatory response in the intestinal mucosa. In fact, DCs isolated from subjects receiving probiotics were not able to respond to a second inflammatory challenge, such as LPS.

We also observed an unexpected non-specific *in vivo* proliferation of all lymphocyte subsets, with the exception of B cells, in the patients receiving probiotics. This effect also appeared to be dose dependent. We speculate that in this specific setting, lymphocytes might have been activated by immune pathways that we did not investigate, such as through monocyte or epithelial cell cross talk<sup>[33,36]</sup>.

The clinical significance and impact of these observations remain to be elucidated. However, the results suggest a role for probiotics in blunting a surgery-induced over-inflammatory response at intestinal and distant sites<sup>[37]</sup>.

In conclusion, our data suggest that *La1*, but not *BB536*, adheres to the colonic mucosa and colonizes stools, affects intestinal microbiota by reducing the con-

centration of pathogens, and modulates local immunity. Further trials are warranted to understand if, in this cohort of patients, better results might be obtained by different single probiotics or by a mixture of probiotic strains, increased dose, longer treatment period or timing of administration. The results of these future trials should be taken into consideration before designing phase III trials.

## COMMENTS

### Background

The therapeutic use of probiotic bacteria is generating great interest in several diseases, but extensive research is required to understand when their administration is really beneficial.

### Research frontiers

The administration of probiotics in patients undergoing resection for colon cancer has been tested for safety and efficacy.

### Innovations and breakthroughs

The results of the present work suggest that giving probiotics before colon surgery might reduce the number of enteric bacteria that might be responsible for postoperative infectious complications. Moreover, probiotics are able to modulate the immune response of the intestine and there is an intensive cross-talk between immune cells and bacteria.

### Applications

These data should be useful to design future clinical studies with a larger number of patients to understand if there is a correlation between metabolic, immunological and microbiological changes and reduction of surgery-related infections.

### Terminology

Probiotics: Mono or mixed cultures of live microorganisms that might beneficially affect the host by improving the characteristics of indigenous microflora.

### Peer review

This is the first clinical trial investigating *in vivo* the interaction among probiotic administration, variation of microbiota, and modulation of intestinal immune function in patients undergoing colorectal resection for cancer. Such studies might be important to better understand the potential mechanisms of action of probiotics and subsequently to design appropriate and safe trials with relevant clinical endpoints.

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## Interaction of the major inflammatory bowel disease susceptibility alleles in Crohn's disease patients

Veronika Csöngéi, Luca Járomi, Enikő Sáfrány, Csilla Sipeky, Lili Magyari, Bernadett Faragó, Judit Bene, Noémi Polgár, Lilla Lakner, Patrícia Sarlós, Márta Varga, Béla Melegh

Veronika Csöngéi, Luca Járomi, Enikő Sáfrány, Csilla Sipeky, Lili Magyari, Bernadett Faragó, Judit Bene, Noémi Polgár, Béla Melegh, Department of Medical Genetics, University of Pécs, Pécs 7624, Hungary

Lilla Lakner, Department of Medicine and Gastroenterology, Markusovszky Hospital, Szombathely 9700, Hungary

Patrícia Sarlós, 3rd Department of Internal Medicine, University of Pécs, Pécs 7624, Hungary

Márta Varga, 3rd Department of Medicine and Gastroenterology, Réthy Pál Hospital, Békéscsaba 5600, Hungary

**Author contributions:** Csöngéi V and Járomi L performed the genotyping and analyzed the data; Sáfrány E, Sipeky C, Magyari L, Faragó B and Bene J designed and performed the genotyping and were also involved in editing the manuscript; Polgár N overviewed the genotyping and revised the paper; Lakner L, Sarlós P and Varga M contributed human subjects and DNA samples; Melegh B designed and overviewed the study; Csöngéi V wrote the manuscript.

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**Correspondence to:** Béla Melegh, MD, PhD, DSc, Professor, Chairman, Department of Medical Genetics, University of Pécs, Pécs 7624, Hungary. [bela.melegh@aok.pte.hu](mailto:bela.melegh@aok.pte.hu)

Telephone: +36-72-536427 Fax: +36-72-536032

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locus (IGR2198a\_1 rs11739135 and IGR2096a\_1 rs12521868), *CARD15* (R702W rs2066845 and L1007fs rs2066847), *ATG16L1* (rs2241880) and *IL23R* (rs1004819, rs2201841) genes were genotyped by PCR-RFLP, the G908R (rs2066844) in *CARD15* was determined by direct sequencing.

**RESULTS:** The association of *ATG16L1* T300A with CD was confirmed [ $P = 0.004$ , odds ratio (OR) = 1.69, 95% CI: 1.19-2.41], and both *IL23R* variants were found to represent significant risk for the disease ( $P = 0.008$ , OR = 2.05, 95% CI: 1.20-3.50 for rs1004819 AA;  $P < 0.001$ , OR = 2.97, 95% CI: 1.65-5.33 for rs2201841 CC). Logistic regression analysis of pair-wise interaction of the inflammatory bowel disease (IBD) loci indicated that *IL23R*, *ATG16L1*, *CARD15* and *IBD5* (IGR2198a\_1) contribute independently to disease risk. We also analysed the specific combinations by pair of individual *ATG16L1*, *IL23R* rs1004819, rs2201841, IGR2198a\_1, IGR2096a\_1 and *CARD15* genotypes for disease risk influence. In almost all cases, the combined risk of susceptibility pairs was higher in patients carrying two different risk-associated gene variants together than individuals with just one polymorphism. The highest OR was found for *IL23R* rs2201841 homozygous genotype with combination of positive *CARD15* status ( $P < 0.001$ , OR = 9.15, 95% CI: 2.05-40.74).

**CONCLUSION:** The present study suggests a cumulative effect of individual IBD susceptibility loci.

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**Key words:** Gene interaction; Interleukin-23 receptor; Autophagy-related 16-like 1; IBD5; Caspase recruitment domain-containing protein 15; Crohn's disease; Inflammatory bowel disease

**Peer reviewers:** Ferenc Sipos, MD, PhD, Cell Analysis Laboratory, 2nd Department of Internal Medicine, Semmelweis

### Abstract

**AIM:** To investigate the interaction of interleukin-23 receptor (*IL23R*) (rs1004819 and rs2201841), autophagy-related 16-like 1 (*ATG16L1*) (rs2241880), caspase recruitment domain-containing protein 15 (*CARD15*) genes, and *IBD5* locus in Crohn's disease (CD) patients.

**METHODS:** A total of 315 unrelated subjects with CD and 314 healthy controls were genotyped. Interactions and specific genotype combinations of a total of eight variants were tested. The variants of *IBD5*

University, Szentkirályi u. 46., Budapest 1088, Hungary; Dr. Morten Frisch, MD, PhD, DSc, Department of Epidemiology Research, Statens Serum Institut, Building 206, Room 317, 5 Artillerivej, DK-2300 Copenhagen S, Denmark

Csóngei V, Járomi L, Sáfrány E, Sipeky C, Magyari L, Faragó B, Bene J, Polgár N, Lakner L, Sarlós P, Varga M, Melegh B. Interaction of the major inflammatory bowel disease susceptibility alleles in Crohn's disease patients. *World J Gastroenterol* 2010; 16(2): 176-183 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i2/176.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i2.176>

## INTRODUCTION

Two main clinical presentations of inflammatory bowel disease (IBD) are Crohn's disease (CD) and ulcerative colitis (UC)<sup>[1]</sup>. IBD is now widely believed to originate from an uncontrolled mucosal immunity of the gastrointestinal tract<sup>[2]</sup>. Twin and family studies have reported that besides environmental factors genetic susceptibility is essential in IBD development<sup>[3]</sup>. Up to now, many novel candidate genes have been found to confer increased risk for the disease, some loci seem to be specific to CD or UC, others have been reported to confer susceptibility to IBD overall; at the moment the most replicated loci are interleukin-23 receptor (*IL23R*) and autophagy-related 16-like 1 (*ATG16L1*).

Duerr *et al*<sup>[4]</sup> identified *IL23R* gene as an IBD-associated gene in a genome-wide association study. Subsequent genome-wide association studies provided replication and confirmed the role of *IL23R* in CD<sup>[5-7]</sup>.

In a European genome-wide association study the coding variant T300A (rs2241880) within the *ATG16L1* gene was reported to be highly associated with CD, and to carry the whole disease risk exerted by this locus<sup>[8]</sup>. The association of *ATG16L1* gene and CD was replicated in numerous studies<sup>[9-12]</sup>. *IL23R* and *ATG16L1* T300A were also proved to be risk variants in the Hungarian CD population<sup>[13]</sup>.

The rising number of CD candidate genes gives us the possibility to evaluate gene-gene interactions among susceptibility genes. Playing a role in biomolecular mechanisms, these interactions, or epistases are ubiquitous features of the genetic architecture of common human diseases<sup>[14]</sup>; their existence has been proved by several studies<sup>[15-17]</sup>. Since gene-gene interactions cannot only enhance but also weaken the individual gene effects, which can explain the lack of replication of single-locus results<sup>[14,18]</sup>, complex gene-gene interactions may be considered more important than independent effects of single susceptibility genes.

Therefore our aim was to join the major susceptibility genes into a gene-gene interaction analysis in the Hungarian CD population: two *IL23R* gene risk variants, namely the intronic rs2201841 and rs1004819, the *ATG16L1* gene variant T300A, the three well-known SNPs (R702W, L1007fs and G908R) of caspase recruit-

ment domain-containing protein 15 (*CARD15*) gene and two markers located in *IBD5* (IGR2198a\_1 and IGR2096a\_1) were tested for statistical interaction.

## MATERIALS AND METHODS

### Patients

We examined 315 unrelated patients with CD (151 males, 164 females, mean age  $38.65 \pm 0.79$  years). The CD group included mixed Caucasian patients who had typical symptoms and diagnosis. A group of 314 clinically healthy subjects (170 males, 144 females, mean age  $40.8 \pm 0.80$  years) with no IBD or other autoimmune disease were collected for the study. The origin of DNA samples was the central Biobank governed by the University of Pecs, as part of the National Biobank Network of Hungary ([www.biobank.hu](http://www.biobank.hu)), which belongs also to the pan-European Biobanking and Biomolecular Resources Research Infrastructure preparatory phase project (<http://bbmri.eu/bbmri/>). The governance, maintenance and management principles of the Biobank had been approved by the national Scientific Research Ethics Committee, Budapest (ETT TUKEB).

During the entire investigation period the guidelines and regulations of the 1975 Helsinki Declaration and the currently operative national laws were followed; the patients gave their informed consent for use of their collected, anonymized DNA samples for research purposes.

### Genotyping

Genomic DNA was extracted from peripheral blood leukocytes with a routine salting out method. For genotyping the variants of *IBD5* locus (IGR2198a\_1 rs11739135 and IGR2096a\_1 rs12521868), *CARD15* (R702W rs2066845 and L1007fs rs2066847), *ATG16L1* (rs2241880) and *IL23R* (rs1004819, rs2201841) genes PCR-RFLP methods were applied, the primers designed and used are given in Table 1. The PCR amplifications were performed on MJ Research PTC-200 thermal cyclers (Bio-Rad, Hercules, CA, USA) using the following conditions: initial denaturation at 96°C for 2 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing for 45 s at 54°C (rs1004819), 55°C (rs2241880 and rs2201841), 58°C (rs11739135, rs12521868), 60°C (rs2066847), and 62°C (rs2066845), extension at 72°C for 45 s and final extension at 72°C for 5 min. Each polymerase chain reaction contained 200 µmol/L of each dNTP, 1 U of *Taq* polymerase, 5 µL of reaction buffer (100 mmol/L Tris HCl, pH = 9.0; containing 500 mmol/L KCl, 15 mmol/L MgCl<sub>2</sub>, 0.2 µmol/L of each primer and 1 µL DNA to be amplified in a final volume of 50 µL. The amplicons were digested by allele-specific restriction endonucleases *Hin*I II (rs11739135), *Tru*I (rs12521868), *Hin*P1 I (rs2066845), *Bsp*LI (rs2066847), *Lve*I (rs2241880), *Taa*I (rs1004819) and *Hpy*F3 I (rs2201841). The amplicon contained an obligate cleavage site of the restriction enzyme for the suitable

Table 1 Primer sequences for the analysed variants

Gene	SNP	Primers (5'-3')
<i>IL23R</i>	rs1004819	F: GCATTCTAGGACCGTTTTGG R: ATCTGGTGGAAATATGTGAAACCTA
<i>IL23R</i>	rs2201841	F: GGCAAAAGGGAATTGAGAGG R: GGCCTATGATTATGCTTTTCCTG <sup>1</sup>
<i>ATG16L1</i>	rs2241880	F: CTCTGTACCATATCAAGCGTGG R: TCTAGAAGGACAGGCTATCAACAGATG
<i>CARD15</i>	rs2066844	F: GAGCCGCACAACTTCAGATC R: ACTTGAGGTGCCCAACATTACAG
<i>CARD15</i>	rs2066845	F: GAGGCCACTCTGGGATTGAG R: TAGACTCTGAAGCTTACCTGCGC <sup>1</sup>
<i>CARD15</i>	rs2066847	F: TGGCTAACTCCTGCAGTCTC R: GATCCTCAAATTCCTGCCATTC
IGR2198a_1	rs11739135	F: AGACACTGGGACATCATCTGTCTG R: GGGCAATTCTATGAGGACATTTAGA
IGR2096a_1	rs12521868	F: CAAGATTTCCTGCCATAGCCTCTT R: GGAGGGTGGTGTAGCCAGAGTAG

<sup>1</sup>Mismatch bases are underlined. F: Forward; R: Reverse; *IL23R*: Interleukin-23 receptor; *ATG16L1*: Autophagy-related 16-like 1; *CARD15*: Caspase recruitment domain-containing protein 15.

visual control of the efficacy of the digestion. The restriction fragments were separated by electrophoresis on 3% agarose gels containing ethidium bromide and visualized by UV transillumination. The genotyping of G908R (rs2066844) in *CARD15* was carried out by direct sequencing by BigDye Terminator labelling with ABI 3100 automatic sequencer (Foster City, CA, USA).

### Statistical analysis

Statistical analysis was carried out using the SPSS 15.0. package for Windows (SPSS Inc., Chicago, IL, USA). The allele frequencies were compared with Pearson's  $\chi^2$  test. Haploview 4.1 was used to test linkage disequilibrium. The  $r^2$  values for IGR2198a\_1 and IGR2096a\_1; and for *IL23R* rs1004819 and rs2201841 were below 0.8 ( $r^2 = 0.63$  and  $r^2 = 0.62$ , respectively). Binary logistic regression analysis was applied to observe the individual contributions of *IBD5*, *CARD15*, *ATG16L1* and *IL23R*, and to test for pairwise statistical interaction. An association was considered significant if a *P* value of < 0.05 was attained. *CARD15* status was classified as - (wild type) or + (at least one mutation in any of the three *CARD15* SNPs). The individual *CARD15* genotypes and *CARD15* status were stratified by *IBD5* genotypes; *IL23R* and *ATG16L1* genotypes were stratified by *CARD15* genotypes, *CARD15* status and *IBD5* genotypes; and *ATG16L1* genotype was stratified by *IL23R* genotypes. The odds ratios (ORs) and confidence intervals for these specific combinations of *IBD5*, *CARD15*, *ATG16L1* and *IL23R* were derived from  $\chi^2$  in 2x2 contingency tables.

## RESULTS

All analysed allele frequencies and genotype distributions were in Hardy-Weinberg equilibrium both in patients and controls; results are shown in Table 2.

Table 2 Case-control genotypes and allele frequencies of variants in *IL23R*, *ATG16L1*, *CARD15* and *IBD5* *n* (%)

	CD ( <i>n</i> = 315)	Controls ( <i>n</i> = 314)	OR (95% CI) <sup>1</sup>	<i>P</i>
<i>IL23R</i> (rs1004819)				
GG	119 (37.8)	151 (48.1)		
GA	152 (48.3)	140 (44.6)		
GA+AA	196 (62.2)	163 (51.9)	1.50 (1.09-2.08)	0.013 <sup>a</sup>
AA	44 (14.0)	23 (7.3)	2.05 (1.20-3.50)	0.008 <sup>a</sup>
RAF	0.381	0.296		0.001 <sup>a</sup>
<i>IL23R</i> (rs2201841)				
TT	131 (41.6)	152 (48.4)		
TC	139 (44.1)	145 (46.2)		
TC+CC	184 (58.4)	162 (51.8)	1.28 (0.93-1.76)	0.14
CC	45 (14.3)	17 (5.4)	2.97 (1.65-5.33)	< 0.001 <sup>a</sup>
RAF	0.363	0.285		0.003 <sup>a</sup>
<i>ATG16L1</i> T300A (rs2241880)				
AA	56 (17.8)	72 (22.9)		
AG	151 (47.9)	163 (51.9)		
AG+GG	259 (82.2)	242 (77.1)	1.45 (0.98-2.17)	0.06
GG	108 (34.3)	79 (25.2)	1.69 (1.19-2.41)	0.004 <sup>a</sup>
RAF	0.583	0.511		0.011 <sup>a</sup>
<i>CARD15</i> R702W (rs2066844)				
CC	275 (87.3)	294 (93.6)		
CT	38 (12.1)	18 (5.7)		
CT+TT	40 (12.7)	20 (6.4)	2.13 (1.19-3.80)	0.011 <sup>a</sup>
TT	2 (0.6)	2 (0.6)	0.62 (0.06-6.98)	0.70
RAF	0.067	0.035		0.011 <sup>a</sup>
<i>CARD15</i> G908R (rs2066845)				
GG	299 (94.9)	305 (97.1)		
GC	16 (5.1)	9 (2.9)		
GC+CC	16 (5.1)	9 (2.9)	1.65 (0.71-3.86)	0.24
CC	0 (0.0)	0 (0.0)	NC	NC
RAF	0.025	0.014		0.17
<i>CARD15</i> L1007fs (rs2066847)				
-	264 (83.8)	291 (92.7)		
- insC	42 (13.3)	23 (7.3)		
- insC+insC	51 (16.2)	23 (7.3)	2.57 (1.51-4.36)	< 0.001 <sup>a</sup>
insC insC	9 (2.9)	0 (0.0)	NC	NC
RAF	0.095	0.037		< 0.001 <sup>a</sup>
<i>CARD15</i> status <sup>2</sup>				
-	220 (69.8)	262 (83.4)		
+	95 (30.2)	52 (16.6)	2.17 (1.46-3.21)	< 0.001 <sup>a</sup>
IGR2198a_1 (rs11739135)				
GG	91 (28.9)	120 (38.2)		
GC	160 (50.8)	144 (45.9)		
GC+CC	251 (79.7)	194 (61.8)	1.54 (1.10-2.15)	0.013 <sup>a</sup>
CC	64 (20.3)	50 (15.9)	1.32 (0.87-1.99)	0.19
RAF	0.457	0.389		0.014 <sup>a</sup>
IGR2096a_1 (rs12521868)				
GG	94 (29.8)	120 (38.2)		
GT	149 (47.3)	142 (45.2)		
GT+TT	296 (94.0)	262 (83.4)	1.44 (1.03-2.02)	0.034 <sup>a</sup>
TT	72 (22.9)	52 (16.6)	1.45 (0.97-2.17)	0.07
RAF	0.465	0.392		0.009 <sup>a</sup>

<sup>1</sup>Adjusted for age and gender; <sup>2</sup>*CARD15* status is denoted - (wild type) and + (at least one mutation in any of the three SNPs). <sup>a</sup>*P* < 0.05 vs controls. RAF: Risk allele frequency; NC: Not calculated; OR: Odds ratio.

The risk allele frequencies of *IL23R* rs1004819 and rs2201841, *ATG16L1* rs2241880, IGR2198a\_1, (rs11739135), IGR2096a\_1 (rs12521868), *CARD15* R702W (rs2066845) and *CARD15* L1007fs (rs2066847) were significantly higher in patients compared to controls (Table 2). After adjusting for age and gender, logistic regression analysis showed that *IL23R* rs1004819 A and



**Table 3** Pairwise analysis of interactions of *CARD15*, *IBD5* (IGR2198a\_1), *ATG16L1* and *IL23R* to risk of CD

Model	OR (95% CI)	P
<i>IL23R</i> rs1004189 * <i>ATG16L1</i>	1.51 (0.73-3.11)	0.26
<i>IL23R</i> rs2201841 * <i>ATG16L1</i>	1.01 (0.29-3.57)	0.99
<i>IL23R</i> rs1004189 * <i>CARD15</i> status <sup>1</sup>	1.20 (0.54-2.67)	0.65
<i>IL23R</i> rs2201841 * <i>CARD15</i> status	1.57 (0.30-8.31)	0.60
<i>IL23R</i> rs1004189 * IGR2198a_1	1.30 (0.71-2.36)	0.40
<i>IL23R</i> rs2201841 * IGR2198a_1	0.46 (0.12-1.75)	0.25
<i>ATG16L1</i> * <i>CARD15</i> status	1.49 (0.59-3.80)	0.40
<i>ATG16L1</i> * IGR2198a_1	0.95 (0.45-2.00)	0.89
<i>CARD15</i> status * IGR2198a_1	0.75 (0.33-1.71)	0.49

<sup>1</sup>*CARD15* status is denoted - (wild type) and + (at least one mutation in any of the three SNPs). CD: Crohn's disease.

rs2201841 C, IGR2198a\_1 C, IGR2096a\_1 T, *CARD15* 702W, *CARD15* 1007fsinsC variants confer risk for CD; OR values ranged from 1.44 to 2.57 ( $P < 0.02$ ).

For *IL23R* rs1004189 and rs2201841, and for *ATG16L1* a highly significant increase in risk was observed in homozygous individuals ( $P = 0.008$ , OR = 2.05, 95% CI: 1.20-3.50 for rs1004189 AA;  $P < 0.001$ , OR = 2.97, 95% CI: 1.65-5.33 for rs2201841 CC;  $P = 0.004$ , OR = 1.69, 95% CI: 1.19-2.41 for *ATG16L1* GG genotype).

The *CARD15* G908R (rs2066845) did not show any association with CD.

Next, we analysed the possible statistical interactions by pairs of *ATG16L1*, *IL23R* variants, *CARD15* status and *IBD5* (IGR2198a\_1). No evidence of interactions between these seven markers was found. None of the  $P$  values was significant; the lowest  $P$  value was 0.25 (Table 3).

Finally, we observed the specific combinations of single markers by pairs, the combined ORs are shown in Tables 4-6. The individual *IL23R* and *ATG16L1* genotypes were stratified by *CARD15* genotypes and *CARD15* status; results are shown in Table 4. *ATG16L1* and both *IL23R* variants showed significant association with CD on the background of a wild type *CARD15* genotype. The rs2201841 CC genotype in itself was found to be a stronger CD risk factor than *CARD15*. The ORs were higher in individuals carrying one of the *CARD15* variants with either the *ATG16L1* GG genotype or *IL23R* rs1004189 variant ( $P < 0.001$ , OR = 3.33, 95% CI: 1.96-5.67 for rs1004189 A carriers; and  $P < 0.001$ , OR = 3.82, 95% CI: 1.86-7.86 for *ATG16L1* GG genotype on the background of *CARD15* + status). The *IL23R* rs2201841 CC genotype and + *CARD15* status together showed by far the highest OR ( $P < 0.001$ , OR = 9.15, 95% CI: 2.05-40.74).

The *IL23R* rs2201841 C variant in homozygous form increased the susceptibility for the disease on the *ATG16L1* nonhomozygous (i.e. wild type and heterozygous subjects together) background significantly; we could not detect a similar effect for rs1004189 A carriers. *ATG16L1* was associated with CD in the absence of *IL23R* rs2201841 CC genotype, but not in rs1004189 noncarriers. We found that bearing the *ATG16L1* GG genotype

together with one of the *IL23R* susceptibility variants enhanced the risk for CD ( $P < 0.001$ , OR = 2.51, 95% CI: 1.55-4.08 for rs1004189;  $P = 0.001$ , OR = 4.68, 95% CI: 1.72-12.78 for rs2201841 homozygous genotype).

The ORs calculated for specific combinations of *IBD5* with *IL23R* and *ATG16L1* genotypes are shown in Table 5. We detected significantly increased risk in patients carrying the *IL23R* rs2201841 CC genotype on a wild type *IBD5* background. The IGRs showed significant association with the disease on an *IL23R* rs2201841 nonhomozygous or *ATG16L1* nonhomozygous background. *ATG16L1* did not significantly influence the susceptibility of CD except in the presence of IGR variants ( $P < 0.001$ , OR = 2.38, 95% CI: 1.46-3.87 for IGR2198a\_1 C; and  $P = 0.001$ , OR = 2.32, 95% CI: 1.42-3.79 for IGR2096a\_1 T background). Moreover, for the combinations of IGRs and *IL23R* rs1004189 we could detect significantly elevated high ORs only in carriers of IGR2198a\_1 C and rs1004189 A, or in patients with IGR2096a\_1 T and rs1004189 A variants ( $P = 0.001$ , OR = 2.44, 95% CI: 1.43-4.15 for IGR2198a\_1 C; and  $P = 0.001$ , OR = 2.41, 95% CI: 1.42-4.09 for IGR2096a\_1 T). The IGRs and *IL23R* rs2201841 CC genotypes together resulted in higher risk than the IGRs in themselves, but this OR value was lower than the OR calculated for the rs2201841 CC genotype alone ( $P < 0.001$ , OR = 3.66, 95% CI: 1.81-7.41 for IGR2198a\_1 C; and  $P < 0.001$ , OR = 3.71, 95% CI: 1.80-7.67 for IGR2096a\_1 T in patients carrying *IL23R* rs2201841 homozygous genotype).

The ORs for individual *CARD15* genotypes stratified by *IBD5* IGR2198a\_1 and IGR2096a\_1 genotypes are summarized in Table 6. Both the *IBD5* markers significantly increased the risk of CD in the absence of *CARD15* mutations; the *CARD15* variants were confirmed to be stronger risk factors for CD than IGRs. The *IBD5* variants showed higher significant risk together with + *CARD15* status ( $P < 0.001$ , OR = 3.19, 95% CI: 1.90-5.37 for IGR2198a\_1; and  $P < 0.001$ , OR = 3.18, 95% CI: 1.87-5.39 for IGR2096a\_1 on the background of *CARD15* + status).

## DISCUSSION

Since the identification of *NOD2/CARD15* as the first susceptibility gene for CD in 2001<sup>[19,20]</sup>, several additional loci have been implicated in CD and confirmed by replication, among others the *IBD5*, *IL23R* and *ATG16L1*<sup>[4-6,8,9,21,22]</sup> loci.

Recently the idea was raised that exploring gene-gene interactions might lead to a better understanding of disease cause and might help the prediction of disease risk. So far numerous studies have assessed the risk for the development of CD by combining information from the known genetic risk variants associated with the disease. Though Hampe *et al.*<sup>[8]</sup> found a modest but significant association between *ATG16L1* and *CARD15* in their pioneer study, no interaction was demonstrated between the two loci in the majority of

**Table 4** Genotype-specific CD odds ratios<sup>1</sup> (with 95% CI) for combinations of variants in *IL23R*, *ATG16L1* and *CARD15*

	<i>CARD15</i> R702W		<i>CARD15</i> G908R		<i>CARD15</i> L1007fs			<i>CARD15</i> status		<i>ATG16L1</i>	
	CC	CT+TT	GG	GC+CC	- -	- insC+insC	insC	-	+	AA+AG	GG
<i>IL23R</i> rs1004189											
GG	1	2.35 (1.03-5.34) <sup>a</sup>	1	1.55 (0.46-5.21)	1	2.85 (1.28-6.35) <sup>a</sup>		1	2.100 (1.17-3.76) <sup>a</sup>	1	1.16 (0.68-1.99)
GA+AA	1.56 (1.12-2.19) <sup>a</sup>	3.18 (1.45-6.97) <sup>a</sup>	1.51 (1.09-2.09) <sup>a</sup>	3.23 (0.99-10.57)	1.57 (1.12-2.20) <sup>a</sup>	3.40 (1.69-6.82) <sup>a</sup>		1.50 (1.04-2.16) <sup>a</sup>	3.33 (1.96-5.67) <sup>a</sup>	1.34 (0.92-1.95)	2.51 (1.55-4.08) <sup>a</sup>
<i>IL23R</i> rs2201841											
TT+TC	1	2.25 (1.26-4.03) <sup>a</sup>	1	1.49 (0.62-3.59)	1	2.43 (1.42-4.18) <sup>a</sup>		1	2.12 (1.42-3.16) <sup>a</sup>	1	1.48 (1.03-2.14) <sup>a</sup>
CC	3.04 (1.67-5.57) <sup>a</sup>	4.75 (0.53-42.81)	2.69 (1.49-4.86) <sup>a</sup>	NC	2.89 (1.57-5.32) <sup>a</sup>	8.52 (1.04-69.74) <sup>a</sup>		2.70 (1.42-5.15) <sup>a</sup>	9.15 (2.05-40.74) <sup>a</sup>	2.67 (1.31-5.44) <sup>a</sup>	4.68 (1.72-12.78) <sup>a</sup>
<i>ATG16L1</i>											
AA+AG	1	2.20 (1.14-4.26) <sup>a</sup>	1	1.44 (0.56-3.72)	1	2.37 (1.29-4.34) <sup>a</sup>		1	2.12 (1.35-3.31) <sup>a</sup>	-	-
GG	1.57 (1.09-2.25) <sup>a</sup>	3.18 (1.11-9.08) <sup>a</sup>	1.51 (1.06-2.14) <sup>a</sup>	6.91 (0.83-57.92)	1.54 (1.07-2.22) <sup>a</sup>	4.27 (1.54-11.79) <sup>a</sup>		1.54 (1.04-2.27) <sup>a</sup>	3.82 (1.86-7.86) <sup>a</sup>	-	-

<sup>1</sup>*CARD15* R702W, G908R and L1007fs: OR relative to wild type genotype; *CARD15* status: OR relative to - (wild type) group; *ATG16L1*: OR relative to wild and heterozygous genotypes together. <sup>a</sup>*P* < 0.05 *vs* controls.

**Table 5** Genotype-specific CD odds ratios<sup>1</sup> (with 95% CI) for combinations of variants in *IBD5*, *IL23R* and *ATG16L1*

	<i>IL23R</i> rs1004189		<i>IL23R</i> rs2201841		<i>ATG16L1</i>	
	GG	GA+AA	TT+TC	CC	AA+AG	GG
IGR2198a_1						
GG	1	1.43 (0.80-2.53)	1	4.83 (1.52-15.37) <sup>a</sup>	1	1.71 (0.94-3.09)
GC+CC	1.45 (0.84-2.48)	2.44 (1.43-4.15) <sup>a</sup>	1.58 (1.11-2.24) <sup>a</sup>	3.66 (1.81-7.41) <sup>a</sup>	1.60 (1.07-2.38) <sup>a</sup>	2.38 (1.46-3.87) <sup>a</sup>
IGR2096a_1						
GG	1	1.55 (0.88-2.73)	1	4.03 (1.39-11.62) <sup>a</sup>	1	1.63 (0.91-2.92)
GT+TT	1.49 (0.87-2.56)	2.41 (1.42-4.09) <sup>a</sup>	1.50 (1.06-2.13) <sup>a</sup>	3.71 (1.80-7.67) <sup>a</sup>	1.50 (1.01-2.24) <sup>a</sup>	2.32 (1.42-3.79) <sup>a</sup>

<sup>1</sup>IGR2198a\_1, IGR2096a\_1 and *IL23R* rs1004189: OR relative to wild type genotype; *ATG16L1* and *IL23R* rs2201841: OR relative to wild and heterozygous genotypes together. <sup>a</sup>*P* < 0.05 *vs* controls.

**Table 6** Genotype-specific CD odds ratios<sup>1</sup> (with 95% CI) for combinations of variants in *IBD5* and *CARD15*

	<i>CARD15</i> R702W		<i>CARD15</i> G908R		<i>CARD15</i> L1007fs			<i>CARD15</i> status	
	CC	CT+TT	GG	GC+CC	- -	- insC+insC	insC	-	+
IGR2198a_1									
GG	1	3.17 (1.15-8.69) <sup>a</sup>	1	1.79 (0.39-8.22)	1	3.04 (1.30-7.14) <sup>a</sup>		1	2.65 (1.36-5.17) <sup>a</sup>
GC+CC	1.60 (1.13-2.27) <sup>a</sup>	2.819 (1.39-5.72) <sup>a</sup>	1.52 (1.08-2.13) <sup>a</sup>	2.69 (0.97-7.45)	1.61 (1.13-2.31) <sup>a</sup>	3.58 (1.80-7.16) <sup>a</sup>		1.63 (1.11-2.39) <sup>a</sup>	3.19 (1.90-5.37) <sup>a</sup>
IGR2096a_1									
GG	1	3.07 (1.20-7.86) <sup>a</sup>	1	2.19 (0.51-9.41)	1	2.43 (1.06-5.59) <sup>a</sup>		1	2.46 (1.29-4.69) <sup>a</sup>
GT+TT	1.55 (1.09-2.20) <sup>a</sup>	2.75 (1.32-5.70) <sup>a</sup>	1.47 (1.05-2.06) <sup>a</sup>	2.41 (0.86-6.77)	1.48 (1.03-2.11) <sup>a</sup>	3.74 (1.85-7.54) <sup>a</sup>		1.54 (1.05-2.26) <sup>a</sup>	3.18 (1.87-5.39) <sup>a</sup>

<sup>1</sup>IGR2198a\_1, IGR2096a\_1, *CARD15* R702W, G908R and L1007fs: OR relative to wild type genotype; *CARD15* status: OR relative to - (wild type) group. <sup>a</sup>*P* < 0.05 *vs* controls.

subsequent Caucasian studies<sup>[10,23,24]</sup>. Prescott *et al.*<sup>[12]</sup> also demonstrated that *ATG16L1* increased the susceptibility for CD irrespective of *CARD15* status; at the same time they detected increased *ATG16L1* G allele frequency in *CARD15* carriers compared with noncarriers, which may indicate a weak interaction between these candidate CD susceptibility genes. Moreover, an additive effect was reported between *ATG16L1* and *CARD15*<sup>[25]</sup>. The independence of *ATG16L1* and *IBD5* variants was also confirmed in IBD<sup>[12,23]</sup>.

Besides the individual risk of *IL23R* variants several

studies examined their epistatic interaction with other IBD genes like *CARD15*<sup>[24-26]</sup>. Mostly the well-replicated *IL23R* R381Q protecting variant was implied in gene-gene interaction analyses and was reported to act independently of *CARD15*. In one study an additive effect was found between this *IL23R* variant and *CARD15* gene<sup>[25]</sup>. The epistasis of the intronic rs1004189 risk variant with *CARD15* and *IBD5* was examined in a German CD population, but no gene-gene interaction was found<sup>[26]</sup>. No evidence of epistatic interaction between *CARD15* and *IBD5* was demonstrated in an Italian IBD study either<sup>[27]</sup>.

Besides combining the most associated CD susceptibility variants by pair, multilocus analyses with three or more loci were also performed. Latiano *et al.*<sup>[24]</sup> did not find any significant interaction between *ATG16L1*, *IL23R*, *CARD15* and *IBD5* by triplets. Prescott *et al.*<sup>[12]</sup> established a combined additive risk for all high risk genotypes in *ATG16L1*, *CARD15* and *IBD5*, which was 20 fold that of the baseline risk for individuals carrying none of the risk alleles. Weersma *et al.*<sup>[28]</sup> found an association between the increase in the number of risk alleles (*ATG16L1*, *IL23R*, *CARD15*, *IBD5* and *DLG5*) and an increased risk for the development of CD.

Here we selected two risk-conferring variants of *IL23R* and *ATG16L1* T300A mutation, and studied them together with the well-replicated *CARD15* and *IBD5* loci in Hungarian CD patients. Besides confirming our previous results with respect to *IL23R* rs2201841<sup>[29]</sup>, we found a significant positive association between *IL23R* rs1004819 variant and CD observed in the Hungarian population for the first time. For *ATG16L1* rs2241880 the estimated risk derived from the 315 CD patients showed a significant 1.7-fold increase in risk for the homozygous genotype. Our data are in line with most previous reports in Hungarian and other Caucasian populations<sup>[12,13]</sup>. Our results also verify that the previously identified R702W and L1007fs alterations in *CARD15* gene act as CD susceptibility factors in the Hungarian population<sup>[30,31]</sup>. Since neither *SLC22A4* nor *SLC22A5* variants seem to confer risk for CD in the Hungarian population<sup>[32]</sup>, we tested two other disease-associated *IBD5* markers, IGR2198a\_1 (rs11739135), IGR2096a\_1 (rs12521868) for gene-gene interactions<sup>[22,33,34]</sup>.

First we observed the statistical pairwise interactions between *IL23R* (rs1004189, rs2201841), *ATG16L1* (rs2241880), *CARD15* status and IGR2198a\_1 (rs11739135). No evidence of epistatic interaction was found by logistic regression suggesting that all examined loci contribute independently to disease risk.

Next, we analysed the specific combinations of individual genotypes by pairs with respect to CD risk. We detected a significant association with CD for IGR2198a\_1 and IGR2096a\_1, respectively, on the background of wild type (-) *CARD15* status, indicating that these two *IBD5* markers and *CARD15* are independent determinants of disease risk. Also high, significant ORs were found for *ATG16L1* and *IL23R* variants (rs1004189, rs2201841), respectively, both in the presence and absence of *CARD15* mutations, suggesting that these genes also act independently on CD risk. We detected a significant association in patients bearing *IL23R* rs2201841 CC genotype and wild type *IBD5* background together, and *vice versa*. In carriers of IGR variants a significantly high risk was detected on *IL23R* rs2201841 nonhomozygous background; accordingly they may play an independent role in CD susceptibility. Similar to the results of previous studies<sup>[12,25]</sup>, *ATG16L1* increased the disease risk both in the presence and absence of *CARD15*, moreover in our study it was independent from *IL23R* rs2201841

genotype, but not from rs1004189 and *IBD5* status. For combinations of *IL23R* rs1004189 and *IBD5* markers, significant association was seen only in individuals carrying together the rs1004189 mutation and one of the two IGR variants.

In almost all specific pairwise combinations, the highest OR was found in patients with two different risk-associated gene variants, this cumulative OR was by far the highest in individuals with *IL23R* rs2201841 CC genotype and + *CARD15* status.

However, we cannot detect significant statistical interaction between the analysed *IL23R*, *ATG16L1*, *CARD15* and *IBD5* risk alleles. The results of the present study suggest that these susceptibility factors may have a possible cumulative effect in the Hungarian CD population. By combining information from the known common risk polymorphisms significant predictive value from genetic markers might be gained; accordingly further large, well-powered studies should be performed to clarify the exact nature of these possible correlations.

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## COMMENTS

### Background

Up to now, strong evidence has been provided that genetic factors play a significant role in determining the susceptibility of individuals to inflammatory bowel disease (IBD), especially for Crohn's disease (CD). After the identification of the disease-associated *NOD2* [caspase recruitment domain-containing protein 15 (*CARD15*)] gene, huge genome-wide linkage-analyses and meta-analyses have reported several CD susceptibility regions like *IBD5* locus, *DLG5*, interleukin-23 receptor (*IL23R*), and autophagy-related 16-like 1 (*ATG16L1*) gene.

### Research frontiers

Besides establishing the risk of carrying single variants, numerous studies have performed gene-gene interaction analyses for the major well-replicated susceptibility genes (*CARD15*, *ATG16L1*, *IL23R* genes and *IBD5* locus). Mostly the independence of these main loci has been reported; nevertheless some studies have found an increased disease risk for carrying two or more certain risk variants together compared to non-carriers or individuals with only one susceptibility variant. In the present study two SNPs of *IL23R*, one of the *ATG16L1*, three of the *CARD15* genes and two of *IBD5* locus were genotyped and involved in interaction analysis in the Hungarian CD population.

### Innovations and breakthroughs

The present study confirms the reported association between *IL23R* rs2201841 and *ATG16L1* rs2241880 variants and CD susceptibility. The authors examined the *IL23R* rs1004189 in the Hungarian CD population for the first time, and found it significantly more frequent in patients compared to healthy controls. The analysis of statistical pairwise interactions between *IL23R*, *ATG16L1*, *CARD15* status and *IBD5* confirmed the independence of these susceptibility genes, while the specific combinations by pair showed the highest odds ratio in patients with two different risk-associated gene variants, suggesting that they may have a cumulative effect in this Hungarian CD population.

### Applications

The exploration of epistatic interactions between the major susceptibility genes and the specification of high risk genotype combinations could support the better understanding of the development of CD and could facilitate the diagnosis of high-risk patients.



## Peer review

The authors investigated the interactions of the major IBD susceptibility alleles in a Hungarian CD cohort. This is of high clinical significance as this methodology could help the diagnosis of high-risk patients.

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## An antioxidant resveratrol significantly enhanced replication of hepatitis C virus

Mitsuyasu Nakamura, Hidetsugu Saito, Masanori Ikeda, Ryota Hokari, Nobuyuki Kato, Toshifumi Hibi, Soichiro Miura

Mitsuyasu Nakamura, Ryota Hokari, Soichiro Miura, Department of Internal Medicine 2, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 3598513, Japan  
Mitsuyasu Nakamura, Hidetsugu Saito, Toshifumi Hibi, Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 1608582, Japan

Masanori Ikeda, Nobuyuki Kato, Department of Molecular Biology, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikada-cho, Okayama 7008558, Japan

**Author contributions:** Nakamura M performed the majority of experiments; Ikeda M and Kato N provided reagents and were involved in editing the manuscript; Hokari R, Hibi T and Miura S provided financial support for this work; Saito H designed the study, provided financial support and wrote the manuscript.

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**Correspondence to:** Hidetsugu Saito, MD, PhD, Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 1608582, Japan. [hsaito@sc.itc.keio.ac.jp](mailto:hsaito@sc.itc.keio.ac.jp)

Telephone: +81-3-33531211 Fax: +81-3-33518705

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**RESULTS:** This is the first report to investigate the effect of RVT and AXN on HCV replication. In contrast to other reported viruses, RVT significantly enhanced HCV RNA replication. Vitamin E also enhanced HCV RNA replication as reported previously, although AXN did not affect replication. IFN and RBV significantly reduced HCV RNA replication, but these effects were dose-dependently hampered and attenuated by the addition of RVT. AXN did not affect antiviral effects of IFN or RBV.

**CONCLUSION:** These results suggested that RVT is not suitable as an antioxidant therapy for chronic hepatitis C.

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**Key words:** Replicon system; Luciferase assay; Ribavirin; Interferon; Polyphenol; Astaxanthin

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### Abstract

**AIM:** To elucidate the effect of antioxidants, resveratrol (RVT) and astaxanthin (AXN), on hepatitis C virus (HCV) replication.

**METHODS:** We investigated the effect of recent popular antioxidant supplements on replication of the HCV replicon system OR6. RVT is a strong antioxidant and a kind of polyphenol that inhibits replication of various viruses. AXN is also a strong antioxidant. The replication of HCV RNA was assessed by the luciferase reporter assay. An additive effect of antioxidants on antiviral effects of interferon (IFN) and ribavirin (RBV) was investigated.

### INTRODUCTION

Chronic liver disease develops in over 70% of those infected with hepatitis C virus (HCV), and HCV is now the most common cause of liver cirrhosis and also hepatocellular carcinoma (HCC), especially in Japan. It has been said that the median time for progression to cirrhosis is 30-40 years, but other factors such as male gender, the age at infection, co-infection with hepatitis

B virus or human immunodeficiency virus (HIV), and alcohol consumption accelerate progression of this chronic disease. Oxidative stress has been postulated to be one of the deleterious factors of chronic hepatitis, and it was reported that antioxidant levels were significantly reduced in chronic hepatitis and cirrhosis<sup>[1]</sup>. Moreover, it was also found that HCV proteins themselves generate oxidative stress, and the additive effect of oxidative stress caused by the inflammatory process in hepatitis and that induced by HCV proteins may further advance the disease stage of chronic hepatitis C<sup>[2]</sup>. Therefore, it is thought that antioxidant therapy has a role in slowing disease progression to cirrhosis and subsequent HCC. In fact, there are studies suggesting a beneficial effect of antioxidant therapy for patients who did not respond to interferon (IFN) therapy, and that a combination of antiviral and antioxidant therapies may enhance the overall response rate of patients with chronic hepatitis C<sup>[3,4]</sup>.

Current recommended therapy for previously untreated and relapsed patients is a combination of pegylated interferon (Peg-IFN) and ribavirin (RBV), resulting in a sustained virological response in around 50% of genotype 1 patients with high viral load<sup>[5]</sup>. Recent studies have shown that protease and polymerase inhibitors possess a strong additive effect on antiviral therapy of Peg-IFN/RBV and seem to be promising<sup>[6]</sup>. However, these regimens are expensive and adverse effects are sometimes severe and frequent, meaning that large numbers of patients give up treatment for a variety of reasons<sup>[7]</sup>. These conditions have led patients with chronic hepatitis C to use complementary and alternative medicine (CAM) including various supplements, and a previous survey found that about 40% of patients of liver disease outpatient clinics in the US used CAM at least once during the preceding month<sup>[8]</sup>. Moreover, a large number of supplements are used by patients universally to maintain their health condition or improve quality of life even if they are cared by medical doctors and they always do not tell doctors whether they used CAM and/or supplements. The most frequent CAM or supplements taken by patients with chronic hepatitis C were antioxidants, which may be beneficial for this disease as described above.

Resveratrol (RVT) was discovered to be a strong activator of *sirtuin*, a gene for longevity<sup>[9]</sup>, and has been implicated as the most important polyphenol responsible for the beneficial effects of red wine consumption, which has been called as the “French Paradox”<sup>[10]</sup>. Polyphenols contained in red wine have shown a strong antioxidative effect on cardioprotection, anti-atherosclerosis and relaxation of vascular endothelium through nitric oxide release. *Sirtuin* is activated when a person undergoes calorie restriction, and RVT is thought to be a surrogate for calorie restriction, which induces stabilization of DNA and also increases longevity by 70%. RVT is also known to improve liver lesions such as acetaminophen-induced hepatic injury and liver fibrosis in the mouse. When RVT was administered to mice fed a high-fat diet, fatty liver induced by this high-

calorie diet was significantly improved<sup>[11]</sup>. In addition to these favorable reactions, it has been reported that RVT inhibited viral replication of several major viruses, such as cytomegalovirus (CMV), varicella-zoster, influenza A, and herpes simplex virus (HSV)<sup>[12-15]</sup>. This supplement is popular and is thought to be one of candidates for the supplemental treatment of chronic hepatitis C.

Another candidate is astaxanthin (AXN: 3,3'-dihydroxy-b, b-carotene-4,4'-dione), which also showed a strong antioxidative effect<sup>[16]</sup>. AXN is the carotenoid responsible for the pink pigmentation in the flesh of salmon, lobster, krill and other aquatic animals and plants. Recent studies have indicated that AXN is more powerful than its carotenoid cousin,  $\beta$  carotene, at neutralizing singlet oxygen<sup>[17]</sup>. This supplement is known to improve the condition of so-called metabolic syndrome<sup>[18,19]</sup>, and is therefore popular. The antiviral effect of this supplement has not been examined so far.

These reports suggested that RVT and AXN might be good candidates for an antioxidative as well as an anti-HCV agent. However, we have no information whether these antioxidants affect HCV replication or not and if they are suitable for patients with chronic hepatitis C. In this study, we tried to assess the effect of these antioxidants on HCV replication using the HCV replicon system as an *in vitro* tool<sup>[20]</sup>. This is the first report to investigate the effect of RVT and AXN on HCV replication *in vitro*.

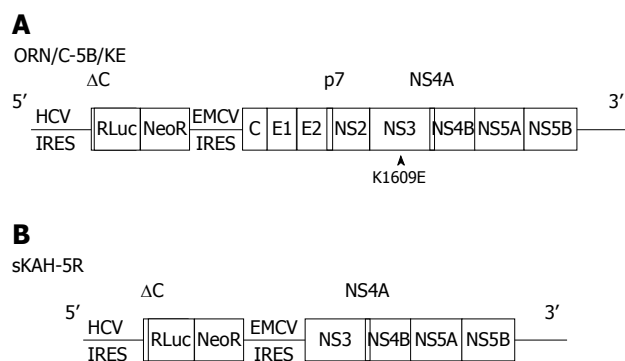
## MATERIALS AND METHODS

### Cells and virus

OR6 cells, a cell line cloned from ORN/C-5B/KE cells<sup>[21]</sup> supporting genome-length HCV RNA (strain O of genotype 1b) encoding the luciferase reporter gene, were used. This cell line was originally derived from a hepatoma cell line, HuH-7, as described elsewhere<sup>[21]</sup>. The schematic organization of the ORN/C-5B/KE gene is shown in Figure 1A. This cell line was cultured and maintained as previously reported<sup>[22]</sup>. Another cell line used was sKAH-5R<sup>[23]</sup>, which was established from a patient with acute hepatitis C, having subgenomic HCV RNA encoding the luciferase reporter gene (Figure 1B). The latter cell line was cultured under the same conditions as the OR6 cells, which includes the gene without a structural region of HCV RNA from the ORN/C-5B/KE gene.

### Chemicals

We evaluated RVT and AXN as new supplements, and vitamin E (VE) was used as a control because its effect on the HCV replicon system was already reported elsewhere<sup>[24]</sup>. RVT (3,5,4'-trihydroxystilbene), RBV (1-b-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide), AXN (3,3'-dihydroxy-b,b-carotene-4,4'-dione), VE and IFN-2b were purchased from Sigma-Aldrich Japan (Tokyo, Japan). AXN, RVT and VE were prepared as 10-20 mg/mL stock solutions in dimethylsulfoxide (DMSO) and stored at -80°C until used. This stock solution was diluted with culture medium. The final



**Figure 1** Organization of hepatitis C virus (HCV) RNA used in the replicon systems. A: Genome-length HCV RNA generated in OR6 cells; B: Subgenomic HCV RNA used in sKAH-5R and s1B-4R cells. Structural region of HCV RNA was deficient in the replicated RNA of sKAH-5R and s1B-4R cells.

concentration of DMSO was 0.2%, which did not interfere with viral replication, the highest concentration of RVT used in this study was 100  $\mu\text{mol/L}$ , that of AXN was 50  $\mu\text{mol/L}$ , and that of VE was 15  $\mu\text{mol/L}$ . To monitor the anti-HCV effects of IFN and RBV on replication, OR6 cells ( $1.5 \times 10^4$  /well) were plated onto 24-well plates at least in triplicate for each assay and cultured for 4 h. Then the cells were treated with IFN at a final concentration of 1, 2, 4, 10, or 20 U/mL or RBV at a final concentration of 10 or 25  $\mu\text{mol/L}$  for 72 h, harvested with renilla lysis reagent (Promega, Madison, WI), and assayed for luciferase activity according to the manufacturer's protocol. The same protocol was applied for sKAH-5R cells. The additive effect of RVB, RVT and AXN on the antiviral effect of IFN (1 U) was studied and compared using luciferase activity.

### Luciferase reporter assay

Approximately  $1$  to  $4.5 \times 10^4$  cells were plated onto 6-well plates and cultured for 24 h. Cells were treated with each agent for 72 h. The cells were then harvested with Renilla lysis reagent and subjected to the Renilla luciferase (RL) assay according to the manufacturer's protocol.

### Cell viability

We tested the toxic effect of RBV as described elsewhere<sup>[22]</sup>. The effect of RVT (5–100  $\mu\text{mol/L}$ ) and AXN (1–50  $\mu\text{mol/L}$ ) on cell viability was investigated. To examine the cytotoxic effect of RVT and AXN on cells with replicon RNA, the cells were seeded at a density of  $2 \times 10^5$  cells per dish onto 6-well plates. After 24 h culture, the cells were treated with RVT at final concentrations of 25 and 50  $\mu\text{mol/L}$  in the absence of G418. After incubation for 72 h, the number of viable cells was counted in an improved Neubauer-type hemocytometer after trypan blue dye (Invitrogen, Carlsbad, CA) treatment.

### Statistical analysis

The difference in relative luciferase activity was tested using the Student's *t*-test and the Mann-Whitney *U*-test as appropriate. *P*-values < 0.05 were considered statistically

significant. Every experiment was performed in triplicate and two independent experiments were done.

## RESULTS

### RVT dose-dependently enhanced HCV RNA replication but AXN inhibited replication

The effect of RVT and AXN was examined in comparison to that of VE, using the OR6 assay system, in which genomic length HCV RNA replication is represented by RL fluorescence activity.

After treatment of OR6 cells with various concentrations of RVT for 72 h, the luciferase activity was dose-dependently increased up to 20  $\mu\text{mol/L}$  (Figure 2A). The activity gradually decreased at higher concentrations, but at 100  $\mu\text{mol/L}$  it was still higher than that without RVT. Since it has been reported that the proliferation of the HCV subgenomic replicon is dependent on host-cell growth, we examined the effect of RVT on cell number and viability of OR6 cells by the trypan blue dye exclusion test. As shown in Figure 2B, RVT did not increase OR6 cells until 15  $\mu\text{mol/L}$ , and the cell viability decreased at higher concentrations than 20  $\mu\text{mol/L}$  of RVT. This decrease seen in higher concentrations paralleled the luciferase activity shown in Figure 2A, and it seemed that RVT further enhanced HCV RNA replication at concentrations higher than 20  $\mu\text{mol/L}$  when estimated by the number of viable cells. Different from the effect of RVT, AXN did not enhance luciferase activity (Figure 2C). The luciferase activity decreased at a concentration more than 10  $\mu\text{mol/L}$ , and this decrease seemed to be due to decrease in cell viability (Figure 2D).

VE dose-dependently increased luciferase activity up to a concentration of 15  $\mu\text{mol/L}$  (Figure 3). This result indicated that VE also upregulates HCV RNA replication in OR6 cells. The cytotoxic effect of VE was not observed in the cell viability test. This result was compatible to the data already reported elsewhere<sup>[24]</sup>.

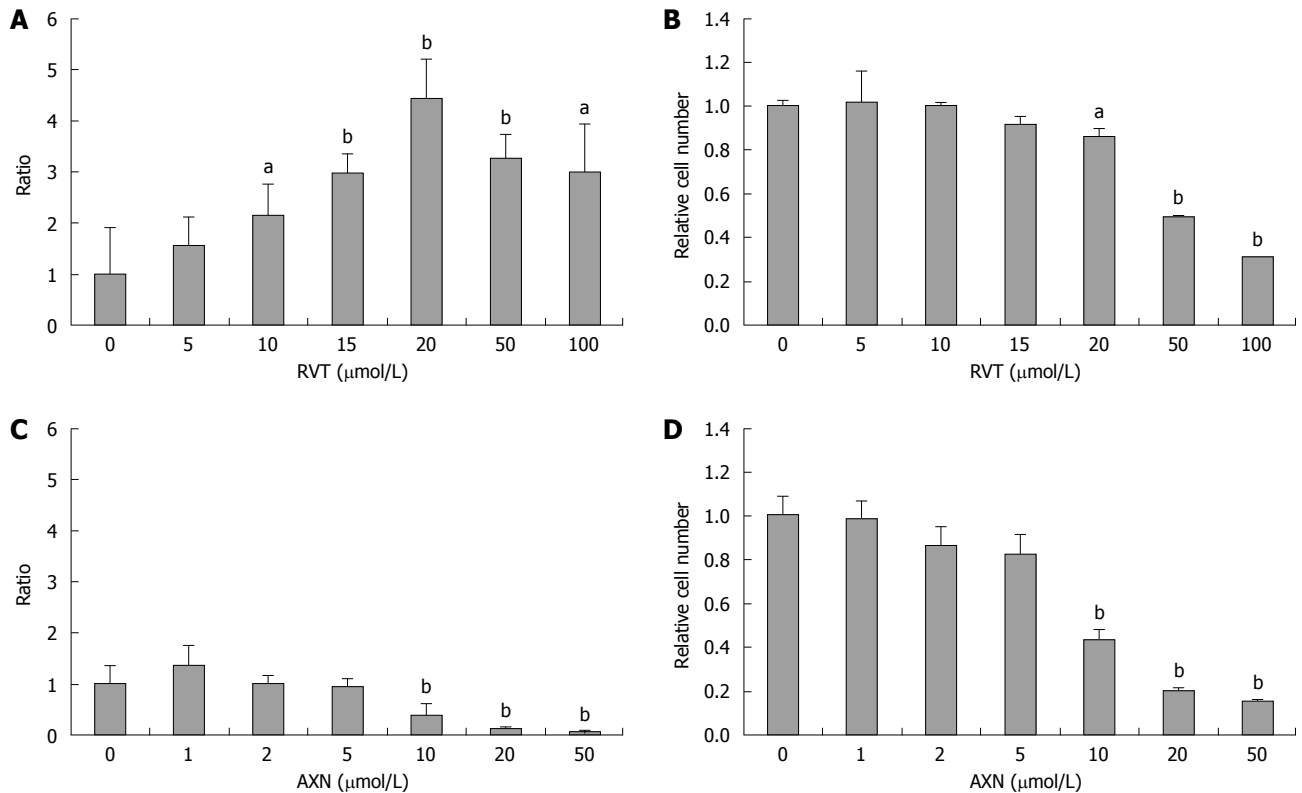
### Proliferative effect of RVT was also observed in the subgenomic replicon

Next, we further investigated the effect of RVT on other clones of HCV RNA. We tested the effect of RVT on the subgenomic HCV RNA-replicating cell, sKAH-5R. RVT also enhanced the replication of sKAH-5R subgenomic HCV RNA at 2–10  $\mu\text{mol/L}$  (Figure 4A). This concentration is not toxic to the sKAH-5R cells, but more than 20  $\mu\text{mol/L}$  of RVT was toxic to sKAH-5R cells (Figure 4B). Thus, subgenomic replicons showed the same results observed in the full-genome length replicon.

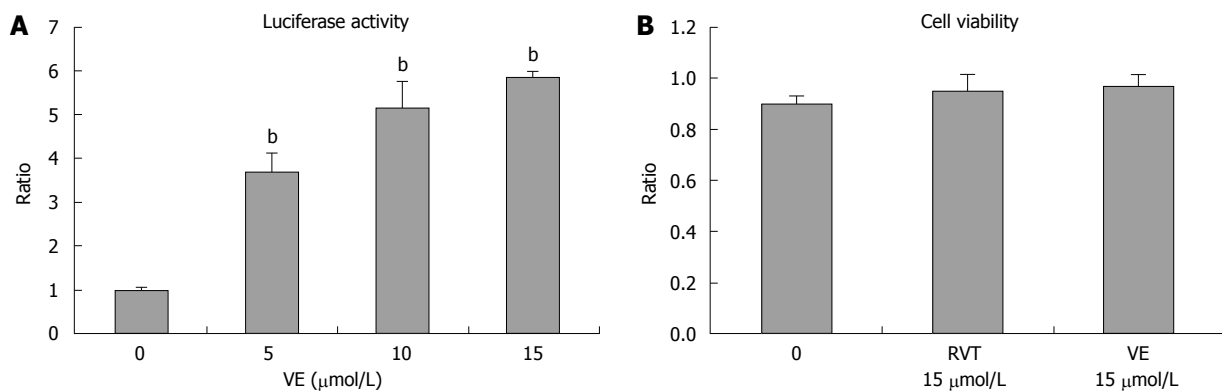
### Anti-viral effects of IFN and RBV

The effects of IFN-2b and RBV were independently applied to these cells to demonstrate that anti-viral agents reduce HCV RNA replication in this cell line. Figure 5A shows the time course of luciferase activity after administration of IFN. Luciferase activity was





**Figure 2** Luciferase activity and cell viability of OR6 cells after addition of resveratrol (RVT) and astaxanthin (AXN) for 72 h. The indicated concentrations of RVT or AXN were added to the culture medium of OR6 cells, and after 72 h of culture, cells were harvested with Runilla lysis buffer and the lysate was subjected to the luciferase assay. Cell viability was evaluated by a trypan blue dye exclusion assay. A: Luciferase activity after addition of RVT; B: Cell viability after addition of RVT; C: Luciferase activity after addition of AXN; D: Cell viability after addition of AXN. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01.



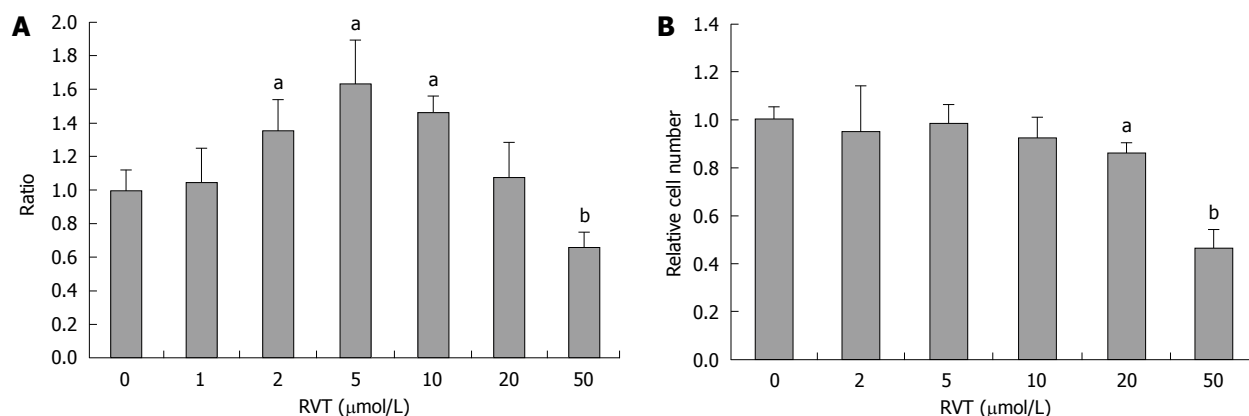
**Figure 3** The effect of vitamin E (VE) on luciferase activity and cell viability of OR6 cells (72 h). Cells were treated with the indicated concentrations of VE for 72 h. A: Luciferase activity after addition of VE; B: Cell viability after addition of RVT and VE. <sup>b</sup>*P* < 0.01.

dose-dependently inhibited by IFN. Figure 5B shows the effect of RBV at concentrations of 10 and 25 μmol/L on luciferase activity of OR6 cells 72 h after addition of RBV in comparison to that of IFN (1 U/mL). RBV also reduced luciferase activity of OR6 cells dose-dependently, but the effect was smaller than that of IFN. Anti-viral effects of IFN and RBV were thus confirmed in this cell line.

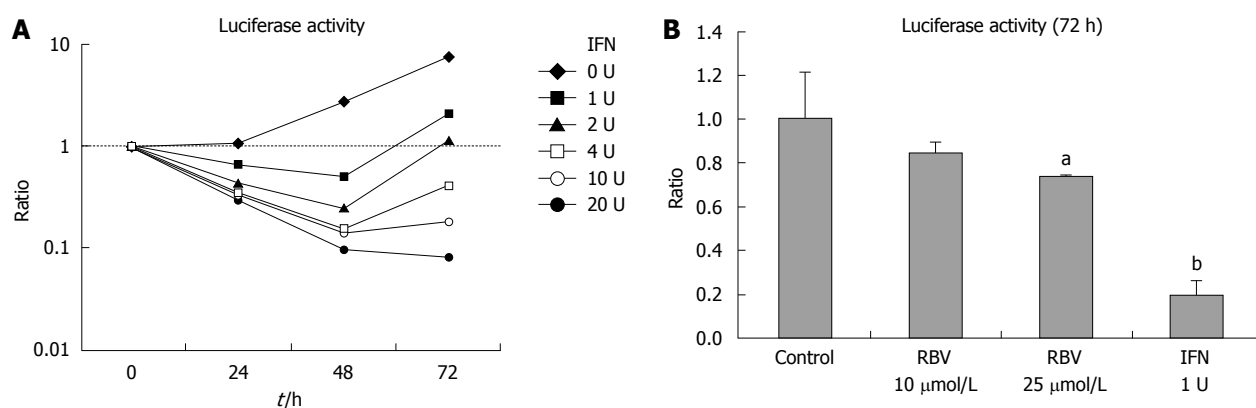
#### **RVT reversed anti-viral activity of IFN and RBV, but AXN did not affect it**

We then investigated whether RVT reverses the anti-viral

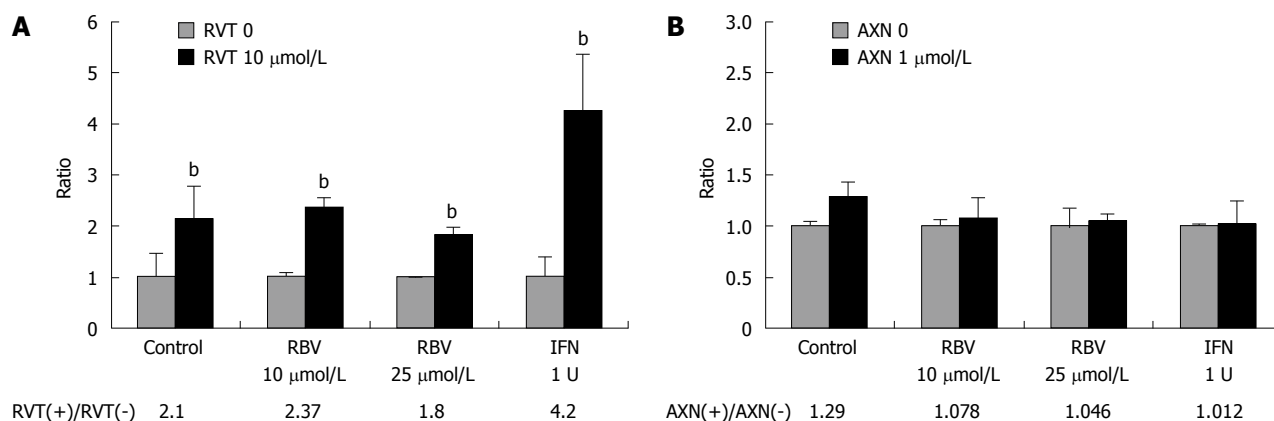
effects of RBV and IFN. Luciferase activity of OR6 cells 72 h after treatment with RBV or IFN was compared to treatment with RBV plus RVT or IFN plus RVT (Figure 6A). The effect of treatment with 10 μmol/L of RVT alone on OR6 cells showed a 2.1-fold increase of luciferase activity. The addition of RVT (10 μmol/L) to RBV- or IFN-treated cells reversed the anti-proliferative effect of RBV on HCV RNA even when the cells were treated with 25 μmol/L of RBV plus 1 U/mL of IFN, which was normally enough to reduce HCV RNA to 1/5 (Figure 5B). Ten μmol/L RVT upregulated luciferase activity in RBV- or IFN-treated OR6 cells around 2-fold



**Figure 4** The effect of resveratrol on subgenomic replicon cells, sKAH-5R. RVT was added to the cells for 72 h and luciferase activity was assayed as was indicated in Figure 2. A: Luciferase activity after addition of RVT; B: Cell viability after addition of RVT. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01.



**Figure 5** Sequential change of luciferase activity of OS6 cells after addition of interferon (IFN). A: IFN was added for the indicated duration and the ratio to the luciferase activity at time 0 was shown. Statistical significance (<sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01) is shown below: at 24 h 0 vs 1<sup>a</sup>, 2<sup>b</sup>, 4<sup>b</sup>, 10<sup>b</sup> and 20<sup>b</sup>; at 48 h, 0 vs 1<sup>b</sup>, 2<sup>b</sup>, 4<sup>b</sup>, 10<sup>b</sup> and 20<sup>b</sup>; 1 vs 2<sup>b</sup>, 4<sup>b</sup>, 10<sup>b</sup> and 20<sup>b</sup>; 20 vs 2<sup>b</sup>, 4<sup>b</sup> and 10<sup>b</sup>; at 72 h, 0 vs 1<sup>b</sup>, 2<sup>b</sup>, 4<sup>b</sup>, 10<sup>b</sup> and 20<sup>b</sup>; 1 vs 2<sup>a</sup>; 1 vs 4<sup>b</sup>, 10<sup>b</sup> and 20<sup>b</sup>; 2 vs 4<sup>b</sup>, 10<sup>b</sup> and 20<sup>b</sup>; 4 vs 10<sup>b</sup> and 20<sup>b</sup>; 10 vs 20<sup>b</sup>; B: Luciferase activity after addition of RBV and IFN for 72 h was shown as the ratio to that without antiviral agents. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01.

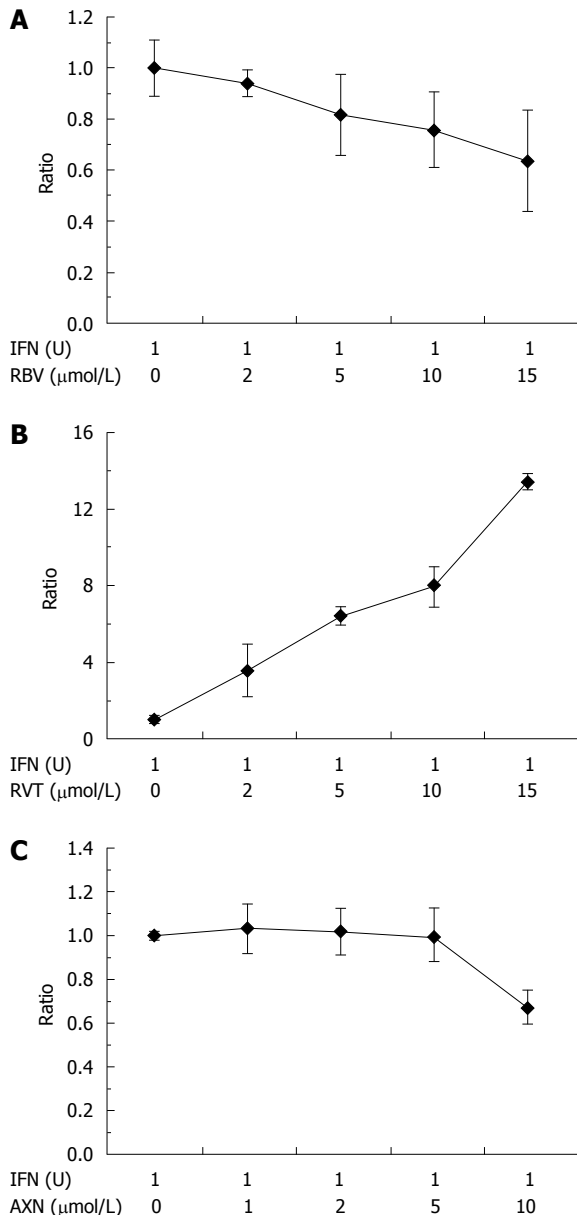


**Figure 6** The enhancing effect of RVT on replication of HCV RNA, even when treated with ribavirin (RBV) and IFN. RVT was simultaneously added to RBV or IFN. A: The ratio was calculated as the ratio of luciferase activity with RVT to that without RVT. <sup>b</sup>*P* < 0.01 vs RVT 0; B: The same procedure was applied in AXN.

and 4.2-fold, respectively (Figure 6A). On the other hand, AXN did not affect the effect of RBV and IFN (Figure 6B), indicating that AXN has no disadvantageous effect on antiviral activity of IFN and RBV.

This proliferative effect of RVT was further emphasized by comparison to the additive effects of RBV

with IFN. We compared the dose-dependent effect of RBV, RVT and AXN on the anti-proliferative effect of IFN (Figure 7). RBV (A), RVT (B) and AXN (C) were added to OS6 cells at concentrations of 0, 1, 2, 5, 10 or 15 μmol/L with 1 U/mL of IFN for 48 h. The ratio between luciferase activity of RBV-, RVT- or AXN-treated



**Figure 7** The comparative effect of RBV (A), RVT (B) and AXN (C) on the luciferase activity of IFN-treated cells. Cells were simultaneously treated with 1 U/mL of IFN and the indicated concentrations of RBV, RVT and AXN were added with IFN.

cells and that without co-treatment is shown. RBV further reduced the IFN-induced decrease in luciferase activity (Figure 7A), while RVT reversed this decrease, and further increased luciferase activity (Figure 7B). This effect was dose-dependent and it is noted that the enhanced ratio was strikingly large. On the other hand, AXN did not affect antiviral effect of IFN (Figure 7C).

## DISCUSSION

We have shown that RVT, a natural polyphenol contained in red wine and peanuts, enhanced *in vitro* replication of HCV RNA without producing significant proliferation of host cells. This is the first report demonstrating a proliferative effect of RVT on HCV. RVT inhibited

replication of HSV-1, HSV-2<sup>[25-27]</sup> human CMV<sup>[13]</sup>, Influenza A and orthomyxo virus<sup>[15]</sup>. Moreover, it was reported that RVT inhibited replication of HIV-1 synergistically with nucleoside analogues<sup>[28]</sup>. These reports suggest that RVT has a broad spectrum of anti-viral activities, and that RVT may selectively target the host, rather than the virus, as a mode of action for inhibiting viral replication. In spite of these inhibitory effects on viral replication, the mechanism of enhancing replication of HCV RNA is unclear. These results suggested that RVT is not suitable for antioxidant therapy of chronic hepatitis C. We also examined the effect of VE on replication of HCV RNA in OS6 cells, and VE enhanced its replication as effectively as RVT. On the other hand, AXN did not enhance replication of HCV RNA and had no effect on antiviral activity of IFN and RBV. These results indicated that we could recommend patients with chronic hepatitis C do not take RVT, especially when they receive antiviral therapy.

RVT is a non-flavonoid polyphenol and exerts anti-oxidative, anti-neoplastic and anti-inflammatory properties<sup>[11]</sup>. Moreover, RVT has received much attention as an agent for prolongation of lifespan by activating silent information regulator 2 proteins, or sirtuins<sup>[25]</sup>, which are implicated in influencing aging and regulating transcription, apoptosis and stress resistance<sup>[29]</sup>. These are causes for the popularity of this supplementation. Therapeutic intervention in liver injury with RVT has been suggested in various liver diseases<sup>[30]</sup>, such as alcohol-induced liver disease<sup>[31]</sup>, drug-induced liver injury<sup>[32]</sup>, ischemia-reperfusion injury<sup>[33]</sup>, and fatty liver diseases<sup>[11,34]</sup>. Furthermore, RVT has been implicated to be favorable for prevention of hepatic fibrosis<sup>[35,36]</sup>. These observations in combination with anti-viral effects indicated that RVT might be therapeutically beneficial or suitable for chronic hepatitis C. However, the direct effect of RVT on HCV RNA replication has not been studied thus far. In spite of our expectation, RVT did not inhibit replication of HCV, and on the contrary, it enhanced replication. Moreover, RVT hampered the anti-viral effect of IFN or RBV, and HCV RNA replication was enhanced even when enough concentration of IFN or RBV was administered to OR6 cells to reduce HCV replication. This condition was quite different from that observed in HIV-1 replication, in which the effect of RVT was synergistic with anti-viral effect of nucleotide analogues. Unlike RVT, AXN did not affect HCV replication and IFN-based antiviral activity, while it possesses strong antioxidant power.

An immunological response against virus-infected cells is an important pathogenic mechanism of chronic viral hepatitis. Reactive oxygen species (ROS) produced by activated macrophages and a consequent rise of lipid peroxidation cause direct activation of hepatic stellate (Ito) cells, leading to hepatic fibrosis and cirrhosis<sup>[37]</sup>. Moreover, HCV core protein directly increases ROS as well as lipid peroxidation products and antioxidant gene expression<sup>[38]</sup>. HCV infection is also associated with liver

iron accumulation<sup>[39]</sup>, which further produces ROS in the liver. These observations suggested that anti-oxidant therapy has an important role in slowing disease progression to cirrhosis in chronic hepatitis C. In consequence of this theory, the use of CAM is common in patients with chronic liver disease<sup>[8]</sup>. Liu *et al.*<sup>[40]</sup> reviewed medical herbs for HCV infection and concluded that some agents may have an effect on liver enzymes, but there is no firm evidence supporting efficacy of CAM. However, few studies have investigated the effect of antioxidants on HCV itself. Yano *et al.*<sup>[24]</sup> investigated the effect of ordinary nutrients on HCV RNA replication using the replicon system, and found that some antioxidants such as  $\beta$ -carotene, vitamin D<sub>2</sub> and linoleic acid inhibited replication. They also showed an effect of VE on HCV RNA replication that was the same as in our study. In our study, AXN did not affect replication of HCV RNA. Thus, there is a group of antioxidants which inhibit replication of HCV, while there is another group of antioxidants which enhance its replication. The precise mechanism of this difference has not been clarified, but the investigation of this mechanism may provide new insights into anti-viral mechanisms. Recently it has been demonstrated that anti-HCV nutrients induce activation of the MEK-ERK1/2 signaling pathway through phosphorylation of ERK1/2<sup>[41]</sup>. Study of this phenomenon may provide clues for a new therapeutic strategy in anti-viral treatment of HCV.

RVT has been shown to have a large number of regulatory biological functions, and Docherty *et al.*<sup>[25-27]</sup> extensively studied the mechanism by which RVT inhibits the replication of HSV. However, even though it has been extensively studied, the molecular mechanism of RVT's action is not clear. Our results were quite different from those of Docherty's. In our study, not all antioxidants but 2 of 3 antioxidants increased the replication of HCV suggesting that the molecular mechanism of each agent is likely variable depending on viruses when we speculate in combination with studies of Docherty *et al.*<sup>[25-27]</sup> and Yano *et al.*<sup>[24]</sup>. On the other hand, reports suggesting a correlation between HCV replication and lipid metabolism have accumulated recently. It has been demonstrated that the cellular lipid droplet is an important structure for replication or assembly of viral components of HCV, especially HCV core protein<sup>[42]</sup>. The inhibitory effect of 3-hydroxyl-3-methylglutaryl coenzyme A reductase inhibitors on HCV have also been reported<sup>[43]</sup>. Moreover, the success of peg-IFN plus RBV combination therapy, that resulted in the disappearance of HCV, affected lipid metabolism thereafter *in vivo*<sup>[44]</sup>. Thus, it is conceivable that HCV genomic structure as well as the intracellular lipid is indispensable for viral replication of HCV. It is thought that RVT and VE affect intracellular lipid metabolism because they are lipid-soluble antioxidants. It is also interesting that HCV itself produces ROS, and that antioxidants affect the replication of HCV.

Bechmann *et al.*<sup>[45]</sup> recently demonstrated that RVT

in response to free fatty acid administration deteriorates fibrogenic activation of human hepatic stellate cells. They showed that RVT upregulated the expression of key mRNAs associated with activated, fibrogenic stellate cells, and also demonstrated that the combined presence of free fatty acids and RVT significantly reduced the hepatic stellate cells' susceptibility to apoptosis. This report was controversial since previous reports<sup>[35,36]</sup> demonstrated favorable effects of RVT on prevention of fibrosis progression. Bechmann *et al.*<sup>[45]</sup> pointed out that the concentration of RVT was different from the previous study, and species' differences (employing rat *vs* human hepatic stellate cells) might be significant. Thus, RVT may have different therapeutic effects at various concentrations, and further investigation is needed to clarify a role of RVT in chronic liver diseases. Their result also suggested that patients with chronic hepatitis C should not take RVT as an additive nutrient, especially when they receive IFN-based antiviral therapy. Further investigations focusing on the enhancing mechanism of RVT on HCV RNA and different responses between RVT and AXN is necessary, and these approaches may develop a new strategy of anti-HCV agents.

In conclusion, we recommend patients with chronic hepatitis C who receive IFN-based antiviral therapy not to take RVT as an antioxidant supplement, although AXN may not affect anti-viral therapy.

## COMMENTS

### Background

Antiviral therapy for chronic hepatitis C has been developing, but the current standard therapy with pegylated interferon (Peg-IFN) and ribavirin combination therapy for 12 mo has achieved around 50% of patients who are infected with genotype 1 hepatitis C virus (HCV). Patients who have not attained viral clearance tend to take several supplementations for this chronic disease with an expectation for retardation of disease progression. A previous survey found that about 40% of patients of liver disease outpatient clinics in the US used complementary and alternative medicine (CAM) at least once during the preceding month. Among CAM, antioxidants have been popularly used by patients with chronic hepatitis C because it is said that oxidative stress deteriorates chronic hepatitis. However, the information about the use of supplementations for chronic hepatitis C was insufficient.

### Research frontiers

Resveratrol (RVT) was discovered to be a strong activator of *sirtuin*, a gene for longevity, and the most important polyphenol responsible for the beneficial effects of red wine, which has been called the "French Paradox". RVT showed a strong antioxidative effect on cardioprotection, anti-atherosclerosis and relaxation of vascular endothelium through nitric oxide release. This information resulted in the popularity of this supplementation for people who suffered from chronic diseases. Since RVT inhibits the replication of other viruses, it is thought that RVT also inhibits HCV replication. However they revealed RVT enhanced HCV replication.

### Innovations and breakthroughs

The investigation on HCV replication has been enabled by using the replicon system, in which HCV RNA replicates but unfortunately viral particles were not produced. Recently, the cell culture system in which HCV particles are produced was developed by Dr. Wakita T, and many new insights of HCV virology have been discovered. This study focused on the effect of taking daily supplementations on viral replication and antiviral therapy of HCV.

### Applications

The authors recommend patients with chronic hepatitis C who receive IFN-



based antiviral therapy not to take RVT as an antioxidant supplement, although astaxanthin (AXN) may not affect anti-viral therapy.

### Peer review

In this study, Nakamura *et al* tried to show the efficacy of antioxidants, RVT and AXN, on HCV replication. Since RVT inhibits the replication of other viruses, it was thought that RVT also inhibited HCV replication. However they revealed RVT enhanced HCV replication. These results are very interesting.

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## Hepatopoietin Cn suppresses apoptosis of human hepatocellular carcinoma cells by up-regulating myeloid cell leukemia-1

Jing Chang, Yang Liu, Dong-Dong Zhang, Da-Jin Zhang, Chu-Tse Wu, Li-Sheng Wang, Chun-Ping Cui

Jing Chang, Dong-Dong Zhang, Da-Jin Zhang, Chu-Tse Wu, Li-Sheng Wang, Chun-Ping Cui, Department of Experimental Hematology, Beijing Institute of Radiation Medicine, 27 Taiping Road, Beijing 100850, China

Yang Liu, Chu-Tse Wu, School of Chemical Engineering and Technology, Tianjin University, Weijin Road #92, Nankai District, Tianjin 300072, China

**Author contributions:** Chang J, Liu Y and Zhang DD contributed equally to this work and performed the majority of experiments; Zhang DJ, Wang LS and Wu CT provided the vital reagents and analytical tools and were also involved in editing the manuscript; Cui CP designed the study and wrote the manuscript. **Supported by** (in part) Grants From the National Natural Science Foundation of China, No. 30800558 and No. 30930041 and the Chinese Major Special Science & Technology Project for Development of Major New Drugs, No. 2009ZX09103-617 **Correspondence to:** Dr. Chun-Ping Cui, Department of Experimental Hematology, Beijing Institute of Radiation Medicine, 27 Taiping Road, Beijing 100850, China. [cui\\_chunping2000@yahoo.com.cn](mailto:cui_chunping2000@yahoo.com.cn)

Telephone: +86-10-68158312 Fax: +86-10-68158311

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### Abstract

**AIM:** To investigate the role of hepatopoietin Cn (HPPCn) in apoptosis of hepatocellular carcinoma (HCC) cells and its mechanism.

**METHODS:** Two human HCC cell lines, SMMC7721 and HepG2, were used in this study. Immunostaining, Western blotting and enzyme linked immunosorbent assay were conducted to identify the expression of HPPCn and the existence of an autocrine loop of HPPCn/HPPCn receptor in SMMC7721 and HepG2. Apoptotic cells were detected using fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide.

**RESULTS:** The HPPCn was highly expressed in human HCC cells and secreted into culture medium (CM). FITC-labeled recombinant human protein (rhHPPCn) could specifically bind to its receptor on HepaG2 cells. Treatment with 400 ng/mL rhHPPCn dramatically increased the viability of HCC-derived cells from 48.1% and 36.9% to 85.6% and 88.4%, respectively ( $P < 0.05$ ). HPPCn silenced by small-interfering RNA reduced the expression and secretion of HPPCn and increased the apoptosis induced by trichostatin A. Additionally, HPPCn could up-regulate the expression of myeloid cell leukemia-1 (Mcl-1) in HCC cells *via* mitogen-activated protein kinase (MAPK) and sphingosine kinase-1.

**CONCLUSION:** HPPCn is a novel hepatic growth factor that can be secreted to CM and suppresses apoptosis of HCC cells by up-regulating Mcl-1 expression.

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**Key words:** Hepatopoietin Cn; Autocrine; Hepatocellular carcinoma; Apoptosis; Myeloid cell leukemia-1

**Peer reviewers:** Yukihiro Shimizu, MD, PhD, Kyoto Katsura Hospital, 17 Yamada-Hirao, Nishikyo, Kyoto 615-8256, Japan; Ezio Laconi, MD, PhD, Professor of General Pathology, Department of Sciences and Biomedical Technologies, Unit of Experimental Pathology, University of Cagliari, Via Porcell, 4 - IV Piano, 09125 - Cagliari, Italy; Qin Su, Professor, Department of Pathology, Cancer Hospital and Cancer Institute, Chinese Academy of Medical Sciences and Peking Medical College, PO Box 2258, Beijing 100021, China

Chang J, Liu Y, Zhang DD, Zhang DJ, Wu CT, Wang LS, Cui CP. Hepatopoietin Cn suppresses apoptosis of human hepatocellular carcinoma cells by up-regulating myeloid cell leukemia-1. *World J Gastroenterol* 2010; 16(2): 193-200 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i2/193.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i2.193>

## INTRODUCTION

Hepatocellular carcinoma (HCC) represents the fifth-most prevalent malignant disease affecting humans worldwide with an increasing incidence<sup>[1]</sup>. There is evidence that the protumorigenic growth factor signaling is dysregulated in human HCC affecting different signaling systems, such as insulin-like growth factor, hepatocyte-growth factor (HGF), transforming-growth factor  $\alpha$  (TGF- $\alpha$ )/epidermal-growth factor (EGF), and TGF- $\beta$ <sup>[2]</sup>.

Hepatopoietin Cn (HPPCn) is a novel hepatic growth factor derived from a hepatocyte-stimulating substance, which was first reported by LaBrecque in 1975<sup>[3,4]</sup>. It has been shown that the HPPCn mRNA level increases in partially hepatectomized mice following liver injury<sup>[5]</sup>. Recombinant human protein (rhHPPCn) specifically stimulates cell proliferation in primary cultures of hepatocytes and HCC-derived cell lines (HepG2, SMMC7721) *in vitro* as well as liver regeneration following partial hepatectomy *in vivo*. In addition, rhHPPCn can protect hepatocytes against ethanol-induced injury<sup>[6]</sup>.

Investigations of HPPCn have been mainly restricted to its remarkable activity in stimulating liver regeneration. However, its mechanism and potential effect on HCC are unclear. In this study, HPPCn was highly expressed in cytoplasm and nuclei of human HCC cells and secreted into the culture medium (CM), and fluorescein isothiocyanate (FITC)-labeled rhHPPCn could specifically bind to its receptor on HepG2 cells, suggesting that there is an autocrine loop of HPPCn/HPPCn receptor in HCC-derived cell lines. Furthermore, exogenous rhHPPCn suppressed trichostatin A (TSA)-induced apoptosis of HCC cells and up-regulated myeloid cell leukemia-1 (Mcl-1) expression in HCC-derived cells *via* the mitogen-activated protein kinase (MAPK) or sphingosine kinase-1 (SPK1).

## MATERIALS AND METHODS

### Cell lines and cell culture

Two human HCC cell lines, SMMC7721 and HepG2, were used in this study. Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma, Saint Louis, MO, USA) supplemented with 10% fetal calf serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin.

### Antibodies and other reagents

Recombinant human HPPCn and anti-HPPCn sera were produced as previously described<sup>[5]</sup>. Primary antibodies, including those against Mcl-1, phos-Erk1/2 (tyr204), phos-Stat3, non-activated Erk1/2, or Stat3, were obtained from Santa Cruz Company (Santa Cruz, CA, USA). Other reagents used in this study were monoclonal anti-mouse/goat/rabbit peroxidase conjugate (Sigma, St. Louis, MO), enhanced chemiluminescence (ECL)

kit, and 3, 3', 5, 5' tetramethylbenzidine (TMB) substrate (Biozol, Eching, Germany).

### Immune staining

Liver tissue samples were fixed with 4% (w/v) freshly prepared paraformaldehyde and cut into 4  $\mu$ m-thick sections with a vibratome (Leica VT1000S; Germany). Non-specific protein binding sites were blocked using 2% bovine serum albumin in phosphate-buffered saline (PBS) for 1 h, and anti-HPPCn serum in a blocking solution was incubated overnight at 4°C in a humidified chamber. The sections were incubated with peroxidase-labeled secondary antibody, treated with 3,3'-diaminobenzidine and hydrogen peroxide, and observed under a microscope.

### Enzyme linked immunosorbent assay (ELISA)

Cells ( $2 \times 10^6$ ) were seeded in a 75 cm<sup>2</sup> tissue culture flask, cultured for 8 h, washed 3 times with a serum-free medium, and cultured for an additional 30 h in 10 mL of a serum-free medium. The supernatant liquid was used as a conditioned medium. To prepare a concentrated conditioned medium, the CM was concentrated to a final volume of 1 mL by ultra-filtration (Millipore, USA) with a 5000-MW-cut-off<sup>[7]</sup>.

In order to detect HPPCn protein in CM, 100  $\mu$ L of the harvested CM was dispensed in a 96-well ELISA plate and incubated overnight at 4°C. The ELISA plate was washed 3 times with PBS, incubated for 2 h with an anti-HPPCn antibody, washed with PBS and then with secondary antibody, incubated for 2 h followed by an additional 15 min in a TMB-substrate. Color intensity was measured with an ELISA-Reader (Dynatech Laboratories, Frankfurt, Germany).

### Western blotting

Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The nitrocellulose membrane was blocked with a 5% solution of non-fat milk in TBS containing 0.1% Tween 20 (TBST), incubated with primary antibody in 5% bovine serum albumin containing TBST at 4°C overnight, washed 3 times with TBST and incubated with secondary antibody in 5% milk containing TBST. After washed 5 times with TBST, the membrane was developed using the ECL method.

### Cell-binding assay

FITC-labeled rhHPPCn was prepared by incubating FITC (Sigma, St. Louis, MO) with recombinant protein (1/20, w/w) in a borate buffer (0.05 mol/L Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>, pH 9.3) at 4°C for 12 h, followed by purification through a Sephadex G-25 column, according to its manufacturer's instructions (Pharmacia Biotech, Alameda, CA)<sup>[8,9]</sup>.

For the analysis of rhHPPCn binding to HepG2, cells ( $2 \times 10^3$ – $4 \times 10^5$ ) were stained with FITC-labeled rhHPPCn in RPMI with 1% BSA for 30 min at 37°C, washed with PBS containing 1% BSA to remove unbound proteins, resuspended and analyzed by flow cytometry



using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). Cells on the flow cytometer were gated by forward *vs* side scatter to eliminate dead cells.

### Apoptosis assay

Apoptotic cells were detected using FITC-conjugated Annexin V (Caltag Laboratories, Burlingame, CA, USA) and propidium iodide (PI). Cells were washed twice with cold PBS and resuspended in an Annexin V-binding buffer containing 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 140 mmol/L NaCl, and 5 mmol/L CaCl<sub>2</sub> at a concentration of  $1 \times 10^6$  cells/mL. The suspension (100  $\mu$ L containing  $1 \times 10^5$  cells), 5  $\mu$ L of Annexin V-FITC and 10  $\mu$ L of PI were added into a 5-mL culture tube. The tube was gently vibrated and incubated for 15 min in the dark at room temperature. After a binding buffer (400  $\mu$ L) was added into the tube, the cells were analyzed by flow cytometry.

### RNA interference

Small interfering RNA (siRNA) for human HPPCn was synthesized by Shanghai Gene Chemical Company (Shanghai, China). The sequences employed in silencing human HPPCn are sense: 5'-CGAACCUCACGCAUCAAAATT-3' and antisense: 5'-UUUAGAUGCGUGAGGUUCGGA-3'. The sequences employed for non-silencing human HPPCn are sense: 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense: 5'-ACGUGACACGUUCGGAGAATT-3'. The HepG2 and SMMC7721 cells were transfected with siRNA using 1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide-cholesterol (Invitrogen, San Diego, CA, USA) as recommended by its manufacturer.

### Sphingosine (Sph) kinase activity assay

Cells were lysed by freeze-thawing (3 times). The lysates were cleared by centrifugation at 12000 *g* for 30 min at 4°C. The protein concentration of cell lysates was measured by bicinchoninic acid assay (Pierce, Rockford, IL, USA). The SPK activity was assayed by incubating the cell lysates with Sph (Sigma, St Louis, MO, USA) and <sup>32</sup>P-ATP (10  $\mu$ Ci, 50 mmol/L) for 30 min at 37°C. The products were separated by thin-layer chromatography on silica gel G60 (Merck KGaA, Darmstadt, Germany) using 1-butanol/methanol/acetic acid/water (80:20:10:20) and observed by autoradiography<sup>[10]</sup>.

### Statistics analysis

Results were presented as mean  $\pm$  SD of three independent experiments. The data were analyzed by unpaired Student's *t* test using SPSS version 10.0 (SPSS Inc, USA). *P* < 0.05 was considered statistically significant.

## RESULTS

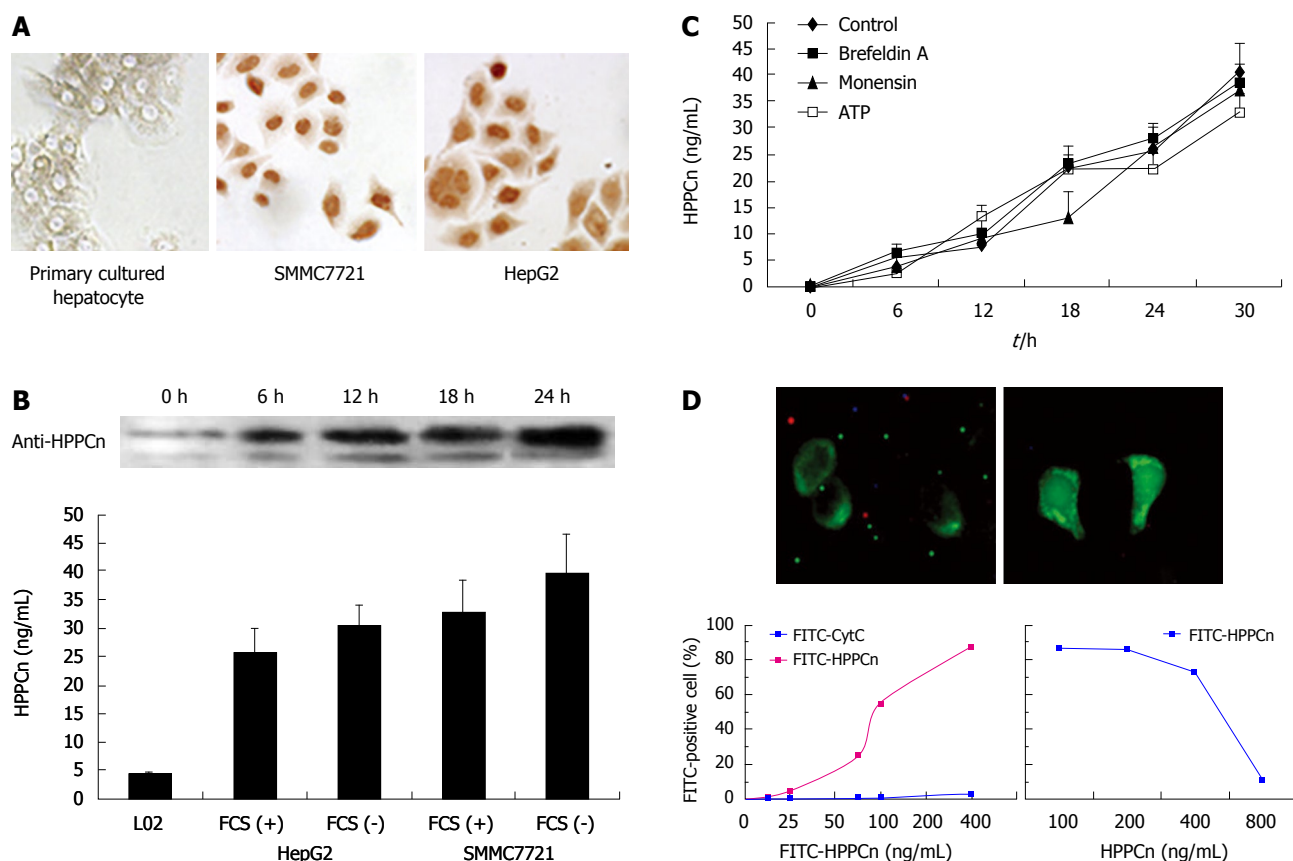
### HPPCn acted as an autocrine factor for HCC cells *in vitro*

Sequence analysis revealed that HPPCn was a nuclear factor and a member of the leucine-rich acidic nuclear

protein family. Since extracellular HPPCn can act as an autocrine factor for HCC cells *in vitro*, we studied its production, secretion, and mechanism of action. The intracellular localization of HPPCn was observed in human HCC cells with immune staining (Figure 1A). The HCC cells (HepG2 and SMMC7721) were strongly stained for HPPCn and the staining was mostly restricted to nuclei and cytoplasm. However, in primarily cultured hepatocytes, the HPPCn staining was weak and mainly restricted to cytoplasm of HCC cells. To determine whether HPPCn is secreted by HCC cells, we analyzed their CM by ELISA and Western blotting, which showed that HPPCn protein was released into CM by HepG2 and SMMC7721 (Figure 1B). The kinetics of HPPCn secretion by HCC cells is shown in Figure 1C. The HPPCn secretion, first detected at 6 h following culture, increased even at 18 and 30 h following culture, suggesting that brefeldin A and monensin, inhibitors of the endoplasmic reticulum (ER)-Golgi pathway, do not affect the HPPCn secretion<sup>[11]</sup>. The secretion of HPPCn triggered by ATP is negligible<sup>[12,13]</sup>. To eliminate the possibility of its leakage (as opposed to secretion) from HCC cells, we assessed the cellular damage with trypan blue staining, which showed that over 99% of the cells were still alive (data not shown), indicating that accumulation of HPPCn in the CM of those cells is caused by its secretion rather than by its leakage from HCC cells. To test the binding of HPPCn to HCC cells, HepG2 cells were incubated with different concentrations of FITC-labeled rhHPPCn and cytochrome C (Cyt-C) at 37°C for 1 h and analyzed by flow cytometry. Fluorescence was found in membrane of HepG2 cells incubated with FITC-labeled HPPCn (Figure 1D) but not in membrane of those incubated with Cyt-C control (results not shown). Furthermore, HCC cells binding to FITC-labeled HPPCn and the density of the bound rhHPPCn tended to reach a plateau when the concentration of FITC-labeled HPPCn was higher than 400 ng/mL (Figure 1D), indicating that the binding of HCC cells to FITC-labeled HPPCn is saturated. In addition, we assessed the reversibility of bound HPPCn with a competitive inhibition curve (Figure 1D), which showed that approximately 30% of HCC cells were bound to FITC-labeled HPPCn following incubation in the presence of 100 ng/mL of unlabeled HPPCn and approximately 10% of HCC cells were bound to FITC-labeled HPPCn when the concentration of unlabeled rhHPPCn was higher than 800 ng/mL, indicating that HCC cells can express and secrete HPPCn and HPPCn can bind to HCC cells in a specific, saturated, and reversible manner and that an autocrine loop of HPPCn/HPPCn receptor is existed in HCC cells.

### HPPCn suppressed apoptosis of HCC cells

HPPCn showed its effect on TSA-induced apoptosis of HCC cells. TSA was initially characterized by an anti-fungal drug and strongly inhibited TSA activity at nanomolar level<sup>[14]</sup>. It has been demonstrated that TSA induces apoptosis of human HCC cell lines (HepG2, Hep3B, and Huh-7) by inducing acetylation of p53 and



**Figure 1** Hepatopoietin Cn (HPPCn) acting as an autocrine factor for hepatocellular carcinoma (HCC) cells *in vitro*. A: Immunohistochemistry showing staining with anti-HPPCn antibody in primarily cultured hepatocytes and HCC cells; B: Western blotting and enzyme-linked immunosorbent assay (ELISA) showing the secretion of HPPCn into culture medium (CM) of HCC-derived cells; C: ELISA showing the kinetics of HPPCn secretion from HCC cells; D: Binding of HPPCn to HCC cells.

histones (H2A, H2B, H3, and H4)<sup>[15-18]</sup>, and inhibits HGF-induced invasion of HepG2 cells but does not affect increased phosphorylation of Erk and Akt in HGF-treated HepG2 cells<sup>[19]</sup>.

The viability of HepG2, SMMC7721 and L02 cells was 48.11%, 36.9% and 31.9%, respectively, 24 h after treatment with 300 nmol/L of TSA (Figure 2A), and increased to 85.6% and 88.4%, respectively, in HCC-derived cells after treatment with 400 ng/mL rhHPPCn ( $P < 0.02$ ). In normal hepatocytes, the protective effect of HPPCn was moderate. HPPCn silenced by small-interfering RNA reduced the expression and secretion of HPPCn (Figure 2B and C) and enhanced TSA-induced apoptosis of HCC cells (Figure 2D), indicating that exogenous HPPCn suppresses apoptosis of HCC cells while HPPCn silenced by siRNA increases the apoptosis of HCC cells.

#### HPPCn induced Mcl-1 expression in HCC cells via SPK/S1P and MEK/ERK pathways

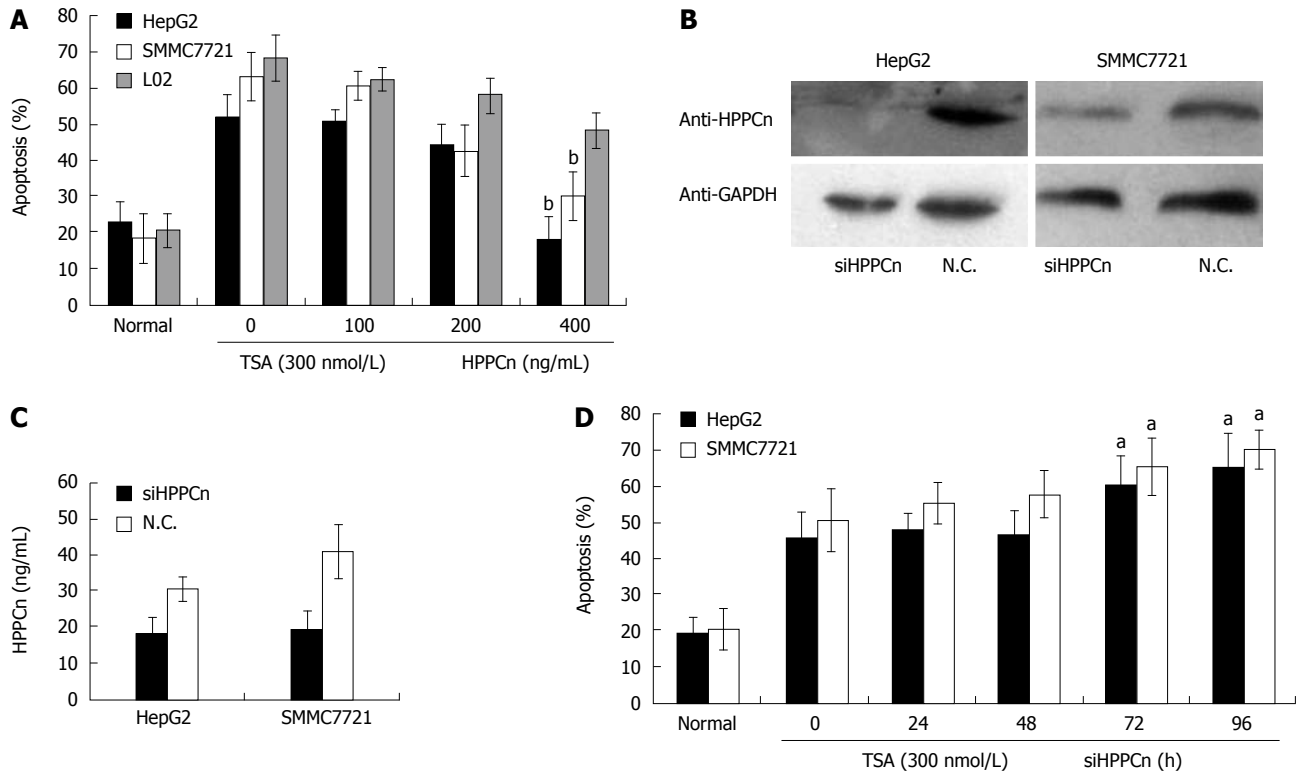
It has been recently demonstrated that Mcl-1, an anti-apoptotic member of the Bcl-2 protein family that interferes with mitochondrial activation, plays a role in the survival of HCC cells<sup>[20]</sup>. The specific up-regulation of Mcl-1 expression in HCC cells could inhibit HCC cell apoptosis induced by chemotherapeutic drugs including histone deacetylase inhibitors<sup>[21]</sup>. However,

Mcl-1 knockdown significantly augments the apoptosis sensitivity of HCC cells toward chemotherapy in combination with PI3K inhibitors<sup>[22,23]</sup>. In addition, certain growth factors (such as HGF and EGF) can induce Mcl-1 expression in HCC cells by activating the PI3K/Akt and MEK/ERK pathways<sup>[24,25]</sup>.

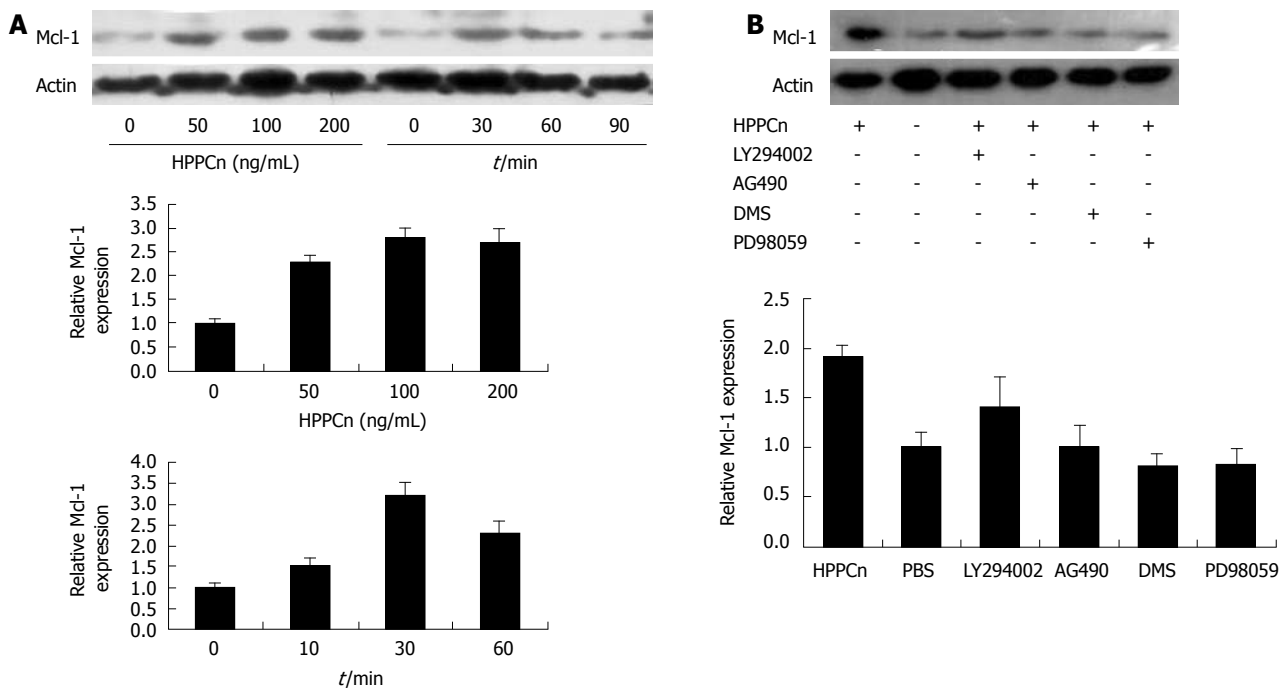
In order to test whether HPPCn influences Mcl-1 expression in HCC cells, HepG2 cells were treated with rhHPPCn at different concentrations for different periods of time. HPPCn induced Mcl-1 expression in a dose- and time-dependent manner (Figure 3A). Furthermore, treatment with rhHPPCn activated the SPK, Stat3, and ERK pathways in HepG2 cells (Figure 4). Thereafter, we determined if the blockage of these pathways can inhibit HPPCn-induced Mcl-1 expression. Cells were pre-incubated with LY294002, PD98059 AG490, or DMS for 1 h, and treated with HPPCn for an additional 3 h. Kinase inhibited by specific inhibitors abrogated the effect of HPPCn on Mcl-1 expression (Figure 3B), demonstrating that HPPCn can activate the signaling pathways involved in survival of HCC cells and up-regulate Mcl-1 expression *via* the MAPK and SPK pathways in HCC-derived cells.

## DISCUSSION

HPPCn is a novel hepatic growth factor with a specific



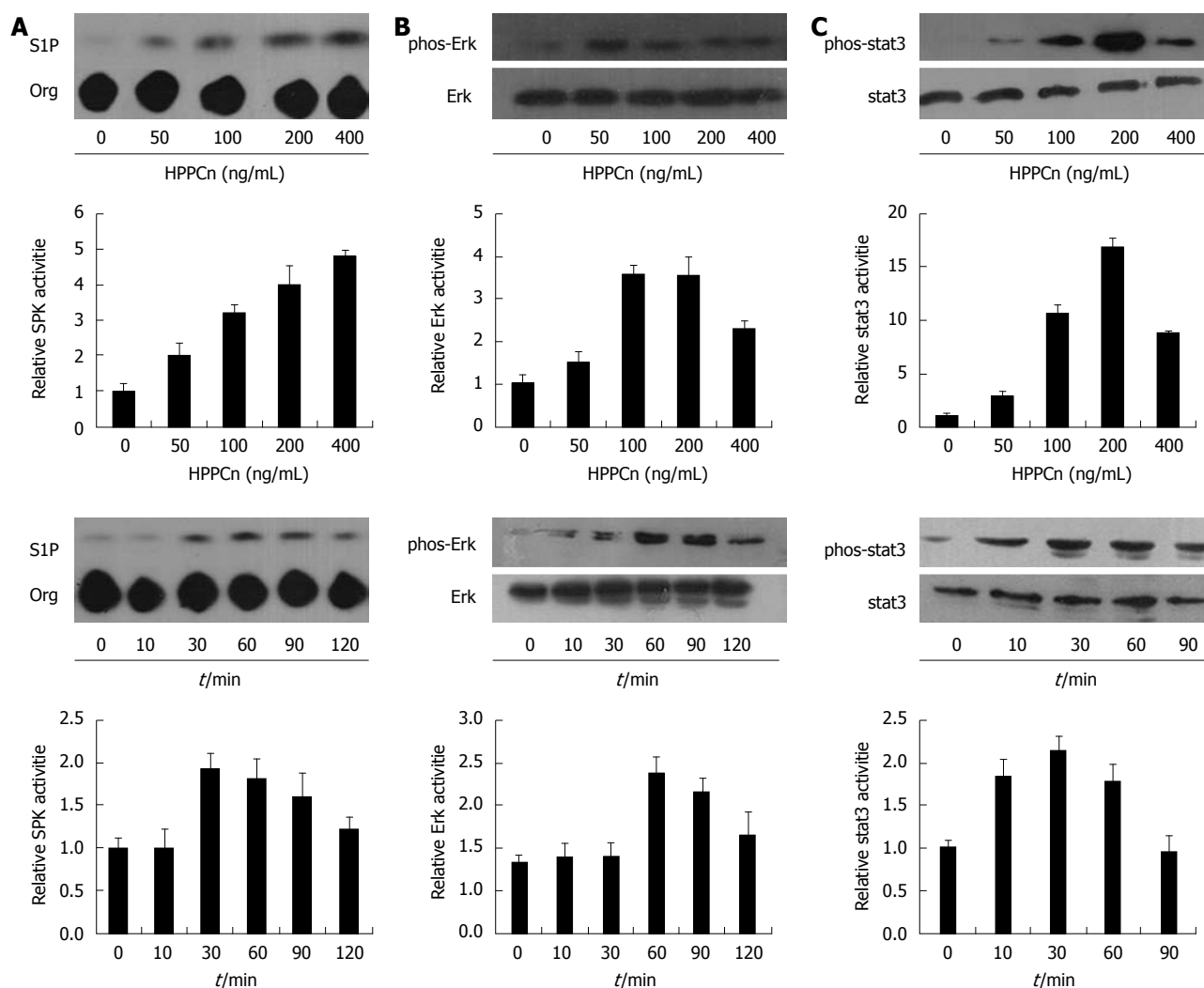
**Figure 2** HPPCn suppressing apoptosis of HCC-derived cells. **A**: Flow cytometry showing dose-dependent suppressing activity of recombinant human protein (rhHPPCn) on apoptosis of HCC cells incubated with 300 nmol/L trichostatin A (TSA) for 24 h prior to treatment with indicated concentrations of rhHPPCn; **B**: Western blotting showing hybridization to anti-HPPCn antibody and anti-GAPDH in HepG2 and SMMC7721 transfected with specific siRNA; **C**: ELISA showing the secretion of HPPCn into CM of HepG2 and SMMC7721; **D**: Flow cytometry showing the percentages of apoptotic cells incubated with 300 nmol/L TSA for 24 h prior to transfection with specific siRNA for an indicated period of time. The data are expressed as mean  $\pm$  SD of 3 independent experiments. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs group without HPPCn/siHPPCn treatment. N.C.: Negative control.



**Figure 3** HPPCn inducing Mcl-1 expression via the MEK/ERK and SPK/S1P pathways. **A**: HPPCn induces Mcl-1 expression in a dose- and time-dependent manner and the densities of signals were determined by densitometry; **B**: Western blotting showing hybridization to anti-Mcl-1 antibody and anti- $\beta$ -actin in HepG2 after the cells were treated with HPPCn for 3 h prior to pre-incubation with DMS, PD98059, LY294002, or AG490 for 1 h.

proliferation stimulating activity and suppresses apoptosis of HCC cells as an autocrine factor. In this study, the

potential nuclear localization sequences and immune staining suggested that HPPCn was a nuclear factor and



**Figure 4** Effects of rhHPPCn on the activities of SPK (A), Jak-Stat3 (B), and Erk1/2 (C). HepG2 cells were treated for 30 min with the indicated concentration of rhHPPCn and 200 ng/mL rhHPPCn for the indicated periods of time. The phosphorylation of Stat3 and Erk1/2 were determined by Western blotting with antibodies against both species of Stat3 and Erk1/2. The densities of signals were determined by densitometry.

localized in nuclei of HCC cells. ELISA and Western blotting showed that the secretion of HPPCn into CM was not influenced by brefeldin A or monensin, two inhibitors of ER-Golgi pathway. Furthermore, FITC-labeled rhHPPCn binding to HCC cells was specific, saturated and reversible, suggesting that HPPCn acts as an autocrine growth factor in HCC cells *in vitro*.

It has been reported there are some autocrine loops in HCC cells, such as basic fibroblast growth factor (bFGF)/flg, TGF- $\alpha$ /erbB2<sup>[26-28]</sup>, and augments of liver regeneration (ALR)<sup>[7]</sup>. However, the action of bFGF and TGF- $\alpha$  is neither hepatocyte-specific nor liver-specific. It has been shown that ALR protects hepatocytes in Eck's fistula but not cultured hepatocytes and hepatic cell lines against atrophy<sup>[29,30]</sup>. Thus, identification of HPPCn autocrine mechanism in HCC cells might elucidate the pivotal process of hepatic carcinogenesis.

It is interesting to note that HPPCn resembles HCC-derived growth factor (HDGF) and high-mobility group box 1 protein (HMGB1). Extracellular HDGF can stimulate the proliferation of cultured hepatoma cells,

fibroblasts, smooth muscle cells, and endothelial cells. However, it has been reported that nuclear localization is a prerequisite for the mitogenic activity of intracellular HDGF<sup>[31,32]</sup>. Similarly, as a nuclear protein, HMGB1 promotes the interaction between DNA and other proteins, and regulates several families of DNA-binding proteins. It has been reported that HMGB1 relocalizes in LPS-activated monocytes from nuclei to secretory organelles, and activates endothelial cells, promotes angiogenesis, and initiates inflammation as a cytokine<sup>[13,33]</sup>. Another similarity between HPPCn, HMGB1, and HDGF is their secondary structure. Motifs within their N-terminal domains, referred to as LRR, HMG box, and HATH, are involved in protein-protein interaction. In addition, these proteins contain 2 or 3 nuclear localization sequences but lack of any signal peptide-like hydrophobic region, indicating that HPPCn is a bifunctional nuclear protein and acts as a growth factor for cells<sup>[34,35]</sup>.

HPPCn activates signaling pathways involved in survival of HCC cells and up-regulates Mcl-1 expression *via* MAPK, SPK1 in HCC cells. It has been recently



demonstrated that Mcl-1, an anti-apoptotic member of the Bcl-2 protein family which interferes with mitochondrial activation, plays a role in the survival of HCC cells. Furthermore, some growth factors, including HGF and EGF, induce Mcl-1 expression in HCC cells by activating the PI3K/Akt and MEK/ERK pathways contributing to the poor prognosis of HCC patients, suggesting that Mcl-1 is one of the important molecules involved in HPPCn action.

In conclusion, HPPCn is a novel hepatic growth factor that can be secreted to CM and suppresses apoptosis of HCC cells by up-regulating Mcl-1 expression.

## COMMENTS

### Background

There is evidence that protumorigenic growth factor signaling is dysregulated in human hepatocellular carcinoma (HCC) affecting different signaling systems such as insulin-like growth factor, hepatocyte-growth factor, transforming-growth factor  $\alpha$  (TGF- $\alpha$ )/epidermal-growth factor, and TGF- $\beta$ .

### Research frontiers

Hepatopoietin Cn (HPPCn) is a novel hepatic growth factor derived from a hepatocyte-stimulating substance (HSS). Recombinant human protein (rhHPPCn) specifically stimulates cell proliferation in primary culture of hepatocytes and HCC-derived cell lines (HepG2, SMMC7721) *in vitro* as well as liver regeneration following partial hepatectomy *in vivo*. In addition, rhHPPCn has been demonstrated to be capable of protecting hepatocytes against ethanol-induced injury.

### Innovations and breakthroughs

In this study, HPPCn was highly expressed in cytoplasm and nuclei of human HCC cells and secreted into the culture medium. Fluorescein isothiocyanate-labeled rhHPPCn could specifically bind to its receptor on HepaG2 cells, suggesting that an autocrine loop of HPPCn/HPPCn receptor is existed in HCC-derived cell lines. Exogenous rhHPPCn suppressed trichostatin A-induced apoptosis of HCC cells. Moreover, HPPCn up-regulated the expression of myeloid cell leukemia-1 (Mcl-1) in HCC-derived cells via the mitogen-activated protein kinase or sphingosine kinase-1.

### Applications

The mechanism of HPPCn underlying liver regeneration the authors observed in this contributes to the diagnosis and treatment of liver diseases.

### Terminology

HSS: A hepatic stimulator substance isolated from rat liver and human fetal liver can increase synthesis of hepatic DNA *in vivo* and *in vitro* as shown by 3H-TdR incorporation. However, its composition still remains unknown.

### Peer review

The manuscript written by Chang *et al* describes autocrine growth of human HCC cells inhibited by HPPCn. The study showed that HPPCn could suppress apoptosis of HCC cells by up-regulating the expression of Mcl-1 and by activating the PI3K/AKT and MEK/ERK pathways, suggesting that HPPCn plays an important role in the hepatocarcinogenesis. The data are interesting and the experiments are well organized.

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## Rupintrivir is a promising candidate for treating severe cases of Enterovirus-71 infection

Xiao-Nan Zhang, Zhi-Gang Song, Ting Jiang, Bi-Sheng Shi, Yun-Wen Hu, Zheng-Hong Yuan

Xiao-Nan Zhang, Zhi-Gang Song, Ting Jiang, Yun-Wen Hu, Zheng-Hong Yuan, Research Unit, Shanghai Public Health Clinical Center, Caolang Road 2901, Shanghai 201508, China  
Bi-Sheng Shi, Zheng-Hong Yuan, Key Laboratory of Medical Molecular Virology, Shanghai Medical College, Fudan University Dongan Road 130, Shanghai 200032, China

**Author contributions:** Zhang XN performed the majority of experiments and molecular simulations; Song ZG provided cDNA samples of EV71 infected patients; Jiang T performed phylogenetic analyses; Shi BS performed the cloning and sequencing of EV71 3C protease fragments; Hu YW and Yuan ZH coordinated and helped design the project.

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**Correspondence to:** Zheng-Hong Yuan, Professor, Research Unit, Shanghai Public Health Clinical Center, Caolang Road 2901, Shanghai 201508, China. [zhyuan@shaphc.org](mailto:zhyuan@shaphc.org)  
Telephone: +86-21-64161928 Fax: +86-21-64227201

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of various outbreaks, including those obtained in our hospital from May to July 2008, were also analyzed to validate the conservation of the drug binding pocket.

**CONCLUSION:** Rupintrivir, whose safety profiles had been proved, is an attractive candidate and can be quickly utilized for treating severe EV71 infection.

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**Key words:** Rupintrivir; Hand foot and mouth disease; Molecular mechanics Poisson-Boltzmann/surface area; Molecular mechanics Generalized-Born/surface area; Homology modeling; Picornavirus

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### Abstract

**AIM:** To evaluate the suitability of rupintrivir against Enterovirus 71 (EV71) induced severe clinical symptoms using computational methods.

**METHODS:** The structure of EV71 3C protease was predicted by homology modeling. The binding free energies between rupintrivir and EV71 3C and human rhinovirus 3C protease were computed by molecular dynamics and molecular mechanics Poisson-Boltzmann/surface area and molecular mechanics generalized-born/surface area methods. EV71 3C fragments obtained from clinical samples collected during May to July 2008 in Shanghai were amplified by reverse-transcription and polymerase chain reaction and sequenced.

**RESULTS:** We observed that rupintrivir had favorable binding affinity with EV71 3C protease (-10.76 kcal/mol). The variability of the 3C protein sequence in isolates

### INTRODUCTION

During March to May 2008, an outbreak of hand, foot and mouth disease (HFMD) erupted in Anhui province, China. Thousands of people, mostly infants and children, were admitted into hospitals with moderate to severe symptoms, e.g. encephalitis, aseptic meningitis and pulmonary edema *etc.*, and over twenty deaths were reported by the health authorities. Laboratory investigations revealed that human enterovirus 71 (EV71) was responsible for this epidemic. Our center also

received 103 cases of HFMD (77 positive for EV71) from May to July 2008 with mild (fever, pneumonia) to severe (meningitis, encephalitis) symptoms and one patient died of viral encephalitis. Currently, treatment options for EV71 infection are limited, individual symptoms such as fever, encephalitis and meningitis are eased by supportive medication.

EV71 belongs to the picornaviridae family, which also include Poliovirus, Rhinovirus, and Coxsackie virus *etc.* It has a 7.4 kb positive-stranded RNA genome encoding one big polyprotein which was cleaved by virally encoded 2A and 3C protease in the process of protein synthesis<sup>[1]</sup>. This results in four structural proteins, VP1, VP2, VP3, VP4, and several non-structural proteins, 2A, 2B, 2C, 3A, 3B, 3C and 3D-Pol. In addition to its role in precursor polyprotein cleavage, the 3C protease is capable of cleaving host factors associated with host cell transcription and binding viral RNA as a constituent of replication complex<sup>[2-4]</sup>. The pivotal role of 3C in the viral life cycle makes it an ideal target for drug design. Indeed, there have been publications on the development of 3C peptidomimetic inhibitors against the picornaviridae family members<sup>[5-7]</sup>, including HRV (Human rhinovirus), an etiological cause of common colds<sup>[8]</sup>. Among them, rupintrivir (Figure 1), an irreversible inhibitor of HRV 3C, is by far the only 3C inhibitor that has entered clinical trials<sup>[9-12]</sup>. Matthews *et al.*<sup>[8]</sup> designed this molecule by taking advantage of covalent adduct formation (Michael reaction) with a reactive serine or cysteine at the active site of the protease such that the inhibitory potency is greatly enhanced. The Phase I and Phase II results, according to the company's press release, showed good safety and pharmacokinetics profiles. Furthermore, reduction in respiratory symptom scores and HRV titer in the upper respiratory tract of experimentally induced HRV infection was also documented.

It has been recently reported that a cell based yeast two hybrid assay system supported the idea that rupintrivir can effectively bind to EV71 3C<sup>[13]</sup>. We attempted to evaluate the affinity of rupintrivir to EV71 3C by homology modeling and atomistic molecular dynamics (MD) simulation. First, a comparative model of EV71 3C was constructed based on the crystal structure of HRV (1CQQ) and Poliovirus 3C protease (1L1N). EV71 3C-rupintrivir was then modeled according to the HRV 3C-rupintrivir complex (1CQQ) and 2 nanosecond MD simulations were performed on both complexes. We observed that rupintrivir can fit into the catalytic core of EV71 3C and is stable during MD. Binding free energy analyses using molecular mechanics Poisson-Boltzmann/surface area and molecular mechanics generalized-born/surface area methods were also performed. The estimated values suggested that rupintrivir has a decreased but still favorable affinity to EV71 3C compared with its HRV homolog. To test whether rupintrivir could fit for the majority of circulating isolates of EV71, we collected the 3C sequences of various epidemics from Genbank together with those obtained from clinical isolates in our center and analyzed the effects on drug-target interaction.

It was found that the residues which make direct contacts with rupintrivir showed minimal sequence variation in 48 divergent isolate sequences. Taken together, we suggest that rupintrivir has a significant affinity and chemical reaction rate with EV71 3C. Given that the pharmacokinetics and safety profiles of this drug have been proved, the findings of this study suggest that rupintrivir may be speedily utilized for treatment of severe EV71 infections, which will be significantly more efficient, in terms of time and resource, than development from a new chemical entity.

## MATERIALS AND METHODS

### Patients

One hundred and three cases of HFMD were admitted into the Shanghai Public Health Clinical Center and tested for EV71 and Coxsackie virus 16 with reverse transcription and polymerase chain reaction during May to July 2008. Seventy seven of these patients tested positive for EV71, in whom, respiratory symptoms and neurological symptoms (meningitis, encephalitis) were documented. There was one death of a 20-mo-old child caused by severe viral encephalitis after being transferred to our hospital.

### Viral culture, sequencing and phylogenetic analysis

Throat swabs and stools were obtained from patients with HFMD in Shanghai public health clinical center. If they tested positive for EV71, samples were grown in RD cells, a human rhabdomyosarcoma cell line, in DMEM with 10% fetal bovine serum, 2 mmol/mL L-glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin at 37°C with 5% CO<sub>2</sub>. If cytopathic effects were observed, culture supernatants were collected and RNA was extracted with TRIzol (Invitrogen) reagent and reverse transcribed with superscript II reverse transcriptase (Invitrogen). cDNA from four isolates (42T, 44T, 61T, 122F) were used as templates to amplify viral 3C sequence. The primers used were as follows, 3C forward: 5'-GATYRTHCCTGAW RCTCCCACCA, 3C reverse 5'-GGGTCTTTACTRKK CAASACWG. The 891 bp fragment covering the entire 3C sequence was amplified and TA-cloned into pGEM-T easy vector (Promega). To ensure accuracy, each cDNA was amplified and TA-cloned twice and each clone was sequenced in both directions and assembled. Nucleotide sequences of the 3C region of the four EV71 strains were submitted to Genbank and were analyzed together with the 44 strains in GenBank. The 48 strains were aligned with CLUSTALX software. Phylogenetic trees were constructed by the neighbor joining method and plotted using the program Tree-view. The robustness of the trees was then tested by bootstrap analysis with 1000 pseudo-replicates.

### Homology modeling of EV71 3C protease

The 3C reference sequence (GenBank EU703812) was from a complete genome of an EV71 isolate in Fuyang, Anhui Province, May 2008 and was reported by National



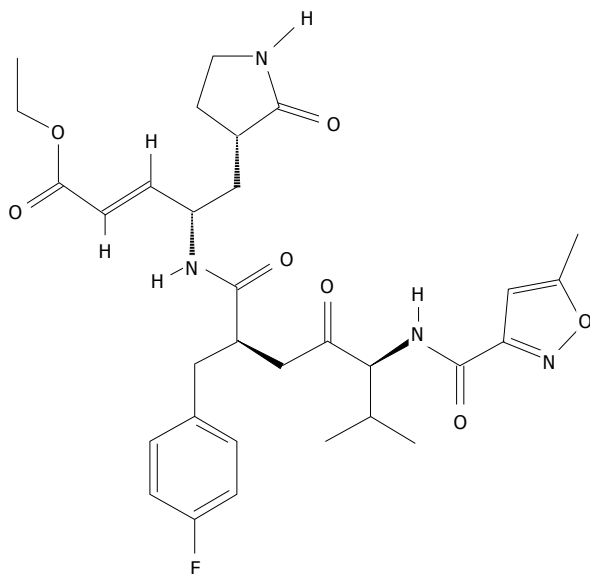


Figure 1 Chemical structure of rupintrivir.

Polio and Measles Lab, Institute for Viral Disease Control and Prevention, China CDC. Two homologous entries in the protein data bank with over 44% and 55% identity, i.e. 1CQQ (HRV 3C-rupintrivir complex, resolution 1.85 Å) and 1L1N (poliovirus 3C, resolution 2.10 Å) were used as templates for modeling. Homology modeling was performed on a SGI Tezro workstation using the *Homology* module in the Insight II 2000 software package. The protein sequences were aligned with PSI-BLAST and were further refined by using structural alignment functionality. This alignment was used for comparative modeling implemented in the *Modeler* module which generates structures by applying spatial restraints and MD refinement<sup>[14]</sup>. Three models were generated with the optimization level set as “high” and the coordinate with the lowest probability density function value was chosen. The quality of the model was assessed by *MolProbity* sever<sup>[15]</sup> and *3D-profile*<sup>[16,17]</sup>.

The initial coordinate for the EV71 3C-rupintrivir complex was constructed in Insight II according to the 1CQQ crystal structure. Briefly, EV71 3C and 1CQQ were superimposed and the covalent bond between the Cys147 thiol group and rupintrivir C19 was broken. After removal of HRV 3C protease, EV71 3C and rupintrivir were merged and subjected to further energy minimization.

### Molecular dynamic simulations

The simulations were performed using the AMBER 9 package with AMBER99SB<sup>[18]</sup> and GAFF<sup>[19]</sup> force fields. The coordinate of rupintrivir was prepared by breaking the bond between the inhibitor and the Cys147 thiol group in 1CQQ and resuming the trans-unsaturated bond. Partial charges and missing force field parameters were generated by the Antechamber program<sup>[19]</sup> in the AMBER suite using the AM1-BCC method. The 1CQQ complex (inhibitor detached from Cys147) and the

EV71 3C-rupintrivir structure as described above were employed as the starting point for energy minimization and MD simulations. Missing hydrogen atoms were added using the Xleap program. The protonation state of the histidine residues was checked by H++ server<sup>[20]</sup>. The complexes were solvated with 8 Å of TIP3P in a truncated octahedron by *solvateOct* command in Xleap. The systems were neutralized by sodium or chlorine ions.

Because of the large VDW repulsion energy caused by the manually built coordinate, the EV71-rupintrivir complex was carefully minimized before starting MD in a four-step manner, with 200 cycles of steepest descent and 800 cycles of conjugate gradient each step and descending restraints (500, 50, 10 and 2 kcal/mol-Å<sup>2</sup>). The 1CQQ complex was minimized with similar parameters except with less restraint (50, 10, 2 and 0 kcal/mol-Å<sup>2</sup>). The particle mesh Ewald method was used to treat long-range electrostatics and a cutoff was set to 12 Å. The SHAKE procedure was utilized to fix all hydrogen atoms, the time step was set to 2.0 fs. The system was heated in 50 ps from 0 to 300 K using Langevin dynamics with a collision frequency of 2.0/ps and 2.0 kcal/mol-Å<sup>2</sup> restraint. A 500 ps equilibration was performed in a NPT ensemble followed by a 2 ns production dynamics. The coordinates were saved every 2 ps.

### Binding free energy analysis

The binding free energy was calculated using the MMPBSA<sup>[21]</sup> and MMGB-SA procedures<sup>[22,23]</sup>. The binding free energy is calculated as follows:  $\Delta G_{\text{binding}} = \Delta E_{\text{gas}} + \Delta G_{\text{sol}} - T\Delta S$ , where  $\Delta E_{\text{gas}} = \Delta E_{\text{ele}} + \Delta E_{\text{vdw}}$ . The gas phase energy, desolvation energy and entropic contributions are averaged over the snapshots generated during MD. The desolvation energy can be further separated into polar and nonpolar parts, that is,  $\Delta G_{\text{sol}} = \Delta G_{\text{PB}}/\Delta G_{\text{GB}} + \Delta G_{\text{SA}}$ . The polar solvation energy can be estimated by Poisson Boltzmann or Generalized Born method implemented in the *mm\_pbsa* script<sup>[23]</sup> in AMBER9. The nonpolar solvation energy ( $\Delta G_{\text{SA}}$ ) is computed with Molsurf. The  $\Delta G_{\text{PB}}$  was calculated with grid spacing set to 0.5 Å and ionic strength was set to 0. The  $\Delta E_{\text{gas}}$  and  $\Delta G_{\text{sol}}$  was calculated with 100 snapshots of the trajectory at time interval of 20 ps. The entropy contribution ( $T\Delta S$ ) was estimated by normal-mode analysis utilizing the *nmode* module, which computes vibrational, rotational, and translational entropies. Each snapshot was minimized 50000 steps until the energy gradient was below 0.0001 kcal/mol Å with a distance-dependent dielectric constant of 4r (r is the distance between two atoms). Ten snapshots at each trajectory were calculated due to the computation cost of normal mode analysis.

### Energy decomposition using MM-GBSA method

Apart from the computation efficiency compared with the PBSA approach, the GBSA energies can be further decomposed on a per residue and even a backbone or side-chain basis which provides a simple means to evaluate energy contributions of each residue. All energy

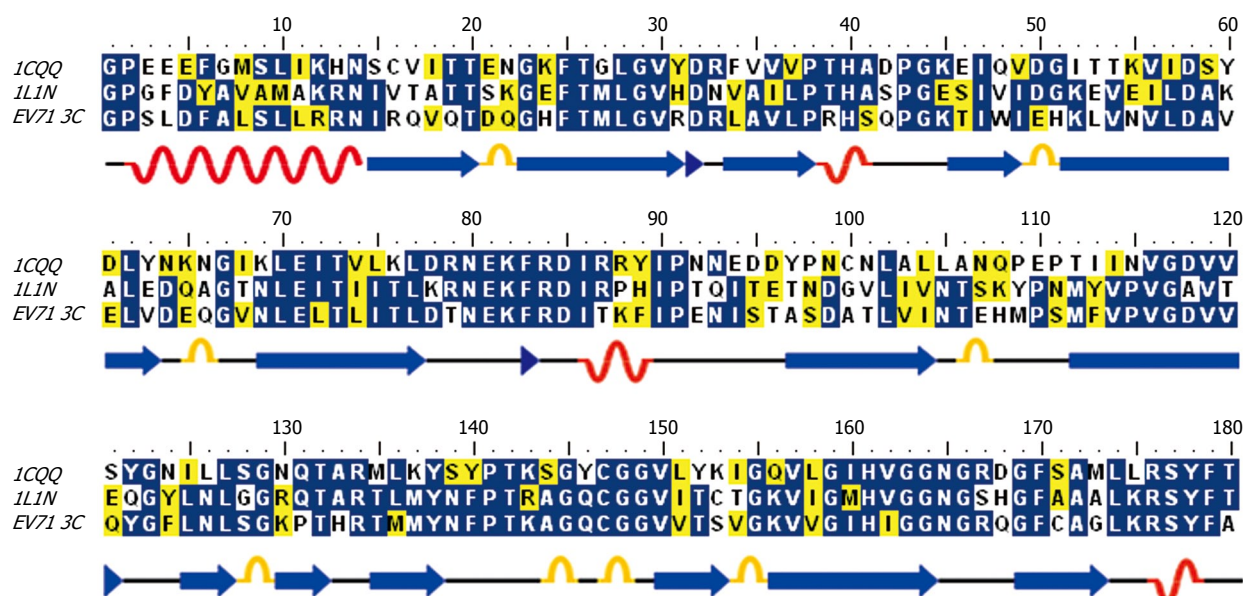


Figure 2 Sequence alignment of EV71 3C protease with HRV 3C (1CQQ) and Poliovirus 3C (1L1N). Secondary structures ( $\alpha$  helices and  $\beta$  sheets) were illustrated.

components were estimated using the decomposition functionality in *mm\_pbsa* module using 100 snapshots of the trajectory at time interval of 20 ps.

### Trajectory analysis and visualization

Trajectories from MD simulation were analyzed by the *ptraj* module. Hydrogen bond formation was analyzed by the *hbond* command and atom-to-atom distance, angle and dihedral angle were measured by *distance*, *angle* and *dihedral* command, respectively. Trajectories were visualized and inspected by VMD<sup>[24]</sup>, the Chimera suite<sup>[25]</sup> was used for graphic presentations of the complexes.

## RESULTS

### Homology modeling of EV71 3C protease

Using PSI-BLAST to search similar PDB sequences, a number of homologous entries were found including 1L1N (poliovirus 3C protease, 55% identity), 2IJ2 (poliovirus 3CD precursor, 54% identity), 2B0F (NMR structure of HRV 3C protease, 49% identity) and 1CQQ (Type 2 HRV 3C with rupintrivir, 44% identity). 1L1N<sup>[26]</sup> and 1CQQ<sup>[8]</sup> were subsequently chosen for template of homology modeling because of higher resolution and the preferable holo coordinate (Figure 2). The predicted structures generated by *Modeler* were then evaluated by *Profile-3D*; one model was selected with a score of 85.38 (expected score 82.94) and a sound folding profile for further analysis. The stereochemical property as indicated by the Ramachandran plot was highly reasonable. 98.9% of the residues were in favored regions and 99.4% were in allowed regions. Only one residue (Asp32) was barely outside of the allowed regions (data not shown).

The structure of EV71 3C protease retained the key features of HRV and poliovirus 3C with a two  $\beta$ -barrels fold and a shallow groove for substrate binding between

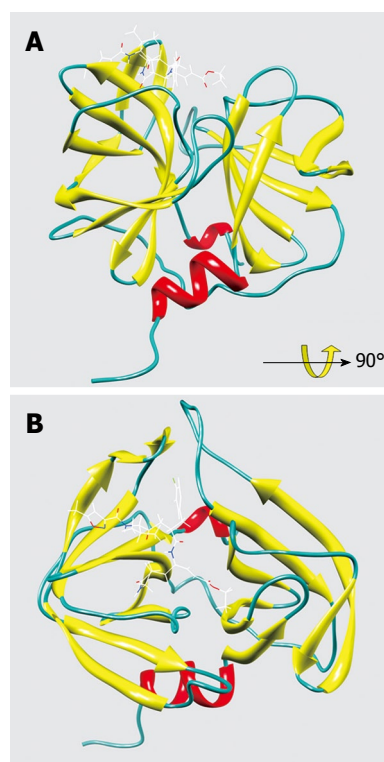


Figure 3 The overall structure of EV71 3C protease complexed with rupintrivir. A: A solid ribbon presentation of EV71 3C with rupintrivir; B: 90° rotation view of the structure.

the two domains (Figure 3). The backbone root mean square deviation (RMSD) between EV71 3C-1L1N and EV71 3C-1CQQ is 0.229 Å and 1.48 Å, respectively

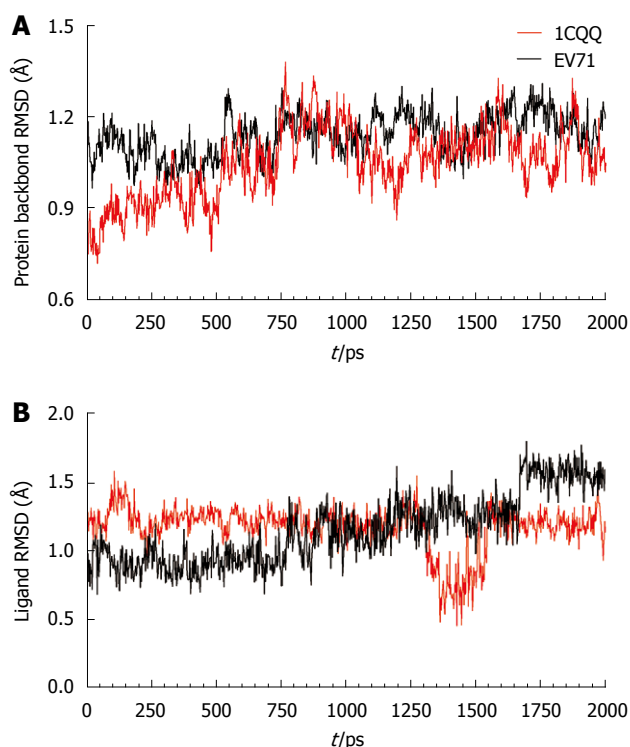
### Stability of the EV71 3C-rupintrivir complex

To further probe the stability of the EV71 3C-rupintrivir complex, we then performed MD simulations and RMSD of  $C_{\alpha}$  atoms during the production phase relative to initial coordinate was plotted (Figure 4A). Compared with 1CQQ complex (RMSD 1.04 Å), the EV71 protein backbone showed relatively higher displacement during

**Table 1** Binding free energies (kcal/mol) of Rhinovirus 3C protease and EV71 3C protease complexed with rupintrivir

Receptor		$\Delta E_{ele}$	$\Delta E_{vdw}$	$\Delta G_{SA}$	$\Delta G_{PB}$	$\Delta G_{GB}$	$-T\Delta S$	$\Delta G_{PBSA}$	$\Delta G_{GBSA}$
Rhino 3C	Mean	-40.70	-64.31	-8.22	64.17	55.67	30.10	-18.96	-27.56
	STD	4.75	3.63	0.37	3.78	3.71	6.26	9.45	9.42
EV71 3C	Mean	-37.74	-60.67	-8.11	66.68	56.24	28.87	-10.76	-21.41
	STD	8.57	3.75	0.16	5.88	5.23	3.85	11.70	11.39

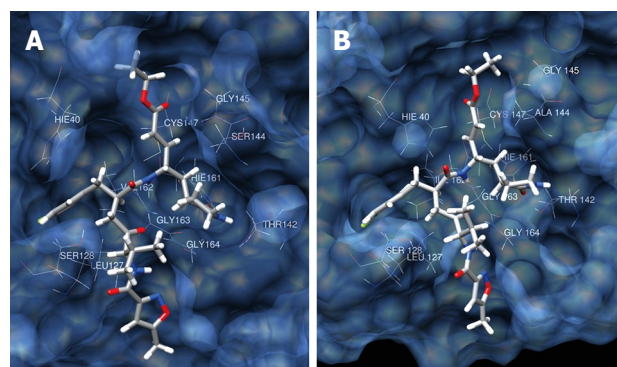
$\Delta E_{ele}$ : Coulombic energy;  $\Delta E_{vdw}$ : Van der Waals energy;  $\Delta G_{SA}$ : non-polar solvation free energy;  $\Delta G_{PB}$ : Poisson-Boltzmann polar solvation energy;  $\Delta G_{GB}$ : Generalized-Born polar solvation energy;  $\Delta G_{PBSA} = \Delta E_{ele} + \Delta E_{vdw} + \Delta G_{SA} + \Delta G_{PB} - T\Delta S$ ;  $\Delta G_{GBSA} = \Delta E_{ele} + \Delta E_{vdw} + \Delta G_{SA} + \Delta G_{GB} - T\Delta S$ ; STD: Standard error of mean values.



**Figure 4** Receptor and ligand backbone displacement. A: Backbone root mean square deviation (RMSD) of the HRV 3C (1CQQ) and EV71 3C protein; B: Ligand backbone RMSD during the 2ns molecular dynamics simulation.

production MD (1.56 Å), which was mainly caused by the five N terminal residues (G<sub>1</sub>PSLD<sub>5</sub>). Unlike its template, 1CQQ, these residues did not adopt a helical conformation and showed very high fluctuation during MD (data not shown). This was presumably caused by the less reasonable starting coordinate assigned during homology modeling. Nevertheless, these residues were not in close contact with the inhibitor and hence did not significantly influence the binding energy. Indeed, if the displacement was measured without these residues, EV71 complex showed displacement similar to that of 1CQQ and reached equilibrium and remained stable during the simulation (Figure 4A). Ligand heavy-atom RMSD plot (Figure 4B) also indicated similar displacement (average, 1CQQ:1.18 Å, EV71: 1.16 Å) but remained stable in a 2ns run (STD, standard deviation, 1CQQ: 0.16 Å, EV71: 0.25 Å).

Both 1CQQ and EV71 MD simulations retained



**Figure 5** Binding mode of AG7088 with 1CQQ (A) and EV71 3C (B). The protein surface is rendered semitransparent with associated backbone and side chain atom.

the major binding mode in the crystal structure (Figure 5). The P1 lactam group filled into the pocket formed by Thr142, His 161 *etc.*, the carboxamide oxygen and the amide nitrogen interacted with these residues *via* hydrogen bonds. The P2 4-fluoro-Phe group fitted very well in the S2 specificity pocket. The isoxazole group was buried in the S4 pocket and the vinyl group was close to and oriented well with the SG atom of Cys147.

#### Binding free energy estimate of EV71-rupintrivir complex

We then estimated the binding free energy of EV71 complex and 1CQQ complex using MM-PBSA and MM-GBSA methods. To save computational cost, the single trajectory approach was used since no significant conformational changes upon complex formation were reported. Indeed, 1L1N (no inhibitor bound) and 1CQQ (rupintrivir bound) have 0.75 Å backbone RMSD with 43.89% sequence identity, and the shapes of catalytic core are essentially the same (catalytic pocket residue backbone RMSD = 1.01 Å). As shown in Table 1, the PBSA method predicted binding free energy of -18.96 kcal/mol for 1CQQ and -10.76 for EV71 complex. However, the GBSA method gave considerably higher free energy (-27.56 kcal/mol for 1CQQ, -21.41 kcal/mol for EV71) because of lower estimation of unfavorable electrostatic solvation energy. The non-polar solvation energy gave a favorable contribution (-8.22 kcal/mol in 1CQQ and -8.11 kcal/mol in EV71) because of reduced surface area. Overall, we observed that the EV71 complex still had



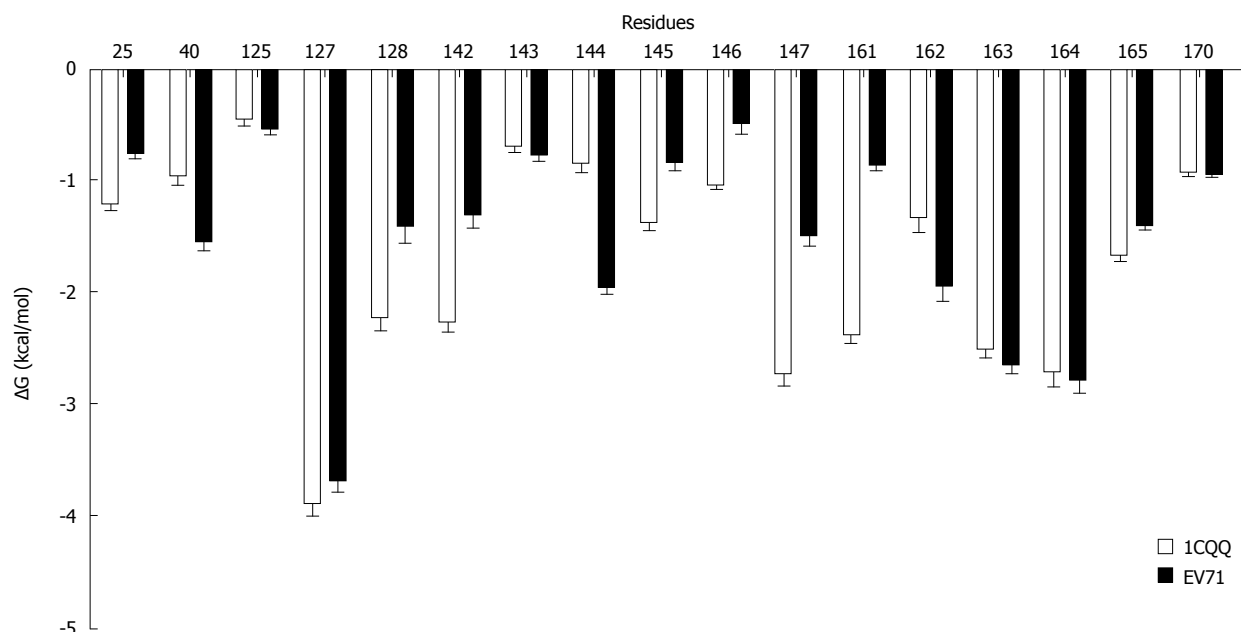


Figure 6 Energy decomposition (using MM-GBSA) of the key interaction residues in 1CQQ and EV71 3C complexes.

favorable binding energy although slightly lower than that in 1CQQ.

### Energy decomposition

To further analyze the energy contributions of individual residues, we decomposed the binding energies of 1CQQ and EV71 complex (Figure 6). It can be concluded from the plot that the decreased binding free energy was not governed by a single or two residues but a sum of cumulative effects. Indeed, residue Phe25, Ser128, Thr142, Gly145, 146Tyr/Gln, Cys147, His161 contributed less favorably in EV71 complex. Notably, all these sites, except for residue 146, have the same amino acid which suggests that the decreased energy contribution is caused by minute differences in the backbone coordinate. Nevertheless, we also observed several sites (His40, Ala144 and Ile162) that had more binding energy contributions than those in 1CQQ. Further analysis indicates that these residues all have preferred VDW and solvation energy.

### Sequence variability of EV71 3C protease and its effect on rupintrivir binding

As RNA viruses typically exhibit a high mutation rate in their replication process which could facilitate drug resistance, we sought to evaluate natural sequence variations in isolates collected in various epidemics and their effect on rupintrivir binding. Four sequences of 3C isolated in our hospital were also included (Figure 7A). Phylogeny analysis indicated that very high similarity between isolates in Shanghai and in Fuyang, Anhui province and 122F has 100% identity with the EU703812 reference sequence (Figure 7A). We then further scrutinized the variability of the residues with considerable contact with rupintrivir. As shown in Figure 8, most strains exhibited high conservation on key residues (red open box) in the binding site (residue 25, 40, 125-128, 142-147, 161-165,

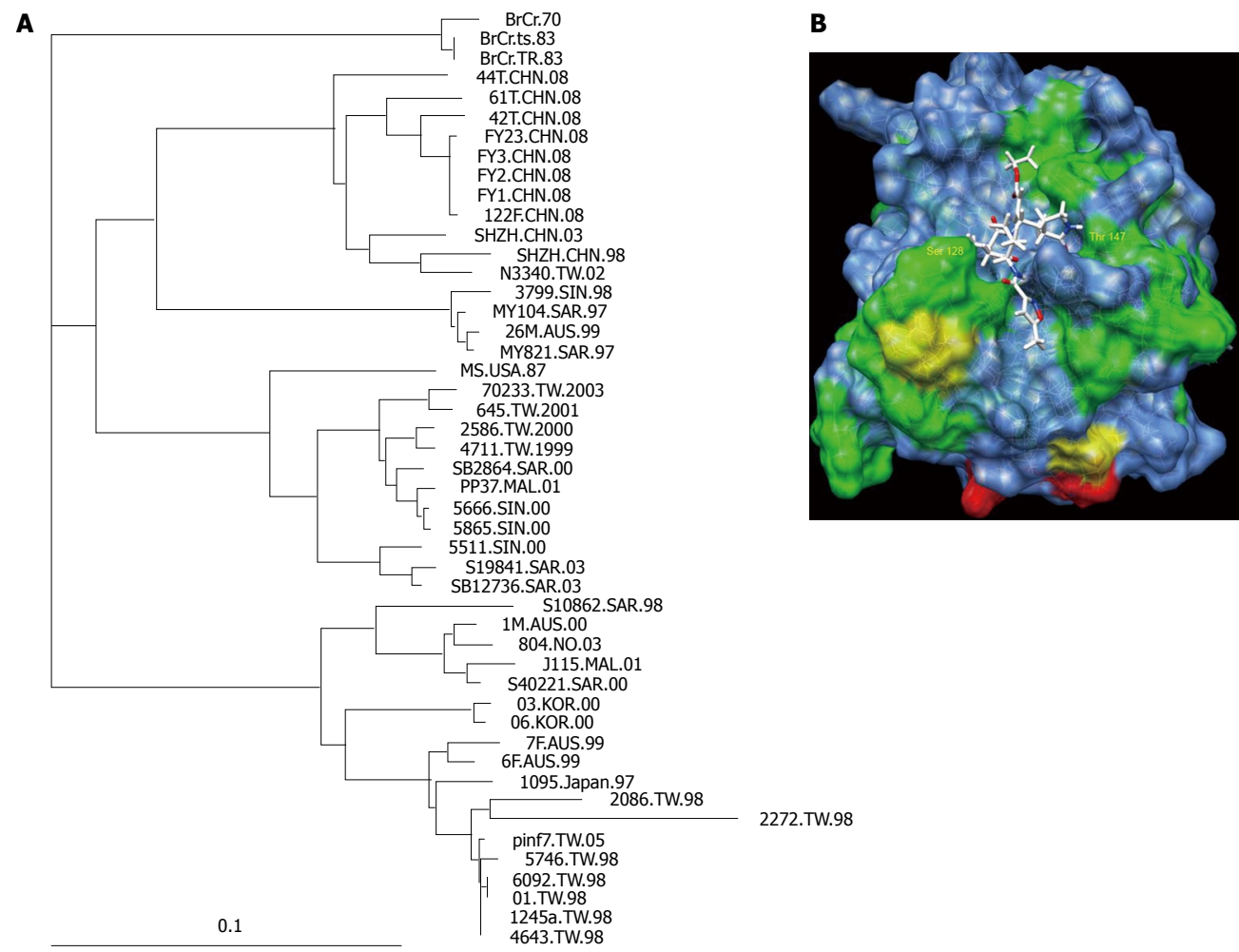
170). However, variations on key sites were repeatedly shown in isolate 2272.TW.98 which was obtained from a patient who died of pulmonary hemorrhage and shock in 1998, Taiwan. Further structural analysis suggests that Arg128 in 2272.TW.98 (Ser in Reference sequence) may create a steric hindrance to the 4-fluoroPhe group of rupintrivir (Figure 7B). In addition, the Pro142 in 2086.TW.98, which replaced the conserved Thr residue important for hydrogen bond formation with the lactam ring, could also drastically affect inhibitor docking. The variability observed in residue 144, 145 in 2272.TW.98 and BrCr.70 is unlikely to abolish rupintrivir's activity as the unsaturated ester group is shown to be quite flexible in simulation (data not shown). Taken together, although the catalytic core of EV71 3C is generally preserved in a wide range of isolates, potential natural drug resistance may exist which would limit the usefulness of rupintrivir.

## DISCUSSION

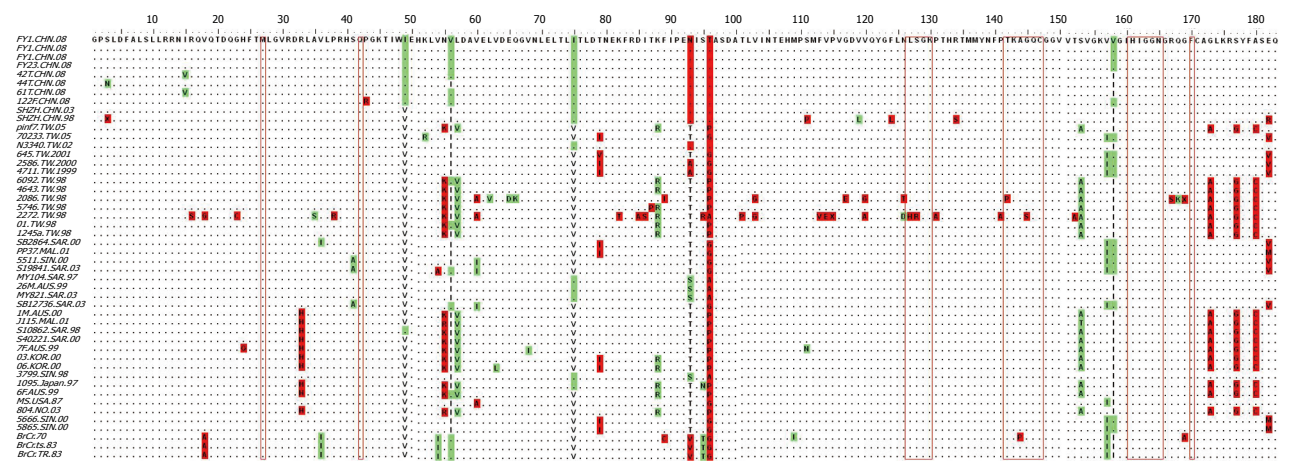
Since the first case of EV71 infection in California in 1969, EV71 has been identified in several small-scale outbreaks in United States, Europe, Australia, Japan, Brazil and Malaysia<sup>[27-32]</sup>. EV71 infection has become a prominent public health issue since the outbreak in Taiwan, China, 1998, with 129106 cases of HFMD and 405 cases of severe disease<sup>[33]</sup>. Notably, a recent EV71 epidemic in Anhui province during March to May 2008, PR China has also caused over 20 deaths and raised public awareness of the HFMD.

There have been some reports on anti-EV71 drug development in recent years<sup>[34-36]</sup>. Notably, Kuo *et al.*<sup>[7]</sup> designed a series of compounds against EV71 3C based on the structure of rupintrivir. Nevertheless, most of these drugs are still in the early phase and need





**Figure 7** Sequence variability of EV71 3C protease and its effect on rupintrivir binding. A: Phylogenetic tree of 48 isolates of EV71 in various outbreaks; B: A colored representation of sequence variation on the surface of 3C protease. Positions with two, three and over three different residues are labeled green, yellow and red, respectively. Residue Ser 128 and Thr 142 were labeled.



**Figure 8** Sequence alignment of 3C protease from isolates collected in various epidemics (CHN: Mainland China; TW: Taiwan, China; FY: Fuyang, Anhui Province, China; SHZN: Shenzhen, China; AUS: Australia; SAR, SIN, MAL: Malaysia; KOR: Korea; NO: Norway). Residues important for rupintrivir are labeled with red open boxes.

further optimization of pharmacokinetics and ADMET (absorption, distribution, metabolism, excretion and toxicity) profiles. EV71 vaccine research is also in an early development stage<sup>[37]</sup> and needs further human

safety tests. The scarcity of available antiviral medication renders clinical intervention of severe cases challenging.

In this report, we suggest that an available drug, rupintrivir, is an attractive candidate for treatment of

severe cases of EV71 infection. MD and MM-PBSA (GBSA) analysis suggested a decreased but still favorable interaction with EV71 3C protease compared with HRV 3C. In addition, rupintrivir had been shown to be effective against all the human HRV serotypes and several human enterovirus strains (CVB2, 5, EV6, 9) because of high conservation in the rupintrivir binding site, although EV71 was not tested<sup>[9]</sup>. Recently, using plaque reduction assay and a real-time fluorescence resonance energy transfer model, rupintrivir showed around 0.8  $\mu\text{mol/L}$  EC50 activity<sup>[38]</sup>. Of note, the EC50 in EV71 (0.8  $\mu\text{mol/L}$ <sup>[38]</sup>) and in type 2 HRV (20 nmol/L<sup>[8,9]</sup>) can be well correlated with free energy estimate in our research (-18.96 kcal/mol in HRV and -10.76 kcal/mol in EV71, MM-PBSA method). Collectively, using molecular simulation and binding free energy estimation, we suggest that rupintrivir can effectively inhibit EV71 3C protease, which is confirmed by recent *in vitro* viral culture study. Utilization of old drugs for treatment of new indications has its intrinsic superiority since all the pre-clinical research and most of clinical research can be obviated. If rupintrivir could prove its efficacy in HFMD patients, this drug could be quickly administered to EV71 patients with severe symptoms and possibly reduce the mortality rate of HFMD in infants.

## COMMENTS

### Background

The enterovirus 71 (EV71) is the major pathogen causing hand foot and mouth disease (HFMD). During March to May 2008, an epidemic of HFMD erupted in Anhui province and the surrounding area, China with significant mortality. The authors' hospital received over one hundred of these patients including several with severe complications.

### Research frontiers

To date, there have been no antiviral drugs available for EV71 infection with severe symptoms. Although, reports of compounds that can inhibit EV71 replication *in vitro* are accumulating, they still need to be tested in pharmacokinetics, ADMET (absorption, distribution, metabolism, excretion and toxicity) and other safety profiles.

### Innovations and breakthroughs

In this article, the authors modeled the EV71 3C protease, which is critical for viral replication, based on the crystal structure of rhinovirus and poliovirus 3C. They further evaluated an irreversible inhibitor of rhinovirus 3C, rupintrivir as a possible drug against EV71 by molecular dynamics and molecular mechanics Poisson-Boltzmann/surface area and molecular mechanics generalized-born/surface area methods. This is the first attempt to computationally evaluate the suitability of rupintrivir in inhibiting EV71 3C protease.

### Applications

With the *in vitro* and computational evidence supporting its anti-EV71 activity together with its safety profile in previous clinical trials, rupintrivir may be quickly administered to EV71 patients with severe symptoms and possibly reduce the mortality rate of HFMD in infants.

### Terminology

EV71 belongs to the picornaviridae family and is the major cause of HFMD. HFMD is a human syndrome caused by intestinal viruses of the picornaviridae family. The most common viruses causing HFMD are Coxsackie A virus and EV71. HFMD usually affects infants and children and mostly causes mild symptoms. However, significant mortality has been observed in Anhui province 2008 and in previous outbreaks in Taiwan in 1998.

### Peer review

The manuscript is a predictive study for the use of rupintrivir against EV71 infection, based on a computer analysis of the relationship of rupintrivir structure

and EV71 structure. Data showed the potential binding of rupintrivir on the EV71 3C protease. The study is well conducted and the analysis appears solid.

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## Liver myofibroblasts activate protein C and respond to activated protein C

Jennifer Gillibert-Duplantier, Anne Rullier, Véronique Neaud, Walter Kisiel, Jean Rosenbaum

Jennifer Gillibert-Duplantier, Anne Rullier, Véronique Neaud, Jean Rosenbaum, INSERM U889, Victor Segalen Bordeaux 2 University, F-33076 Bordeaux, France  
Walter Kisiel, Department of Pathology, University of New Mexico School of Medicine, Albuquerque, NM 87131, United States

**Author contributions:** Gillibert-Duplantier J and Rullier A performed the majority of the experiments; Neaud V performed the RT-PCR experiments; Kisiel W provided vital reagents; Rosenbaum J designed the study and wrote the manuscript.  
**Correspondence to:** Jean Rosenbaum, MD, INSERM U889, Victor Segalen Bordeaux 2 University, F-33076 Bordeaux, France. [jean.rosenbaum@gref.u-bordeaux2.fr](mailto:jean.rosenbaum@gref.u-bordeaux2.fr)  
**Telephone:** +33-5-57571594 **Fax:** +33-5-56514077  
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### Abstract

**AIM:** To study the protein C activation system in human liver myofibroblasts, and the effects of activated protein C (APC) on these cells.

**METHODS:** Human liver myofibroblasts were obtained by outgrowth. Expression of protease activated receptor 1 (PAR-1), endothelial protein C receptor (EPCR) and thrombomodulin (TM) was analyzed by flow cytometry. Extracellular signal-regulated kinase (ERK)1/2 activation was assessed by Western blotting using anti-phospho-ERK antibodies. Collagen synthesis was studied with real-time reverse transcription-polymerase chain reaction (RT-PCR). Activation of protein C was studied by incubating liver myofibroblasts with zymogen protein C in the presence of thrombin and detecting the generation of APC with a colorimetric assay using a peptide substrate.

**RESULTS:** Primary cultures of human liver myofibroblasts expressed EPCR on their surface, together with PAR-1 and TM. This receptor system was functional since exposure of myofibroblasts to APC induced

ERK1/2 phosphorylation in a dose- and time-dependent manner. Furthermore, APC significantly upregulated the expression of collagen mRNA, as shown by real-time RT-PCR. Collagen upregulation was controlled through the ERK pathway as it was inhibited when using the mitogen-activated protein/extracellular signal-regulated kinase kinase inhibitor PD98059. Finally, using a cell-based colorimetric assay, we showed that intact myofibroblasts converted protein C into APC in the presence of thrombin.

**CONCLUSION:** These data suggest that APC is a new modulator of liver myofibroblast activity and contributes to the pathophysiology of chronic liver diseases.

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**Key words:** Liver fibrosis; Thrombin; Activated protein C; Protease-activated receptor

**Peer reviewer:** Dr. Katja Breitkopf, Department of Medicine II, University Hospital Mannheim, University of Heidelberg, Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany

Gillibert-Duplantier J, Rullier A, Neaud V, Kisiel W, Rosenbaum J. Liver myofibroblasts activate protein C and respond to activated protein C. *World J Gastroenterol* 2010; 16(2): 210-216 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i2/210.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i2.210>

### INTRODUCTION

Liver myofibroblasts are key players in the pathogenesis of chronic liver diseases such as fibrosis and cancer. Liver fibrosis is a major health problem as it complicates every chronic liver disease, whether due to hepatitis viruses, alcohol, non-alcoholic steatohepatitis or other causes. Liver fibrosis may progress to cirrhosis, which is an important cause of morbidity. The cell types responsible for the deposition of the excess extracellular



matrix characteristic of fibrosis have been identified as being predominantly hepatic stellate cells and portal fibroblasts<sup>[1,2]</sup>. During liver injury, these cells differentiate into myofibroblasts that proliferate and synthesize fibrosis components. Besides liver fibrosis, a number of studies have shown that the stroma of hepatocellular carcinoma, the major type of primary liver cancer, is populated with myofibroblasts that play major roles in promoting, for instance, extracellular matrix deposition and tumor cell invasion<sup>[3-7]</sup>.

Recently, we and others have demonstrated that the serine proteinase thrombin, a key effector of blood coagulation, affects the phenotype of liver myofibroblasts<sup>[8-11]</sup> and behaves as a pro-fibrogenic molecule in the liver<sup>[12,13]</sup>. Experiments using either antagonists<sup>[12]</sup>, or knock-out animals<sup>[14]</sup> have shown that the pro-fibrogenic effects of thrombin are dependent largely upon signal transduction through protease activated receptor-1 (PAR-1). Upon binding to PAR-1, thrombin cleaves the N terminus of the receptor and the new N terminus behaves as a tethered ligand that activates another PAR1 molecule, which leads to intracellular signaling<sup>[15]</sup>. Besides thrombin, PAR-1 is also a receptor for several other molecules that include matrix metalloproteinase-1<sup>[16]</sup> and especially activated protein C (APC)<sup>[17]</sup>.

APC is generated from protein C (PC) by cleavage with thrombin. This requires that thrombin is bound to thrombomodulin (TM), and PC to its specific cell surface receptor, endothelial protein C receptor (EPCR). The best-known functions of APC are its anticoagulant properties through proteolytic inactivation of factors V a and VIIIa. However, cellular effects of APC have also been shown, which depend on PAR-1 signaling upon interaction with EPCR-bound APC. EPCR is deemed endothelium-specific, therefore, these effects have been studied mostly in endothelial cells where APC is credited with a cytoprotective effect. Recently, EPCR expression also has been demonstrated on a few other cell types, such as lung epithelial cells<sup>[18]</sup>, leukocytes<sup>[19]</sup>, keratinocytes<sup>[20]</sup>, and vascular smooth muscle cells<sup>[21]</sup>. Liver myofibroblasts exhibit many common features with vascular smooth muscle cells, and it is already known that they express functional PAR-1, therefore, we questioned whether they express other cell surface components of the APC system and have functional responses to APC.

## MATERIALS AND METHODS

### Materials

Human PC was purified from plasma as described previously<sup>[22]</sup>. APC was generated using the *Agkistrodon contortrix contortrix* C snake venom protease that recognizes the thrombin cleavage site in PC<sup>[23]</sup>. Monoclonal anti-EPCR antibodies (JRK 1494 and HEPER 1489) and anti-TM antibody (CTM 1009) were kindly provided by Dr. Charles Esmon. Anti-PAR-1 was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA; clone ATAP2), and anti-smooth muscle  $\alpha$ -actin (ASMA) from

Dako (clone 1A4). Human thrombin (1000 NIH U/mg) was from Sigma (St Louis, MO, USA; T4393).

### Cell culture

Human hepatic myofibroblasts were obtained from explants of non-tumor liver resected during partial hepatectomy, and characterized as described previously<sup>[24,25]</sup>. Specifically, the procedure, based on the selective growth advantage of myofibroblasts in the culture conditions used, allowed for a 100% pure myofibroblast population, as shown by positive staining for ASMA and vimentin, and negative staining for CD68 (a Kupffer cell marker), von Willebrand factor (an endothelial cell marker) or cytokeratin (an epithelial cell marker). Myofibroblasts were used between the 3rd and the 6th passage, and were grown in DMEM that contained 5% fetal calf serum, 5% pooled human serum and 5 ng/mL epidermal growth factor (EGF). EGF was removed from the medium at least 3 d before the experiments were conducted.

### Flow cytometry

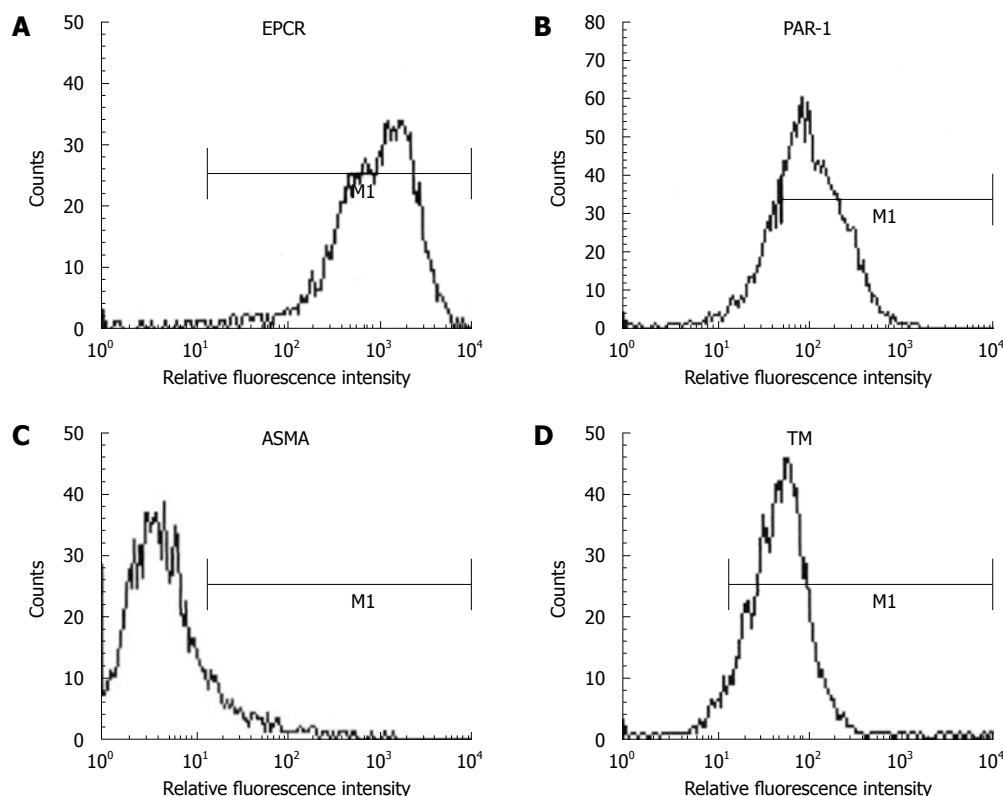
Myofibroblasts were detached from culture plates by incubation in 2 mmol/L EDTA for 15 min at 37°C and collected by centrifugation. One to two hundred thousand cells were incubated with anti-EPCR (JRK 1494, 1/1000), anti-TM (1/50), or anti-PAR-1 (1/50) antibodies. Following a wash with PBS/0.1% BSA, cells were incubated with a secondary phycoerythrin-coupled antibody (1/200) for 15 min at 4°C. After a final wash, the cells were resuspended in PBS/0.1% BSA for analysis.

### Mitogen-activated protein kinase (MAPK) phosphorylation

Extracellular signal-regulated kinase (ERK) phosphorylation was measured essentially as described previously<sup>[10]</sup>. Briefly, cells were grown to confluency and serum-starved for 2 d, and subsequently exposed to the required agonists in serum-free Waymouth medium. At the end of the incubation, cell lysates were prepared in the presence of proteases and phosphatase inhibitors as described previously<sup>[4]</sup>. Equivalent amounts of proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and analyzed by Western blotting for MAPK phosphorylation using phospho-ERK antibody (Cell Signaling Technology, Beverly, MA, USA). The blots were washed and the appropriate peroxidase-conjugated secondary antibody was applied. Immuno-detected proteins were visualized by using an enhanced chemiluminescence assay (Amersham Biopharmacia, Orsay, France). Membranes were stripped and reblotted using antibody to total-ERK. Signals were acquired on a Macintosh computer connected to a Kodak Digital Science DC120 camera and were quantified by using NIH Imaging software.

### APC generation

APC generation from PC by human liver myofibroblasts was demonstrated with a colorimetric method using



**Figure 1** Human liver myofibroblasts express endothelial protein C receptor (EPCR) (A), protease activated receptor 1 (PAR-1) (B), anti-smooth muscle  $\alpha$ -actin (ASMA) (C) and thrombomodulin (TM) (D). Fixed unpermeabilized cells were labeled with antibodies to ASMA and thrombomodulin, and analyzed by flow cytometry. The signal obtained with a control antibody was superimposable with that of ASMA and was not shown for clarity.

**Table 1** Primer sequences used for real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Name	Primer sequence	Amplicon size (bp)
RLP0	F: 5'-GGCGACCTGGAAGTCCAAC-3' R: 5'-CCATCAGCACCACAGCCTTC-3'	149
Collagen $\alpha 1(I)$	F: 5'-ATGTTTCAGCTTTGTGGACCT-3' R: 5'-CAGCTGACTTCAGGGATGT-3'	92

commercial reagents (Spectrozyme aPC plasma specific chromogenic substrate; American Diagnostica, Greenwich, CT, USA). Briefly, confluent quiescent myofibroblasts were incubated for 30 min with purified PC with or without added thrombin 0.1 U/mL (1.8 nmol/L) in HBSS/0.1% BSA. Supernatants were collected and incubated with an APC chromogenic substrate. The optical density of the solution was measured at 405 nm in a Dynatech microplate reader (MTX Lab Systems, Inc., Vienna, VA, USA).

#### Reverse transcription-polymerase chain reaction (RT-PCR) for collagen I

Total RNA was extracted from liver samples using Nucleospin RNA II (Macherey Nagel, Düren, Germany). RNA was reverse transcribed using Superscript II (Promega, Charbonnières-les-Bains, France). Nucleotide sequences of primers for collagen  $\alpha 1(I)$  and for RLP0 (which

encodes the human acidic ribosomal phosphoprotein P0, used as a control) are shown in Table 1. Controls without template or reverse transcriptase were also performed.

PCR reactions were performed using a Stratagene  $\times$  4000 thermocycler (Stratagene, Amsterdam, The Netherlands) and the SYBR Green PCR Core reagents kit (Bio-Rad, Marnes-la-Coquette, France). Five microliters of diluted cDNA samples (produced from 3 ng RNA) were added to 20  $\mu$ L PCR master mix, and experiments were performed in duplicate. Data analysis was performed using Mx4000 version 4.2 (Stratagene). Gene expression results were first normalized to internal control RLP0<sup>[26]</sup>, and the relative levels of expression were quantified by calculating  $2^{-\Delta\Delta C_t}$ <sup>[27]</sup>.

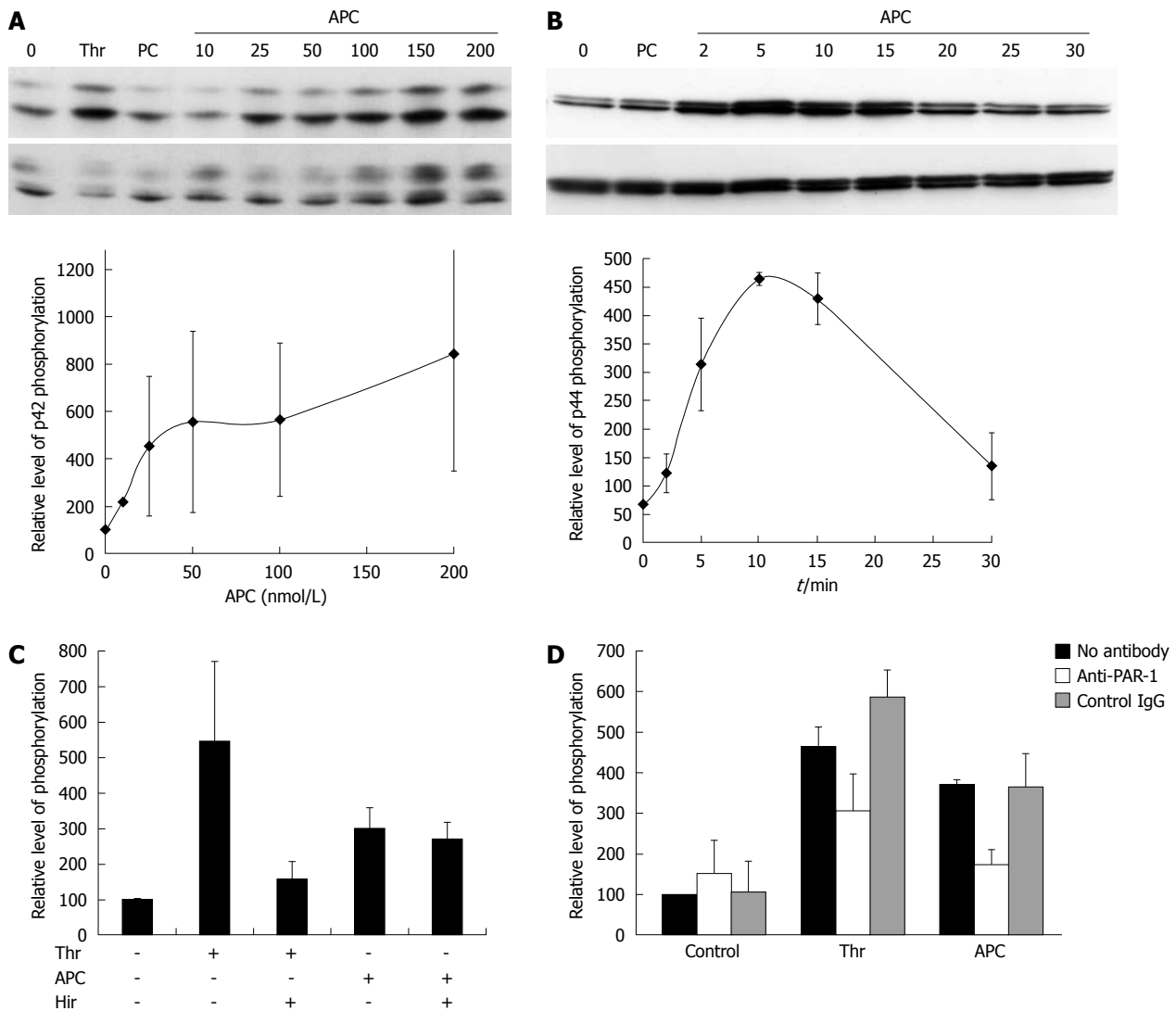
#### Statistical analysis

Differences between multiple means were analyzed by the non-parametric Kruskal-Wallis test using the StatCrunch software (<http://www.statcrunch.com>). A value of  $P < 0.05$  was considered significant.

## RESULTS

### Cultured myofibroblasts express the APC activating complex

APC signaling requires the presence of EPCR and PAR-1 at the cell surface. We thus examined the expression of these two receptors on cultured human liver

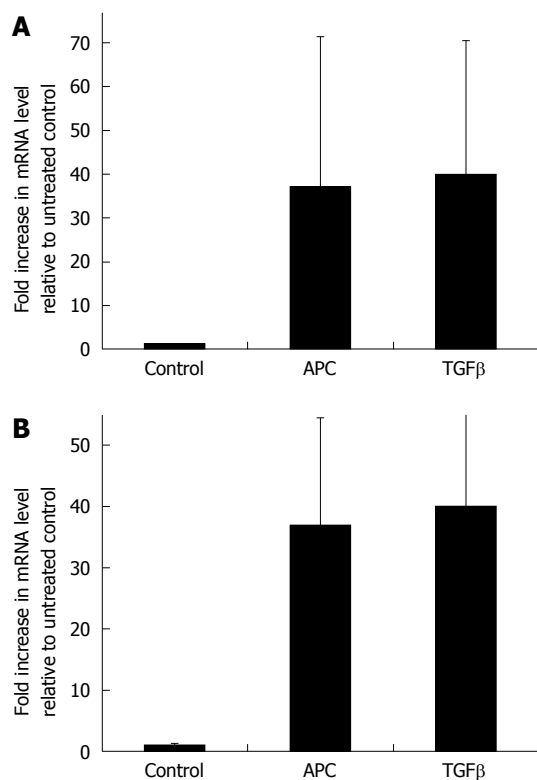


**Figure 2** Effect of activated protein C (APC) on extracellular signal-regulated kinase (ERK) phosphorylation. A: Dose-response of APC. Cells were exposed for 10 min to 27 nmol/L thrombin (Thr), 100 nmol/L nonactivated protein C (PC), or APC. ERK phosphorylation was assessed using Western blotting. The upper panel shows a blot labeled with an antibody to phospho-ERK, and the lower panel shows the same blot re-hybridized with an antibody to total ERK. The graph shows the quantification of p42 phosphorylation in three similar independent experiments. Results are shown as mean  $\pm$  SE. Phospho-p42 signals were normalized first to total p42 signals, and the results were expressed as a percentage of the non-stimulated control. Similar results were obtained when quantitating p44 phosphorylation; B: Kinetics of APC induced phosphorylation of ERK. Cells were exposed to 100 nmol/L APC. The upper panel shows a blot labeled with an antibody to phospho-ERK, and the lower panel shows the same blot re-hybridized with an antibody to total ERK. The graph shows the quantification of p44 phosphorylation in three similar independent experiments. Phospho-p44 signals were normalized first to total p44 signals, and the results were expressed as a percentage of the non-stimulated control. Similar results were obtained when quantitating p42 phosphorylation; C: APC effect is not due to contaminating thrombin. Cells were exposed for 10 min to 27 nmol/L Thr or 100 nmol/L APC. They were pretreated with or without hirudin. The graph shows the quantification of combined ERK1/ERK2 phosphorylation in four similar independent experiments; D: APC signals through PAR-1. Cells were exposed for 10 min to 27 nmol/L Thr or 100 nmol/L APC. They were preincubated with the indicated antibodies. The graph shows the quantification of combined ERK1/ERK2 phosphorylation in three similar independent experiments.

myofibroblasts, using flow cytometry on unpermeabilized cells. As shown in Figure 1, liver myofibroblasts expressed easily detectable EPCR and PAR-1. As a control to show that we detected only cell-surface-exposed molecules, we similarly labeled the cells with an antibody to ASMA, an abundant cytoplasmic protein, and found no signal (Figure 1C), although this protein could be demonstrated easily by Western blotting of cell extracts (data not shown). Thus, myofibroblasts express APC receptors on their surface. In addition, we also showed the expression of TM, the thrombin receptor required for PC cleavage (Figure 1D).

### APC induces intracellular signaling

As a readout for the effects of APC, we measured the phosphorylation of the MAPKs ERK1 and ERK2, which is a hallmark of signaling *via* PAR-1. Western blotting demonstrated a dose-dependent increase in phosphorylation of ERK1/2. The maximum level of phosphorylation obtained at a concentration of 100 nmol/L was comparable with that obtained with 27 nmol/L thrombin (1 IU/mL) (Figure 2A). Non-activated PC had no detectable effect. The effect of APC reached a maximum after 10 min of stimulation (Figure 2B). Although the preparation of APC from PC avoided the



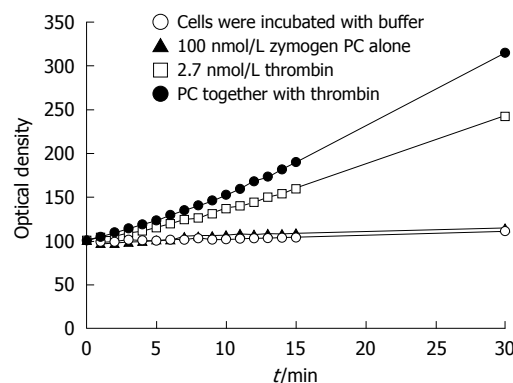
**Figure 3 APC upregulates collagen transcripts.** A: Cells were exposed for 24 h to 100 nmol/L APC or 1 ng/mL transforming growth factor- $\beta$ 1. Collagen transcripts were measured by real-time reverse transcription-polymerase chain reaction and normalized to those of the RPL0 gene. Results are expressed as fold increase over control values and are the mean  $\pm$  SE of five experiments; B: Cells were exposed for 6 h to 100 nmol/L APC, in the presence or absence of the mitogen-activated protein kinase (MAPK) inhibitor PD98059 (50  $\mu$ mol/L), before measuring collagen transcripts. Results are the mean  $\pm$  SE of three experiments.

use of thrombin, we made sure that the effects of APC were not due to trace amounts of thrombin. Thus, cells were pretreated with the specific thrombin inhibitor, hirudin, prior to APC addition. Although hirudin abolished the effects of thrombin on ERK1/2 phosphorylation, it did not alter those of APC, which indicated that they were not caused by contaminating thrombin (Figure 2C).

In order to know whether the effects of APC required PAR-1, cells were incubated with blocking antibodies to PAR-1 or control IgG before adding APC. The PAR-1 antibody blocked by 56.0%  $\pm$  8.7% the effect of APC on ERK1/2 phosphorylation, and as expected, also that of thrombin (37.6%  $\pm$  10.9%,  $P = 0.03$  in both cases) (Figure 2D).

#### APC signaling leads to increased collagen synthesis

We found that exposure of cells to APC significantly increased the amount of  $\alpha$ 1(I) collagen transcripts as compared to the control conditions. Actually, under the conditions used, the effects of 100 nmol/L APC were equivalent to those of transforming growth factor- $\beta$ , a prototypic inducer of extracellular matrix synthesis, used at a concentration of 1 ng/mL (Figure 3A). In order



**Figure 4 Human liver myofibroblasts can generate APC from PC.** Supernatants were collected and incubated with an APC chromogenic substrate. The graph shows the optical density at 405 nmol/L, which resulted from cleavage of the substrate, which was proportional to the amount of APC generated. It represents the mean of two separate experiments.

to know whether this effect of APC involved MAPK signaling, experiments were repeated with or without addition of the MAPK inhibitor PD98059. As shown in Figure 3B, treatment with PD98059 abrogated the effect of APC on  $\alpha$ 1(I) expression.

#### Myofibroblasts generate APC from PC

Human liver myofibroblasts expressed all components of the APC-generating system, therefore, we asked whether they provided a suitable surface for APC generation from PC. We thus incubated cells with the PC zymogen alone or together with thrombin. As shown in Figure 4, incubation of myofibroblasts with PC in the absence of thrombin did not lead to cleavage of the chromogenic substrate. Thrombin alone had a known nonspecific effect<sup>[28]</sup>, which was abolished by hirudin (data not shown). However, when PC and thrombin were added, the amount of peptide cleavage was considerably greater, which indicated that APC was generated.

## DISCUSSION

We show here that human liver myofibroblasts express EPCR. EPCR is functional since treatment of myofibroblasts with APC leads to enhanced ERK phosphorylation, a hallmark of PAR-1 activation<sup>[29]</sup>. We found that APC upregulated collagen mRNA levels, which suggests its role in extracellular matrix metabolism, which is one of the major functions of liver myofibroblasts. It has been shown previously that APC signaling *via* PAR-1 and EPCR leads to extracellular matrix remodeling<sup>[20]</sup>. It is thus possible that APC contributes, together with thrombin, to the liver wound-healing response.

We further showed that myofibroblasts also express TM, the thrombin receptor required for activation of PC to APC. This raised the hypothesis that myofibroblasts could provide a suitable surface for generation of APC from PC in the presence of thrombin. This was indeed proven in the cell-based assay that we ran. Thus, liver myofibroblasts, in addition to endothelial cells, are among



the rare cell types that can generate APC. APC generation by liver myofibroblasts could prove extremely important for these cells to respond to APC under physiological or pathophysiological conditions. Indeed, APC and thrombin share the same signaling receptor, PAR-1, but efficient activation by APC requires concentrations about 10 000-fold higher than those of thrombin<sup>[30]</sup>. It is thus not yet entirely clear how APC, which requires thrombin for its generation, can still activate PAR-1 in the presence of thrombin. However, recent evidence suggests that APC signaling is much more efficient when APC is locally generated from PC than when provided exogenously<sup>[31]</sup>. This may be linked to the observation that receptors for PC activation and APC signaling are co-localized within lipid rafts<sup>[32]</sup>. Our finding that human liver myofibroblasts can generate APC makes it very likely that they can respond efficiently to endogenously generated APC. There is evidence that thrombin, which is required for PC activation, is generated in the fibrotic liver, as demonstrated by the presence of fibrin deposition in experimental<sup>[33]</sup> or human liver disease<sup>[34]</sup>. Therefore, our data suggest that APC can indeed contribute to the pathophysiology of chronic liver diseases through effects on liver myofibroblasts, in addition to endothelial cells.

## ACKNOWLEDGMENTS

We thank Dr. C Esmon for antibodies.

## COMMENTS

### Background

A number of recent studies have emphasized the interface between liver fibrosis and the blood hemostasis system, by showing the deleterious role of thrombin and its cellular receptors on the progression of liver fibrosis.

### Research frontiers

Activated protein C (APC) is a major inhibitor of coagulation but also has cytokine-like properties by interacting with a complex of cell surface receptors. Besides endothelial cells, few cells have been shown to express such functional receptors.

### Innovations and breakthroughs

In this study, the authors showed that human liver myofibroblasts, the major fibrogenic cell type in the liver, can bind and activate protein C (PC). Furthermore, they demonstrate that APC triggers collagen synthesis in these cells, which suggests that the local generation of APC by myofibroblasts in the liver may modulate the liver wound-healing process.

### Applications

This study gives new insight into the interplay between blood hemostasis components and liver fibrogenesis.

### Terminology

PC is a circulating protein that can be activated following binding to the endothelial protein C receptor by thrombin, itself bound to thrombomodulin. APC is a major inhibitor of blood coagulation. It can also bind to cell surface protease activated receptor-1 and induce intracellular signals, like a cytokine.

### Peer review

The manuscript is clear and the results are convincing. The data add important new details about pro-fibrogenic activity of liver myofibroblasts.

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## Mobilization of hematopoietic progenitor cells in patients with liver cirrhosis

Ursula M Gehling, Marc Willems, Kathleen Schlagner, Ralf A Benndorf, Maura Dandri, Jörg Petersen, Martina Sterneck, Joerg-Matthias Pollok, Dieter K Hossfeld, Xavier Rogiers

Ursula M Gehling, Marc Willems, Martina Sterneck, Joerg-Matthias Pollok, Xavier Rogiers, Department of Hepatobiliary Surgery and Transplant Surgery, University Hospital Hamburg-Eppendorf, 20246 Hamburg, Germany

Kathleen Schlagner, Maura Dandri, Jörg Petersen, Dieter K Hossfeld, Department of Medicine, University Hospital Hamburg-Eppendorf, 20246 Hamburg, Germany

Ralf A Benndorf, Institute of Experimental and Clinical Pharmacology and Toxicology, University Hospital Hamburg-Eppendorf, 20246 Hamburg, Germany

Xavier Rogiers, Gent University Hospital and Medical School, 9000 Gent, Belgium

**Author contributions:** Gehling UM, Willems M and Rogiers X designed the study; Gehling UM and Schlagner K performed the research; Benndorf RA, Dandri M and Petersen J contributed to analysis and interpretation of the data; Sterneck M, Pollok JM and Hossfeld DK contributed to data acquisition; Gehling UM wrote the manuscript.

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**Correspondence to:** Ursula M Gehling, MD, Department of Hepatobiliary Surgery and Transplant Surgery, University Hospital Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany. [gehling@uke.uni-hamburg.de](mailto:gehling@uke.uni-hamburg.de)

Telephone: +49-40-428039586 Fax: +49-40-428035898

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**RESULTS:** Progenitor cells with a CD133<sup>+</sup>/CD45<sup>+</sup>/CD14<sup>+</sup> phenotype were observed in 61% of the patients. Between 1% and 26% of the peripheral blood mononuclear cells (MNCs) displayed this phenotype. Furthermore, a distinct population of c-kit<sup>+</sup> progenitor cells (between 1% and 38 % of the MNCs) could be detected in 91% of the patients. Additionally, 18% of the patients showed a population of progenitor cells (between 1% and 68% of the MNCs) that was characterized by expression of breast cancer resistance protein-1. Further phenotypic analysis disclosed that the circulating precursors expressed CXC chemokine receptor 4, the receptor for SDF-1. In line with this finding, elevated plasma levels of SDF-1 were present in all patients and were found to correlate with the number of mobilized CD133<sup>+</sup> progenitor cells.

**CONCLUSION:** These data indicate that in humans, liver cirrhosis leads to recruitment of various populations of hematopoietic progenitor cells that display markers of intrahepatic progenitor cells.

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**Key words:** CD133 antigen; CD14 antigen; c-kit protein; Breast cancer resistance protein-1 protein; Progenitor cells; CXC chemokine receptor 4; Stromal cell-derived factor-1; Liver cirrhosis

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### Abstract

**AIM:** To test the hypothesis that liver cirrhosis is associated with mobilization of hematopoietic progenitor cells.

**METHODS:** Peripheral blood samples from 72 patients with liver cirrhosis of varying etiology were analyzed by flow cytometry. Identified progenitor cell subsets were immunoselected and used for functional assays *in vitro*. Plasma levels of stromal cell-derived factor-1 (SDF-1) were measured using an enzyme linked immunosorbent assay.

Gehling UM, Willems M, Schlagner K, Benndorf RA, Dandri M, Petersen J, Sterneck M, Pollok JM, Hossfeld DK, Rogiers X. Mobilization of hematopoietic progenitor cells in patients with liver cirrhosis. *World J Gastroenterol* 2010; 16(2): 217-224 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i2/217.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i2.217>

## INTRODUCTION

Since 2000, several studies have suggested that after transplantation of bone-marrow-derived hematopoietic stem and progenitor cells, such cells have the capacity to migrate into the liver and differentiate into functional hepatocytes<sup>[1-4]</sup>. However, recent data have indicated that cell fusion, but not transdifferentiation, is the principle mechanism by which bone-marrow-derived cells acquire the features of hepatocytes<sup>[5-7]</sup>, and that the myelomonocytic progeny rather than the bone marrow stem cells are the major fusion partners of hepatocytes<sup>[8,9]</sup>.

In a previous study, we have demonstrated that partial hepatectomy (PH) in healthy human liver donors induces a significant mobilization of CD133<sup>+</sup> hematopoietic progenitor cells into the peripheral blood<sup>[10]</sup>. Besides their hematopoietic potential, these cells can differentiate into cells with a hepatocytic morphology and phenotype *in vitro*, which suggests that the mobilized progenitor cells participate in liver repair *in vivo*. The released progenitor cells display a uniform monocytic phenotype, and with respect to their hematopoietic differentiation capacity, represent myelomonocytic precursor cells. Hence, these cells might be endowed with a high fusion potential. It has been shown that a subset of peripheral blood CD14<sup>+</sup> cells can differentiate into multiple cell lineages of all three germ layers<sup>[11,12]</sup>, therefore, it is also conceivable that the CD133<sup>+</sup> progenitor cells identified in our study are the ancestor cell of this subset, with a similar differentiation plasticity.

By hypothesizing that PH-induced CD133<sup>+</sup> cells also occur in other clinical situations of liver injury, we analyzed peripheral blood samples of patients with liver cirrhosis for the presence of these precursors. As in our previous study, we used antibodies against the stem cell markers c-kit and breast cancer resistance protein-1 (Bcrp-1) to characterize further the phenotype of circulating CD133<sup>+</sup> cells. Using this approach, we found different populations of hematopoietic progenitor cells, including the CD133<sup>+</sup> population observed after PH.

## MATERIALS AND METHODS

### Materials

Phycoerythrin (PE)-conjugated anti-CD133 monoclonal antibody (MoAb; clone AC141), anti-CD133 MoAb-conjugated super paramagnetic beads, anti-c-kit MoAb-conjugated super paramagnetic beads, and anti-IgG2b secondary microbeads were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-BCRP-1 MoAb and PE-anti-Bcrp-1 were purchased from eBioscience (Vienna, Austria). PE-anti-c-kit MoAb was from DAKO Cytomation (Hamburg, Germany). Fluorescein isothiocyanate (FITC)-conjugated anti-CD34 MoAb, anti-CD45 MoAb, anti-CD14 MoAb, as well as PE- and FITC-conjugated isotype-matched mouse immunoglobulins were from BD Pharmingen (Heidelberg, Germany), and FITC-anti-CXC chemokine receptor

4 (CXCR4) MoAb from R&D Systems (Wiesbaden, Germany). The stromal cell-derived factor-1 (SDF-1) enzyme linked immunosorbent assay (ELISA) kit was purchased from R&D Systems. Fibronectin was from Gibco Life Technologies (Karlsruhe, Germany), and chamber slides were from Becton Dickinson. Methylcellulose and methylcellulose supplemented with hematopoietic growth factors were purchased from Cell Systems (St. Katharinen, Germany). Paraformaldehyde and methanol were from Sigma (Deisenhofen, Germany). Anti-multi-cytokeratin antibody (rabbit anti-human polyclonal) was from Novocastra (Newcastle, UK). Anti-rabbit immuno alkaline-phosphatase polymer (N-Histofine) and the New Fuchsin substrate (N-Histofine Substrate Kit) were purchased from Medac (Hamburg, Germany).

### Collection of peripheral blood samples

The study involved peripheral blood samples from 72 patients with liver cirrhosis of varying etiology. All patients were enrolled into University Hospital Hamburg-Eppendorf Liver Transplant Protocols and gave informed consent for their blood samples to be used for experimental purposes. The study protocol was approved by the University Hospital's Research Committee and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. All samples were collected in heparinized tubes and immediately prepared for analysis.

### Flow cytometry

After lysis with hemolytic buffer (0.155 mol/L NH<sub>4</sub>Cl, 0.012 mol/L NaHCO<sub>3</sub>, 0.1 mmol/L EDTA, pH 7.2) for 5 and 2 min,  $1 \times 10^6$  cells were incubated with PE-conjugated anti-CD133 MoAb in combination with either FITC-conjugated anti-CD34 MoAb, anti-CD45 MoAb, anti-CD14 MoAb or anti-CXCR4 MoAb. Other combinations included FITC-anti-CD34 MoAb, FITC-anti-CD14 MoAb, or FITC-anti-CXCR4 with either PE-anti-Bcrp-1 or PE-anti-c-kit. Isotype-matched mouse immunoglobulins (BD Pharmingen) served as controls. All incubations were performed at 4°C in the presence of normal goat serum. Two-color flow cytometry was accomplished using a FACSCalibur flow cytometer (Becton Dickinson) and CellQuest software (Becton Dickinson). Each analysis included at least 50 000 events. By using isotype controls for PE and FITC, gates for analysis were set such that the lower left panel contained at least 98% of the total cells. The percentage of positive cells was assessed after correction for the percentage of cells that was reactive with the respective isotype control.

### Isolation of mobilized progenitor cells by magnetic cell sorting

Cells were incubated with either anti-CD133 MoAb, anti-c-kit MoAb conjugated super paramagnetic beads or with anti-Bcrp-1 MoAb and anti-IgG2b secondary microbeads, washed, and processed through a MACS magnetic



separation column (Miltenyi Biotec), as previously described<sup>[13]</sup>. An aliquot of the purified cells was analyzed by flow cytometry.

### Quantification of SDF-1 by ELISA

The plasma levels of SDF-1 were measured using a sandwich ELISA (R&D Systems) according to the manufacturer's instructions. Absorbance at 450 nm was determined by an automated ELISA reader (THERMOMax; Molecular Devices, Ismaning, Germany). Regression curve was used to convert OD units to picograms per milliliter of SDF-1. Patients' samples were run in triplicate.

### Suspension cultures

Immunoselected cells were cultured in fibronectin-coated chamber slides at a density of  $2 \times 10^6$  cells/mL in stem cell growth medium, as previously described<sup>[13]</sup>. Hepatocytic differentiation was induced as previously described<sup>[10]</sup>. Cells were incubated for up to 28 d at 37°C in 5% CO<sub>2</sub>. Additional feeding was performed on alternate days. The supernatant was then removed by gentle pipetting and replaced with fresh medium.

### Clonogenic assays for hematopoietic progenitors

Purified cells were plated at  $1 \times 10^3$  cells/mL in semisolid growth medium (methylcellulose) that was supplemented with various hematopoietic growth factors, as previously described<sup>[13]</sup>. All cultures were performed in duplicate, incubated at 37°C in 5% CO<sub>2</sub> and 95% humidity, and scored after 14 d culture, using an inverted microscope.

### Immunocytochemical analysis of CD133-derived cells

Cultured CD133-derived cells were fixed with 4% paraformaldehyde for 10 min at room temperature followed by fixation with methanol for 2 min at -20°C or with ice-cold acetone for 10 min. After blocking with 10% goat serum for 20 min, specimens were incubated with an anti-multi-cytokeratin antibody overnight at room temperature. The reactivity was detected by using an anti-rabbit immuno alkaline-phosphatase polymer in combination with the New Fuchsin substrate. Specimens were counterstained with hematoxylin. Negative controls included replacement of primary antibody with isotypes or PBS, as well as staining of peripheral blood smears. Positive controls were performed using cytopspins with freshly thawed primary human hepatocytes (own preparations from resected liver tissue).

### Statistical analysis

Distribution of data was tested with the Kolmogorov-Smirnov test. Variables are expressed as mean  $\pm$  SE. Bivariate correlations were analyzed with Spearman's rho as indicated.  $P < 0.05$  was considered significant. For all statistical analysis, SPSS version 13.0 was used.

## RESULTS

### Patients' characteristics

The characteristics of the patients studied are shown in

Table 1 Patient characteristics

	AIH	ALD	Hepatitis B	Hepatitis C	Other
<i>n</i>	11	30	9	13	9
Sex (M/F)	3/8	21/9	7/2	8/5	5/4
Age (yr, $\pm$ SE)	47.4 $\pm$ 7	52.9 $\pm$ 8	52.5 $\pm$ 5	50.5 $\pm$ 8	51.8 $\pm$ 9
Child A	6	11	7	6	4
Child B	3	17	2	6	4
Child C	2	4	0	1	1

AIH: Autoimmune hepatitis (including primary biliary sclerosis and primary sclerosing cholangitis); ALD: Alcoholic liver disease. Other includes patients with polycystic liver and with non-specified cirrhosis.

Table 2 Types and frequencies of circulating progenitor cells in patients with liver cirrhosis

Cell subset	Positive/total number of patients	% of the MNCs
CD133 <sup>+</sup>	44/72 (61.2%)	5.8 $\pm$ 4.9
c-kit <sup>+</sup>	65/72 (90.9%)	9.2 $\pm$ 3.2
Bcrp-1 <sup>+</sup>	12/72 (17.9%)	4.1 $\pm$ 3.7

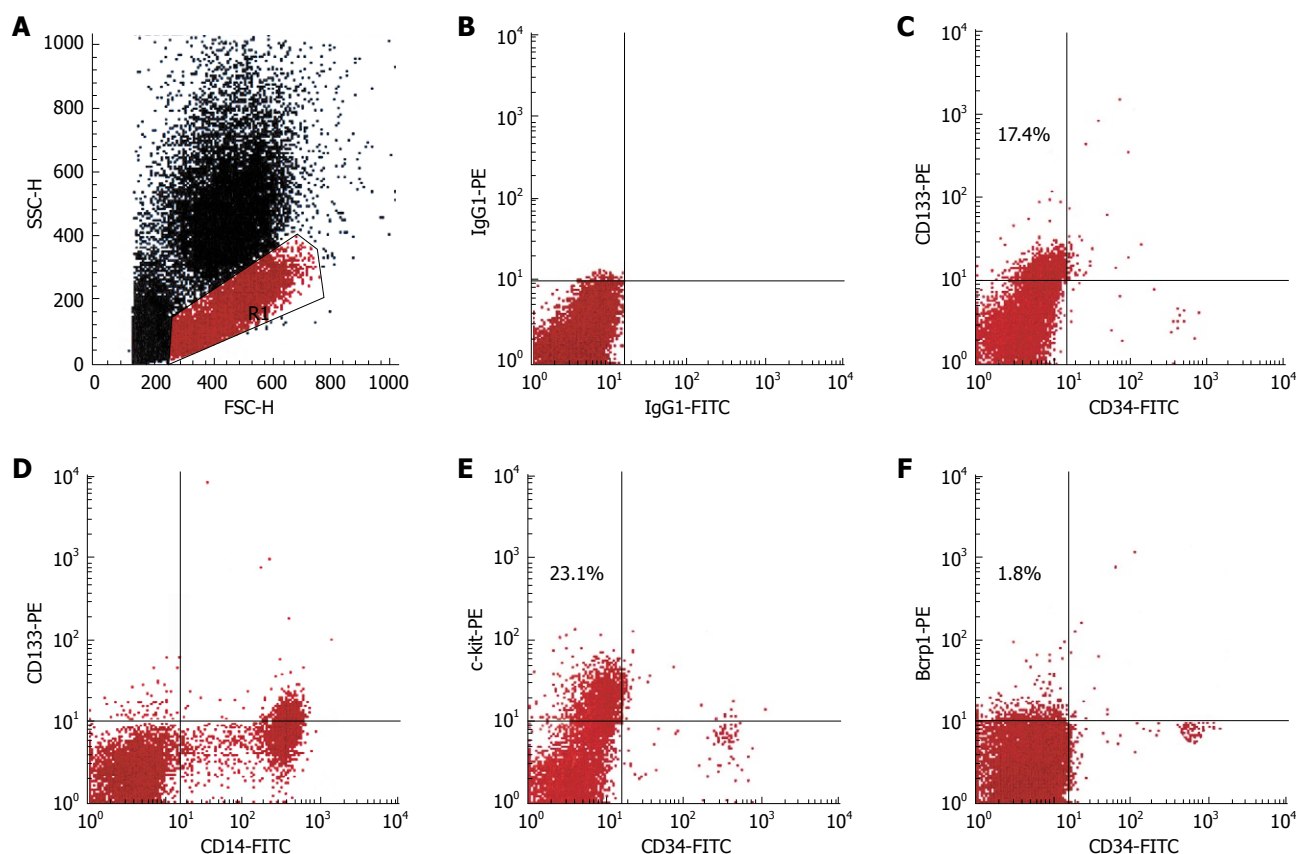
Table 1. Cirrhosis was caused by autoimmune inflammation (11 patients), alcoholic liver disease (ALD, 30 patients), hepatitis B (9 patients), hepatitis C (13 patients), or to non-specified etiology. Autoimmune-mediated cirrhosis was observed more frequently in female patients, whereas male patients were predominantly affected by ALD and viral hepatitis. The subgroups did not significantly differ in age. With respect to the Child score, advanced liver cirrhosis was most prominent in the ALD group.

### Mobilization of CD133<sup>+</sup>, c-kit<sup>+</sup> and Bcrp-1<sup>+</sup> populations

As revealed by flow cytometry, CD133<sup>+</sup> cells were observed in 61% (44/72) of all patients (Table 2). On average, 5.8% of the peripheral blood mononuclear cells (MNCs) expressed this marker. Further phenotypical characterization showed that the vast majority of these cells coexpressed CD14 (Figure 1) and CD45 but not CD34 (data not shown), which indicated that this population was identical to PH-induced progenitor cells. Unexpectedly, a distinct population of c-kit<sup>+</sup> cells was found in > 90% of the patients studied. Between 1% and 38% of the MNCs displayed this phenotype. In 12 patients, an additional population of Bcrp-1<sup>+</sup> cells was detectable, which on average, represented 4.1% of the MNCs (Table 2). All three subsets coexpressed CD45, whereas coexpression of CD34 and/or CD14 was variable in c-kit<sup>+</sup> and Bcrp-1<sup>+</sup> populations (data not shown).

### Lack of correlation between phenotypes and numbers of mobilized progenitor cells and clinical parameters

Analysis of peripheral blood samples from the same patient at different time points showed that progenitor cell mobilization is not a permanent phenomenon. The phenotypes and numbers of circulating progenitors varied in the same patient in an irregular timely manner. Thereby, no correlation was found with any clinical parameters,



**Figure 1** Characterization of circulating progenitor cell populations by flow cytometry. A: Forward/sideward scatter analysis; B: Isotype controls; Representative two-color flow cytometry analysis of the peripheral blood mononuclear cell (MNC) fraction, demonstrating CD133<sup>+</sup> (C and D), c-kit<sup>+</sup> (E), and Bcrp-1<sup>+</sup> (F) populations.

such as liver enzymes, bilirubin, serum albumin, leukocyte count, and platelet count or with the etiology or stage of disease (data not shown). However, numbers of circulating CD133<sup>+</sup> progenitor cells inversely correlated with patients' age (Figure 2A). In addition, there was a significant positive correlation between the numbers of CD133<sup>+</sup>/CD34<sup>+</sup> and Bcrp-1<sup>+</sup>/CD34<sup>+</sup> peripheral blood cells (Figure 2B).

### Mobilization involves the SDF-1/CXCR4 chemokine receptor system

To investigate the molecular mechanisms that mediate progenitor cell mobilization, peripheral blood progenitor cells were analyzed for the expression of CXCR4, the receptor for SDF-1. Virtually all mobilized CD133<sup>+</sup> cells coexpressed this receptor, whereas in the c-kit<sup>+</sup> populations, on average, half of the cells stained positive for CXCR4 (Figure 3). As mentioned before, Bcrp-1<sup>+</sup> populations were only observed in a few patients. In the set of experiments in which coexpression of CXCR4 was studied, no patient showed elevated numbers of Bcrp-1<sup>+</sup> cells, therefore, the expression of CXCR4 on these cells remains to be explored. In view of the finding that the CD133<sup>+</sup> and c-kit<sup>+</sup> population were found to exhibit CXCR4, the plasma levels of SDF-1 were measured ( $n = 44$ ). Elevated SDF-1 levels were noted in all patients studied. Statistical analysis revealed a significant positive correlation of the plasma levels with the number of mobilized CD133<sup>+</sup>/CD34<sup>+</sup> cells (Figure 2C).

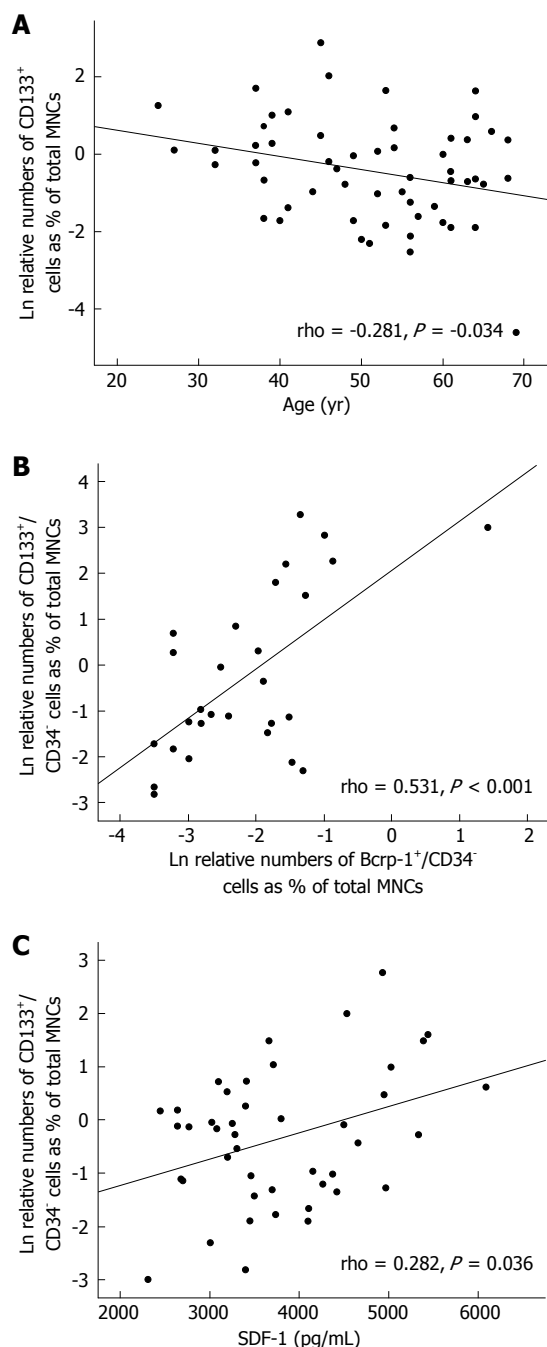
**Table 3** Clonogenic potential of circulating hematopoietic progenitor cell populations (mean  $\pm$  SE)

Cells	BFU-E	CFU-E	CFU-GEMM	CFU-GM	CFU-G	CFU-M
c-kit	5 $\pm$ 2	13 $\pm$ 5	3 $\pm$ 3	19 $\pm$ 8	53 $\pm$ 11	27 $\pm$ 9
Bcrp-1	8 $\pm$ 3	9 $\pm$ 2	4 $\pm$ 1	9 $\pm$ 7	37 $\pm$ 14	13 $\pm$ 5
CD133	0	0	0	5 $\pm$ 3	46 $\pm$ 13	63 $\pm$ 15

The results of three independent experiments per populations are shown, each experiment was performed in duplicate. BFU-E: Burst-forming unit erythrocyte; CFU-E: Colony-forming unit erythrocyte; CFU-GEMM: Colony-forming unit granulocyte-erythrocyte-macrophage-megakaryocyte; CFU-GM: Colony-forming unit granulocyte-macrophage; CFU-G: Colony-forming unit granulocyte; CFU-M: Colony-forming unit macrophage.

### In vitro functional properties of the mobilized populations

To evaluate the clonogenic potential of the cirrhosis-induced progenitor cells, each subset was enriched by immunoselection and transferred to a standard colony assay for hematopoietic stem and progenitor cells. As shown in Table 3, c-kit<sup>+</sup> populations and Bcrp-1<sup>+</sup> cells had the capacity to produce colonies of the erythroid, granulocytic, and macrophage/monocytic lineage, as well as mixed colonies. In line with our previous study, CD133<sup>+</sup> populations solely gave rise to colonies of the granulocytic and macrophage/monocytic lineage. The three progenitor cell populations were also evaluated for their potential to differentiate into the hepatocytic lineage, using culture conditions that were suitable for stimulating hepatocytic



**Figure 2 Significant correlations.** Correlation of numbers of circulating CD133<sup>+</sup> cells with patient age (A). Correlation of the numbers of circulating CD133<sup>+</sup>CD34<sup>+</sup> cells with the numbers of circulating Bcrp-1<sup>+</sup>/CD34<sup>+</sup> cells (B) and with stromal cell-derived factor-1 (SDF-1) plasma levels (C).

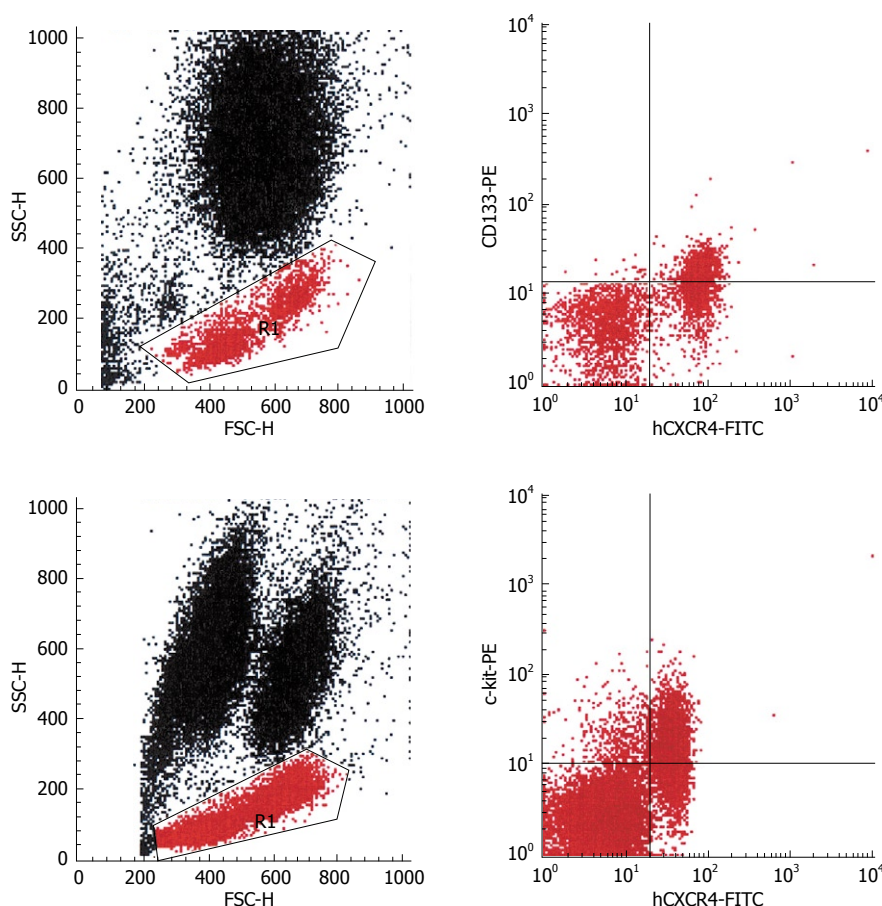
differentiation of PH-induced CD133<sup>+</sup> progenitor cells<sup>[10]</sup>. CD133<sup>+</sup> populations reproducibly generated an adherent layer of cytokeratin-expressing cells, while c-kit<sup>+</sup> and Bcrp-1<sup>+</sup> progenitor cells did not differentiate towards the hepatocytic lineage and could only be maintained in culture for 5 d (data not shown).

## DISCUSSION

We demonstrated in this study that liver cirrhosis is associated with mobilization of CD133<sup>+</sup>, c-kit<sup>+</sup>, and

Bcrp-1<sup>+</sup> populations of hematopoietic progenitor cells. Our previous studies have shown that liver resection leads to a mobilization of a unique population of CD133<sup>+</sup> progenitor cells with a myelomonocytic phenotype and hematopoietic as well as hepatocytic differentiation potential *in vitro*. The finding that identical cells are present in the peripheral blood of patients with liver cirrhosis is highly indicative of a certain role of these cells in liver regeneration. In this context, there is increasing evidence that the intrahepatic compartment of progenitor cells, which are referred to as “oval cells” in rodents and as “ductular reactions” in humans, and which can only be observed as proliferates in diseased liver, expresses CD133<sup>[14-18]</sup>. A recent study has suggested that the human liver contains CD133<sup>+</sup> stem cells that differ from the “ductular reactions” with respect to functional properties, and the fact that the CD133<sup>+</sup> stem cells can be isolated from healthy livers<sup>[19]</sup>. The demonstration of circulating c-kit<sup>+</sup> and Bcrp-1 progenitor cell populations in patients with liver cirrhosis might also be of functional significance, because both markers also have been shown to be expressed on human intrahepatic progenitor cells<sup>[20,21]</sup>. However, functional analyses *in vitro* have suggested that the c-kit<sup>+</sup> and Bcrp-1<sup>+</sup> populations identified in this study are multi-lineage hematopoietic progenitor cells without hepatocytic potential, whereas the CD133<sup>+</sup> subset represents hematopoietic progenitor cells committed to the myelomonocytic lineage, which possess hepatocytic potential. Alternatively, it is possible that the c-kit<sup>+</sup> and Bcrp-1<sup>+</sup> cells require other stimuli than used to differentiate into hepatocytic cells.

Progenitor cell mobilization in patients with liver cirrhosis was found not to be a permanent phenomenon. Only a minority of patients showed all three populations at the same time point. Unexpectedly, the occurrence of mobilized progenitor cells did not correlate with any clinical parameter. This led us to the hypothesis that mobilization and recruitment, respectively, of progenitor cells in these patients is triggered by intra-organ hypoxia. It has been shown that: (1) hypoxia induces mobilization of bone-marrow-derived endothelial progenitor cells for neovascularization<sup>[22]</sup> and the production of SDF-1 in endothelial cells<sup>[23]</sup>, and (2) SDF-1 is involved in stress-induced stem cell recruitment to the liver<sup>[24,25]</sup>. Therefore, we investigated expression of the SDF-1 receptor CXCR4 on the progenitor cell populations and assayed the plasma levels of SDF-1 in these patients as a first approach to test our hypothesis. Virtually all mobilized CD133<sup>+</sup> cells coexpressed CXCR4, whereas only a portion of c-kit<sup>+</sup> cells displayed this receptor. Coexpression of CXCR4 by Bcrp-1<sup>+</sup> cells could not be evaluated because the patients included in this set of experiments did not mobilize Bcrp-1<sup>+</sup> cells. However, the number of circulating CD133<sup>+</sup> cells was found to correlate positively with SDF-1 plasma levels, which were elevated in all patients studied, and the numbers of circulating CD133<sup>+</sup>/CD34<sup>+</sup> cells correlated with the numbers of Bcrp-1<sup>+</sup>/CD34<sup>+</sup> cells, which indicated that



**Figure 3** Coexpression of CXC chemokine receptor 4 (CXCR4), the receptor for SDF-1, on circulating CD133<sup>+</sup> cells and c-kit<sup>+</sup> cells as revealed by flow cytometry.

the numbers of Bcrp1<sup>+</sup>/CD34<sup>+</sup> cells might also correlate with SDF-1 plasma levels. Hence, we hypothesize that within the Bcrp1<sup>+</sup> population, Bcrp1<sup>+</sup>/CD34<sup>+</sup> cells coexpress CXCR4. Interestingly, the numbers of c-kit<sup>+</sup>/CD34<sup>+</sup> cells did not show a correlation with SDF-1 plasma levels. This finding is in line with the observation that not all c-kit<sup>+</sup> cells coexpressed CXCR4.

There are many hints in the literature that suggest that hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) plays a major role in progenitor cell recruitment to injured liver tissue. Using a mouse model of soft tissue ischemia, Ceradini *et al.*<sup>[26]</sup> have shown that HIF-1 $\alpha$  regulates progenitor cell trafficking in a hypoxic-dependent manner *via* induction of SDF-1 expression in endothelial cells of the ischemic tissue. The authors have hypothesized that hypoxia is a fundamental requirement for progenitor cell trafficking and function. We do not believe that hypoxia is a prerequisite for progenitor cell recruitment in all situations of tissue injury, because numerous studies have shown that expression of HIF-1 $\alpha$  can be induced independently of hypoxia by many cytokines, growth factors and hormones<sup>[27]</sup>. Nevertheless, we believe that intrahepatic hypoxia is the initial event that triggers progenitor cell recruitment in liver injury. Hence, progenitor cell mobilization after PH could arise *via* an acute state of intrahepatic hypoxia, because large parts of the hepatic endothelium are removed. Liver cirrhosis most likely causes intermittent hypoxia each time when progressive fibrosis ligates relatively large intrahepatic blood vessels.

This would explain the intermittent occurrence of mobilized progenitor cells in these patients.

The origin of mobilized progenitor cells after PH and in patients with liver cirrhosis remains to be determined. The finding that the number of circulating CD133<sup>+</sup> cells correlated inversely with patient age suggests that at least this population is mobilized from the bone marrow, because it is known that the reserve pool of hematopoietic bone marrow stem and progenitor cells decreases with age<sup>[28]</sup>. No correlation was found between the number of c-kit<sup>+</sup> cells and age, which indicates that the c-kit<sup>+</sup> cells do not solely derive from the bone marrow. The c-kit<sup>+</sup> population might also comprise liver-derived angioblasts<sup>[19]</sup> that occur in the circulation because of hypoxia-induced proliferation of these cells in the liver. With respect to Bcrp1<sup>+</sup> cells, the number of patients that showed an increased number of these cells did not suffice to calculate the statistical significance of the correlation with age.

In summary, we have shown that liver cirrhosis is associated with an intermittent mobilization of various populations of hematopoietic progenitor cells into the circulation. One of the populations, characterized by expression of CD133, is identical to the progenitor cell population observed in peripheral blood after liver resection. Coexpression of CXCR4 on CD133<sup>+</sup> cells and c-kit<sup>+</sup> cells suggests their origin from the bone marrow. Furthermore, our data indicate an involvement of the SDF-1/CXCR4 chemokine receptor system in the mobilization process. Animal studies are needed to evaluate



the exact role of each progenitor cell population in liver regeneration.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

The liver is known to possess enormous regenerative potential. Nevertheless, the cellular mechanisms that govern human liver regeneration are not understood completely. There is an increasing body of evidence to suggest that various types of stem and progenitor cells play a role in this process.

### Research frontiers

The authors have demonstrated recently that liver resection in humans leads to a significant increase in the number of circulating cells that express the hematopoietic stem cell marker CD133 and the leukocyte markers CD45 and CD14. This was the first study to indicate that CD133<sup>+</sup> progenitor cells might contribute to liver regeneration. Meanwhile, several studies have shown that the human liver contains various populations of CD133<sup>+</sup> stem and progenitor cells. In addition, these cells are believed to be involved in hepatocarcinogenesis, a process that results from chronic liver regeneration.

### Innovations and breakthroughs

The present study further supports the hypothesis that the CD133<sup>+</sup> hematopoietic precursor cells observed after liver resection contribute to liver regeneration, because circulating cells with an identical phenotype were found in a large number of patients with liver cirrhosis of varying etiology. Furthermore, this study is believed to be the first to demonstrate the presence of two additional populations of hematopoietic progenitor cells in the peripheral blood of these patients. Both populations are characterized phenotypically by the expression of stem cell markers that are also known to be expressed on human intrahepatic progenitor cells.

### Applications

Understanding the cellular mechanisms of liver regeneration is a prerequisite for the development of novel strategies for treating liver disease. Further studies are needed to define the exact role of the three identified progenitor cell populations in liver regeneration and to evaluate their therapeutic potential.

### Terminology

CD133 is an antigen that is expressed on hematopoietic stem and progenitor cells, endothelial progenitor cells, neural progenitor cells, intrahepatic stem and progenitor cells, epithelial cells and various cancer stem cells. The physiological function of this molecule is not known yet. The antigen c-kit is the receptor for the hematopoietic cytokine stem cell factor and is expressed on hematopoietic stem and progenitor cells, endothelial and intrahepatic progenitor cells, as well as on monocytes, mast cells and dermal melanocytes. Breast cancer resistance protein-1 is a membrane protein, which was initially shown to be expressed on breast cancer cells. Functionally, it belongs to the ATP-binding cassette transporters that serve as protective efflux pumps for many cells, including hematopoietic stem cells and intrahepatic progenitor cells.

### Peer review

The article is well written, with interesting results that provide sufficient experimental evidence to draw the conclusions. The figures reflect the major findings of the study, and are presented appropriately. The discussion is well organized. References are appropriate, relevant and up to date.

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## Prevalence of occult hepatitis B virus infection in haemodialysis patients from central Greece

Paraskevi Mina, Sarah P Georgiadou, Christos Rizos, George N Dalekos, Eirini I Rigopoulou

Paraskevi Mina, Sarah P Georgiadou, Christos Rizos, George N Dalekos, Eirini I Rigopoulou, Department of Medicine and Research Laboratory of Internal Medicine, Medical School, University of Thessaly, 41110 Larissa, Thessaly, Greece  
George N Dalekos, Eirini I Rigopoulou, Research Group of Investigational Medicine, Institute of Biomedical Research and Technology, Centre for Research and Technology-Thessaly (CE. RE. TE. TH), 41222 Larissa, Thessaly, Greece

**Author contributions:** Dalekos GN and Rigopoulou EI had the original idea for the study, wrote the study protocol, and along with Mina P wrote the paper; Mina P, Rizos C and Georgiadou SP did the laboratory work, collected the clinical and biochemical data, and performed the statistical analysis; Dalekos GN and Rigopoulou EI wrote the final version of the paper; all authors have seen and approved the final draft of the paper.

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**Correspondence to:** George N Dalekos, MD, PhD, Professor and Chairman, Department of Medicine and Research Laboratory of Internal Medicine, Medical School, University of Thessaly, 41110 Larissa, Thessaly, Greece. [dalekos@med.uth.gr](mailto:dalekos@med.uth.gr)  
Telephone: +30-2410-2285 Fax: +30-2410-671863

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**RESULTS:** HBV-DNA was detected in 15/366 patients (4.1%) and HBsAg in 20/366 (5.5%). The prevalence of occult HBV infection was 0.9% (3/346 HBsAg-negative patients). Occult HBV was not associated with a specific marker of HBV infection or anti-HCV or anti-HEV reactivity. There was no significant difference in HBV-DNA titres, demographic and biochemical features, between patients with occult HBV infection and those with HBsAg-positive chronic HBV infection.

**CONCLUSION:** In central Greece, 4% of ESRF patients had detectable HBV-DNA, though in this setting, the prevalence of occult HBV seems to be very low (0.9%).

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**Key words:** Hepatitis B virus-DNA; Occult hepatitis B virus infection; Haemodialysis; Hepatitis B; Real-time polymerase chain reaction

**Peer reviewers:** Guang-Wen Cao, MD, PhD, Professor and Chairman, Department of Epidemiology, Second Military Medical University, 800 Xiangyin Rd., Shanghai 200433, China; Zeinab Nabil Ahmed, Professor of Microbiology, Microbiology & Immunology Department, Faculty of Medicine (for girls), Al-Azhar University, Nasr City, Cairo, Egypt

### Abstract

**AIM:** To assess the hepatitis B virus (HBV)-DNA and the prevalence of occult HBV infection in end-stage renal failure (ESRF) patients from Central Greece.

**METHODS:** Sera from 366 ESRF patients attending five out of six dialysis units from Central Greece were investigated for HBV-DNA by real-time polymerase chain reaction. Only serum samples with repeatedly detectable HBV-DNA were considered positive. IgG antibodies to hepatitis C virus (anti-HCV) were tested by a third generation enzyme linked immunosorbent assay (ELISA), while IgG antibodies to hepatitis E virus (anti-HEV) were tested by two commercially available ELISAs.

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### INTRODUCTION

Infections with hepatitis B virus (HBV) and hepatitis C virus (HCV) are well-known and important causes of liver disease in end-stage renal failure (ESRF) patients on haemodialysis (HD)<sup>[1-4]</sup>. The adoption of preventive

measures and extensive infection control guidelines, along with a decreased need for transfusions after the introduction of erythropoietin, and the development of an effective HBV vaccine, have significantly contributed to the progressive reduction of HCV and HBV prevalence in HD patients<sup>[2,4]</sup>. However, the relatively low response rates to HBV vaccination in this group of patients might contribute, under some specific circumstances, to the ongoing HBV transmission in this setting.

On the other hand, three studies from different parts of Greece have reported a high prevalence of antibodies against the hepatitis E virus (HEV) -an icosahedral non-enveloped single stranded RNA virus- in ESRF patients on HD, compared to the healthy population<sup>[5-7]</sup>, suggesting that the oral-faecal route may not be the only mode of transmission in HD. However, a similar prevalence of anti-HEV antibodies in Greek patients who have undergone open-heart surgery might indicate that past infections, and not viral exposure during HD or other interventional procedures, are responsible for the acquisition of HEV<sup>[8]</sup>.

In chronic renal failure patients undergoing maintenance HD, the diagnosis of liver disease based on biochemical tests is difficult; taking into account that aminotransferase levels in HD patients are usually suppressed<sup>[9]</sup>. It has been hypothesized that reduced immune competence of chronic uremic patients is a possible cause of attenuated inflammatory reactions in the liver and consequently reduced hepatocyte destruction<sup>[2,4,9]</sup>. Therefore, the quantitative detection of HBV-DNA has been shown to be the most efficient method to evaluate viral replication in HD patients infected with HBV<sup>[2]</sup>. Besides, several studies using sensitive HBV-DNA polymerase chain reaction (PCR) assays have revealed the presence of HBV-viremia in patients who cleared HBsAg from either acute self-limited or chronic HBV infection, or even after a successful anti-HBV treatment<sup>[10]</sup>. Demonstration of this clinical entity has resulted in the introduction of the concept of "occult" HBV infection, which is characterised by undetectable serum HBsAg but detectable HBV-DNA in serum or the liver<sup>[11]</sup>. Occult HBV infection has been reported in patients with chronic HCV infection<sup>[12-15]</sup>, hepatocellular carcinoma, and cryptogenic or autoimmune liver diseases<sup>[16,17]</sup>. Data on occult HBV infection among patients on long term HD is scarce<sup>[18-23]</sup>, as most of the studies have investigated the presence of occult HBV infection in the context of chronic HCV infection<sup>[21-23]</sup>. Greece belongs to the intermediate endemicity countries with a wide variance of HBsAg seropositivity among different regions and various populations, ranging from 3%-5% and markers of previous HBV infection between 17% and 25%<sup>[24-28]</sup>. Unpublished data from our group (Gatselis, Stefanos, Koukoulis and Dalekos, data in preparation), have estimated the prevalence of HBV infection in 2006, in a representative sample of the general population of the region of Thessaly to be 4.26% (range 3.6%-5.2%, age range 18-80 years,  $n = 1174$ ).

For these reasons, we conducted a large study in Thessaly, Central Greece to: (1) determine the presence of HBV-DNA in all 366 consecutive ESRF patients with or without chronic HBV infection attending the five out of six Nephrology and Dialysis units in Thessaly region; and (2) assess the prevalence of occult HBV infection in this cohort. Additionally, the possible clinical impact of HBV-DNA positivity in these patients (either in the context of chronic or occult HBV infection) was assessed by comparing epidemiological, clinical, laboratory and HBV, HCV and HEV serological markers between HBV-DNA-positive and -negative patients.

## MATERIALS AND METHODS

Thessaly is one of the thirteen regions of Greece and covers the largest part of central Greece. The prefectures of Larissa (capital city Larissa), Magnesia (capital city Volos), Trikala (capital city Trikala) and Karditsa (capital city Karditsa) constitute the Thessaly region<sup>[25,29]</sup>. The population is approximately 800 000 people (census 2001; 7.5% of the national population and 0.22% of the EU population). All 366 ESRF patients (243 men, 123 women; mean  $\pm$  age  $60.5 \pm 14$  years and mean duration of HD  $49.2 \pm 48.2$  mo) attending five out of the six Nephrology and Dialysis units of Thessaly region in Central Greece were studied. Sixty patients were from the Nephrology and Dialysis unit of the General Hospital in the city of Larissa (unit 1), 70 from the only private dialysis centre in the city of Larissa (unit 2), 119 from the dialysis unit of the General Hospital in the city of Volos (unit 3), 56 from the dialysis unit of the General Hospital in the city of Trikala (unit 4) and 61 from the dialysis unit of the General Hospital in the city of Karditsa (unit 5). The aetiology of renal failure was as follows: diabetic nephropathy (16.7%), idiopathic glomerulonephritis (14.5%), vascular renal disease (9.3%), urinary tract obstruction (9.3%), polycystic kidney disease (7.7%), systemic diseases (3.3%), tubulointerstitial diseases (1.6%), other causes (3.3%), and unknown (34.3%). The patients' characteristics are shown in Table 1.

Serum samples were collected between May and August 2001 (before HD) and then stored at  $-80^{\circ}\text{C}$  in aliquots until tested. Serological markers of HBV infection (HBsAg, anti-HBs, anti-HBc, HBeAg, and anti-HBe) were determined using standard third generation commercially available enzyme immunoassays (Abbott GmbH, Wiesbaden-Delkenheim, Germany). The samples were investigated for the presence of HBV-DNA using a sensitive commercially available real time PCR kit (COBAS Taqman HBV Test; cut-off of detection: 6 IU/mL)<sup>[30]</sup>. The COBAS TaqMan HBV Test is an *in vitro* nucleic acid amplification test for quantitation of HBV in human serum or plasma, using the High Pure Viral Nucleic Acid kit for manual specimen preparation and the COBAS TaqMan 48 Analyzer for automated amplification and detection. The highly conserved HBV precore/core region was



Table 1 Characteristics of 366 ESRF patients (mean  $\pm$  SD)

	ESRF patients (n = 366)
Sex (M/F)	243/123
Age (mean/range, yr)	60.5/17-85
Haemodialysis duration (mo) <sup>1</sup>	49.2 $\pm$ 48.2
Dialysis type (haemodialysis/peritoneal dialysis)	352/14
Known history of viral hepatitis (yes/no) <sup>2</sup>	51/315
History of transfusions (yes/no)	129/247
Number of blood units	6.8 $\pm$ 4.7
Duration of erythropoietin treatment (mo)	46.2 $\pm$ 35.8

<sup>1</sup>Haemodialysis duration was calculated from the date of the first haemodialysis procedure to the date of the collection of the sera; <sup>2</sup>Proven history of viral hepatitis, either icteric or anicteric. M: Male; F: Female; ESRF: End-stage renal failure.

amplified. Every sample with detectable HBV-DNA by real time PCR was tested again in another experiment. Only serum samples with repeatedly detectable HBV-DNA were considered positive for HBV-DNA.

Anti-HCV antibody was tested by a third generation enzyme linked immunosorbent assay (ELISA) (HCV 3.0 ELISA Ortho, Raritan, NJ) according to the manufacturers' instructions. For the detection of anti-HEV IgG antibodies two commercially available ELISAs (Abbott Diagnostika, Wiesbaden-Delkenheim, Germany and Genelabs Diagnostics, Singapore Science Park, Singapore) were used. These assays included two recombinant antigens, which correspond to the structural regions of the HEV genome<sup>[7]</sup>. Both ELISAs were performed according to the manufacturers' instructions. Only repeatedly reactive samples in both assays were considered positive for the presence of anti-HEV IgG. According to the above criteria, initially reactive specimens that were non-reactive on retesting were considered negative.

Levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined using standard techniques. Informed consent was obtained from all patients involved in the study. The Local Ethical Committee of the Medical School, University of Thessaly, approved the study protocol.

### Statistical analysis

Results were expressed as mean  $\pm$  SD or median and range as appropriate. Data were analyzed by unpaired *t*-test,  $\chi^2$  (two by two with Yates' correction), and Fisher's exact test, where applicable. A two-sided *P* value < 0.05 was considered as statistically significant. Confidence intervals (95% CI) were determined using the formula  $P = p \pm 1.96 (pq/n)^{1/2}$  where *p* is the frequency, *q* is 1 - *p* and *n* is the number of individuals tested from each group.

## RESULTS

In total, HBV-DNA was detected in 15 of the 366 patients with ESRF (4.1%; 95% CI: 2%-6.1%). Twenty patients (5.5%) were HBsAg positive (overt HBV infection) and

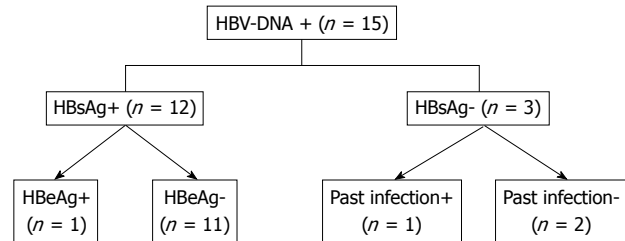


Figure 1 Flowchart of the profile of 15 hepatitis B virus (HBV)-DNA positive haemodialysis patients.

245 were anti-HBs positive (66.9%) (Table 2). Eighty-eight (24%) and 15 (4.1%) were anti-HCV and anti-HEV positive, respectively (Table 2). The prevalence of HBV-DNA seropositivity ranged from 1.4%-10% among the five Dialysis units (*P* = 0.163) (Table 2). The median HBV-DNA titre was 25 IU/mL (range: 7-10 584 IU/mL). HBV-DNA seropositivity was not associated with age, sex, history of hepatitis, history of blood transfusions, the number of blood units transfused, the dialysis type, or with the aetiology of renal failure, aminotransferase activity or anti-HEV reactivity. As expected, there was a significant association between the presence of HBV-DNA and HBsAg, anti-HBc, anti-HBe positivity (*P* < 0.05 for all, Table 3). The absence of HBV-DNA was associated with the presence of anti-HBs and anti-HCV (*P* < 0.05 for both, Table 3).

Amongst the 15 HBV-DNA positive patients, 12 had overt HBV infection (80%), including one HBeAg positive, 10 anti-HBe positive and one anti-HBe positive patient who was also anti-HBs positive. Of the three remaining HBV-DNA positive patients, one had markers of past HBV infection (anti-HBc and anti-HBs positive) and two had no serological markers of present or past HBV infection (Figure 1). Thus, the overall prevalence of occult HBV infection amongst the 346 HBsAg negative ESRF patients in the five Nephrology and Dialysis units in the Thessaly region was 0.9% (3/346; 95% CI: 0%-1.9%). Actually, the prevalence of occult HBV infection in Volos Dialysis Unit was 2.6% (3/116) compared to zero prevalence in all other Dialysis Units in the Thessaly region (*P* = 0.199). HBV-DNA titres (median titre: 19 IU/mL, range: 7-37 IU/mL) in patients with occult HBV infection were not significantly different compared to those with overt HBV infection (median: 27 IU/mL, range: 9-10 584 IU/mL, *P* = 0.391). Occult HBV infection was associated neither with age, sex, history of hepatitis, history of blood transfusions, the number of blood units transfused, biochemical parameters (AST, ALT) nor with the aetiology of renal failure (data not shown). Moreover, occult HBV-DNA detection was not associated with a specific marker of past HBV infection and anti-HCV or anti-HEV reactivity. No statistical significance was found comparing the age, HD duration, biochemical parameters, and serological markers of HCV and HEV infections or the duration of erythropoietin treatment

**Table 2** Prevalence of HBV, HCV, and HEV serological markers, as well as HBV-DNA, in individual haemodialysis units *n* (%)

	Unit 1 ( <i>n</i> = 60)	Unit 2 ( <i>n</i> = 70)	Unit 3 ( <i>n</i> = 119)	Unit 4 ( <i>n</i> = 56)	Unit 5 ( <i>n</i> = 61)	Total ( <i>n</i> = 366)
HBsAg	9 (15)	2 (2.9)	3 (2.5)	2 (3.6)	4 (6.6)	20 (5.5)
Anti-HBs	41 (68.3)	39 (55.7)	87 (73.1)	38 (67.9)	40 (65.6)	245 (66.9)
Anti-HBc	32 (53.3)	40 (57.1)	39 (32.8)	35 (62.5)	29 (47.5)	175 (47.8)
HBeAg	0	0	1 (0.8)	1 (1.8)	0	2 (0.5)
Anti-HBe	13 (21.7)	14 (20)	15 (12.6)	17 (30.4)	14 (23)	73 (19.9)
HBV-DNA	6 (10)	1 (1.4)	3 (2.5)	2 (3.6)	3 (4.9)	15 (4.1)
Anti-HCV	7 (11.7)	27 (38.6)	27 (22.7)	20 (35.7)	7 (11.5)	88 (24)
Anti-HEV	3 (5)	1 (1.4)	4 (3.4)	1 (1.8)	6 (9.8)	15 (4.1)

HEV: Hepatitis E virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

**Table 3** Epidemiological, biochemical and serological markers of HBV, HCV and HEV in 366 patients with ESRF according to HBV-DNA positivity (mean  $\pm$  SD)

	HBV-DNA		<i>P</i> value
	Positive ( <i>n</i> = 15)	Negative ( <i>n</i> = 351)	
Sex (M/F)	12/3	231/120	NS
Age (yr)	66 $\pm$ 13	60 $\pm$ 13	NS
Dialysis type (haemodialysis/ peritoneal dialysis)	15/0	337/14	NS
History of hepatitis (yes/no)	0/15	51/300	NS
History of transfusions (yes/no)	4/11	125/226	NS
Number of blood units	3.5 $\pm$ 1.9	4.9 $\pm$ 5.2	NS
Duration of erythropoietin treatment (mo)	30 $\pm$ 27	46 $\pm$ 36	NS
AST (UNL 40 U/L)	17 $\pm$ 8	22 $\pm$ 19	NS
ALT (UNL 40 U/L)	18 $\pm$ 11	26 $\pm$ 20	NS
HBsAg (pos/neg)	12/3	8/343	< 0.001
Anti-HBc (pos/neg)	13/2	162/189	0.003
Anti-HBs (pos/neg)	2/13	243/108	< 0.001
HBeAg (pos/neg)	1/14	1/350	NS
Anti-HBe (pos/neg)	11/4	62/289	< 0.001
Anti-HCV (pos/neg)	0/15	88/263	0.03
Anti-HEV (pos/neg)	1/14	14/337	NS

NS: Not significant; UNL: Upper normal limit.

between patients with occult and overt HBV infection (data not shown). Serological markers of HBV, HCV and HEV infections in the 15 HBV-DNA positive patients are presented in Table 4.

## DISCUSSION

The present study demonstrated that 4.1% of Greek patients with ESRF from Central Greece had detectable HBV-DNA by real-time PCR, including 0.9% with occult HBV infection. Interestingly, 5.5% of HD patients had chronic HBV infection (overt HBV infection) but only 60% of them (thus 3.3% of total ESRF patients) were HBV-DNA positive. HBsAg prevalence in patients on maintenance HD varies among studies and geographical areas, from 0.8% to 17%, remaining very high in less developed countries<sup>[2,18,31,32]</sup>. The prevalence of anti-HBc was relatively high in our study (48%). As anti-HBc positivity indicates previous exposure to HBV infection, this finding in our HD patients in association with the 5.5%

**Table 4** HBV, HCV, and HEV serology in 15 HBV-DNA positive patients *n* (%)

HBsAg+	12 (80)
HBsAg+, anti-HBc+, anti-HBe+	10 (66.7)
HBsAg+, anti-HBc+, HBeAg+	1 (6.7)
HBsAg+, anti-HBc+, anti-HBe+, anti-HBs+	1 (6.7)
Anti-HCV+	0 (0)
Anti-HEV+	0 (0)
HBsAg-	3 (20)
HBsAg-, anti-HBc+, anti-HBs+	1 (6.7)
HBsAg-, anti-HBc-, anti-HBs-	2 (13.3)
Anti-HCV+	0 (0)
Anti-HEV+	0 (0)

prevalence of HBsAg might suggest contact with HBV during adulthood, though this cannot be safely ascertained if it has taken place in an HD setting. Nevertheless, a similar prevalence of markers of previous HBV infection has already been reported in our country for several high-risk groups of HBV infection, such as HD patients, alcoholics, and heroin addicts, while in refugees this prevalence can be as high as 70%<sup>[22,26,33-35]</sup>. Furthermore, with the development of sensitive PCR-based testing for HBV-DNA, studies in dialysis units have demonstrated that the prevalence of occult HBV infection ranges from 0% to 58% in published reports<sup>[18-23]</sup>. These variations could be attributed to differences in the sensitivity of the methods used for the detection of HBV-DNA, in the patients' sample investigated in each study, and in geographic variations of HBV prevalence. Though our aim was to strictly assess the prevalence of occult HBV infection in a very large cohort of HD patients, a recent publication on Greek blood donors with occult HBV infection was able to successfully perform the molecular characterization of HBV strains in less than half of occult cases, revealing several amino acids substitutions related to diagnostic escape and antiviral resistance<sup>[30]</sup>.

It should be stated herein, however, that the detection of HBV-DNA in serum samples rather underestimates the true prevalence of occult HBV infection<sup>[11]</sup>. Indeed, the most correct and precise methodological approach for the determination of the prevalence of occult HBV infection is the analysis of liver DNA extracts. This is further supported by a recent study from our group,

which assessed the prevalence of occult HBV infection in patients with autoimmune liver diseases<sup>[17]</sup>. However, the availability of liver tissues is often limited by restrictions on the performance of liver biopsies, which in the setting of HD is often very difficult and usually contraindicated.

In agreement with previous reports, we found that both groups of ESRF patients with chronic HBV infection and occult HBV infection had low levels of HBV-DNA<sup>[2,18,23]</sup>. HBV-DNA titres have been reported to remain low and stable over time, which can probably explain the low mortality rates due to liver disease in ESRF patients in developed countries<sup>[2]</sup>. The mechanisms responsible for the inhibition of HBV activity are as yet undefined in this specific group of patients. The passage of HBV-DNA from serum into the dialysate compartment during HD session, the destruction of HBV genome during HD procedure, and the interplay between HBV and the immune system could all play a role<sup>[2]</sup>. The low viral load observed in these subjects could also be attributed to multiple amino acid substitutions in the polymerase region, which might give rise to less fit viral strains, as was recently demonstrated in Greek blood donors with occult HBV infection<sup>[30]</sup>. The longer half-life of interferon in dialysis patients is another important factor, though none of the patients in our study was receiving antiviral treatment. Moreover, viral interactions can maintain HBV infection in a latent state, as in ESRF patients with chronic HCV infection and occult HBV viremia<sup>[22,23]</sup>. In these cases, a negative interference has been considered between the two viruses, leading to low HBV-DNA levels<sup>[36,37]</sup>. In our study, HBV-DNA positivity was negatively associated with HCV infection, further supporting the possibility of reciprocal replicative suppression of the two viruses. In this context, all three HD patients with occult HBV infection in our study were negative for anti-HCV and HCV-RNA by a transcription mediated amplification assay<sup>[38]</sup>. On the contrary, HBV-DNA positivity was not associated with markers of HEV infection.

The emerging evidence of the potential clinical significance of occult HBV infection is the main reason for the growing interest in this topic. Accumulating data shows that occult HBV infection might be transmitted in cases of blood transfusions, in the event of organ transplantations, and in particular, in cases of orthotopic liver transplantation as an obvious consequence of the fact that hepatocytes are the reservoir of the viral strains<sup>[11]</sup>. However, the risk of occult HBV transmission in cases of kidney transplantation seems to be low<sup>[39-41]</sup>. Nevertheless, virological and clinical reactivations of occult HBV infection have been repeatedly observed in several clinical conditions, where an immunosuppressive status evolves, including haematological or other malignancies, HIV infection, haematopoietic stem cell transplantation, and organ transplantation<sup>[11]</sup>. Therefore, the theoretical basis of HBV reactivation in HD cases with occult hepatitis B, who had undergone kidney transplantation and are under immunosuppression, is still unresolved.

In conclusion, approximately 4% of ESRF patients on HD from Central Greece have detectable HBV-DNA levels, amongst which 20% have occult HBV infection, which might indicate that the absence of HBsAg in the blood of HD patients is not sufficient to ensure lack of circulating HBV. However, in the total group of HBsAg negative HD patients, the prevalence of occult HBV infection in our region seems to be one of the lowest worldwide (0.9%). The demographic and biochemical features of HBV-DNA positive subjects, including those with occult HBV infection, do not help to distinguish these individuals from those who are HBV-DNA negative. We believe that longitudinal studies including even larger numbers of patients are needed to further clarify the clinical significance and outcome of occult HBV infection in this setting.

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## COMMENTS

### Background

The prevalence and clinical significance of occult hepatitis B virus (HBV) infection in the haemodialysis (HD) setting in large series of patients with end-stage renal failure (ESRF) on HD has not been adequately addressed.

### Research frontiers

This situation might have considerable implications, either from the public health point of view (due to the risk transmission of the virus among individuals in the hemodialysis units) or from the clinical point of view, as attested by well-known cases of acute and severe exacerbations of HBV infection under some circumstances, such as transplantation and the subsequent immunosuppressive treatment.

### Innovations and breakthroughs

Occult HBV infection in HD patients ranges from 0% to 58% in published reports. Though their large study in 366 ESRF patients on HD from Central Greece revealed the presence of HBsAg in 5.5% and HBV-viremia in approximately 4%, the actual presence of occult HBV infection was identified in only 0.9% of HBsAg negative patients (one of the lowest frequencies of occult HBV infection in HD patients worldwide). These discrepancies in the frequencies of occult HBV infection in a HD setting could be ascribed to differences in the number of patients tested, the sensitivity of the methods used for the detection of HBV-DNA, the patients' specimen investigated in each study (serum or liver tissue), and geographical variations regarding in HBV prevalence.

### Applications

Based on the potential clinical significance of occult HBV infection in the HD setting, the authors believe that further multicentre longitudinal studies, including larger numbers of patients, are needed to further clarify whether occult HBV infection is a major clinical problem in this setting or not.

### Terminology

Occult HBV infection, is a clinical entity characterised by undetectable HBsAg in the serum but detectable HBV-DNA in serum or the liver. The use of sensitive HBV-DNA polymerase chain reaction assays in serum samples or, preferably, in liver tissues when available using the highly conserved HBV precore/core regions for amplification, appears to be the assay of choice for the diagnosis of this condition.

### Peer review

This study described the occult HBV infection in 366 ESRF patients on HD in Nephrology and Dialysis units of the Thessaly region in Central Greece. The authors concluded that the prevalence of occult HBV infection was 0.9%, although the HBsAg prevalence was 5.5% in this cohort. This study provides



basic epidemiological data, which is of great interest, as it includes some original and useful data for this patients setting.

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## Predictors of disease severity in ulcerative colitis patients from Southwestern Ontario

Lee S Roth, Nilesh Chande, Terry Ponich, Maya L Roth, James Gregor

Lee S Roth, Nilesh Chande, Terry Ponich, James Gregor, Division of Gastroenterology, London Health Sciences Centre, Victoria Hospital, 800 Commissioner's Road East, London, Ontario, N6A 5W9, Canada

Maya L Roth, Operational Stress Injury Clinic, Parkwood Hospital, London, Ontario, N6C 5J1, Canada

Author contributions: Roth LS, Ponich T, Chande N and Gregor J designed the research; Roth LS performed the research; Roth ML analyzed the data; Roth LS wrote the paper; all authors contributed towards editing the manuscript.

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Correspondence to: Lee S Roth, MD, FRCPC, Division of Gastroenterology, London Health Sciences Centre, Victoria Hospital, 800 Commissioner's Road East, London, Ontario, N6A 5W9, Canada. [lee.roth@londonhospitals.ca](mailto:lee.roth@londonhospitals.ca)

Telephone: +1-519-6858500-76843 Fax: +1-519-6676620

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### Abstract

**AIM:** To understand the demographic characteristics of patients in Southwestern Ontario, Canada with ulcerative colitis (UC) in order to predict disease severity.

**METHODS:** Records from 1996 to 2001 were examined to create a database of UC patients seen in the London Health Sciences Centre South Street Hospital Inflammatory Bowel Disease Clinic. To be included, patients' charts were required to have information of their disease presentation and a minimum of 5 years of follow-up. Charts were reviewed using standardized data collection forms. Disease severity was generated during the chart review process, and non-endoscopic Mayo Score criteria were collected into a composite.

**RESULTS:** One hundred and two consecutive patients'

data were entered into the database. Demographic analyses revealed that 51% of the patients were male, the mean age at diagnosis was 39 years, 13.7% had a first degree relative with inflammatory bowel disease (IBD), 61.8% were nonsmokers and 24.5% were ex-smokers. In 22.5% of patients the disease was limited to the rectum, in 21.6% disease was limited to the sigmoid colon, in 22.5% disease was limited to the left colon, and 32.4% of patients had pancolitis. Standard multiple regression analysis which regressed a composite of physician global assessment of disease severity, average number of bowel movements, and average amount of blood in bowel movements on year of diagnosis and age at time of diagnosis was significant,  $R^2 = 0.306$ ,  $F(7, 74) = 4.66$ ,  $P < 0.01$ . Delay from symptoms to diagnosis of UC, gender, family history of IBD, smoking status and disease severity at the time of diagnosis did not significantly predict the composite measure.

**CONCLUSION:** UC severity is associated with younger age at diagnosis and year of diagnosis in a longitudinal cohort of UC patients, and may identify prognostic UC indicators.

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**Key words:** Demographic; Disease severity; Prognosis; Ulcerative colitis

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Roth LS, Chande N, Ponich T, Roth ML, Gregor J. Predictors of disease severity in ulcerative colitis patients from Southwestern Ontario. *World J Gastroenterol* 2010; 16(2): 232-236 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i2/232.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i2.232>

## INTRODUCTION

Canada has one of the highest rates of ulcerative colitis (UC) in the world, with an annual incidence of approximately 3500 cases and a prevalence of roughly 60 000<sup>[1]</sup>. Each patient with UC is faced with a chronic disease with an uncertain course, experiencing periods of relapse and remission, and often requiring long-term medical therapy. In some cases, however, the disease remains mild or becomes more severe but it is difficult to predict the ultimate course of UC without simply monitoring the patient for years.

Many therapies have been studied over the past 60 years for the treatment of UC, starting with sulfasalazine and hydrocortisone<sup>[2]</sup>, through newer 5-aminosalicylate products<sup>[3]</sup>, immunosuppressives<sup>[4,5]</sup>, and now biological therapies. A treatment pyramid has been proposed, with less potent and more tolerable agents at the bottom and more powerful agents with greater potential toxicities at the top<sup>[6]</sup>. The recent introduction of infliximab has provided another treatment option for UC patients with refractory disease, but the long-term safety and efficacy of this therapy is unknown<sup>[7]</sup>. In addition, the substantial cost of biological therapies makes using these agents as initial therapy difficult to justify given the large number of patients who respond to more inexpensive treatments.

Studies examining groups of UC patients have been published from various geographic regions over a number of decades, with many looking at cohorts of UC patients longitudinally<sup>[8-11]</sup>. Many of these studies, however, were published prior to the introduction of newer therapies and may lack applicability to current UC patients. Furthermore, few studies have looked at Canadian UC patients, and therefore the relevance of these results to our patient population is unknown. The aim of this study was to examine the UC population of Southwestern Ontario (SWO), Canada in an effort to gather information on the natural history of the disease and determine predictors of future disease severity at the time of diagnosis.

## MATERIALS AND METHODS

Patients with UC seen in the London Health Sciences Centre South Street Hospital Inflammatory Bowel Disease Clinic from 1996-2001 were identified by a search of diagnostic codes. Individual clinic charts for these patients were reviewed to determine if they met the study inclusion criteria, which included a new diagnosis of UC, details of disease presentation at diagnosis, and documentation of follow up for at least 5 years.

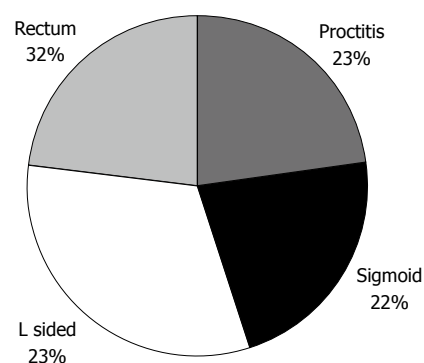
Patient data were collected retrospectively from clinic charts using standardized data collection forms created specifically for this study. Demographic data including age, sex, year of diagnosis, age at diagnosis, smoking status, and a family history of inflammatory bowel disease (IBD) as well as annual measurements of disease severity and course of disease were recorded.

Disease severity was measured using a modified

**Table 1** Demographic data of the UC patient cohort *n* (%)

Gender (male/female)	52 (51)/50 (49)
Mean age at diagnosis (yr)	39 (range 9-85; SD = 17.9)
Family history of IBD in 1st degree relative	14 (13.7)
Non-smokers/ex-smokers	63 (61.8)/25 (24.5)

UC: Ulcerative colitis; IBD: Inflammatory bowel disease.



**Figure 1** Extent of disease in the ulcerative colitis (UC) patient cohort.

version of the Mayo Scoring System for assessment of UC activity<sup>[12]</sup>, which excluded the endoscopic component. Values from 0 to 3 were recorded for each of: frequency of bowel movements, amount of blood in bowel movements, and physician assessment of disease severity.

Statistical analysis was carried out using SPSS 15.0 for a multiple regression analysis of demographic factors on the disease severity composite. Microsoft Excel was used for the data management and averages.

This study was approved by the Research Ethics Board at The University of Western Ontario.

## RESULTS

One hundred and two consecutive patient charts who met the inclusion criteria were reviewed. Demographic analyses demonstrated that 51% of the cohort was male, 13.7% had a family history of IBD, and 61.8% were nonsmokers (Table 1). Average delay from time of symptom onset to time of diagnosis was 1.8 years (range 0-34 years; SD = 4.5 years).

Data examining the extent of disease are shown in Figure 1. Colectomy rates were calculated at 14.7% of the cohort (15 patients), and are plotted against time in Figure 2. Figure 3 outlines the indications for colectomy, while Figure 4 displays the demographics of this group. Seven of these patients underwent an ileal pouch-anal anastomosis with the remaining 8 patients undergoing an ileostomy. Of those who underwent ileal pouch anal-anastomosis, 5 (71%) had at least 1 subsequent episode of pouchitis. In the patients who had a colectomy, 10 patients (66.6%) had pancolitis while disease extent was limited to the sigmoid colon in 4 patients (26.7%) and to the left colon in 1 patient (6.7%). The average composite severity score for this group was 3.24 across

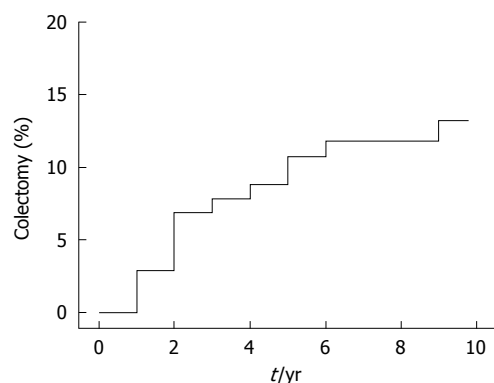


Figure 2 Kaplan Meier curve of colectomy over time.

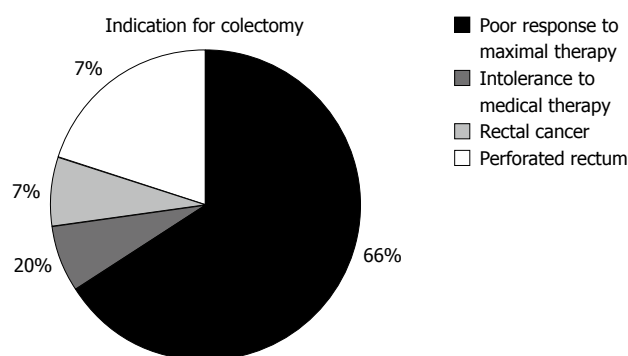


Figure 3 Indications for colectomy in 15 patients in the UC cohort.

at least 5 years of follow up (1.15 for number of bowel movements, 0.85 for blood in bowel movements, and 1.24 for physician global assessment of severity).

Three patients died during the follow up period, two from unrelated malignancies (melanoma and squamous cell lung cancer) and one from congestive heart failure.

Disease severity was determined for each patient during each year of follow up. All patients except one had complete data for at least 5 years of follow up after diagnosis, with the reviewer unable to assign severity status to one patient during 1 year of follow up. Data are listed in Figure 5 with severity divided into three groups: low (modified Mayo Score 0-2), moderate (modified Mayo Score 3-5), and high (modified Mayo Score 6-9).

The composite of disease severity was averaged across the number of years with UC, with a mean value for the cohort of 2.12 (standard deviation 1.18; minimum 0.36; maximum 6.22). When grouped into categories of severity, 77.5% (79 patients) had an average score of less than three, 21.5% (22 patients) had an average score between three and six, and 1% (1 patient) had an average score of greater than six.

Pearson product moment correlation analyses were conducted in order to evaluate the extent of interrelationship between the composite value of disease severity and demographic variables. Demographic variables included year of diagnosis, delay from symptom onset to diagnosis, gender, smoking status at time of diagnosis, family history of IBD in a first degree relative, severity

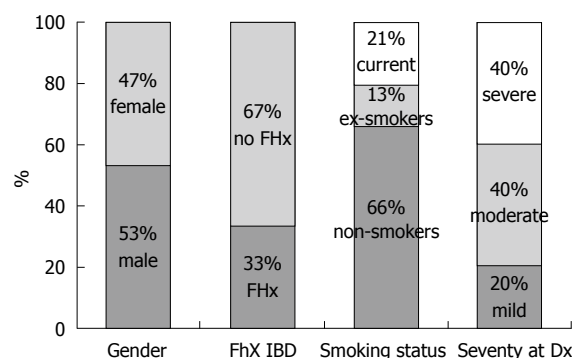


Figure 4 Demographic data on UC patients who underwent colectomy.

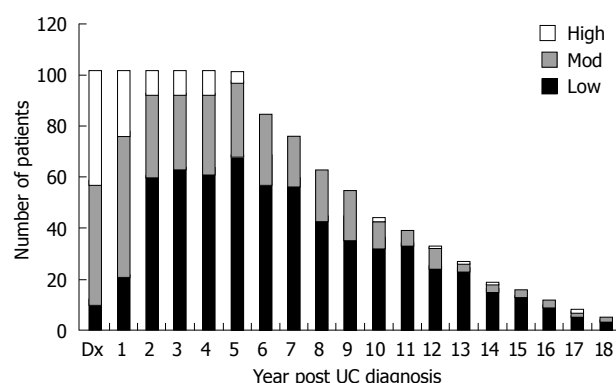


Figure 5 Ulcerative colitis disease severity at time of diagnosis and during follow up divided into 3 categories: low severity (modified Mayo Score 0-2), moderate severity (modified Mayo Score 3-5), high severity (modified Mayo Score 6-9).

at diagnosis, and age at diagnosis. Year of diagnosis was positively associated with the composite of disease severity [ $r(102) = 0.35$ ,  $P < 0.01$ ], such that a more recent year of diagnosis was associated with a higher composite score. Smoking status at time of diagnosis [ $r(97) = -0.22$ ,  $P < 0.05$ ] and age at diagnosis [ $r(102) = -0.32$ ,  $P < 0.01$ ] were negatively associated with the composite score of disease severity, such that current smokers and younger patients had a higher composite score.

Regression analysis was performed on the composite value of disease severity on various demographic variables. Standard multiple regression analyses on year of diagnosis and age at time of diagnosis were significant,  $R^2 = 0.306$ ,  $F(7, 74) = 4.66$ ,  $P < 0.01$ . The correlation indicated that more severe disease was present in those diagnosed at an earlier age, and those diagnosed more recently had a worse course of disease. Delay from symptoms to diagnosis of UC, gender, family history of IBD, smoking status and disease severity at the time of diagnosis did not significantly predict the composite measure.

## DISCUSSION

UC is a chronic disease with a variable and uncertain course. Patients diagnosed with the disease have no means to predict whether their disease will be ongoing,



consist of severe relapses, or remain indolent. Previous studies have looked at the natural course of UC, however, this study has the added benefit of following a fairly large cohort of patients with UC for a minimum of 5 years. Demographics such as disease extent, gender, and presence of a family history of IBD compare favorably with the cohort from Farmer's 1992 study of over 1000 individuals from the Cleveland Clinic<sup>[8]</sup>. Previously reported colectomy rates range from 8.7% in a recent European trial<sup>[9]</sup>, to 45% in a Swedish study from 1990<sup>[11]</sup>. Our value of 15% lies well within this range, and indeed, is comparable to the Northern European portion of the aforementioned study. This allows some quality assurance that patients in the SWO IBD clinic have similar demographics and outcomes to other areas of the world.

The Mayo Scoring System<sup>[12]</sup> is a validated tool that measures disease activity in patients with UC. We applied this tool to UC patients at diagnosis and during follow-up in an effort to determine which demographic variables could predict long term disease activity. The endoscopy score was excluded from this tool as sigmoidoscopy/colonoscopy was not routinely performed on a regular basis in most patients. Thus the score was out of a maximum of 9 rather than 12. Some scales, such as the Simple Clinical Colitis Activity Index<sup>[13]</sup> and Seo *et al*<sup>[14]</sup> index do not rely on endoscopic scores, while other scores such as the Ulcerative Colitis Disease Activity Index and St. Mark's index<sup>[15]</sup> incorporate an endoscopic component into their values. In a comparison between those scores that contain endoscopy related elements against those that did not, it was found that endoscopy added little additional information to the assessment of disease activity<sup>[16]</sup>. Therefore, the use of the Mayo score without the endoscopic system is likely a valid alternative.

We found that younger age at diagnosis predicts a more severe future disease course. Previous studies have shown that patients with early onset UC are more prone to colorectal cancer<sup>[17]</sup>, however the finding that severity of future disease is predicted is not widely prevalent in the current literature. One theory that has arisen in discussion as well as at presentations is that the more severe the disease, the earlier it unmask itself clinically. This then leads to more symptoms during the patient's follow up and a worse course clinically for the patient.

While younger age at diagnosis seems to play a significant role in predicting disease severity, so does the year of diagnosis. It is difficult to tease out the fine nuances of younger age and year of diagnosis, as these obviously affect each other. It would seem from the analysis, however, that the year independently predicts the outcome, and therefore we have to consider that as our arsenal for UC has grown, perhaps our treatment of this condition has not improved as much as we originally thought. An alternative explanation could be found in the fact that all patients in this study were seen in a rapidly growing IBD clinic in a tertiary care referral centre, and perhaps the severity of cases has been high with mild or moderate cases being managed by community practitioners.

The above variables predicted future disease severity,

however, there were a number of factors not found to be predictive. One of the most intriguing was smoking status, as multiple previous studies have found that smoking was a protective factor<sup>[18]</sup>, while non- or ex-smokers had a higher risk of relapsing disease<sup>[19]</sup>. In addition, disease severity at presentation did not correlate with the severity index over the disease course, nor did delay from diagnosis to treatment. This was unexpected, as literature by Sandborn<sup>[20]</sup> found that classifying presentations into one of four severity categories was useful for prognosticating disease. In addition, gender and family history all seemed to have a minimal role in predicting future disease.

The limitations of the study make a large sweeping commentary on our prediction of UC difficult. It is retrospective in design, and relies heavily on chart review. The number of patients in the study compares favorably with many previous reviews of UC and contains patients with similar disease extent and severity, but with just over 100 patients it is difficult to be certain of the effect in a larger group. Additionally, the geographic nature of the population may make it hard to extract information on other populations in Canada or the rest of the world.

The information provided by the demographic variables is valuable in many ways. Firstly, to the patient this may alleviate much of the sense of unknown, and allow them to cope with the diagnosis and treatment more effectively. In addition to this, however, it allows the clinician to triage which patients may progress more quickly and customize their therapy accordingly. For instance, if a younger patient presents with confirmed UC, one may be more willing to adopt a "top down" approach and start them on anti-TNF agents immediately. This may help save time, patient discomfort, and reduce the risk of outcomes such as colectomy in that severe symptoms do not have the potential to develop. Rather than navigate patients through the pyramid of UC agents, this would guide patients to the agent most suited to their specific condition.

This study provides evidence that predictors of disease severity in UC may exist in simple demographic information that is available to any clinician and may help ease the unpredictability of this condition as well as help physicians treat patients with tailored therapy. Additional studies looking prospectively at patients with UC in an effort to help predict disease severity would be most useful and easily conducted.

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## COMMENTS

### Background

Ulcerative colitis (UC) is an inflammatory disease of the large intestine that

affects many Canadians. The individuals, often young and in the prime of their life, experience symptoms such as diarrhea, bleeding from the bowels, and abdominal discomfort. They can expect periods of health interrupted by worsening of their disease, with some having very serious symptoms and others having minimal to no symptoms.

### Research frontiers

The natural history of the disease has been looked at in previous studies, but predicting which patients will have severe disease and which will have mild disease has not been fully examined.

### Innovations and breakthroughs

This article describes a Canadian population of patients with UC that have been followed for at least 5 years, and looked back to see if any of their characteristics, such as age, gender, smoking status, delay to diagnosis, year of diagnosis or the manner in which they presented, predicted how they will progress in coming years. Age at diagnosis and year of diagnosis seemed to predict the disease course, with a younger age at diagnosis and a more recent year of diagnosis predicting a more severe disease pattern.

### Applications

These findings allow physicians to give patients newly diagnosed with UC an idea of what to expect in the coming years. Although not completely predictive, those patients who present with UC who are younger may expect a more severe disease course. Physicians treating these patients may be more inclined to start stronger therapies initially in these patients.

### Peer review

The original manuscript by Roth *et al* is a very interesting and a very well written manuscript. It is a retrospective study, with a good attempt to find out the predictors of severity from the demographic parameters. The paper is nicely laid out with a very good selection of references and nicely addressed limitations. A retrospective study, it is a good attempt to find out the predictors of severity from the demographic parameters.

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## Endoscopic ultrasonography can diagnose distal biliary strictures without a mass on computed tomography

Yasuyuki Saifuku, Michiko Yamagata, Takero Koike, Genyo Hitomi, Kazunari Kanke, Hidetaka Watanabe, Toshimitsu Murohisa, Masaya Tamano, Makoto Iijima, Keiichi Kubota, Hideyuki Hiraishi

Yasuyuki Saifuku, Michiko Yamagata, Takero Koike, Genyo Hitomi, Kazunari Kanke, Hidetaka Watanabe, Toshimitsu Murohisa, Masaya Tamano, Makoto Iijima, Hideyuki Hiraishi, Department of Gastroenterology, Dokkyo Medical University, 880 Kitakobayashi, Mibu, Shimotsuga-gun, Tochigi 321-0293, Japan

Keiichi Kubota, Second Department of Surgery, Dokkyo Medical University, Tochigi 321-0293, Japan

Author contributions: Saifuku Y performed the research and analyzed the data; Yamagata M designed the study, analyzed the data and wrote the paper; Koike T, Hitomi G, Kanke K, Watanabe H, Murohisa T, Tamano M, Iijima M and Kubota K performed the clinical work; Hiraishi H reviewed the manuscript.

Correspondence to: Michiko Yamagata, MD, PhD, Department of Gastroenterology, Dokkyo Medical University, 880 Kitakobayashi, Mibu, Shimotsuga-gun, Tochigi 321-0293, Japan. [myamagat@dokkyomed.ac.jp](mailto:myamagat@dokkyomed.ac.jp)

Telephone: +81-282-861111 Fax: +81-282-867761

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### Abstract

**AIM:** To assess the diagnostic ability of endoscopic ultrasonography (EUS) for evaluating causes of distal biliary strictures shown on endoscopic retrograde cholangiopancreatography (ERCP) or magnetic resonance cholangiopancreatography (MRCP), even without identifiable mass on computed tomography (CT).

**METHODS:** The diagnostic ability of EUS was retrospectively analyzed and compared with that of routine cytology (RC) and tumor markers in 34 patients with distal biliary strictures detected by ERCP or MRCP at Dokkyo Medical School Hospital from December 2005 to December 2008, without any adjacent mass or eccentric thickening of the bile duct on CT that could cause biliary strictures. Findings considered as benign strictures on EUS included preservation of the normal

sonographic layers of the bile duct wall, irrespective of the presence of a mass lesion. Other strictures were considered malignant. Final diagnosis of underlying diseases was made by pathological examination in 18 cases after surgical removal of the samples, and by clinical follow-up for > 10 mo in 16 cases.

**RESULTS:** Seventeen patients (50%) were finally diagnosed with benign conditions, including 6 "normal" subjects, while 17 patients (50%) were diagnosed with malignant disease. In terms of diagnostic ability, EUS showed 94.1% sensitivity, 82.3% specificity, 84.2% positive predictive value, 93.3% negative predictive value (NPV) and 88.2% accuracy for identifying malignant and benign strictures. EUS was more sensitive than RC (94.1% vs 62.5%,  $P = 0.039$ ). NPV was also better for EUS than for RC (93.3% vs 57.5%,  $P = 0.035$ ). In addition, EUS provided significantly higher sensitivity than tumor markers using 100 U/mL as the cutoff level of carbohydrate antigen 19-9 (94.1% vs 53%,  $P = 0.017$ ). On EUS, biliary stricture that was finally diagnosed as malignant showed as a hypoechoic, irregular mass, with obstruction of the biliary duct and invasion to surrounding tissues.

**CONCLUSION:** EUS can diagnose biliary strictures caused by malignant tumors that are undetectable on CT. Earlier detection by EUS would provide more therapeutic options for patients with early-stage pancreaticobiliary cancer.

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**Key words:** Computed tomography; Cytology; Endoscopic retrograde cholangiopancreatography; Endoscopic ultrasound; Indeterminate biliary stricture; Magnetic resonance cholangiopancreatography; Tumor marker

**Peer reviewers:** Tarkan Karakan, Associate Professor, Department of Gastroenterology, Gazi University, Ankara, 06500,

Turkey; Dr. Ching-Chung Lin, Gastroenterology Division, Mackay Memorial Hospital, 5F, No 28, Lane 286, Shidong Road, Taipei 11154, Taiwan, China; Jon Arne Soreide, Professor, Department of Surgery, Stavanger University Hospital, Stavanger, N-4068, Norway

Saifuku Y, Yamagata M, Koike T, Hitomi G, Kanke K, Watanabe H, Murohisa T, Tamano M, Iijima M, Kubota K, Hiraishi H. Endoscopic ultrasonography can diagnose distal biliary strictures without a mass on computed tomography. *World J Gastroenterol* 2010; 16(2): 237-244 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i2/237.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i2.237>

## INTRODUCTION

Determining the etiology of a distal biliary stricture is crucial to the provision of appropriate therapy. However, such determination can be particularly problematic in cases of biliary strictures without an identifiable mass that could cause the stricture on computed tomography (CT) or magnetic resonance imaging (MRI). Empiric resection may be necessary to differentiate benign and malignant strictures, and the decision to operate has traditionally been made on the basis of clinical history and cholangiographic appearance of the stricture<sup>[1]</sup>. However, determining the cause of a stricture on the basis of morphologic features and brush cytology is unreliable<sup>[2]</sup>.

Intraductal ultrasonography (IDUS) with wire-guided, thin-caliber, high-frequency probes is promising for the diagnosis of biliary stricture<sup>[3,4]</sup>. This technique is easily performed as an adjunct to endoscopic retrograde cholangiopancreatography (ERCP) without significant lengthening of the procedure. For the diagnosis of biliary stricture without an identifiable mass, however, data from IDUS are insufficient<sup>[4,6]</sup>. In addition, the number of patients for whom IDUS can be performed has decreased, since magnetic resonance cholangiopancreatography (MRCP) has been replacing the role of diagnostic ERCP. Moreover, if patients with an identifiable mass on CT or MRI are excluded, the proportion of benign strictures is about 30%-50%<sup>[4,5,7,8]</sup>. This relatively high percentage of benign strictures complicates the decision to perform IDUS in all patients with biliary strictures of indeterminate etiology, as IDUS sometimes causes complications such as ERCP-induced pancreatitis and cholangitis.

The utility of endoscopic ultrasonography (EUS) for diagnosing unexplained biliary strictures thus warrants urgent discussion, since EUS is now the first choice in screening for small pancreatic tumors that cannot be detected by other imaging modalities and is not associated with ERCP-related complications<sup>[9]</sup>. However, only 1 paper by Lee *et al.*<sup>[1]</sup> has mentioned the utility of EUS features in patients with unexplained biliary stricture. According to Lee *et al.*<sup>[1]</sup>, sonographic features of EUS, including the presence of a pancreatic mass, an irregular bile duct wall, or bile duct thickness > 3 mm, could improve the diagnosis of unexplained stricture.

The aim of this study was to assess the ability of EUS to diagnose distal biliary strictures for which cross-sectional imaging modalities such as CT and MRI could not detect a causative mass or bile duct thickness. Moreover, we report the sonographic appearance of malignant and benign strictures using EUS, as the lack of easily recognizable morphologic criteria has partly caused the limited availability of EUS for diagnosing biliary strictures of indeterminate etiology.

## MATERIALS AND METHODS

### Inclusion criteria

This retrospective analysis was performed on 34 patients who underwent EUS at Dokkyo Medical School Hospital from December 2005 to December 2008 for evaluation of strictures in the biliary tract that were detected by ERCP or MRCP and who did not have an identifiable mass lesion causing the stricture on cross-sectional CT or MRI. Patients with strictures at the proximal bile duct were excluded from this study, since EUS is already known to be less accurate for strictures of that portion<sup>[10]</sup>. All patients provided written consent for the procedure performed.

### EUS examination

EUS and ERCP were performed with the patient under conscious sedation by 1 of 2 operators (S.Y. and Y.M.). EUS was performed using a 7.5-MHz US probe (UM-200; Olympus, Tokyo, Japan) connected to a standard EUS processor (EU-30; Olympus). This probe provides radial scanning perpendicular to its axis. For the purposes of this study, EUS images were reviewed to identify extrinsic compression at the stricture site without knowledge of the final diagnosis. Evaluation points were: (1) presence of a mass that could create extrinsic compression at the site of the stricture; (2) disruption of the normal 2 or 3 sonographic layers of the bile duct wall<sup>[11]</sup>; and (3) continuation of a mass into adjacent structures, referring to previously defined criteria for evaluation of biliary strictures on IDUS<sup>[3,10,12]</sup>. Findings considered as a benign stricture on EUS included preservation of the normal sonographic layers of the bile duct wall, irrespective of the presence of a mass lesion. Other strictures were considered malignant.

Thirty patients underwent dynamic CT with a liver protocol using the method reported by Yanaga *et al.*<sup>[13]</sup>. All scans were performed on a multidetector helical CT scanner (SOMATOM Sensation 64; Siemens, Tokyo, Japan). A multiphasic scanning technique was used and 3-phase contrast-enhanced CT was performed during the hepatic arterial phase, portal phase and equilibrium phase. For the remaining 4 patients who could not use contrast medium due to renal dysfunction, only plain CT was performed. Images were reconstructed at an 8-mm slice thickness, and interpreted by CT radiologists who specialize in body imaging.

In 25 patients, MRCP was performed using 1.5-T



MRI (MAGNETOM Symphony; Siemens) or 3-T MRI (MAGNETOM Trio, A Tim System; Siemens). For ERCP, the procedure was performed in a standard fashion with a side-viewing therapeutic duodenoscope (Olympus JF 260V; Olympus) using standard iodinated contrast medium. Brushings of the biliary stricture were obtained during ERCP. The remainder of the ERCP procedure was then performed according to the individual needs of each patient. When a drainage tube was placed for palliative treatment in patients suffering from obstructive jaundice, bile pooled for 24 h was collected for cytopathological analysis 3 times. For routine cytology (RC), specimens obtained from brushings and bile collected from a drainage tube were reviewed by dedicated gastrointestinal cytopathologists.

As tumor markers, carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) were examined at admission, and CEA > 5.0 ng/mL or CA19-9 > 37 U/mL were considered to indicate malignancy.

### Final diagnosis and follow-up

The final diagnosis was based on definitive cytologic studies diagnostic for malignancy or surgical removal followed by pathological examination in 18 patients and clinical follow-up for > 10 mo in 16 patients. Repeat ERCP, biliary brushing and bile collection were performed as clinically indicated. Clinical follow-up included imaging with abdominal CT, EUS and MRCP. Follow-up information was also obtained from hospital medical records, by contacting primary care physicians.

### Statistical analysis

Continuous variables are expressed as median (range). Each test is expressed in proportions in terms of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy. Analysis was performed using StatView software (Hulinks, Inc., Tokyo). Comparisons between continuous variables were performed using non-parametric tests. Comparisons between qualitative variables were performed using a  $\chi^2$  test for independence or Fisher's exact probability test. Values of  $P < 0.05$  were considered statistically significant.

## RESULTS

### Patient characteristics

Thirty-four patients (17 men, 17 women) met the inclusion criteria. The median age of the patients was 71 years (range, 23-90 years). Jaundice (total bilirubin > 2 mg/dL) was evident at presentation in 13 patients. In the 21 patients without jaundice, abnormal liver blood tests were found in 8 patients, and 6 patients complained of abdominal pain. Abnormal hepatobiliary images on transabdominal US at medical check-up were detected in 7 patients, who had neither abnormalities in blood data nor clinical symptoms. No significant difference in clinical characteristics was seen between patients with benign stricture, normal cases and those with malignant strictures (Table 1).

Table 1 Patient characteristics

Final diagnosis	Benign stricture and normal case ( <i>n</i> = 17)	Malignant stricture ( <i>n</i> = 17)	Statistical significance
Age (yr)	71.5 (23-90)	69.0 (56-84)	NS
Sex (male/female)	9/8	8/9	NS
Presenting symptoms			NS
Jaundice	5	8	
Elevated liver enzymes	2	6	
Abdominal pain	5	1	
Abnormal imaging	5	2	

NS: Not significant.

Table 2 Final diagnosis

	<i>n</i>
Benign bile duct stricture	11
Fibrotic or inflammatory stricture	4
Dilatation of the bile duct	3
Chronic pancreatitis	2
Anomalous arrangement of pancreaticobiliary duct	1
Congenital choleductal cyst	1
Malignant bile duct stricture	17
Peripancreatic cancer	8
Bile duct cancer	7
Carcinoma of the papilla of Vater	2
Normal study	6

### Final diagnosis

There were 17 malignant strictures, 11 benign strictures and 6 normal cases. Among 8 cases of peripancreatic cancer (tumor located in the head of the pancreas), the diagnosis was confirmed as pancreatic cancer by pathological examinations using surgical specimens in 6 cases and 2 lesions were considered malignant based on clinical follow-up. Two patients who were clinically diagnosed as peripancreatic cancer died of liver failure 4 mo after EUS examination, accompanied by CA19-9 elevation. Among 7 cases of biliary cancer, the diagnosis was confirmed by pathological examination using surgical specimens in 5 cases and 2 lesions considered malignant on clinical follow-up were located in the middle duct. One patient who was clinically diagnosed with biliary cancer gradually became emaciated and died within 1 year of EUS examination. In another patient, peritoneal dissemination was found at surgery 4 mo after EUS examination. One patient was diagnosed with carcinoma of the papilla of Vater by pathological examination using surgical specimens. In another patient who was clinically diagnosed with carcinoma of the papilla of Vater, cytology taken from the orifice of the papilla was suspicious of adenocarcinoma and the duodenum finally became stenotic 2 years after EUS examination (Table 2).

The diagnosis of benign strictures was confirmed by surgery in 4 and by clinical follow-up in 7. Patients clinically diagnosed as benign did not show changes in images or clinical exacerbation. Six cases were considered normal for which strictures detected on ERCP or

**Table 3** Malignant strictures correctly judged as malignant on EUS

Final diagnosis	EUS findings	n (%)
Peripancreatic cancer (n = 8)	Mass adjacent to the stricture site in the pancreas head	8 (100)
	Disruption of the bile duct by the mass	6 (75)
	Continuation into adjacent structures	6 (75)
	Invasion of the main pancreatic duct	5 (62.5) <sup>a</sup>
	LN swelling	4 (50)
	Ascites	1 (12.5)
Biliary cancer (n = 6)	Mass adjacent to the stricture site in the pancreas head	6 (100)
	Disruption of the bile duct by the mass	5 (83.3)
	Continuation into adjacent structures	5 (83.3)
	Invasion of the main pancreatic duct	0 (0)
	LN swelling	3 (50)
Cancer of the ampulla of Vater (n = 2)	Mass mainly located on the luminal side	2 (100)
	Infiltration of the muscularis propria by the mass	2 (100)
	LN swelling	1 (50)

<sup>a</sup>P = 0.026 *vs* Biliary cancer. EUS: Endoscopic ultrasonography; LN: Lymph nodes.

MRCP were either not reproducible or were due to compression by Oddi's sphincter (Table 2).

These results suggest that the proportion of malignant strictures was 50% even if lesions which could cause the stricture were not detected on CT and/or MRI. When cases with normal images on EUS were excluded, the proportion of malignancy reached 58.6%.

### **EUS for recognizing causes of biliary strictures shown on ERCP or MRCP**

While EUS is now routinely performed without insertion of apparatus into the biliary tree, few reports have used EUS to evaluate biliary strictures without an identifiable mass on CT. We thus examined the diagnostic ability of EUS, and conducted comparisons with the diagnostic abilities of RC and tumor markers.

Malignancy was correctly diagnosed in 16 of 17 patients. Ultrasonographic findings in patients with malignant strictures correctly judged as malignancy on EUS are summarized in Table 3. A mass accompanied by invasion of surrounding tissues may be suggestive of malignancy. The proportion of invasion of the main pancreatic duct was significantly higher in patients with peripancreatic cancer than in patients with biliary cancer (62.5% *vs* 0%,  $P = 0.026$ ), while no significant difference was seen in terms of diagnostic ability, biliary disruption and invasion of adjacent tissues by the tumors. In addition to the sonographic findings in the mass and the bile duct, EUS also detected enlarged lymph nodes > 10 mm in diameter in 50% of patients with malignant strictures. Ascites were observed in 1 patient with peripancreatic cancer. In 1 false-negative case with distal biliary cancer, no mass was detectable and was judged as benign. In this case, the mass may have been hidden by the acoustic shadow formed by impacted stones in the bile duct.

Correct diagnosis of benign disease was made in 14 of 17 patients. Ultrasonographic findings in patients

with benign strictures correctly judged as benign on EUS are summarized in Table 4. Smooth tapering of the bile duct, preservation of the normal layered structure of the bile duct wall and no mass adjacent to the stricture site may be suggestive of benign disease. There were 3 false-positive patients with fibrotic strictures, diagnosed as systemic lupus erythematosus in 1 patient and unknown etiology in 2. In these cases considered to have malignancy on EUS, a drainage tube was inserted into the bile duct. In 2 patients, a solid mass was found at the distal end of the duct. The duct wall was thickened, losing the layered structure. In 1 patient, the distal end of the bile duct was filled with an irregular mass that could not be distinguished from the duct wall on EUS. The irregular mass looked extended from the duct, which looked like invasion of the adjacent tissues on imaging. Fibrotic change was ascertained when surgery was performed, while masses could not be confirmed during surgery, probably because the substance that looked like masses was debris.

Six strictures identified by ERCP or MRCP were ultrasonographically considered as benign and finally diagnosed as "normal" duct. Four strictures were not reproducible during clinical follow-up. Diagnoses on EUS were calcification on the duct wall in 2 patients, cuneate deformity of the duct wall in 1 patient and normal biliary tree in 1 patient. Two were considered as strictures due to compression by Oddi's sphincter, accompanied by acute cholangitis which improved and was not recurrent.

To summarize, EUS identified lesions that could cause biliary strictures, irrespective of pathological features, in 32 of 34 patients (sensitivity 90.5%, specificity 100%, PPV 100%, NPV 86.7%, accuracy 94.1%) (Table 5). In terms of pathological features, EUS correctly identified causes in 16 of 17 malignant strictures and in 14 of 17 benign lesions. With regard to the proportion of correct diagnosis, no significant difference was seen between patients with malignant and benign lesions (94.1% *vs* 82.4%,  $P = 0.60$ ). The diagnostic abilities of EUS were 94.1% sensitivity, 82.3% specificity, 84.2% PPV, 93.3% NPV and 88.2% accuracy (Table 6). Following RC, adequate samples were obtained in 24 patients (16 malignant, 8 benign strictures), and RC correctly identified the causes in 8 of 14 malignant strictures and in 8 of 16 benign strictures. In terms of the proportion of correct diagnosis, no significance difference was evident between patients with malignant and benign lesions (62.5% *vs* 100%,  $P = 0.066$ ). The diagnostic abilities of RC were 62.5% sensitivity, 100% specificity, 100% PPV, 57.2% NPV, and 75% accuracy.

Tumor markers were measured in 34 patients, and correctly identified malignancy in 13 of 17 malignant strictures and correctly identified a benign disease in 12 of 17 benign strictures. In terms of the proportion of correct diagnosis, no significant difference was seen between patients with malignant and benign lesions (76.5% *vs* 70.6%,  $P > 0.999$ ). The diagnostic abilities of tumor

Table 4 Benign strictures correctly judged as benign on EUS

Final diagnosis	Reason to diagnose as benign stricture	EUS findings
Inflammatory stricture clinically diagnosed as acute cholangitis ( <i>n</i> = 1)	No exacerbation during follow-up (> 23 mo)	Stenosis of the distal end of the bile duct The normal layered structure of the bile duct wall No mass adjacent to the stricture site
Biliary dilation ( <i>n</i> = 3)	No change for > 18 mo	The dilated bile duct gradually tapering at the ampulla of Vater ( <i>n</i> = 2) A 1-cm long narrowing portion at the distal end of the duct smoothly continuous from the dilated proximal duct ( <i>n</i> = 1)
Chronic pancreatitis including 1 autoimmune pancreatitis ( <i>n</i> = 2)	No exacerbation during follow-up (> 10 mo)	Smooth tapering of the distal end of the bile duct without a mass adjacent to the stricture site (in case of autoimmune pancreatitis) Marked calcification at the stricture site ( <i>n</i> = 1)
Anomalous arrangement of the pancreaticobiliary duct ( <i>n</i> = 1)	Confirmed by MRCP	Connection of the pancreatic duct to the biliary duct outside the papilla of Vater
Congenital choleductal cyst ( <i>n</i> = 1)	Confirmed by surgery	Cystic dilatation at the distal end of the bile duct

MRCP: Magnetic resonance cholangiopancreatography.

Table 5 EUS for recognizing various features of biliary obstruction

Diagnostic ability	%
Sensitivity	90.5
Specificity	100
PPV	100
NPV	86.7
Accuracy	94.1

PPV: Positive predictive value; NPV: Negative predictive value.

Table 6 Diagnosis of malignant *vs* benign causes (%)

	Sensitivity	Specificity	PPV	NPV	Accuracy
EUS ( <i>n</i> = 34)	94.1	82.3	84.2	93.3	88.2
Brushing ( <i>n</i> = 24)	62.5 <sup>a</sup>	100	100	57.2 <sup>c</sup>	75.0
Tumor marker (CA19-9 > 37 U/mL) ( <i>n</i> = 34)	76.5	70.6	72.2	75.0	73.5
Tumor marker (CA19-9 > 100 U/mL) ( <i>n</i> = 34)	53.0 <sup>b</sup>	82.4	75.0	63.6	67.7

<sup>a</sup>*P* = 0.039 *vs* EUS; <sup>b</sup>*P* = 0.017 *vs* EUS; <sup>c</sup>*P* = 0.035 *vs* EUS. CA19-9: Carbohydrate antigen 19-9.

markers were 76.5% sensitivity, 70.6% specificity, 72.2% PPV, 75% NPV and 73.5% accuracy.

The diagnostic ability of CA19-9 has been reported to be improved when the cutoff level is increased to 100 U/mL<sup>[14]</sup>, for the level of CA19-9 increases in patients with cholestasis<sup>[15]</sup>. Analysis of tumor markers was thus performed using 100 U/mL as the cutoff level for CA19-9. The results were 53% sensitivity, 82.4% specificity, 75% PPV, 63.6% NPV and 67.7% accuracy. With regard to the proportion of correct diagnoses using 100 U/mL as the cutoff level of CA19-9, no significant difference was seen between patients with malignant and benign lesions (53.0% *vs* 82.4%, *P* = 0.14).

Diagnostic sensitivity and NPV were superior for EUS than for RC (*P* = 0.039 and 0.035, respectively). Compared with tumor markers using 100 U/mL as the cutoff level for CA19-9, EUS showed significantly higher sensitivity (*P* = 0.017).

Table 7 Classification of EUS Imaging

	Benign stricture ( <i>n</i> = 11)	Malignant stricture ( <i>n</i> = 17)	Statistical significance ( <i>P</i> )
Mass			0.0069
+	5	16	
-	6	1	
Size of mass (mm)			NS
≤ 10	3	3	
> 10	2	13	
Shape			0.025
Round	4	3	
Irregular	1	13	
Internal echo			0.004
Hyperechoic	4	1	
Hypo or mixedechoic	1	15	
Disruption of the common bile duct			0.0013
+	1	13	
-	10	4	
Invasion to surrounding tissue			< 0.001
+	1	16	
-	10	1	

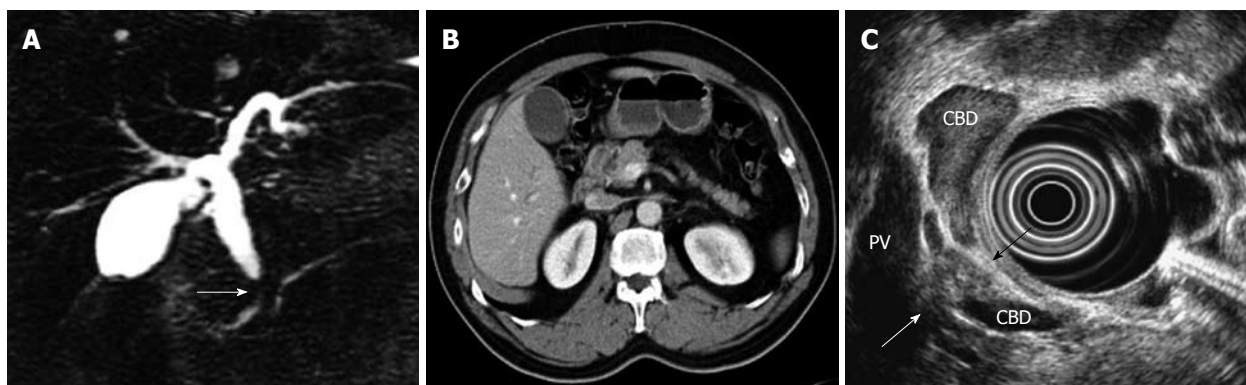
### Ultrasonographic findings on EUS and frequency of malignancy on final diagnosis

Hypoechoic masses with irregular margins, tumor size > 10 mm and mixed echogenicity reportedly imply malignancy on IDUS<sup>[3,10,12]</sup>. Thus, (1) the presence of a mass, (2) tumor size > 10 mm, (3) the margin and internal echo of a mass; (4) disruption of the normal sonographic layers of the bile duct wall, and (5) continuation of a mass into adjacent structures were analyzed to study the relationship between EUS findings and final diagnosis (Table 7).

The proportion of patients with malignancy was significantly higher when a mass was detected (*P* = 0.0069), especially with an irregular margin (*P* = 0.025) and hypoechoic or mixed echoic pattern (*P* = 0.004), the bile duct was accompanied by disruption (*P* = 0.0013) and a case showed invasion to surrounding tissues (*P* < 0.001). There was no significant difference between malignant and benign strictures in terms of tumor size.

To summarize, the data suggest that biliary stricture can be diagnosed as malignancy on EUS when a mass is





**Figure 1** A 62-year-old man with biliary cancer. A: Magnetic resonance cholangiopancreatography (MRCP) showing 11-mm long stricture (arrow) in the distal common bile duct; B: Computed tomography (CT) showing the normal pancreas head; C: Endoscopic ultrasonography (EUS) showing an irregularly shaped mass (black arrow) accompanied by disruption of the bile duct and portal vein invasion (white arrow). CBD: Common bile duct; PV: Portal vein.

adjacent to the stricture, has an irregular margin, a hypoechoic or mixed echoic pattern, disruption of the bile duct, and invasion to surrounding tissue. A typical case is shown in Figure 1.

## DISCUSSION

Consensus has yet to be reached on the management of biliary strictures with unknown etiology. Even after excluding biliary strictures with an identifiable mass on cross-sectional imaging, our data indicate that the risk of malignant stricture is 50%, consistent with previous data<sup>[4,5,7,8]</sup>. The frequency of malignancy is not negligible, allowing surgical exploration as a pre-emptive approach. In particular, when accompanied by obstructive jaundice, the majority of biliary strictures are believed to be malignant and surgery is routinely performed<sup>[7]</sup>. However, the remaining 50% of biliary strictures are benign or are eventually diagnosed as normal when no identifiable mass is seen on CT/MRI. In addition, our data show that 38.5% of biliary strictures are benign, even if accompanied with jaundice. Since the frequency of benign strictures is quite high and these strictures can be managed by endoscopic therapies, recommending exploratory surgery in all patients may not be justifiable. To differentiate patients with a low risk of malignancy from those with a high risk of malignancy who would benefit from surgical exploration, a new diagnostic approach should be provided for the management decision.

IDUS has recently seen clinical use at the time of ERCP for patients with painless jaundice and no identifiable mass on CT/MRI<sup>[5,16]</sup>. In previous studies, IDUS has proven quite accurate in distinguishing benign and malignant strictures, with reported accuracies of 76%-90%<sup>[3,6,17]</sup>. In addition, for indeterminate biliary strictures of unknown etiology, IDUS has provided approved diagnostic ability<sup>[5,8]</sup>. According to Stravropoulos, the sensitivity and specificity of IDUS are both 83%. When IDUS is used with ERCP, IDUS increases the diagnostic accuracy of ERCP from 58% to 90%<sup>[5]</sup>. According to Domagk, IDUS provides correct preoperative diagnosis in 83% of cases<sup>[8]</sup>.

For indeterminate biliary strictures, our study shows that the diagnostic abilities of EUS were 94.1% sensitivity, 82.3% specificity, 84.2% PPV, 93.3% NPV and 88.2% accuracy, comparable to the abilities of IDUS. In addition, compared with RC and tumor markers, EUS showed significantly higher sensitivity and NPV to distinguish benign and malignant strictures. These results suggest that EUS offers high diagnostic ability for distal biliary strictures even without a mass on CT, detecting pancreaticobiliary cancers at a very early stage.

Despite a long search, the only previously published paper on the diagnostic ability of EUS for biliary strictures with unknown etiology was that by Lee *et al*<sup>[1]</sup>. Their diagnostic criteria for malignancy included the finding of a pancreatic head mass and/or irregular bile duct wall, similar to the criteria in our study. According to Lee *et al*<sup>[1]</sup>, EUS for malignancy offers 88% sensitivity, 100% specificity, 100% PPV, and 84% NPV. Compared with our results, Lee *et al*<sup>[1]</sup> achieved much higher specificity. Unlike our patients, however, almost all patients in their study had a stent in place at the time of referral for EUS, possibly making the bile duct wall thicker and/or irregular even in patients with benign stricture. Irregularity of the duct wall might thus have been diagnosed as malignant when the wall was extremely irregular, which would have made the diagnostic criteria for malignancy stricter than expected.

Patients with a final diagnosis of “normal” reaped the greatest benefits from this study, in which the definition of benign strictures was strict. In “normal” cases, EUS could exclude the possibility of malignancy, avoiding unnecessary follow-up and invasive procedures such as surgical exploration.

In our institution, patients with operable lesions judged as malignant based upon our EUS “criteria” and no known contraindication underwent surgery. Patients with lesions judged as benign on EUS and low clinical suspicion for malignancy were treated endoscopically or clinically followed at the outpatient clinic. When clinical data were suggestive of malignancy, further diagnostic procedures such as surgical exploration were performed,



irrespective of EUS results. EUS plus MRCP could replace ERCP with IDUS as the first choice to direct management decisions for indeterminate biliary strictures with no identifiable mass on cross-sectional imaging, especially in cases showing low clinical suspicion for malignancy.

Three false-positive patients showed biliary stricture caused by benign fibrosis. When these patients were referred for EUS, endoscopic biliary drainage tubes had been inserted. Biliary drainage tubes could have caused recurrent cholangitis due to tube occlusion by biliary sludge, leading to thickening of the bile duct wall. In 1 case of benign stricture caused by fibrosis, a hypoechoic mass was detected at the end of the bile duct on EUS, but was not found during surgery. This case indicates that biliary sludge should be considered as a possible diagnosis when a drainage tube is inserted<sup>[18,19]</sup>. The introduction of contrast-enhanced EUS to search for vascular images inside a mass may lead to differentiation of biliary sludge from tumor.

Our study did not perform EUS-FNA, which could have increased the diagnostic ability. According to Lee *et al.*<sup>[1]</sup>, however, EUS-FNA shows 47% sensitivity, 100% specificity, 100% PPV, and 50% NPV, suggesting that sonographic features of biliary strictures are more accurate for diagnosis than the results of EUS-FNA cytology. While some authors have reported EUS-FNA as highly diagnostic for biliary strictures<sup>[20,21]</sup>, the lesions that could explain strictures in those papers were large enough to be detected on CT/MRI, possibly increasing the diagnostic ability of cytology.

RC examination could have compensated for false-negative results in 1 patient with bile duct cancer. In this patient, we could not detect the tumor on EUS, as the tumor was behind impacted stones in the bile duct. This case supports previous reports that malignancy is frequently accompanied by biliary stones<sup>[22]</sup>, recommending cytology examination to prevent false-negative diagnosis, particularly in cases with stones in the biliary tree.

Our results indicate that tumor markers using 100 U/mL as the cutoff level for CA19-9 are less diagnostic than imaging features of biliary strictures detected on EUS, when 100 U/mL is considered as malignancy<sup>[14]</sup>. This is compatible with other studies<sup>[15,23]</sup> noting that tumor markers specific to cancers along the biliary tract are unavailable. When 37 U/mL was used as the cutoff level for CA19-9, no significant difference was seen between the diagnostic abilities of EUS and tumor markers. This is probably because the CA19-9 level increases in cases of cholestasis<sup>[15]</sup>, leading to false-positive cases.

Although preliminary, this study may be the first report to suggest criteria for EUS to classify biliary strictures as benign or malignant. Limited information is available on the criteria for EUS, partly due to the limited availability of EUS<sup>[7]</sup>, while definite criteria for IDUS to classify biliary strictures as benign or malignant have almost been established. According to Menzel, hypoechoic masses with irregular margins and inhomogeneous echo-poor areas

invading surrounding tissue on IDUS are suggestive of malignancy<sup>[10]</sup>. Tamada *et al.*<sup>[3]</sup> reported that the presence of a hypoechoic mass with irregular margins, or infiltration of surrounding tissues, size > 10 mm, and disruption of normal sonographic layers of the bile duct wall, is predictive of malignancy. These data using IDUS are compatible with our results on EUS, suggesting that the presence of a mass adjacent to the biliary stricture, particularly with irregular margins, hypo- or mixed-echoic pattern, disruption of the bile duct, and invasion to surrounding tissue imply malignancy. In our study, mass size was not correlated with the pathological diagnosis of strictures. In the study by Tamada *et al.*<sup>[3]</sup>, malignant masses may have been larger than benign masses, as lesion size was not among the inclusion criteria for subjects in that study. Since our objectives were biliary strictures for which CT could not identify a mass, the lesions should have been much smaller than those studied in other investigations. Malignant and benign lesions may thus have shown no significant difference in size in the present study.

A major limitation in this study was that dynamic CT was performed according to the routine protocol in all cases. If thinner section thickness (e.g. 1–2 mm instead of 8 mm) was used or 3-dimensional analysis instead of horizontal analysis alone was performed, the causative mass could have been detected. If the dynamic study had been adjusted to the location of each biliary stricture, more detailed information could also have been obtained<sup>[24]</sup>. However, no imaging modalities have shown detectability of small pancreatic cancer superior to EUS<sup>[9]</sup>. In extrahepatic bile duct carcinoma, particularly without thickening of biliary walls, identification of local extension or depth using CT alone is known to be difficult<sup>[25]</sup>. In the diagnosis of cancer of the papilla of Vater, depicting tumors by CT is accepted as difficult<sup>[14]</sup>. These reports indicate that detectability of lesions that can cause biliary stricture remains insufficient using CT alone.

In conclusion, sonographic appearance on EUS offers high diagnostic ability for extrahepatic biliary strictures, even if cross-sectional imaging modalities cannot depict the causative lesion. Particularly in cases of benign stricture where the final diagnosis is “normal”, invasive procedures such as surgical exploration or excessive follow-up investigations may be avoided. Furthermore, earlier diagnosis of malignant strictures by EUS would provide more therapeutic options for patients with very early-stage pancreaticobiliary cancer.

## COMMENTS

### Background

There is no consensus regarding how to manage distal biliary stricture without an identifiable mass on cross-sectional imaging, since benign and malignant strictures cannot be definitely distinguished. For indeterminate biliary strictures, intraductal ultrasonography (IDUS) has recently yielded a diagnostic accuracy of around 80%–90%. However, the ability of endoscopic ultrasonography (EUS) to diagnose unexplained strictures has not been fully examined.

### Research frontiers

The authors demonstrated that EUS can diagnose biliary strictures caused by

malignant tumors that are undetectable on computed tomography scan. They also gave a summary of the sonographic features of malignant strictures, which could increase the diagnostic sensitivity of EUS.

### Innovations and breakthroughs

There is only one previously published study on the diagnostic utility of EUS for biliary strictures with an unknown etiology. In that study, however, almost all patients had a stent in place, possibly making the bile duct wall thicker and irregular even in patients with benign strictures. Since this clinical study included many patients without a stent, sonographic features between benign and malignant strictures could be compared, providing characteristic EUS images for malignancy.

### Applications

EUS plus magnetic resonance cholangiopancreatography could be a first-line, noninvasive examination for the diagnosis of distal biliary stricture, followed by IDUS/endoscopic retrograde cholangiopancreatography examinations or histological confirmation, when the latter are required, especially in cases showing low clinical suspicion for malignancy.

### Peer review

This is an interesting retrospective study of clinical relevance. In spite of some limitations, this paper adds to the core knowledge related to diagnostic challenges in patients with a bile duct stricture.

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## Comparing upper gastrointestinal X-ray and endoscopy for gastric cancer diagnosis in Korea

Hoo-Yeon Lee, Eun-Cheol Park, Jae Kwan Jun, Kui Son Choi, Myung-Il Hahm

Hoo-Yeon Lee, Eun-Cheol Park, Jae Kwan Jun, Kui Son Choi, National Cancer Control Research Institute, National Cancer Center, 111 Jungbalsan-ro, Ilsandong-gu, Goyang-si, Gyeonggi-do 410-769, South Korea

Myung-Il Hahm, Department of U-Healthcare Management, College of Medical Science, SoonChunHyang University, 646 Eupnae-ri, Shinchang-myeon, Asan-si, Chungnam 336-745, South Korea

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**Correspondence to:** Eun-Cheol Park, MD, PhD, National Cancer Control Research Institute, National Cancer Center, 111 Jungbalsan-ro, Ilsandong-gu, Goyang-si, Gyeonggi-do 410-769, South Korea. [ecpark@ncc.re.kr](mailto:ecpark@ncc.re.kr)

Telephone: +82-31-9202003 Fax: +82-31-9202189

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*via* upper endoscopy was 2.9-fold higher than *via* upper GI X-ray. The unit costs of screening using upper GI X-ray and upper endoscopy were \$32.67 and \$34.89, respectively. In 2008, the estimated cost of identifying one case of gastric cancer was \$53094.64 using upper GI X-ray and \$16900.43 using upper endoscopy. The cost to detect one case of gastric cancer was the same for upper GI X-ray and upper endoscopy at a cost ratio of 1:3.7.

**CONCLUSION:** Upper endoscopy is slightly more costly to perform, but the cost to detect one case of gastric cancer is lower.

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**Key words:** Gastric cancer; Mass screening; Endoscopy; Early diagnosis; X-ray diagnosis

**Peer reviewer:** Luis Bujanda, Professor, Department of Gastroenterology, CIBEREHD, University of Country Basque, Donostia Hospital, Paseo Dr. Beguiristain s/n, 20014 San Sebastián, Spain

Lee HY, Park EC, Jun JK, Choi KS, Hahm MI. Comparing upper gastrointestinal X-ray and endoscopy for gastric cancer diagnosis in Korea. *World J Gastroenterol* 2010; 16(2): 245-250 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i2/245.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i2.245>

### Abstract

**AIM:** To compare the cost and accuracy of upper gastrointestinal (GI) X-ray and upper endoscopy for diagnosis of gastric cancer using data from the 2002-2004 Korean National Cancer Screening Program (NCSP).

**METHODS:** The study population included 1503646 participants in the 2002-2004 stomach cancer screening program who underwent upper GI X-ray or endoscopy. The accuracy of screening was defined as the probability of detecting gastric cancer. We calculated the probability by merging data from the NCSP and the Korea Central Cancer Registry. We estimated the direct costs of the medical examination and the tests for upper GI X-ray, upper endoscopy, and biopsy.

**RESULTS:** The probability of detecting gastric cancer

### INTRODUCTION

Gastric cancer is the second most common cause of cancer death worldwide, and countries in East Asia, such as China and Japan, have a high incidence of gastric cancer. Korea has 66 cases per 100000 in men and 34 cases per 100000 in women. Although the incidence has declined in recent decades, gastric cancer remains the most frequent cancer diagnosis in Korea<sup>[1,2]</sup>. The

prevention and early detection of gastric cancer is therefore a high priority.

Many Asian countries have no national guidelines or recommendations for gastric cancer screening. In high-risk regions such as Japan and Korea, where gastric cancer is highly prevalent, screening is recommended for individuals over 40 years of age. Japan utilizes mass X-ray screening programs to detect gastric cancer in its early stages<sup>[1,3]</sup>. In recent years, mass screening with upper endoscopy has replaced upper gastrointestinal (GI) X-ray in several cities in Japan, and is considered to be a superior technique by the Japanese medical system<sup>[4,5]</sup>. Upper endoscopy is a highly effective screening method that detects early gastric cancer at a higher rate than upper GI X-ray. However, there are cost issues associated with upper endoscopic mass screening, in that it is nearly 4-fold more expensive than upper GI X-ray in the Japanese medical system<sup>[5,6]</sup>. In addition, primary screening endoscopy was proposed as a cost-effective method to screen for gastric cancer in an intermediate-risk population such as in Singapore<sup>[7]</sup>.

Despite this promising result, direct evidence about the effectiveness of upper endoscopy among individuals at average risk of gastric cancer is still not sufficient to justify its use for routine screening, and no nationwide screening program is available<sup>[2,8]</sup>. Upper endoscopy may be the most cost-effective screening program for Korea, given the relatively low cost of the technique and the high incidence of gastric cancer.

Korea has an organized population-based screening program in which people within a specified age range regularly receive a personal letter inviting them to undergo cancer screening. In 1999, Korea began screening for gastric cancer as a part of the National Cancer Screening Program (NCSP). The target population was enrolled in Medicaid and had National Health Insurance with a premium below 50%. Currently, NCSP provides screening services free of charge. The NCSP recommends biennial upper GI X-ray or upper endoscopic gastric cancer screening for men and women older than 40<sup>[9]</sup>. Although the NCSP offers either upper GI X-ray or upper endoscopy examination as an initial screening method for gastric cancer, there is a lack of agreement about the recommendation of screening method among the government, physicians, and the general population<sup>[2]</sup>.

Given the limited healthcare resources, policy-makers require estimates of the effectiveness and costs of alternative prevention programs. Ideally, policy decisions should be based on the long-term effectiveness and cost-effectiveness of gastric cancer screening<sup>[10]</sup>. However, screening for gastric cancer is not commonly practiced, and there is a paucity of data to lend support to such a program. Few data are currently available regarding the long-term effectiveness of alternative gastric cancer screening strategies. The evaluation of the effects of gastric cancer screening on prognosis is incomplete and epidemiologic data on the survival, mortality, and distribution of gastric cancer stage are still insufficient in Korea. In the present study, we examined the short-

term cost-effectiveness. The data presented here are preliminary, and we were unable to show that the screening programs examined are associated with a decrease in gastric cancer mortality.

## MATERIALS AND METHODS

### *Measures of effectiveness*

We estimated an intermediate outcome: the cost per case detected<sup>[10,11]</sup>. The effectiveness of screening was defined as the probability of detecting gastric cancer. We compared other accuracy indicators between upper GI X-ray and upper endoscopy, such as sensitivity and specificity. We calculated accuracy by merging data from the NCSP and the Korea Central Cancer Registry (KCCR), a nationwide hospital-based cancer registration program that covers more than 90% of the cancer cases in Korea<sup>[12]</sup>.

We used data from the NCSP collected from 2002 to 2004. In total, 1 608 810 participated in the stomach cancer screening program. Of the 1 608 810 participants, male and female subjects who were free of gastric cancers and had complete information related to gastric cancer screening results and identification number were included in this study. The data from 1 503 646 participants in the stomach cancer screening program were finally included in this analysis.

The screening results of the NCSP were reported as follows: “normal”, “benign”, “suspicious”, or “cancer”. We defined both “suspicious” and “cancer” as positive results in each screening method. Outcomes of stomach cancer incidence were based on a 1-year follow-up using the KCCR as a gold standard. We defined cancer incidence by the International Classification of Diseases, code C16 (ICD-10:C16). This research was approved by the Institutional Review Board (IRB) committee.

### *Cost calculation*

We used 2 models to calculate the total cost of gastric cancer screening. One model included the additional costs for diagnostic follow-up after obtaining abnormal results to consider the costs of upper endoscopy incurred after the upper GI X-ray, which was outside the scope of the NCSP. The costs for diagnostic follow-up were based on the payment system for clinics. The other model included only the direct costs of providing preventive cancer screening services (i.e. only the cost a screening program would incur).

We estimated the screening costs of the medical examination and the tests for upper GI X-ray, upper endoscopy, and biopsy. When the upper GI X-ray identified an abnormal result, the diagnosis had to be confirmed with upper endoscopy and biopsy, even if the final result was a false positive. Therefore, the patient may incur expenses in addition to the initial screening test. We performed 2 cost analyses: in model I (the standard model), we calculated the cost of diagnosis confirmation when the results of the upper GI X-ray were positive (“suspicious” or “cancer”), whereas model II represented



**Table 1** Characteristics of gastric cancer screening participants (2002–2004) *n* (%)

	Upper GI X-ray	Upper endoscopy	Total (%)
Year			
2002	290 410 (75.0)	96 637 (25.0)	387 047 (100.0)
2003	393 217 (70.9)	161 736 (29.1)	554 953 (100.0)
2004	383 751 (68.3)	177 895 (31.7)	561 646 (100.0)
Total (%)	1 067 378 (71.0)	436 268 (29.0)	1 503 646 (100.0)

GI: Gastrointestinal.

**Table 2** Results of gastric cancer screening (2003–2005)

	Upper GI X-ray ( <i>n</i> )			Upper endoscopy ( <i>n</i> )		
	Cancer	No cancer	Total	Cancer	No cancer	Total
Positive	892	108 178	109 070	1041	16 105	17 146
Negative	1227	957 081	958 308	724	418 398	419 122
Total	2119	1 065 259	1 067 378	1765	434 503	436 268

the expense to the screening program and only included the cost of the screening test.

The stomach cancer screening costs were based on the 2008 National Cancer screening payment system. Costs were expressed in US dollars (USD), based on the average exchange rate of 1121.88 won for 1 USD in 2008. We varied the cost-ratio of upper GI X-ray and upper endoscopy screening programs, to explore the change in cost-effectiveness when the cost of upper endoscopy was higher than that of upper GI X-ray, as in other countries.

## RESULTS

### Characteristics of the gastric cancer screening participants

Between January 2002 and December 2004, 1 503 646 people took part in the NCSP (Table 1). Of those, 71% were screened using upper GI X-ray and 29% were screened with upper endoscopy. Of the participants who were screened in 2002, 75.0% of the participants underwent upper GI X-ray and 25.0% underwent upper endoscopy. In 2004, 68.3% underwent upper GI X-ray and 31.7% underwent upper endoscopy.

### Gastric cancer screening test accuracy

During a 1-year follow-up period, 3884 new gastric cancer cases were reported, and the gastric cancer incidence was 258.3 per 100 000 people. Of the 1 067 378 people screened with the upper GI X-ray, 109 070 positive results were obtained: 892 were true positives and 108 178 were false positives. In contrast, of the 436 268 participants screened with upper endoscopy, 17 146 positive results led to 1041 true positives and 16 105 false positives (Table 2).

The probability of finding a true positive for gastric cancer in 1 503 646 participants was 836 per 100 000 for the upper GI X-ray screening test and 2386 per 100 000 for upper endoscopy (Table 3). Therefore, the probability

**Table 3** Estimated costs and effectiveness of gastric cancer screening programs

	Model I		Model II	
	Upper GI X-ray	Upper endoscopy	Upper GI X-ray	Upper endoscopy
Probability of gastric cancer ( <i>n</i> )	0.000836	0.002386	0.000836	0.002386
Unit cost, US\$ (a)	44.37	40.33	37.14	40.33
Cost to detect one case of gastric cancer ( <i>a/n</i> )	53 094.64	16 900.43	44 445.78	16 900.43
Reduction in cost to detect one case of gastric cancer		36 194.21		27 545.35

Model I : Including screening test costs and confirmation test costs, if the results of upper GI X-ray were abnormal; Model II : Including screening test costs only.

**Table 4** Unit cost in 2008 for the gastric cancer screening tests

	Units	Cost in USD
Screening cost	Medical examination	\$4.47
	Upper gastrointestinal X-ray	\$32.67
	Upper endoscopy	\$34.89
	Biopsy	\$24.54
Confirmation cost		\$70.73

Confirmation cost: the cost of endoscopic examination with biopsy when the results of the upper GI X-ray were positive ("suspicious" or "cancer").

of detecting gastric cancer with upper endoscopy was about 2.9-fold higher than with upper GI X-ray. The sensitivities of upper GI X-ray and upper endoscopy were 42.1% and 59.0%, respectively, and the specificities were 89.8% and 96.3%, respectively. The positive predictive value was 0.008 for upper GI X-ray and 0.061 for upper endoscopy, and the negative predictive values were 0.999 and 0.998, respectively.

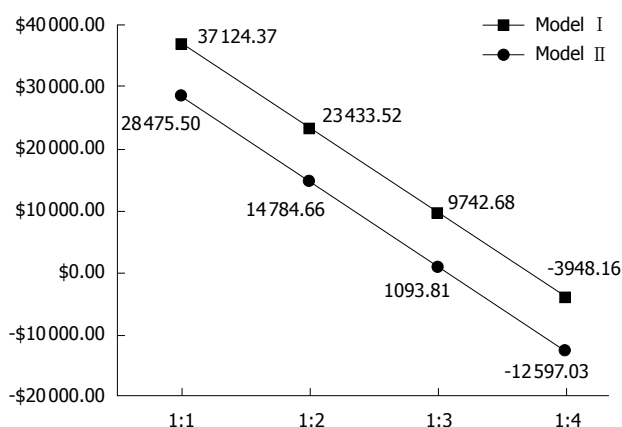
### Cost of gastric cancer screening tests

Table 4 shows the unit costs for gastric cancer screening in 2008. Screening using upper GI X-ray and upper endoscopy cost \$32.67 and \$34.89, respectively, and the cost ratio was 1:1.07.

### Cost-effectiveness of the screening tests

The cost of identifying one case of gastric cancer was estimated to be \$53 094.64 with upper GI X-ray screening and \$16 900.43 with upper endoscopy in 2008. Therefore, using upper endoscopy as the initial screening test would reduce the cost of detecting one case of gastric cancer by \$36 194.21 in model I and \$27 545.35 in model II.

Figure 1 shows the difference in cost-effectiveness according to various cost ratios for the upper GI X-ray



**Figure 1** Changes in the reduction in costs (in USD) according to the cost ratios of upper GI X-ray and upper endoscopy. Model I : Including screening test costs and confirmation test costs, if the results of the upper gastrointestinal X-ray were abnormal; Model II : Including screening test costs only.

and upper endoscopy screening programs. This was analyzed to explore the change in cost-effectiveness when the cost of upper endoscopy exceeded that of upper GI X-ray, as in other countries. At a ratio of 1:1, we can save \$37124.37 in model I. However, when upper endoscopy screening was 4-fold more expensive than upper GI X-ray, it cost \$3948.16 more to conduct an upper endoscopy test than an upper GI X-ray. In model I, the cost to detect one case of gastric cancer was the same for upper GI X-ray and upper endoscopy at a cost ratio of 1:3.7.

## DISCUSSION

We found that the upper endoscopy screening test was more accurate and the detection rate was higher than for upper GI X-ray. A few studies have examined the accuracy of upper endoscopy in detecting cancer<sup>[1,4,5,13-16]</sup>. A study conducted in Niigata, Japan found that the detection rate of gastric cancer when using endoscopy was 2.7-4.6-fold higher than when using upper GI X-ray or photofluorography<sup>[1,5]</sup>. These investigators reported an endoscopic sensitivity of 77.8% based on a 3-year follow-up using the cancer registry system in Fukui prefecture<sup>[14]</sup>. Another study based on a follow-up survey of individual participants reported that the sensitivity of endoscopy was 84%<sup>[16]</sup>. Our findings for sensitivity and specificity were lower than those reported by others for both upper endoscopy and the upper GI X-ray.

It is difficult to directly compare the results of different studies because of differences in target populations and gastric cancer screening systems. For example, our target population was a general population and included people from across the nation. The other studies mentioned here were conducted in municipalities within a prefecture and were hospital-based. Moreover, in Japan photofluorography is the initial assessment for gastric cancer screening followed by upper GI X-ray and upper endoscopy<sup>[1,3,4,13,17]</sup>, whereas in Korea upper GI X-ray or upper endoscopy are the initial tests in organized gastric cancer screening programs.

Despite the diagnostic advantages of upper endoscopy, it is more expensive and requires more staff and technological expertise than upper GI X-ray. In financial terms, the test is not as effective if the cost is high. The cost of upper endoscopy is 3-4-fold more expensive than upper GI X-ray in Japanese gastric cancer screening programs<sup>[5,6]</sup>. However, Japanese studies showed that even when the ordinary examination fee is charged, upper endoscopy was still the most cost-effective method for detecting gastric cancer<sup>[1,5,6]</sup>. Nevertheless, it is unlikely that upper endoscopy would be feasible as a mass screening program, even in highly developed countries such as Japan, because of a lack of experienced endoscopists<sup>[1,5]</sup>.

In contrast, there are a number of skilled endoscopists in Korea, making upper endoscopy mass screening more practical. In 2008, there were more than 4000 board-certified endoscopic specialists in Korea who were members of the Korean Society of Gastrointestinal Endoscopy, and their ranks have been increasing by about 150 persons annually. Upper endoscopy mass screening for gastric cancer has a higher detection probability than upper GI X-ray, and upper endoscopy is a more cost-effective screening method in Korea, given the relatively low cost of this technique (about the same as upper GI X-ray) and the high incidence of gastric cancer<sup>[1,18]</sup>. The results of our study indicate that upper endoscopy will be a more cost-effective mass screening program than upper GI X-ray until the cost per cancer case detected becomes 3.7-fold more expensive than with upper GI X-ray.

The majority of people who undergo cancer screening receive no benefit from the procedure and may actually be exposed to additional health risks as a result of screening. These risks arise from complications that can result in hospitalization or even death, from false positive results that lead to unnecessary, invasive follow-up procedures, and from over diagnosis (i.e. the treatment of cancers that, in the absence of screening, would neither have been detected in the patient's lifetime nor caused death). Therefore, mass screening is a tradeoff between major benefits to a few and small risks to many<sup>[19]</sup>.

Calculation of the total cost of gastric cancer screening must include the costs of upper endoscopy incurred after the initial assessment, as well as those of complications that infrequently result from the procedure. The balance of cost-effectiveness includes not only financial costs, but also non-financial costs including anxiety, emotional distress, and inconvenience. We did not include these non-financial costs in our study. Upper endoscopy is an invasive, but relatively safe procedure with an extremely low complication rate (mortality, 1 in 3300-40000)<sup>[7,13,20]</sup>. Upper endoscopy enables simultaneous biopsy for histological confirmation of malignancy. Early detection with upper endoscopy permits curative management by surgical resection and endoscopic mucosal resection<sup>[4,17]</sup>. Upper endoscopy can detect gastric cancer at an earlier stage than upper GI X-ray. However, we were unable to compare the stage distribution of gastric cancer detected by upper endoscopy or upper GI X-ray, because NCSP does not require the reporting

of detailed clinical information including the stage of gastric cancer detected by screening.

Another weakness in our study was the inability to control self-selection bias, because we did not have information about socioeconomic status such as income level, education level, or job status and risk factors. These problems should be resolved in a prospective study that we have planned for the future. In a previous study, respondents with higher income levels were more likely to have an upper endoscopy test. Under the Korean health insurance system, the cost of the upper endoscopy test is almost the same as that for the upper GI X-ray. In addition, the NCSP offers a free-of-charge endoscopy test for gastric cancer screening. Despite these programs, disparities in the use of endoscopy vary with household income, possibly suggesting that misconceptions exist about the cost of endoscopy. In addition, the higher rates of endoscopy use in those with a family history of gastric cancer might indicate that the endoscopy test was the preferred test for a high-risk population<sup>[2]</sup>.

Screening large numbers of people is a costly undertaking. Healthcare providers and policy-makers are increasingly interested in the efficient allocation of medical resources, including those used for cancer screening programs. It is essential to have a framework that compares the health benefits and resource expenditures associated with competing medical and public health interventions that will allow decision-makers to identify cost-effective interventions. A complete cost-effectiveness analysis of alternative screening strategies depends on the treatment cost and its effect in terms of life years saved (or quality-adjusted life years saved)<sup>[10,19]</sup>. Longer follow-up is required to generate the mortality results critical for decision-making. However, decisions about whether and how to screen for cancer cannot always be delayed until mortality data are available. Some degree of modeling is frequently required to translate intermediate trial outcomes to long-term outcomes, making cost-effectiveness analysis necessary<sup>[19]</sup>.

The data presented here are preliminary, and we were unable to show that the screening programs examined were associated with a decrease in gastric cancer mortality. Follow-up studies are needed to further investigate this. However, the intermediate outcomes used here indicate that, in Korea, upper endoscopy is a more cost-effective screening program than upper GI X-ray. Although the incidence and mortality of gastric cancer has decreased in Korea during the last decade, gastric cancer screening continues to be a major issue because incidence and mortality remain high.

The best test is a matter of personal preference, which should be considered when physicians make recommendations for screening. Upper endoscopy is gaining acceptance as a standard method for gastric cancer screening and the preferred gastric cancer screening method according to a population-based survey<sup>[2,21]</sup>. The providers' assessment of individuals' screening preferences, in combination with intervention strategies to promote performance of the preferred screening method, may increase compliance with gastric cancer screening recommenda-

tions<sup>[22,23]</sup>. It will be imperative to consider these results when making decisions about population-based screening strategies<sup>[24]</sup>. Evidence-based screening should be promoted in the future to prevent premature death from gastric cancer. To achieve this goal, it is necessary to determine the effectiveness of upper GI X-ray and upper endoscopy mass screening in reducing mortality.

## COMMENTS

### Background

In high-risk regions such as Japan and Korea, where gastric cancer is highly prevalent, screening is recommended for individuals over 40 years of age. However, many Asian countries have no national guidelines or recommendations for gastric cancer screening.

### Research frontiers

In recent years, mass screening with upper endoscopy has replaced upper gastrointestinal (GI) X-ray in several cities in Japan, and is considered to be a superior technique by the Japanese medical system. However, there are cost issues associated with upper endoscopic mass screening. In addition, primary screening endoscopy was proposed as a cost-effective method to screen for gastric cancer in an intermediate-risk population such as in Singapore. Despite this promising result, direct evidence about the effectiveness of upper endoscopy among individuals at average risk of gastric cancer is still not sufficient to justify its use for routine screening, and no nationwide screening program is available.

### Innovations and breakthroughs

The authors identified that upper endoscopy slightly more costly to perform, but the cost to detect one case of gastric cancer was lower. Their study showed the usefulness of endoscopy as an organized screening technique across the nation.

### Applications

Upper endoscopy mass screening is a more cost-effective screening method than upper GI X-ray in Korea, and with its relatively low cost, the high incidence of gastric cancer, and the number of skilled endoscopists, upper endoscopy mass screening is more practical for gastric cancer screening.

### Peer review

The work is an interesting study to be accepted for publication.

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## Reinfection rate and endoscopic changes after successful eradication of *Helicobacter pylori*

Kum Hei Ryu, Sun Young Yi, Youn Ju Na, Su Jung Baik, Su Jin Yoon, Hae-Sun Jung, Hyun Joo Song

Kum Hei Ryu, Center for Cancer Prevention and Detection, National Cancer Center, Madu1-dong, Ilsandong-gu, Goyang-si, Gyeonggi-do, 410-769, South Korea

Sun Young Yi, Youn Ju Na, Su Jung Baik, Su Jin Yoon, Hae-Sun Jung, Hyun Joo Song, Departments of Internal Medicine, Ewha Womans University School of Medicine, 911-1 Mokdong, Yancheon-gu, Seoul, 158-710, South Korea

Author contributions: Yi SY designed the study and performed the major role of collecting patients; Na YJ, Baik SJ, Yoon SJ, Jung HS and Song HJ collected patients' data and were involved in editing the manuscript; Ryu KH was also involved in collecting the data and wrote the manuscript.

Correspondence to: Sun Young Yi, MD, PhD, Department of Internal Medicine, Ewha Womans University School of Medicine, 911-1 Mokdong, Yancheon-gu, Seoul, 158-710, South Korea. [syy@ewha.ac.kr](mailto:syy@ewha.ac.kr)

Telephone: +82-2-26505575 Fax: +82-2-26552076

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**CONCLUSION:** The reinfection rate in Korea is 9.1% which represents a decreasing trend. There was no relationship between *H. pylori* infection status and changes in endoscopic findings. There was also no recurrence or aggravation of ulcers.

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**Key words:** *Helicobacter pylori*; Eradication; Reinfection; Endoscopy

**Peer reviewer:** Annemarie de Vries, MD, PhD, Department of Gastroenterology and Hepatology, Erasmus MC, Room Ba 393, PO Box 2040, 3000 CA, Rotterdam, The Netherlands

Ryu KH, Yi SY, Na YJ, Baik SJ, Yoon SJ, Jung HS, Song HJ. Reinfection rate and endoscopic changes after successful eradication of *Helicobacter pylori*. *World J Gastroenterol* 2010; 16(2): 251-255 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i2/251.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i2.251>

### Abstract

**AIM:** To determine the long-term outcomes regarding reinfection with *Helicobacter pylori* (*H. pylori*) and endoscopic changes after successful *H. pylori* eradication.

**METHODS:** From June 1994 to January 2007, 186 patients (M:F = 98:88; mean age 50.0 ± 11.4 years), in whom *H. pylori* had been successfully eradicated, were enrolled. The mean duration of follow up was 41.2 ± 24.0 mo.

**RESULTS:** *H. pylori* reinfection occurred in 58 patients (31.2%). The average annual reinfection rate was 9.1% per patient year. No recurrence of peptic ulcer was detected at the follow up endoscopy. There were no significant differences between the *H. pylori* eradication regimens for the reinfection rate and no significant differences in endoscopic findings between the *H. pylori*-recurred group and the *H. pylori*-cured group.

### INTRODUCTION

*Helicobacter pylori* (*H. pylori*) is known to cause many gastrointestinal diseases including peptic ulcers, gastric carcinoma and mucosa-associated lymphoid tissue lymphoma<sup>[1]</sup>. Successful eradication of *H. pylori* infection is important in the prevention of recurrent peptic ulcer disease and gastric cancer<sup>[2-4]</sup>. Reinfection rate of *H. pylori* infection after successful eradication is an important problem in the management of peptic ulcer disease. The reinfection rate of *H. pylori* varies considerably among different studies. Reported rates of *H. pylori* recurrence in western countries range from 0.5% to 2.5%<sup>[3,5,6]</sup>. The high prevalence of *H. pylori* infection in Korea suggests the possibility that the reinfection rate might be higher than in western countries. The high prevalence of *H. pylori* infection may possibly be associated with high

rates of reinfection after eradication because of the high risk of re-exposure to infection<sup>[7]</sup>. The aims of this study were firstly to determine the rate of *H. pylori* reinfection and secondly to determine endoscopic changes after successful eradication of *H. pylori* and after subsequent reinfection in an endemic area such as Korea.

## MATERIALS AND METHODS

Patients, who were treated for *H. pylori* infection between June 1994 and January 2007 at Ewha Womans University Mokdong Hospital, Seoul, Korea and who had a negative <sup>14</sup>C urea breath test (UBIT<sup>®</sup>, Otsuka, Japan) 1 mo after eradication, were enrolled. Informed consent was obtained from the patients and ethical approval was given by Ewha Womans University Mokdong Hospital ethical committee (approval No. 187-16). After successful eradication of *H. pylori* infection and cessation of acid-suppression therapy, patients were offered endoscopic examination routinely every year. Follow up endoscopic examination and rapid urease testing (ASAN Helicobacter Test<sup>®</sup>, Asan Pharmaceutical, Korea) were performed. The initial and follow up endoscopies were performed by one endoscopist. The presence and grading of reflux esophagitis, atrophic gastritis, and gastric intestinal metaplasia were determined according to LA classification and Kimura-Takemoto classification of atrophic pattern<sup>[8]</sup>. Atrophic gastritis and gastric intestinal metaplasia were also evaluated histologically. Three endoscopic specialists reviewed the images of the endoscopic findings to reduce the inter-observer variation. Improvements and aggravation were determined by endoscopic and histologic findings. Rapid urease test was performed on biopsy specimens from the body of the stomach at the greater curvature.

Follow up duration was defined as being the length of time from successful eradication until the final test in each patient. Reinfection was defined as being when *H. pylori* recurrence took place at least 1 year after eradication therapy. The cumulative and average annual reinfection rates were calculated. Demographics and mucosal changes were compared between recurred patients (*H. pylori*-recurred group) and those remaining successfully eradicated (*H. pylori*-cured group) using Student's *t*-test and  $\chi^2$  with significance set at  $P < 0.05$ .

## RESULTS

### Subjects

One hundred and eighty six patients (98 men and 88 women) were enrolled. Sixteen patients showed recurrence of *H. pylori* within 1 year post eradication, and these were excluded from the study. Mean age was  $50.0 \pm 11.4$  years. The reasons for the initial endoscopy were; epigastric pain (40.9%), indigestion (25.3%), bleeding (5.9%) and routine check (28.0%). At the initial endoscopy, 19 patients had gastric ulcers, 79 patients had duodenal ulcers and 8 patients had gastroduodenal

**Table 1** Recurrence of *Helicobacter pylori* (*H. pylori*) after successful eradication therapy *n* (%)

Follow up period	<i>n</i>	Recurrence
1 ≤ yr < 2	186	14 (7.5)
2 ≤ yr < 3	133	15 (11.3)
3 ≤ yr < 4	90	8 (8.9)
4 ≤ yr < 5	62	8 (12.9)
5 ≤ yr < 6	40	3 (7.5)
6 ≤ yr < 7	27	6 (22.2)
7 ≤ yr < 8	13	2 (15.4)
8 ≤ yr < 9	7	2 (28.5)
9 ≤ yr < 10	1	0 (0.0)
Total		58 (31.2)

**Table 2** Recurrence rate according to the *H. pylori* eradication regimen *n* (%)

Therapeutic regimens	<i>n</i>	Recurrence
Omeprazole + clarithromycin + amoxicillin	137	41 (30.0)
Bismuth subcitrate + ranitidine + metronidazole + amoxicillin	39	14 (35.9)
Omeprazole + bismuth subcitrate + metronidazole + tetracycline	10	3 (30.0)

ulcers. The other mucosal findings at initial examination were; 23 had reflux esophagitis (12.4%), 91 had chronic superficial gastritis (48.9%), 60 had erosive gastritis (32.3%), 21 had atrophic gastritis (11.3%) and 14 patients had gastric intestinal metaplasia (7.5%).

Post eradication follow up varied from 13 to 112 mo and mean follow up duration was  $41.2 \pm 24.0$  mo.

### Reinfection of *H. pylori* after successful eradication

Reinfection of *H. pylori* after successful eradication occurred in 58 of 186 patients (31.2%). The follow up period and the time when recurrences were found are summarized in Table 1. The annual reinfection rate was 9.1% per patient year (58/638.8 patient years).

One hundred and thirty seven patients were treated with proton pump inhibitor-based triple regimens, 41 of these patients (30.0%) had recurrence. Thirty nine patients were treated with bismuth-based quadruple regimens, 14 of these (35.9%) had recurrence. Among 10 patients who were treated with proton pump inhibitor-based quadruple regimens, 3 (30.0%) patients had recurrence. There was no significant difference among the regimens for reinfection rate (Table 2).

### Endoscopic mucosal changes and comparison between *H. pylori*-recurred group and *H. pylori*-cured group

A comparison between the *H. pylori*-cured group and recurred group is summarized in Table 3. In the recurred group, mean follow up period was 42.5 mo (range 13-104 mo) and in the cured group, mean follow up was 40.6 mo (range 13-112 mo).

At the initial endoscopy, 23 cases of reflux esophagitis, 21 cases of atrophic gastritis and 14 cases of gastric intestinal metaplasia were noted. Peptic ulcers including

**Table 3** Comparisons between the *H. pylori*-cured and -recurred group (mean  $\pm$  SD) *n* (%)

	Cured group ( <i>n</i> = 128)	Recurred group ( <i>n</i> = 58)	<i>P</i>
Age (yr)	49.4 $\pm$ 11.2	51.5 $\pm$ 11.9	NS
Male:Female	65:63	33:23	NS
Follow up (mo)	40.6 $\pm$ 24.3	42.5 $\pm$ 23.7	NS
Endoscopic findings			
Reflux esophagitis			
Presence at initial investigation	19 (15.0)	3 (5.2)	NS
Newly developed or aggravated at follow up	25 (19.7)	8 (13.8)	NS
Improved at follow up	9 (7.1)	3 (5.2)	NS
Atrophic gastritis			
Presence at initial investigation	11 (8.7)	8 (13.8)	NS
Newly developed or aggravated at follow up	7 (5.5)	2 (3.4)	NS
Improved at follow up	3 (2.4)	1 (1.7)	NS
Metaplastic gastritis			
Presence at initial investigation	12 (9.4)	4 (6.9)	NS
Newly developed or aggravated at follow up	5 (3.9)	2 (3.4)	NS
Improved at follow up	7 (5.5)	2 (3.4)	NS

NS: Not significant.

scar stage were observed in 106 cases. Six cases of ulcer were in the acute or healing stage and 100 cases were in the scar stage. At the follow up endoscopy, there were no cases of recurrence or aggravation of ulcers. Newly developed or aggravated cases of reflux esophagitis at the follow up endoscopy were seen in 33 subjects and improved cases were seen in 12 subjects. Nine subjects were newly developed or aggravated and 4 subjects were improved in the atrophic gastritis group and for the gastric intestinal metaplasia group, 7 were newly developed or aggravated and 9 were improved at the follow up endoscopy. There were no significant differences in mucosal changes at the initial and follow up endoscopies between *H. pylori*-recurred group and *H. pylori*-cured group.

## DISCUSSION

Infection with *H. pylori* occurs worldwide, but the prevalence varies greatly between countries and among population groups within the same country. The overall prevalence of *H. pylori* infection is strongly correlated with socioeconomic conditions<sup>[2]</sup>. The prevalence is over 80% in many developing countries, and in Korea the prevalence of *H. pylori* infection is also high at 69.4%, as compared with 20% to 50% in industrial countries<sup>[2,5,6,9]</sup>.

The reinfection rate after eradication therapy for *H. pylori* is extremely low in developed countries such as Europe and the USA. Here, the annual reinfection rates are reported to be around 1%. In contrast to the low rates of *H. pylori* reinfection reported in western populations, high recurrence rates have been reported in such developing countries as Peru, Brazil, Chile,

Vietnam and Bangladesh, all of which are countries with a high prevalence of *H. pylori* infection<sup>[1,5-7,10-12]</sup>. The high prevalence of *H. pylori* infection may possibly be associated with high recurrence of infection after eradication because of the high risk of re-exposure to infection<sup>[7]</sup>.

In 1998, Kim *et al.*<sup>[13]</sup> reported that *H. pylori* reinfection rate in patients followed for up to 5 years was 12.8%. In our study, the patients were followed for up to 9 years and the average annual reinfection rate was 9.1% per patient year. The *H. pylori* reinfection rate was lower than the rate of the previous report but still higher than that of developed countries<sup>[14]</sup>. This high rate of reinfection could be explained by the high prevalence of *H. pylori* infection among asymptomatic Korean adults which may facilitate the transmission of *H. pylori* within families and may affect the high reinfection rates. Since the Korean economy has developed and sanitary conditions have been improved, recent reports have shown that rates of reinfection are lower than in previous reports<sup>[3,15]</sup>.

Recurrence of *H. pylori* is thought to occur *via* two distinct mechanisms, recrudescence and reinfection. Recrudescence reflects reappearance of the original strain of *H. pylori* following its temporary suppression rather than successful eradication. True reinfection occurs when, after successful eradication, a patient becomes infected with either the original strain or a new strain of *H. pylori*<sup>[16]</sup>. Many investigators have found that recurrence rates during the first 3-12 mo after cure are due to late recrudescence. Demonstrated *H. pylori* negativity for 1 year post-treatment is a reliable indicator of successful eradication without recrudescence<sup>[3,7,15,17]</sup>. Recrudescence is related to the efficacy of the regimen used. Studies showed that recurrence of *H. pylori* infection more frequently occurred in patients treated with a low-efficacy treatment regimen than in those treated with a high-efficacy regimen. It seems that low-efficacy therapy does not actually cure *H. pylori* infection in the gastric mucosa but only temporarily suppresses it and does not completely eradicate it from the host<sup>[3,5,15,17]</sup>.

In our study 16/241 patients (6.6%) showed recurrence of *H. pylori* within 1 year post-eradication and this rate was higher than in any previous reports<sup>[3]</sup>. There was no significant difference between regimens in recrudescence and, in addition, there was no significant difference between regimens in recurrence rate. Recent investigations have suggested that long-term effectiveness of antimicrobial therapy may be limited by high rates of recrudescence and reinfection, due to high levels of antibiotic resistance and pressure from high levels of infection<sup>[3,12]</sup>.

The eradication of *H. pylori* infection results in a marked reduction of ulcer relapse. Many studies have shown that peptic ulcer recurrence is usually caused by *H. pylori* reinfection<sup>[6,13]</sup>. However, in our study, no recurrence of peptic ulcer was detected at the follow up endoscopy. Probably the reason why no such recurrence was detected in our study was that the initial stages of ulcer activity, which we observed at initial endoscopy,

were mostly at scar status. Since there was no recurrence of ulcers, we should carefully consider that *H. pylori* eradication might not be necessary at the ulcer scar stage of disease.

Some reports have shown a significantly lower prevalence of *H. pylori* infection among patients with gastroesophageal reflux disease than among those without this disease, suggesting that *H. pylori* infection might protect against reflux esophagitis<sup>[18]</sup>. However, in our study, there was no difference in incidence of this disease between *H. pylori*-cured patients and *H. pylori*-recurred patients. This suggests that there is no relationship between *H. pylori* infection and reflux esophagitis.

*H. pylori* infection may have a role to play in the progression of atrophy and intestinal metaplasia. The rate of glandular atrophy and intestinal metaplasia was found to be higher in *H. pylori*-positive patients but it was very low in those without *H. pylori* infection<sup>[19-25]</sup>. There was no significant difference in atrophic mucosal change between *H. pylori*-recurred and *H. pylori*-cured groups in our study. This could suggest that *H. pylori* reinfection does not affect the progression of the atrophy. We suggest the need for a comparative study of *H. pylori*-positive patients, *H. pylori*-recurred patients, *H. pylori*-negative patients and *H. pylori*-cured patients with regard to endoscopic and histologic findings. Also, polymorphic DNA fingerprinting is needed to detect whether the re-infecting strain is the identical or a different strain.

In conclusion, the reinfection rate in Korea is 9.1%, which represents a decreasing trend, but this is still higher than in the developed countries<sup>[25]</sup>. This may be due to the high prevalence of *H. pylori* infection which may influence high rates of reinfection after eradication. The endoscopic mucosal changes, including reflux esophagitis, metaplasia or atrophy, showed no significant differences between the *H. pylori*-recurred group and *H. pylori*-cured group. There was no incidence of recurred or aggravated ulcers.

## COMMENTS

### Background

Successful eradication of *Helicobacter pylori* (*H. pylori*) is important for the prevention of recurrent peptic ulcer disease and gastric cancer. Reinfection rate of *H. pylori* infection after successful eradication is also an important problem in the management of peptic ulcer disease.

### Research frontiers

The reinfection rate after eradication therapy for *H. pylori* is extremely low in developed western countries. In contrast to this, high recurrence rates have been reported in developing countries such as Peru, Brazil, Chile, Vietnam and Bangladesh, which are countries with a high prevalence of *H. pylori* infection. The high prevalence of *H. pylori* infection may possibly be associated with high rates of reinfection after eradication because of the high risk of re-exposure to infection. In this study, the authors demonstrate the *H. pylori* reinfection rate and endoscopic changes after successful eradication in Korea.

### Innovations and breakthroughs

The high prevalence of *H. pylori* infection in Korea suggests the possibility that the reinfection rate might be higher than western countries. This study showed that the reinfection rate is 9.1% in Korea and that the endoscopic mucosal changes, including reflux esophagitis, metaplasia or atrophy, showed no significant differences between the *H. pylori*-recurred group and *H. pylori*-cured group.

## Applications

The reinfection rate in Korea is 9.1% which represents a decreasing trend but which is still higher than in the developed countries. This may be due to the high prevalence of *H. pylori* infection which may influence high rates of reinfection after eradication. *H. pylori* infection may have a role to play in the progression of atrophy and intestinal metaplasia, but there was no significant difference in atrophic mucosal changes between *H. pylori*-recurred and *H. pylori*-cured groups which suggests that *H. pylori* reinfection does not affect the progression of the atrophy.

## Terminology

Recurrence of *H. pylori* is thought to occur via two distinct mechanisms, recrudescence and reinfection. Recrudescence means reappearance of the original strain of *H. pylori* following its temporary suppression rather than successful eradication. Reinfection occurs when a patient becomes infected with either the original strain or a new strain of *H. pylori* after successful eradication. Demonstrated *H. pylori* negativity for 1 year post-treatment is a reliable indicator of successful eradication without recrudescence.

## Peer review

This manuscript describes an endoscopic cohort study of patients infected with *H. pylori* receiving eradication therapy. The authors aimed to investigate the reinfection rate after eradication therapy and the endoscopic findings after eradication, after a mean follow-up of 41 mo. They observed a reinfection rate of 9% per year, after confirmation of eradication at 1 year follow-up.

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## Analysis of demographic characteristics in 3242 young age gastric cancer patients in Korea

Hye Won Chung, Sung Hoon Noh, Jong-Baeck Lim

Hye Won Chung, Department of Gastroenterology, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemun-gu, 120-752 Seoul, South Korea

Sung Hoon Noh, Department of Surgery, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemun-gu, 120-752 Seoul, South Korea

Jong-Baeck Lim, Department of Laboratory Medicine, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemun-gu, 120-752 Seoul, South Korea

Author contributions: Chung HW and Lim JB designed and performed this research, analyzed the data and wrote the paper; Noh SH performed surgical procedures for patients and provided the database.

Correspondence to: Dr. Jong-Baeck Lim, MD, PhD, Department of Laboratory Medicine, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemun-gu, 120-752 Seoul, South Korea. [jlim@yuhs.ac](mailto:jlim@yuhs.ac)

Telephone: +82-2-22282447 Fax: +82-2-3641583

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( $P = 0.00065$ ), and more frequent exposure to harmful occupational environments ( $P = 0.00072$ ) were observed in all age groups and both genders in young age GC. These relationships were weaker in females compared to males of the same age, and were stronger as the age of patients increased. However, in group C of young age GC patients, environmental factors played important roles in females and males with a similar body weight. In females, older age at first delivery ( $> 35$  years), lack of lactation history, nulliparity, and poor nutritional status during pregnancy were significantly associated with an increased risk of GC ( $P = 0.00034$ ). In this study, 252 patients (7.8%) had a family history of GC with high odds ratio (OR) (3.22-4.21). In particular, family history was more closely associated with GC in males (OR, 4.21 in male vs 3.46 in female) and more advanced cases ( $P = 0.00051$ ).

**CONCLUSION:** Hormonal associated factors were more commonly associated with females whereas environmental factors were more commonly associated with males in young age GC patients.

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**Key words:** Young age gastric cancer; Epidemiology; Risk factor; Age; Gender

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### Abstract

**AIM:** To evaluate the epidemiologic features of young age gastric cancer (GC).

**METHODS:** Retrospectively, a total of 3242 patients with GC between 18 and 45 years of age and 3000 sex- and age-matched controls were reviewed. All subjects were stratified into 3 groups based on age (A, 18-30 years; B, 31-40 years; C, 41-45 years). Epidemiologic characteristics and risk factors were investigated with reference to their age and gender.

**RESULTS:** Compared to controls, more frequent intake of high risk diet ( $P = 0.00075$ ), history of heavy smoking ( $P = 0.00087$ ), intake of heavy alcohol ( $P = 0.00091$ ), lower social economic status ( $P = 0.00083$ ), body mass index  $> 30$  ( $P = 0.00097$ ), urban residence

Chung HW, Noh SH, Lim JB. Analysis of demographic characteristics in 3242 young age gastric cancer patients in Korea. *World J Gastroenterol* 2010; 16(2): 256-263 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i2/256.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i2.256>

## INTRODUCTION

Although gastric cancer (GC) is considered to be a disease of the middle aged and elderly<sup>[1]</sup>, 2%-15% of patients with GC are younger than 45 years of age (defined as young age GC)<sup>[2-5]</sup>, and there has been an increase in the relative proportion of young age GC compared with older age GC<sup>[6-8]</sup> especially in young females<sup>[8]</sup>. Until now, a lot of studies about epidemiologic and clinicopathologic features of GC, including risk factors, have been conducted in the elderly. However, in young age GC, only a few studies with small-sized samples have been conducted. The results of these studies were widely variable within each study<sup>[1-8]</sup>, which may be due to bias or low statistical power. In this study, we reviewed our institution's experience of the demographic and clinicopathologic features of GC in young patients between 18 and 45 years of age (defined as young age GC) from January 1990 to April 2008. Simultaneously, a total of 3000 sex- and age-matched healthy controls living in the same period were enrolled, all of whom received endoscopy at the Department of Gastroenterology and Health Care Center for the evaluation of the risk of young age GC. This retrospective, large-scale, and population-based study with abundant epidemiologic and clinicopathologic information in a single institution may reduce the limitation and bias of small sample-sized studies like those performed previously and confirm more accurate data.

The question of whether young age GC is different from that of older patients has been raised but remains unresolved. According to epidemiologic studies of GC, a marked variation was seen in the incidence of GC according to sex and ethnics<sup>[1-8]</sup>. This fact suggests that sex hormones may modulate GC risk because the status of sex hormones is dependent on age. Therefore, sex as well as age may be considered simultaneously to precisely evaluate the epidemiologic study of GC including risk factors. Additionally, according to previous data, the demographic and clinicopathologic features of young age GC were somewhat different between patients younger and older than 30 years of age<sup>[9]</sup>. Therefore, further epidemiologic evaluation of young age GC should be done after subdivision of age into younger and older than 30 years old. Based on these assumptions, we investigated the epidemiologic characteristics and risk factors of young age GC with reference to age and sex in Korea, a country with a high rate of GC.

## MATERIALS AND METHODS

### Subjects

From January 1990 to April 2008, a total of 21 738 patients were diagnosed with GC at Severance Hospital, Seoul, Korea. Among them, 3 242 patients between 18 and 45 years of age (14.9%) were enrolled in the current study. In same period, a total of 3000 sex- and age-matched individuals who received endoscopy with or

without gastrointestinal symptoms at the Department of Gastroenterology and Health Care Center were also enrolled as healthy controls to evaluate the risk of young age GC. All subjects were stratified into 3 groups based on age (A, 18-30 years; B, 31-40 years; C, 41-45 years) at initial diagnosis.

### Epidemiologic and clinicopathologic characteristics

This study included data on each subject's age, sex, life-style (e.g. smoking or alcohol, and diet pattern), occupational environment, place of residence, body mass index (BMI), family history of GC, socioeconomic status, and combined diseases. For women, history of oral contraceptive use, pregnancy/delivery (time of first pregnancy/delivery, nutrition conditions during pregnancy, and parity status), lactation, and menstruation were also investigated. Socioeconomic conditions were stratified into 3 groups based on household income epidemiology in Korea. For evaluation of nutritional status during pregnancy, weight gain, electrolyte imbalances and the level of albumin and hemoglobin during pregnancy were investigated. The level of weight gain was estimated according to prepregnant BMI categories as recommended by the Institute of Medicine<sup>[10]</sup>.

For all subjects, hematological (total blood count), biochemical (routine chemistry), and serological [IgG for *Helicobacter pylori* (*H. pylori*)] evaluations including tumor markers [carcinoembryonic antigen,  $\alpha$ -fetoprotein ( $\alpha$ FP), and cancer antigen (CA125)] were conducted. For GC, tumor location, number of lesions, growth type (Lauren classification), histological type (World Health Organization classification), and TNM stage were analyzed at initial diagnosis, and treatment-related results including overall survival were assessed.

### Specimen histology

Tissue samples were stained with hematoxylin and eosin solution. Giemsa staining was also performed to detect *H. pylori* infection. The degree of gastritis, combined glandular atrophy with intestinal metaplasia (IM), and *H. pylori* infection were graded according to the updated Sydney classification<sup>[11]</sup>.

### Statistical analysis

The data are presented as median  $\pm$  SD. Analysis of variance with multiple comparison (Scheffe post hoc) and  $\chi^2$  analysis (Pearson) were conducted to compare data among age groups in each different gender using the Statistical Package for the Social Sciences (SPSS/PC+ 13.0, Chicago, IL, USA). Analysis of survival was performed using the Kaplan-Meier method. For comparison of laboratory findings between the cancer groups and controls, *t*-test was conducted. Epidemiologic risk factors of young age GC were evaluated by comparing them with age-matched controls in each different gender using logistic regression with odds ratios (OR) and 95% confidence intervals (CI). A *P* value of less than 0.05 was considered statistically significant.

**Table 1** Demographic and clinicopathologic features of young age in control group *n* (%)

Features	Group A ( <i>n</i> = 350)	Group B ( <i>n</i> = 1500)	Group C ( <i>n</i> = 1150)	Whole ( <i>n</i> = 3000)	<i>P</i> -value
Age (median, yr)	24 ± 1.3	34 ± 2.4	44 ± 3.7	35 ± 4.9	NS
Sex					< 0.05
Male	150 (42.9)	750 (50.0)	750 (65.2)	1650 (55.0)	
Female	200 (52.1)	750 (50.0)	400 (34.8)	1350 (45.0)	
Indication of endoscopy					NS
No symptom	117 (33.4)	514 (34.2)	499 (43.4)	1130 (37.7)	
Dyspepsia	211 (60.3)	798 (53.2)	687 (59.7)	1696 (56.5)	
Weight loss	13 (4.7)	26 (1.7)	28 (2.4)	67 (2.2)	
Nausea/poor oral intake	54 (15.4)	155 (10.3)	308 (26.8)	517 (17.2)	
Others	17 (4.9)	37 (2.5)	28 (2.4)	82 (2.7)	
<i>H. pylori</i> (+) & combined IM					< 0.05
Male	5 (3.3)	65 (4.3)	74 (9.9)	144 (8.7)	
Female	11 (5.5)	89 (11.9)	56 (14.0)	156 (11.6)	

Group A, 18-30 years of age; Group B, 31-40 years of age; Group C, 41-45 years of age; Whole, 18-45 years of age. NS: Not significant; IM: Intestinal metaplasia; *H. pylori*: *Helicobacter pylori*.

**Table 2** Demographic and clinicopathologic features of young age GC patients *n* (%)

Features	Group A ( <i>n</i> = 371)	Group B ( <i>n</i> = 1584)	Group C ( <i>n</i> = 1287)	Whole ( <i>n</i> = 3242)	<i>P</i> -value
Age (median, yr)	26 ± 3.2	36 ± 2.8	43 ± 1.4	38 ± 5.6	NS
Sex					< 0.05
Male	144 (38.8)	798 (50.4)	805 (62.5)	1747 (53.9)	
Female	227 (61.2)	786 (49.6)	482 (37.5)	1495 (46.1)	
Survival (median, mo) <sup>1</sup>	29.8 ± 4.2	31.7 ± 5.3	33.2 ± 3.8	32.4 ± 3.3	NS
Tumor size (mean, cm)	5.6 ± 3.2	5.2 ± 3.8	4.9 ± 2.9	5.14 ± 4.4	NS
Number					< 0.05
Multiple	1 (0.2)	5 (0.3)	8 (0.6)	14 (0.4)	
Single	370 (99.8)	1579 (99.8)	1279 (99.4)	3228 (99.6)	
Site of disease					< 0.05
Antrum	71 (19.1)	442 (27.9)	617 (48.0)	1130 (34.9)	
Body (lower and middle)	84 (22.6)	365 (23.0)	308 (23.9)	757 (23.3)	
Upper body and cardia	140 (37.8)	428 (27.1)	218 (16.9)	786 (24.2)	
Diffuse	76 (20.5)	349 (22.0)	144 (11.2)	569 (17.6)	
Pathology					< 0.05
Adenocarcinoma					
Male	63 (43.8)	401 (50.3)	499 (62.0)	963 (55.1)	
Female	87 (38.3)	347 (44.1)	250 (51.9)	684 (45.8)	
Signet ring cell carcinoma					
Male	81 (56.2)	397 (49.7)	306 (38.0)	784 (44.9)	
Female	140 (61.7)	439 (55.9)	232 (48.1)	811 (54.2)	
Stage of disease					< 0.05
Localized to stomach	34 (9.2)	190 (12.0)	181 (14.1)	405 (12.5)	
Regional metastasis <sup>2</sup>	71 (19.1)	491 (31.0)	475 (36.9)	1037 (32.0)	
Distant metastasis	266 (71.7)	903 (57.0)	631 (49.0)	1800 (55.5)	
Coincidence of IM					< 0.05
Male	9 (6.3)	168 (21.1)	249 (31.0)	426 (24.4)	
Female	13 (5.7)	121 (15.4)	97 (20.1)	231 (15.5)	
<i>H. pylori</i> (+)	25 (6.7)	378 (23.9)	472 (36.7)	875 (27.0)	< 0.05

<sup>1</sup>Survival analysis was performed using the Kaplan-Meier method; <sup>2</sup>This included node metastasis.

## RESULTS

### Demographic and clinicopathologic features

The demographic and clinicopathologic features of all subjects enrolled in this study are summarized in Tables 1 and 2. All subjects were Korean. The median age of cancer patients was 38 years and that of controls was 35 years. The male-to-female ratio of young age GC was 1.2:1.0 on the whole, but it was 1.0:1.6 in group A, 1.0:1.0 in group B, and 1.7:1.0 in group C, respectively, with female predominance when patients were younger.

No statistically significant distinctive laboratory findings were observed in young age GC patients compared with controls except slightly decreased hemoglobin and albumin levels in all genders and elevated  $\alpha$ FP and CA125 levels in young women with carcinomatosis (data was not shown).

Histologically, young age GC was frequently located in the upper third or whole stomach diffusely as patients were younger (Table 2). The ratio of adenocarcinoma-to-signet ring cell carcinoma was 1.0:1.1 on the whole. However, in females, the ratio was 1.0:1.8 in group A,



1.0:1.4 in group B, and 1.1:1.0 in group C, respectively, whereas in males, it was 1.0:1.3 in group A, 1.0:1.0 in group B, and 1.6:1.0 in group C, respectively. These results may indicate that undifferentiated histology is more predominant when patients are younger ( $A > B > C$ ,  $P < 0.05$ ) in both genders. This phenomenon is notably more predominant in younger females. With regard to concomitant gastric pathology around cancer, IM was rarely observed in patients younger than 30 years of age (group A) whereas it was frequently observed in group C (Table 2,  $P < 0.05$ ). IM change was more frequently observed in males compared to females within the same age group (Table 2,  $P < 0.05$ ).

Overall, patients with stage IV cancer presented with poor prognosis. However, in resectable cancer, particularly in EGC, prognosis was not poorer compared to that of the elderly reported in previous studies (data was not shown). Females presented with more advanced features compared to males of the same age. Among 1495 female patients, 37 (2%) were diagnosed with Krukenberg tumor.

There was no significant difference in survival among age groups (Table 1,  $P > 0.05$ ) on the whole although younger patients and females presented with relatively more advanced stage and poor prognosis. Females with a history of recent pregnancy and delivery showed poorer prognosis.

### Risk factors

We evaluated the risk factors of young age GC by comparison of demographic and clinicopathologic features of young age GC with those of sex- and age-matched controls using logistic regression with OR and 95% CIs (Tables 3 and 4). The evaluations were performed after all people were stratified by age and sex with the assumption that older patients may be more frequently exposed to environmental carcinogens and exposed to them for longer than younger patients and that sex hormones may modulate the development of GC. However, we evaluated the influence of hormonal circumstances on the development of GC only in females in this study because a marked variation of sex hormones was rarely observed in young males under 45 years old whereas it was frequently observed in females of reproductive age with regular menstrual changes (Table 4).

In cancer patients, more frequent intake of beef and canned, smoked, and salted food and less frequent intakes of fresh fruit/vegetables (defined as high-risk diet), history of heavy smoking (defined as more than 20 pack-years) and history of heavy alcohol intake (defined as more than 60 g/d) were observed compared with controls in all age groups and both genders, as described previously<sup>[12]</sup> (Table 3,  $P < 0.05$ ). However, these relationships were somewhat weaker in females compared to males of the same age, and these relationships were stronger as the age of patients increased (Table 3,  $P < 0.05$ ). Lowest socioeconomic

status, BMI  $> 30$ , and urban residence increased the risk of GC in all groups and both genders (Table 3,  $P < 0.05$ ). Frequent exposure to harmful industrial and occupational environments (excessive electromagnetic waves, toxic chemicals such as asbestos, lead, sulfur granules, and toxic gases such as CO, NO, methane gas, *etc.*) was also closely associated with increased cancer risk in all age groups and both genders (Table 3,  $P < 0.05$ ). However, these associations were stronger in older patients ( $A < B < C$ ) and males.

*H. pylori* infection is considered as a very important epidemiologic risk factor of GC in both the young and the old<sup>[13,14]</sup>. However, in the current study, *H. pylori* positivity was infrequently observed in group A and B, and *H. pylori* positivity alone was not related to increased cancer risk (Table 2). Additionally, *H. pylori* positivity was not different between genders although the prevalence of young age GC was different according to gender. Only concomitant IM change combined with *H. pylori* positivity was related to increased cancer risk (Table 3,  $P < 0.05$ ). This risk factor was weighted in patient older than 40 years of age and males in whom IM change was relatively frequently observed compared to patients younger than 30 years and females in whom these changes were rarely observed (Table 3,  $P < 0.05$ ).

We also found that in females, frequent use of oral contraceptives without progesterone, older age at first delivery ( $> 35$  years), lack of lactation history, and nulliparity were significantly associated with an increased risk of GC (Table 4,  $P < 0.05$ ). Poor nutritional status during pregnancy (defined as weight gain during pregnancy which is lower than the normal level according to prepregnant BMI categories as recommended by the Institute of Medicine<sup>[10]</sup>) was also associated with an increased risk of GC (Table 4,  $P < 0.05$ ). However, age at menarche and the state of menopause did not influence GC (Table 4,  $P > 0.05$ ). The incidence of other estrogen-associated gynecologic malignancies, such as ovarian, breast, and uterine cancers, were also evaluated and revealed no association with young age GC (Table 4,  $P > 0.05$ ).

In the current study, 252 patients (7.8%) had a family history of GC, which is similar to previous studies<sup>[15]</sup>. In particular, family history was more closely associated with GC in males and more advanced cases (Tables 3 and 4,  $P < 0.05$ ) but the reason is not known.

Overall, environment factors were significantly associated with an increase of GC in all age groups and both genders although these relationships were somewhat weaker in females compared to males of the same age, and these relationships were stronger as the age of patients increased. However, in group C, environmental factors played important roles in females and males with similar weight. In females, hormonal factors associated with reproductive factors, but not menstrual factors, were significantly associated with an increase in GC.

**Table 3** Frequency of control group and young age GC, age-adjusted OR estimates and 95% CI by demographic and clinical characteristics, Korea, 1990 to 2008 *n* (%)

Risk factors	GCs		Controls		Age-adjusted OR		95% CI		P-value	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
High-risk diet <sup>1</sup>										
Whole	694 (39.7)	440 (29.4)	341 (20.7)	265 (19.6)	2.53	1.71	2.17-2.95	1.34-2.03	< 0.001	< 0.001
Group A	54 (37.5)	40 (27.8)	31 (20.7)	25 (16.7)	2.30	1.50	1.37-3.87	0.87-2.57	0.002	0.034
Group B	311 (39.0)	239 (30.4)	153 (20.4)	151 (20.1)	2.49	1.73	1.99-3.13	1.37-1.29	< 0.001	< 0.001
Group C	329 (40.9)	161 (33.4)	157 (22.0)	89 (22.3)	2.61	1.75	2.08-3.27	1.307-2.37	< 0.001	< 0.001
Heavy smoking <sup>2</sup>										
Whole	528 (30.2)	241 (16.1)	264 (16.0)	137 (10.1)	2.27	1.70	1.93-2.69	1.36-2.13	< 0.001	< 0.001
Group A	51 (35.4)	34 (15.0)	30 (20.0)	19 (9.5)	2.19	1.68	1.30-3.72	0.92-3.05	0.003	0.031
Group B	244 (30.6)	143 (18.2)	119 (15.9)	83 (11.1)	2.34	1.79	1.82-2.99	1.34-2.39	< 0.001	< 0.001
Group C	233 (28.9)	64 (13.2)	115 (15.3)	35 (8.75)	2.25	1.60	1.75-2.89	1.03-2.47	< 0.001	0.035
Heavy alcohol drinking <sup>3</sup>										
Whole	608 (34.8)	168 (11.2)	301 (18.2)	91 (6.7)	2.39	1.52	2.04-2.81	1.34-2.29	< 0.001	< 0.001
Group A	54 (37.5)	26 (11.5)	28 (18.7)	12 (6.0)	2.61	2.02	1.54-4.45	0.99-4.13	< 0.001	0.052
Group B	293 (36.7)	81 (10.3)	142 (18.9)	47 (6.3)	2.48	1.72	1.97-3.14	1.18-2.50	< 0.001	0.005
Group C	261 (32.4)	61 (12.7)	131 (17.5)	32 (8.0)	2.14	1.73	1.68-2.72	1.60-2.54	< 0.001	0.019
Low socioeconomic condition <sup>4</sup>										
Whole	414 (23.7)	323 (21.6)	279 (16.9)	206 (15.3)	1.53	1.53	1.29-1.81	1.26-1.86	< 0.001	< 0.001
Group A	28 (19.4)	44 (19.3)	21 (14)	27 (13.5)	1.42	1.54	0.76-2.64	0.99-3.03	0.003	0.009
Group B	191 (23.9)	168 (21.4)	135 (18.0)	122 (16.3)	1.43	1.40	1.12-1.84	1.082-1.81	0.004	0.011
Group C	195 (24.2)	111 (23.0)	123 (16.4)	57 (14.2)	1.63	1.80	1.27-2.10	1.27-2.56	< 0.001	0.001
Urban residence										
Whole	947 (54.2)	805 (53.8)	711 (43.1)	574 (42.5)	1.56	1.58	1.37-1.79	1.36-1.83	< 0.001	< 0.001
Group A	84 (58.3)	132 (58.1)	67 (44.7)	99 (49.5)	1.48	1.42	0.93-1.23	0.973-2.08	0.002	0.073
Group B	454 (56.9)	434 (55.2)	331 (44.1)	322 (42.9)	1.67	1.64	1.37-2.04	1.34-2.00	< 0.001	< 0.001
Group C	409 (50.8)	239 (49.6)	313 (38.9)	153 (38.2)	1.44	1.60	1.18-1.76	1.21-2.08	0.003	0.001
Occupational environment										
Whole	588 (33.6)	331 (22.1)	315 (19.1)	181 (13.4)	2.15	1.84	1.84-2.52	1.51-2.24	< 0.001	< 0.001
Group A	44 (30.6)	47 (20.7)	24 (16.0)	29 (14.5)	2.31	1.54	1.32-4.05	0.93-2.56	0.004	0.094
Group B	263 (33.0)	181 (23.0)	140 (18.7)	98 (13.1)	2.14	1.99	1.69-2.71	1.52-2.61	< 0.001	< 0.001
Group C	281 (34.9)	103 (21.4)	151 (20.1)	54 (13.5)	2.13	1.74	1.69-2.68	1.22-2.50	< 0.001	0.003
Family history of GC <sup>5</sup>										
Whole	150 (8.6)	102 (6.8)	36 (2.2)	28 (2.1)	4.21	3.46	2.91-6.10	2.26-5.29	< 0.001	< 0.001
Group A	11 (7.6)	14 (6.2)	3 (2.0)	4 (2.0)	4.05	3.22	1.10-14.84	1.04-9.95	0.035	0.042
Group B	68 (8.5)	53 (6.7)	17 (2.3)	15 (2.4)	4.01	3.54	2.34-6.90	1.98-6.34	< 0.001	< 0.001
Group C	71 (8.8)	35 (7.2)	16 (2.1)	9 (2.3)	4.17	3.40	2.43-7.15	1.62-7.17	< 0.001	0.001
<i>H. pylori</i> (+) & combined IM										
Whole	367 (21.0)	231 (15.5)	144 (8.7)	156 (11.6)	2.78	1.40	2.26-3.42	1.13-1.74	< 0.001	< 0.001
Group A	11 (7.6)	13 (5.7)	5 (3.3)	11 (5.5)	2.40	1.04	0.71-7.08	0.46-2.39	0.051	0.192
Group B	155 (19.4)	121 (15.4)	65 (4.3)	89 (11.9)	2.54	1.35	1.87-3.46	1.00-1.81	< 0.001	0.045
Group C	201 (25.0)	97 (20.1)	74 (9.9)	56 (14.0)	3.04	1.55	2.28-4.06	1.08-2.22	< 0.001	0.017
BMI > 35										
Whole	374 (21.4)	270 (18.1)	201 (12.4)	159 (11.8)	1.94	1.65	1.63-2.37	1.34-2.04	< 0.001	< 0.001
Group A	30 (20.8)	35 (15.4)	18 (12.0)	19 (9.5)	1.93	1.74	1.09-2.35	0.96-3.15	0.043	0.066
Group B	176 (22.1)	139 (17.7)	95 (12.7)	89 (11.9)	1.95	1.60	1.49-2.56	1.20-2.12	< 0.001	0.001
Group C	168 (20.9)	96 (19.9)	92 (12.3)	51 (12.8)	1.89	1.70	1.43-2.49	1.78-2.46	< 0.001	0.005

<sup>1</sup>High-risk diet is defined as more frequent intake of beef and canned, smoked, and salted food and less frequent intake of fresh fruit/vegetables; <sup>2</sup>Heavy smoking is defined as more than 20 pack-years; <sup>3</sup>Heavy alcohol intake is defined as more than 60 g/d; <sup>4</sup>Socioeconomic conditions were evaluated by household income, stratified into 3 groups based on income epidemiology in Korea. This condition comes under the lowest income group; <sup>5</sup>Family history was defined as first-degree relative with GC. The 3000 normal controls included 1650 males and 1350 females. Group A, 18-30 years of age, *n* = 350; Group B, 31-40 years of age, *n* = 1500; Group C, 41-45 years of age, *n* = 1150; Whole, 18-45 years of age, *n* = 3000. The 3242 young age GC included 1747 males and 1495 females. Group A, 18-30 years of age, *n* = 371; group B, 31-40 years of age, *n* = 1584; group C, 41-45 years of age, *n* = 1287; whole, 18-45 years of age, *n* = 3242. GC: Gastric carcinoma; OR: Odds ratio; CI: Confidence interval; BMI: Body mass index.

## DISCUSSION

Although epidemiologic characteristics of young age GC were varied according to geographical regions and ethnicity, near-universal findings were commonly demonstrated in each article for epidemiologic studies of young age GC, which are as follows: (1) female dominance, (2) located in the upper area, (3) diffuse

growth types, (4) undifferentiated histology (particularly signet ring cell carcinoma), and (5) advanced stage and poor prognosis, which were different from those of the elderly<sup>[1-8]</sup> although some studies showed different results. On the whole, we also observed similar results to those described previously. However, there are several different points in our study compared with previous studies.

**Table 4** Frequency of control group and young age GC, age-adjusted OR estimates and 95% CI by hormonal and reproductive characteristics, Korea, 1990 to 2008 *n* (%)

Risk factors	GCs	Controls	OR	95% CI	P-value
Frequent use of oral contraceptives	432 (28.9)	201 (14.0)	2.50	2.06-3.00	< 0.001
Age at first pregnancy ( $\geq 5$ mo) <sup>1</sup>					
Age < 25	225 (15.1)	243 (17.0)	0.86	0.70-1.05	0.127
Age $\geq 25$ , < 35	848 (56.7)	977 (68.3)	1.05	0.73-1.50	0.438
Age $\geq 35$	422 (28.2)	211 (14.7)	2.27	1.89-2.73	< 0.001
Parity (live births)					
Nullipara or few (1-2)	779 (52.1)	487 (34.0)	2.11	1.82-2.45	< 0.001
Many ( $\geq 3$ )	431 (28.8)	629 (44.0)	0.52	0.44-0.60	< 0.001
History of lactation	313 (20.9)	414 (28.9)	0.68	0.58-0.79	< 0.001
Poor nutritional status <sup>2</sup>	252 (16.9)	122 (8.5)	2.12	1.73-2.73	< 0.001
Age at menarche (yr)					
Age < 12	308 (20.6)	258 (18.0)	1.18	0.98-1.14	0.078
Age $\geq 12$ , < 16	956 (63.9)	930 (65.0)	0.95	0.62-1.44	0.102
Age $\geq 16$	231 (15.5)	243 (17.0)	0.89	0.73-1.09	0.262
Premenopausal state (preoperative)	134 (9.0)	138 (9.6)	0.92	0.72-1.18	0.526
Other gynecologic malignancy <sup>3</sup>	19 (1.3)	16 (1.1)	1.14	0.58-2.22	0.237

<sup>1</sup>Pregnancy was maintained for at least 5 mo; <sup>2</sup>This condition was during pregnancy; <sup>3</sup>These conditions included estrogen-associated cancers such as breast, ovarian, and uterine. GC vs control = 1495 vs 1431.

In this study, we evaluated the epidemiologic features of young age GC after subdividing young patients into 3 age groups and 2 genders under assumption that the effects of sex hormones and environmental factors may be influenced by age; hormonal activities reach a peak around 30 years of age and decline slightly with age, and exposure of environmental carcinogens may be more frequently and longer in the old based on previous studies<sup>[16-20]</sup>.

For demographic risk factors, our study supports the previous results<sup>[12]</sup>. Environmental factors such as life style, socioeconomic status, occupation, resistance, and IM change with *H. pylori* infection in gastric mucosa were statistically significantly associated with young age GC, particularly in males and older patients. Generally, environmental factors might play important roles in the initiation of cancer development. Several life style factors, such as a history of smoking or alcohol use, and diet, are common weighty risk factors<sup>[12]</sup>. Low socioeconomic conditions were particularly associated with the intestinal type of GC as those in this income bracket are more likely to be exposed to poor quality food, which may cause intestinalization of the gastric mucosa at a relatively young age. Additionally, few have access to health care services and notification of IM changes in stomach may become delayed. Over the past few decades, people have been exposed to harmful industrial occupational materials called “endocrine disruptors” as societies have undergone rapid industrialization. These endocrine disruptors are thought to be carcinogens. Therefore, the industrialized occupational environment may influence the increase of GC development at a younger age<sup>[13]</sup>. In same period, females also were exposed to similar harmful environment circumstances. However, these associations were weaker in aged-matched females. We guess the reasons that male patients might have more frequent and

prolonged exposure to environmental carcinogens than females is due to social positions<sup>[14]</sup>. Some epidemiologic data have pointed to an association between *H. pylori* infection and increased risk of young age GC<sup>[21,22]</sup>. Of course, *H. pylori* infection is a critical environmental factor of GC in old age, particularly intestinal type<sup>[16]</sup>, and IM change was combined in most *H. pylori*-associated GC. However, the prevalent histology of young age GC was diffuse-type cancer, and prevalence of IM change was rare in young age GC. In this study, the association between *H. pylori* infection and increased risk of young age GC was more common in older patients and males than in younger patients and females. Therefore, we suggest that *H. pylori*-associated risk factors may play a limited role in the development of young age GC according to age and gender.

GC incidence varies considerably according to studies<sup>[1-8]</sup>. In our series, the male-to-female ratio was 1.2:1.0 on the whole, but it was 1.0:1.6 in group A, 1.0:1.0 in group B, and 1.7:1.0 in group C, respectively, with female predominance as patients were younger (Table 2). The reason for this higher number of female patients in the younger group is not yet known. However, the role of the sex hormones, especially estrogen, has been suggested<sup>[16-20]</sup> although the results have varied among different studies. Some investigators asserted their protective effects on GC whereas others emphasized the opposite. The differences may be derived from geographical or ethnic differences, or the relatively small-sized sample number<sup>[23,24]</sup>. Our study may support the harmful role of estrogen in young age GC in females. In accordance with these results, we observed a close relationship between GC development and hormonal circumstances in young females (Table 4). Additionally, the effect of counter action of progesterone was not noted in many studies asserting the protective effects of estrogen on GC development. Our results may imply

that excessive exposure to estrogen without counter exposure to progesterone is related to an increase in the development of GC in young females.

In conclusion, our study demonstrated that the epidemiologic characteristics including risk factors of young age GC were different according to age and gender. Hormonal factors were more commonly associated with females, particularly in the younger age group, whereas environmental factors were more commonly associated with males, particularly in the older age group.

The development of GC is influenced by a combination of environmental factors and specific genetic alterations including hormonal factors and the role of genetics is considered to be greater in younger patients than older patients<sup>[25]</sup>. Thus, further investigations of the molecular genetics of young age GC are needed to support the results of our study.

## COMMENTS

### Background

Two percent to fifteen percent of patients with gastric cancer (GC) are younger than 45 years of age and there has been an increase in the relative proportion of young age GC compared with older age GC, especially in young females. The question of whether young age GC is different from that of older patients has been raised but remains unresolved. Thus, an epidemiologic study about young age GC is significantly important clinically.

### Research frontiers

The development of GC is influenced by a combination of environmental factors and specific genetic alterations including hormonal factors. The role of genetics is considered to be greater in younger patients than older patients. According to epidemiologic studies of GC, a marked variation was seen in the incidence of GC according to sex and ethnicity. Sex hormones are considered to modulate the risk of development of GC. Also, according to previous data, the demographic and clinicopathologic features of young age GC were somewhat different between patients younger and older than 30 years of age.

### Innovations and breakthroughs

Until now, only a few studies with small-sized samples have been conducted in young age GC patients. However, this retrospective, large-scale, and population-based study with abundant epidemiologic and clinicopathologic information in a single institution may reduce the limitations and bias derived from small sized sample studies like those conducted previously, and confirm more accurate data. The authors also evaluated the epidemiologic features of young age GC after subdividing young patients into 3 age groups and 2 genders under the assumption that the effects of sex hormones and environmental factors may be influenced by age; hormonal activities reach a peak around 30 years of age and decline slightly with age, and exposure to environmental carcinogens may be more frequent and longer in the old, based on previous studies.

### Applications

The authors' study demonstrated that the epidemiologic characteristics and the development risk of young age GC were affected by both environmental factors and hormonal factors, especially sex hormones, with different factors contributing a different degree of risk according to age and gender. Their study demonstrated that hormonal factors were more commonly associated with females, particularly in the younger age group, whereas environmental factors were more commonly associated with males, particularly in the older age group. This knowledge may help to clarify the exact pathophysiology of young age GC and help devise an appropriate treatment approach.

### Peer review

This paper is thought to be an excellent epidemiologic study surveying risk factors for stomach cancer in patients aged 45 or younger. It is an interesting study of significant clinical importance. However, there are some limitations to the study that require attention from the authors.

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## Transarterial chemoembolization as initial treatment for unresectable hepatocellular carcinoma in southern China

Ming Shi, Ji-An Chen, Xiao-Jun Lin, Rong-Ping Guo, Yun-Fei Yuan, Min-Shan Chen, Ya-Qi Zhang, Jin-Qing Li

Ming Shi, Ji-An Chen, Xiao-Jun Lin, Rong-Ping Guo, Yun-Fei Yuan, Min-Shan Chen, Ya-Qi Zhang, Jin-Qing Li, Department of Hepatobiliary, Cancer Center, Sun Yat-sen University, 651, Dongfeng Road East, Guangzhou 510060, Guangdong Province, China

**Author contributions:** Li JQ designed the study and analyzed data; Shi M and Chen JA took care of patients, collected and analyzed data, performed statistical analysis and wrote the manuscript (Shi M and Chen JA made equal contributions); Lin XJ, Guo RP, Yuan YF, Chen MS and Zhang YQ took care of patients, performed TACE treatments and contributed to the discussion.

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**Correspondence to:** Jin-Qing Li, Professor, Department of Hepatobiliary, Cancer Center, Sun Yat-sen University, 651, Dongfeng Road East, Guangzhou 510060, Guangdong Province, China. [lijinq@mail.sysu.edu.cn](mailto:lijinq@mail.sysu.edu.cn)

Telephone: +86-20-87343116 Fax: +86-20-87343480

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nodes metastasis, Child-Pugh class, macrovascular invasion, greatest dimension,  $\alpha$ -fetoprotein (AFP), Hepatitis virus B, tumor capsule, and nodules were independent prognostic factors. Patients with multimodality therapy have better survival than those with TACE treatment only.

**CONCLUSION:** Tumor status, hepatic function reserve, AFP, and hepatitis virus B status were independent prognostic factors for unresectable HCC. Distant metastasis might not be a contraindication to TACE. Multimodality therapy might improve survival.

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**Key words:** Hepatocellular carcinoma; Transarterial chemoembolization; Palliative treatment; Prognosis

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### Abstract

**AIM:** To identify prognostic factors from pretreatment variables of the initial transarterial chemoembolization (TACE) procedure in unresectable hepatocellular carcinoma (HCC).

**METHODS:** One thousand and five hundred and sixty-nine patients with unresectable HCC underwent TACE as initial treatment were retrospectively studied. Pretreatment variables of the initial TACE procedure with a *P* value less than 0.05 by univariate analysis were subjected to Cox proportional hazards model.

**RESULTS:** The median overall survival time and 1-, 5-, 10-year survival rates were 10.37 mo, 47%, 10%, and 7%, respectively. A Cox proportional hazard model showed that 8 pretreatment factors of regional lymph

### INTRODUCTION

Hepatocellular carcinoma (HCC) constitutes the majority of liver cancers which is the sixth most common cancer and the third most common cause of cancer death worldwide<sup>[1]</sup>. While curative therapies include surgical resection, transplantation and percutaneous ablation<sup>[2]</sup>, these are not suitable for use in a great number of HCC patients due either to advanced stage of disease or to poor liver function at the time of diagnosis<sup>[3,4]</sup>. Transarterial chemoembolization (TACE) has become the most popular modality for

palliative treatment among these patients. Two randomized trials from Europe and Asia have confirmed better survival associated with TACE as compared with conservative treatment in selected patients<sup>[5,6]</sup>.

However, considerable controversies remain surrounding definition of suitable candidates for TACE. Many published studies attempted to delineate the survival and identify the prognostic factors related to TACE<sup>[7-14]</sup> and have shown varying patient baseline characteristics and outcomes across a wide range of geographical distribution, probably because of different inclusion criteria, epidemiological variations in hepatitis B and C infections, and local surveillance for HCC.

Globally, more than half of new HCC cases occur in China<sup>[15]</sup>. However, to the best of our knowledge, the baseline characteristics, outcomes and prognostic factors among TACE-treated HCC patients in China have not been fully described. In the present study, we conducted a 13 year retrospective analysis of patients with unresectable HCC who underwent TACE as initial treatment and to identify prognostic factors from variables prior to the initial TACE procedure. We hypothesized that findings of this study should help to select patients who would benefit most from TACE, and help to design and estimate samples for further prospective randomized studies.

## MATERIALS AND METHODS

### Ethics

This study was approved by the Ethical Committee of the Cancer Center of Sun Yat-Sen University and it followed the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from every patient before treatment.

### Patients

From September 1991 to December 2004, 1830 patients with unresectable HCC underwent TACE at the Cancer Center of Sun Yat-sen University. All patients received routine preoperative investigations, including liver function tests,  $\alpha$ -fetoprotein (AFP), hepatitis serology, chest radiography, abdominal ultrasonography, and abdominal CT scanning with contrast-enhancement of images in the arterial and portal venous phases. AFP > 200 ng/mL (normal, < 20 ng/mL) refers to an abnormal elevation of the tumor marker. Further investigations were performed in those with suspected extrahepatic metastases. Only patients who met the following inclusion criteria were enrolled: (1) the hepatic lesion was clinicopathologically diagnosed as HCC based on the diagnostic criteria used by the European Association for the Study of the Liver<sup>[16]</sup>; (2) there was not any treatment prior to TACE; and (3) the HCC was considered to be unresectable by our leading investigator (Professor Jin-Qing Li). Unresectable disease was defined as extensive bilobular involvement of the liver by a large solitary tumor or by multiple tumors, or invasion of major blood vessels including the main portal vein, hepatic veins, inferior vena cava, and main hepatic

artery. Patients with any of the following were excluded: (1) obstructive jaundice; (2) hepatic encephalopathy; (3) Child-Pugh score > 11; and (4) poor data integrity.

Finally, 1569 patients were recruited, comprising 1423 (90.7%) males and 146 (9.3%) females with a mean age of  $49.36 \pm 11.62$  (mean  $\pm$  SD) years. Diagnosis of HCC was based on biopsy in 131 (8.3%) patients, AFP plus radiology in 1167 (74.4%), and radiology alone in 271 (17.3%), respectively.

There were 1338 (86.0%) patients who tested positive for Hepatitis virus B surface antigen (HbsAg) and 48 (3.1%) positive for hepatitis virus C antibody. The mean tumor size was  $10.53 \pm 4.09$  cm (range, 1.0-28.0 cm) and macrovascular invasion was seen in 293 (18.7%) patients. Tumor(s) with direct invasion of adjacent organs was seen in 30 (1.9%) patients. The rate of regional lymph node involvement was 3.2% ( $n = 50$ ) according to the CT-revealed threshold size (larger than 1.0 cm). In total, 269 (17.1%) patients were found with distant metastases to lung [221 (82.3%)], bone [24 (9.0%)], adrenal gland [17 (6.4%)], pelvic cavity [3 (1.1%)], kidney [2 (0.7%)], respectively. According to the TNM classification<sup>[17]</sup>, 502 (32.0%) patients were classified as Stage I, 91 (5.8%) as Stage II, 659 (42.0%) as Stage IIIa, 14 (0.9%) as Stage IIIb, 34 (2.2%) as Stage IIIc, and 269 (17.1%) as Stage IV. According to the Child-Pugh classification, 1413 (90.8%) patients were Child-Pugh class A, 144 (9.2%) were class B, and none were class C.

### Treatment procedure

All patients gave informed consent before any procedure. TACE was performed using the Seldinger technique. A selective 5 French catheter was introduced and visceral angiography was carried out to assess the arterial blood supply to the liver and to confirm patency of the portal vein. Depending on the size, location and arterial supply of the tumor, the tip of the catheter was advanced into the right or left hepatic artery, or tumor-feeding branches (as there was a need for super selective catheterization in some cases).

Hepatic artery infusion chemotherapy was performed using one drug or combinations of mitomycin C, carboplatin, and fluorouracil. After that, chemoembolization was performed using doxorubicin mixed with 5 mL of lipiodol (Lipiodol Ultra-Fluide; Andre Guerbet Laboratories, Aulnay-Sous-Bois, France). Pure lipiodol or gelatin sponge particles were then injected if the chemoembolized artery territory did not show stagnant flow. The dose of anticancer agent-lipiodol emulsion and the pieces of embolic materials used for TACE were determined based on the tumor size, extension of the lesions and tumor blood supply.

### Follow-up

After the initial TACE treatment, patients were followed up by CT scan every 1 to 2 mo to evaluate their tumor status. The survival of TACE-treated patients was calculated from the date of procedure through to the end of follow-up on March 17, 2008. All patient deaths, irrespective of causes, were deemed as the end point.

The mean length of follow-up was 16.62 mo (range, 0.07-133.27). TACE-related death was defined as death within 1 mo of the initial therapy.

New TACEs were performed every 4-10 wk until CT scans and AFP levels suggested the stabilization of the tumor, and unless the patient rejected further treatment or it was not technically feasible either because of hepatic artery occlusion or impaired liver function. The patients were started on multimodality therapy (including surgery, local ablation, radiotherapy, and systemic chemotherapy) when treatment options other than TACE were indicated.

There were 838 (53.4%) patients treated with only one session of TACE, 447 (28.5%) with two sessions, 156 (9.9%) with three sessions, and 128 (8.2%) with more than three sessions. On average, 1.79 sessions (range, 1 to 11) of TACE were given to each patient. There were 359 patients on multimodality therapy, among which 107 patients underwent exploratory laparotomy. Surgical resection was performed in 97 (6.2%), 7 underwent liver transplantation, 3 underwent lung resection, 217 (13.8%) underwent local ablation (including alcohol injection, radiofrequency, microwave), 40 (2.5%) underwent radiotherapy and 60 (3.8%) underwent systemic chemotherapy.

### Statistical analysis

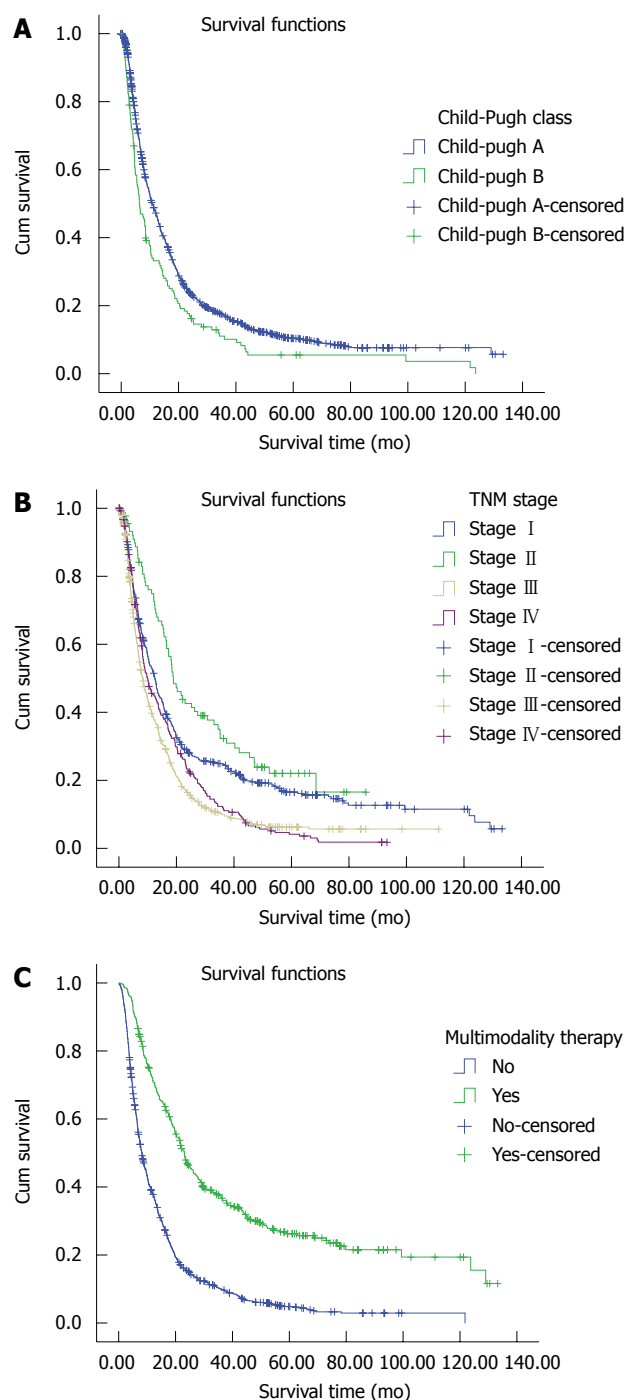
Pretreatment variables with possible prognostic significance were analyzed. Each variable was stratified into 2-4 strata. The survival rates were obtained by the Life Table method. Median survival lengths were obtained by the Kaplan-Meier method and compared by the log-rank test. Multivariate analysis was performed with the Cox proportional hazards model. All variables with a *P* value less than 0.05 by univariate analysis were subjected to multivariate analysis. All significance tests were rendered 2-tailed, and a *P* value less than 0.05 was considered statistically significant. All statistical processing was performed using the Statistical Package for Social Science V8.0 (SPSS, Inc., Chicago, IL, USA).

## RESULTS

During a 13 years follow-up, the total numbers of patient deaths, patients lost to follow-up (in our institute the follow-up is once a year; if a patient cannot be followed up after 1 year, they are considered to be lost to follow-up) and alive cases were 1301 (82.9%), 203 patients (12.9%) and 65 (4.1%), respectively. The annual distribution of deaths was 798 in year 1, 328 in year 2, 85 in year 3, 56 in year 4, 17 in year 5, 8 in year 6, 5 in year 7, 1 in year 9, and 3 in year 12, respectively. The number of patients lost to follow-up was 106 in year 1, 18 in year 2, 27 in year 3, 12 in year 4, 8 in year 5, 4 in year 6, 11 in year 7, 11 in year 8, 3 in year 9, and 1 each in years 10 through 12, respectively.

### Survival rates

Of the 1569 patients who underwent TACE as initial treatment, the overall median length of survival and 1-, 2-, 3-, 4-, 5-, 10-, and 12-year survival rates were



**Figure 1** Survival rates (Kaplan-Meier method). A: Patients with Child-Pugh classes A and B with statistical significance (*P* = 0.000); B: Comparison among TNM stages; C: Comparison of survival rates stratified by multimodality therapy with statistical significance (*P* = 0.000).

10.37 mo, 47%, 24%, 17%, 12%, 10%, 7%, and 4%, respectively. Kaplan-Meier analysis showed a significant difference in median survival length between patients with Child-Pugh class A and those with class B (Figure 1A) but not among patients with different TNM stages. Patients with Stage II disease showed the best survival rate (Figure 1B).

The median length of survival and 1-, 2-, 3-, 4-, 5-, 10-, 12-year survival rates among patients on



Table 1 Univariate analysis of pretreatment variables

Variable	Patients <i>n</i> (%)	Survival time (%)			Median survival length (mo)	<i>P</i> value
		1 yr	5 yr	10 yr		
Age (yr)						
≤ 50	848 (54.0)	46	9	7	9.63	0.003
> 50	721 (46.0)	49	11	7	11.60	-
HBV						
HBsAg(-)HBeAg(-)	215 (13.8)	55	15	12	13.70	0.000
HBsAg(+)HBeAg(-)	811 (52.2)	50	11	9	11.43	-
HBsAg(+)HBeAg(+)	529 (34.0)	40	7	3	8.27	-
AFP (ng/mL)						
≤ 20	292 (19.0)	61	18	-	15.80	0.000
21-200	296 (19.3)	56	13	9	13.50	-
> 200	946 (61.7)	41	7	6	8.53	-
Child-Pugh grade						
A	1413 (90.8)	49	11	8	10.97	0.000
B	144 (9.2)	34	6	4	6.40	-
Nodules						
≤ 3	855 (54.5)	52	12	8	12.30	0.000
> 3	714 (45.5)	42	8	-	8.57	-
Greatest dimension (cm)						
≤ 5.0	164 (10.5)	71	25	16	19.03	0.000
5.1-10	624 (39.8)	50	11	6	11.77	-
10.1-15	606 (38.6)	43	8	7	9.10	-
> 15	175 (11.2)	29	3	-	6.10	-
Tumor capsule						
No	818 (52.8)	41	9	5	8.40	0.000
Yes	732 (47.2)	55	11	9	12.50	-
Macrovascular invasion						
No	1276 (81.3)	50	12	8	11.70	0.000
Yes	293 (18.7)	34	3	-	7.10	-
Regional lymph node metastasis						
No	1519 (96.8)	48	10	7	10.50	0.032
Yes	50 (3.2)	31	8	-	7.10	-
Distant metastasis						
No	1302 (82.9)	48	12	8	10.40	0.183
Yes	269 (17.1)	46	4	-	10.13	-

Table 2 Relative risk of survival for significant pretreatment variables

Variable	<i>P</i> value	Hazard ratio (95% CI)
Regional lymph nodes metastasis	0.001	1.736 (1.272-2.370)
Child-Pugh class	0.017	1.254 (1.041-1.510)
Macrovascular invasion	0.002	1.253 (1.083-1.450)
Greatest dimension	0.000	1.250 (1.167-1.340)
AFP	0.000	1.237 (1.149-1.333)
HBV	0.000	1.232 (1.130-1.343)
Tumor capsule	0.004	1.192 (1.059-1.343)
Nodules	0.012	1.164 (1.034-1.310)

AFP:  $\alpha$ -fetoprotein; HBV: Hepatitis B virus.

multimodality therapy were 23.13 mo, 72%, 47%, 37%, 30%, 26%, 19%, and 12%, compared with 8.23 mo, 40%, 16%, 11%, 7%, 5%, 3%, and 0, respectively, among those treated with TACE alone. There was a statistical difference in survival between these two subsets of patients ( $P = 0.000$ ) (Figure 1C).

### Factors related to survival of patients

Univariate analysis revealed 9 pretreatment factors as

prognostic variables: age, hepatitis B virus (HBV), AFP, Child-Pugh class, nodules, greatest dimension, tumor capsule, macrovascular invasion, and regional lymph node metastasis (Table 1). Multivariate analysis showed the following as independent prognostic factors: regional lymph nodes metastasis, Child-Pugh class, macrovascular invasion, greatest dimension, AFP, HBV, tumor capsule, and nodules with hazard ratios ranging from 1.164 to 1.736 (Table 2).

### Complications and TACE-related mortality

Severe complications occurred in 353 (22.5%) patients after the initial TACE, including abnormal embolization in 9 patients, ascites in 235, pleural effusion in 32, acute renal failure in 4, hepatic failure in 38, jaundice in 197, upper gastrointestinal bleeding in 12, respiratory tract infection in 16, and abdominal infection in 3. Among these, there were 22 (1.4%) cases of TACE-related death with 20 caused by hepatic failure and 2 by upper gastrointestinal bleeding.

### DISCUSSION

For patients of unresectable HCC, the overall median survival was 8.7 wk<sup>[18]</sup> and 3 mo<sup>[19]</sup> in two untreated

populations. The goal of TACE as a palliative treatment is to prolong survival and to enhance quality of life. Unlike neoplasms of other organs, the survival of HCC depends not only on tumor status, but also on hepatic functional reserve, performance status, and response to treatment<sup>[14,20,21]</sup>.

In the current study, performance status was not assessed because of data unavailability. Given that all pre-TACE HCCs were unresectable in our cohort, the eligibility for other treatment modalities after initial TACE may indirectly reflect patient response to treatment. In our effort to screen for prognosis-related factors using univariate analysis of pretreatment factors indicated as significant, subsequent multivariate analysis stratified by multimodality therapy showed 8 factors as independently prognostic: regional lymph nodes metastasis, Child-Pugh class, macrovascular invasion, greatest dimension, AFP, HBV, tumor capsule, and nodules. These factors could be classified into 3 groups in nature: tumor status, hepatic function reserve, AFP and HBV. While AFP level has been shown to be an independent prognostic factor in many studies<sup>[7,10,13,14,20,22,23]</sup>, the cutoff values of AFP level varied widely. In the current study, survival rates appeared statistically different among patients with AFP being less than 20, 21-200, and greater than 200 ng/mL. As an important causal factor of HCC in the current study, HBV infection was seen in most of the patients with a prevalence (86.2%) similar to data from Hong Kong<sup>[5]</sup> (80.0%), but higher than those from Japan<sup>[7]</sup> (11.0%), Spain<sup>[6]</sup> (6.3%), and America<sup>[24]</sup> (49.0%). However, HBV status as an independent prognostic factor has seldom been reported<sup>[25]</sup>. As shown by Kaplan-Meier analysis in our study, the survival rates in HBsAg-negative and HBsAg-positive HBeAg-negative individuals did not differ from each other ( $P = 0.062$ ), but were statistically higher compared with HBeAg-positive patients ( $P = 0.000$ ). These findings suggested the potential benefits of antiviral therapy in HBeAg-positive patients. As with almost all reports related to TACE, in the current study Child-Pugh class was an independent prognostic factor. As to the tumor status, macrovascular invasion, greatest dimension, and nodules were proven by a large prospective cohort study<sup>[7]</sup>. Tumor capsule has been rarely noted as a prognostic factor<sup>[23]</sup>. Regional lymph node metastasis as independent factor was not assessed fully due to lack of data in most literature.

Surprisingly, adjacent organs invasion and distant metastasis, two of major components in TNM staging system, were not independent prognostic factors. Zhang *et al.*<sup>[26]</sup> showed that 67.5% Stage IV HCC patients died of liver failure caused by the progressive intrahepatic lesions and only 20% of those died of respiratory failure due to metastatic lesions. These results may explain why the survival rates of distant metastasis patients were not different from that of local unresectable HCC patients who underwent TACE. The progressive treatment for intrahepatic lesions may be of importance for Stage IV HCC. Our data supported the concept that the validity of the TNM stage system had only been assessed in patients who underwent resection<sup>[27]</sup>.

In the current study, tumor status and liver function were main prognostic factors. Since only 144 (9.2%) patients were Child-Pugh class B, the overall survival rates were largely determined by tumor status. Overall, the median survival length and survival rates in our study appeared somewhat dissatisfactory as compared with results from a meta-analysis<sup>[28]</sup>, which revealed survival rates of  $62\% \pm 20\%$ ,  $30\% \pm 15\%$ , and  $19\% \pm 16\%$  at 1, 3, and 5 years, along with a mean survival time of  $18 \pm 9$  mo. This may be explained by a higher percentage of patients with locally advanced lesions included in our study than in others. To the best of our knowledge, the mean greatest dimension ( $10.53 \pm 4.09$  cm) in the current study may be greater than any lesions reported before. However, the 10-year survival rate up to 7% in the whole group and 3% in TACE group supported the concept of heterogeneity of HCC in terms of survival.

In the current study, 353 (22.5%) patients developed severe complications after the initial TACE, which was mostly correlated to liver function. TACE-related death occurred in 22 (1.4%) patients, in which 20 deaths were caused by hepatic failure and 2 by upper gastrointestinal bleeding. While acute liver function impairment may arise from TACE treatment, Caturelli *et al.*<sup>[29]</sup> showed that TACE did not induce significant long term worsening of liver function in patients with class A or B cirrhosis. Hence, the relations among TACE variables, interval of TACE treatment, survival, and complications need to be further explored.

In conclusion, tumor status, hepatic function reserve, AFP, and HBV status were independent prognostic factors for unresectable HCC treated by TACE. Distant metastasis might not be a contraindication for TACE treatment. For a better survival, once the tumor status allows treatments other than TACE, multimodality therapy should be recommended.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC) constitutes the majority of liver cancers, which is the sixth most common cancer and the third most common cause of cancer death worldwide. A great number of HCC patients are suitable for curative therapy due either to advanced stage of disease or to poor liver function at the time of diagnosis. Transarterial chemoembolization (TACE) has become the most popular modality for palliative treatment among these patients. However, suitable candidates for TACE are still difficult to determine.

### Research frontiers

Two randomized trials from Europe and Asia have confirmed better survival associated with TACE compared with conservative treatment in selected patients. But the survival and prognostic factors of different studies varied due to the difference in patient baseline characteristics, treatment procedure, and regimens used. Hence, it is important to identify the prognostic factors associated with different geographical distributions of HCC.

### Innovations and breakthroughs

In the current study, the pretreatment prognostic factors of regional lymph node metastasis, Child-Pugh class, macrovascular invasion, greatest dimension,  $\alpha$ -fetoprotein, Hepatitis virus B, tumor capsule, and nodules were similar to other studies. However, three important items were indicated: (1) distant metastasis might not be a contraindication to TACE; (2) antiviral therapy in HBeAg-positive patients might benefit survival; (3) multimodality therapy might improve survival.

## Applications

The finding of this study might help to select patients who would benefit most from TACE, and help to design and estimate samples for further prospective randomized studies.

## Peer review

In this manuscript, the authors retrospectively reviewed their experience with TACE in unresectable HCC. This experience is quite extensive, as it concerns 1569 patients over a 13-year period.

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## Single-incision laparoscopic cholecystectomy: Single institution experience and literature review

Yasumitsu Hirano, Toru Watanabe, Tsuneyuki Uchida, Shuhei Yoshida, Kanae Tawaraya, Hideaki Kato, Osamu Hosokawa

Yasumitsu Hirano, Toru Watanabe, Tsuneyuki Uchida, Shuhei Yoshida, Kanae Tawaraya, Hideaki Kato, Osamu Hosokawa, Department of Surgery, Yokohama Sakae Kyouzai Hospital, 132 Katsura-cho, Sakae-ku, Yokohama, Kanagawa 247-8581, Japan

Author contributions: Hirano Y, Watanabe T, Uchida T, Yoshida S, Tawaraya K, Kato H and Hosokawa O contributed equally to this work.

Correspondence to: Yasumitsu Hirano, MD, Department of Surgery, Yokohama Sakae Kyouzai Hospital, 132 Katsura-cho, Sakae-ku, Yokohama, Kanagawa 247-8581, Japan. [yasumitsu@yahoocorp.jp](mailto:yasumitsu@yahoocorp.jp)

Telephone: +81-45-8912171 Fax: +81-45-8958351

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### Abstract

Single-incision laparoscopic surgery is a rapidly evolving field as a bridge between traditional laparoscopic surgery and natural orifice transluminal endoscopic surgery. We report one of the initial clinical experiences in Japan with this new technique. Four cases of gallbladder diseases were selected for this new technique. A single curved intra-umbilical 25-mm incision was made by pulling out the umbilicus. A 12-mm trocar was placed through an open approach, and the abdominal cavity was explored with a 10-mm semi-flexible laparoscope. Two 5-mm ports were inserted laterally from the laparoscope port. A 2-mm mini-loop retractor was inserted to retract the fundus of the gallbladder. Dissection was performed using an electric cautery hook and an Endograsper rotator. There were two women and two men with a mean age of 50.5 years (range: 40-61 years). All procedures were completed successfully without any perioperative complications. In all cases, there was no need to extend the skin incision. Average operative time was 88.8 min. Postoperative follow-up did not reveal any umbili-

cal wound complication. Single-incision laparoscopic cholecystectomy is feasible and a promising alternative method as scarless abdominal surgery for the treatment of some patients with gallbladder disease.

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**Key words:** Laparoscopic Cholecystectomy; Incision; Single-incision laparoscopic cholecystectomy; Single-incision laparoscopic surgery; Single-incision endoscopic surgery; Minimally invasive surgery

**Peer reviewer:** Dr. Tamara Alempijevic, Associate Professor, Clinic for Gastroenterology and Hepatology, Clinical Center of Serbia, 2 Dr Koste Todorovica St., 11000 Belgrade, Yugoslavia

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### INTRODUCTION

Since the introduction of laparoscopic cholecystectomy as the gold standard procedure to remove the gallbladder, many surgeons have attempted to reduce the number and size of ports in laparoscopic cholecystectomy to decrease parietal trauma and improve cosmetic results.

These efforts are some of the fundamentals of the natural orifice transluminal endoscopic surgery (NOTES) approach<sup>[1-4]</sup>, which removes transabdominal incisions completely, but NOTES is technically challenging and current instruments need to be further improved. As a bridge between traditional laparoscopic surgery and NOTES, the recent focus has been on the development of single-incision laparoscopic surgery (SILS) to further



minimize the invasiveness of laparoscopic surgery by reducing the number of incisions.

SILS was described as early as 1992 by Pelosi *et al*<sup>[5]</sup> who performed a single-puncture laparoscopic appendectomy, and in 1997, by Navarra *et al*<sup>[6]</sup> who performed a laparoscopic cholecystectomy *via* two transumbilical trocars and three transabdominal gallbladder stay sutures. SILS can be performed using refinements of existing technology, and surgeons can perform SILS without any new instruments, specific competence, or training. SILS may offer the advantages of reducing postoperative pain, and virtually scarless surgery.

We report our initial experience with four patients who underwent single-incision laparoscopic cholecystectomy, and review the previous literature.

## CASE REPORT

Four cases of gallbladder disease were selected for this new technique from June to July 2009. Indications included chronic cholecystitis and symptomatic cholelithiasis. There were two women and two men with a mean age of 50.5 years (range: 40-61 years). One patient had previously undergone appendectomy for acute appendicitis. Body mass index was 19.4-26.6 (mean: 23.0). All procedures were completed successfully without any perioperative complications. In all cases, there was no need to extend the skin incision. Average operative time was 88.8 min. Characteristics of patients and operative data are included in Table 1. The procedures were performed by the same surgeon.

### Surgical technique

A single curved, intra-umbilical, 25-mm incision was made by pulling out the umbilicus. After exposing the fascia, a 12-mm trocar was placed through an open approach, and the abdominal cavity was explored with a 10-mm semi-flexible laparoscope (LTFVH; Olympus). Pneumoperitoneum was induced and maintained at 8 mmHg with carbon dioxide. Two 5-mm ports were inserted through the anterior sheet of the abdominal rectus muscle, each placed 1 cm laterally from the laparoscope port. The patient was put in an anti-Trendelenburg position and rotated to the left, as in standard laparoscopic cholecystectomy. A 2-mm mini-loop retractor (Mini-loop retractor II; Hakkou-shoji) was inserted through an extra incision in the right subcostal space to retract the fundus of the gallbladder (Figures 1 and 2). Dissection was performed as a normal retrograde cholecystectomy using an electric cautery hook in the left trocar and an Endograsper rotulator (Rotulator Endograsp II, 5 mm; Autosuture) in the other trocar. On the occasion when the optimal exposure of the Triangle of Calot was inadequate, we inserted the hook and the grasper for traction. The cystic artery and duct were first exposed, then separately clipped with a standard 5-mm clip applicator (Endoclip III 5-mm clip applicator; Autosuture) and excised using an endoshear rotulator (Rotulator

Table 1 Patient characteristics and operative data

	Age (yr)	Sex	BMI	Previous history	History of cholecystitis	Operation time (min)
1	61	M	22.2	Appendectomy, HT	+	105
2	40	M	26.6	DM	+	102
3	41	F	19.4	None	-	64
4	60	F	23.7	DM	-	82

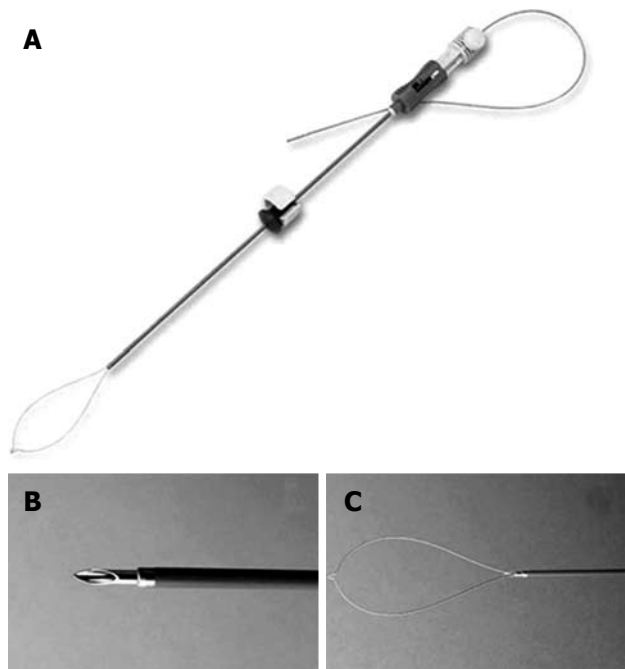


Figure 1 Mini-loop retractor II (Hakkou-shoji). A: Mini-loop retractor II; B: Needle for puncture; C: Loop wire.

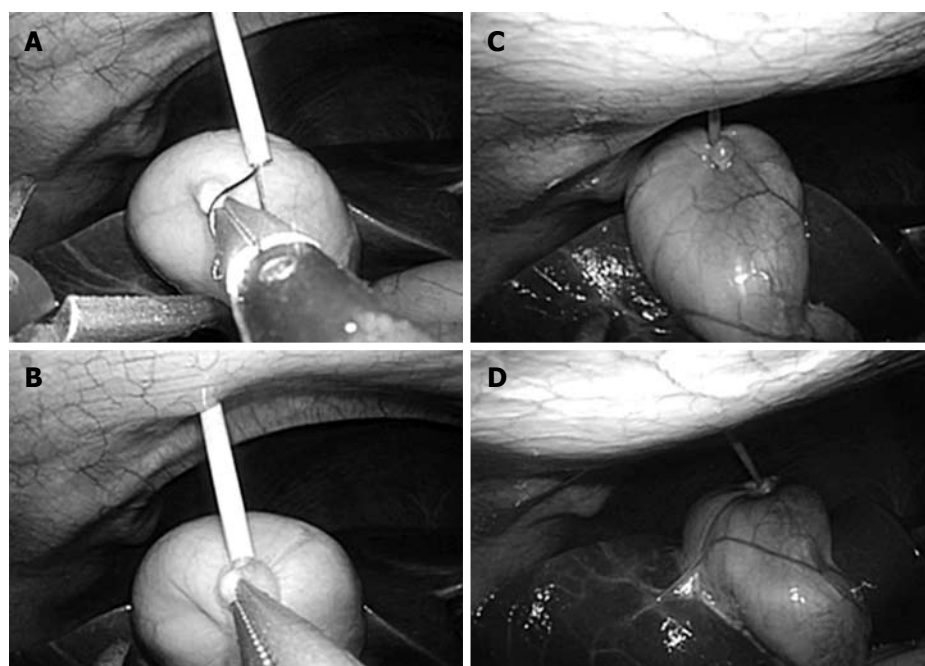
Endo Mini-Shears; Autosuture). The gallbladder was extracted with a standard endocatch (Endocatch Gold, 10 mm; Autosuture) through the umbilical site. Careful control of homeostasis was achieved, and a Penrose drain was placed in the cholecystectomy lodge through the 2-mm incision for the mini-loop retractor II. Finally the 25-mm trocar site was closed with an absorbable suture, and the umbilicus was restored to its physiological position.

Patients received food orally at 24 h postoperatively, and were mobilized. Drains were removed on the first postoperative day. All cases were discharged between the third and fifth postoperative day. Postoperative follow-up did not reveal any umbilical wound complications.

## DISCUSSION

Laparoscopic surgery is a well-established alternative to open surgery across disciplines. Although the magnitude of impact varies by procedure, generally the benefits of laparoscopy on postoperative pain, cosmetics, hospital stay, and convalescence are recognized widely.

Many surgeons have attempted to reduce the number



**Figure 2** Gallbladder suspension using the Mini-loop retractor II. The fundus of the gallbladder was suspended with this retractor (C and D), which was only tightened with a looped wire (A and B).

**Table 2** All published reports of single-incision laparoscopic cholecystectomies

Authors	Publication year	n	Conversion to standard LC (%)	Complication (%)	Average operating time (min)
Piskun <i>et al</i> <sup>[8]</sup>	1999	10	0	0	NR
Tacchino <i>et al</i> <sup>[7]</sup>	2009	12	0	2 (16.7)	55
Cuesta <i>et al</i> <sup>[9]</sup>	2008	10	0	0	70
Rao <i>et al</i> <sup>[10]</sup>	2008	20	3 (15)	0	40
Romanelli <i>et al</i> <sup>[11]</sup>	2008	1	0	0	68
Merchant <i>et al</i> <sup>[13]</sup>	2009	21	1 (4.8)	0	45-90
Palanivelu <i>et al</i> <sup>[14]</sup>	2008	10	4 (40)	1 (10)	148
Navarra <i>et al</i> <sup>[15]</sup>	2008	30	0	0	123
Cugura <i>et al</i> <sup>[17]</sup>	2008	1	0	0	NR
Bucher <i>et al</i> <sup>[16]</sup>	2009	11	0	0	52
Ersin <i>et al</i> <sup>[18]</sup>	2009	20	1 (5)	0	94
Nguyen <i>et al</i> <sup>[19]</sup>	2009	1	0	0	70
Langwieler <i>et al</i> <sup>[20]</sup>	2009	14	0	0	53-115
Podolsky <i>et al</i> <sup>[21]</sup>	2009	5	0	0	121
Zhu <i>et al</i> <sup>[12]</sup>	2009	26	0	0	62
Guo <i>et al</i> <sup>[22]</sup>	2008	1	0	0	158
Gumbs <i>et al</i> <sup>[23]</sup>	2009	2	0	0	< 60
Hong <i>et al</i> <sup>[24]</sup>	2009	15	0	0	79
Kuon Lee <i>et al</i> <sup>[25]</sup>	2009	37	5 (13.5)	2 (5.4)	83.6
Our cases	2009	4	0	0	83

and size of ports in laparoscopic surgery to decrease parietal trauma and improve cosmetic results, and recently two innovations have been developed: NOTES, which removes transabdominal incisions completely and SILS, which completes laparoscopic procedures by trocars located at one umbilical incision.

SILS, however, is not a new concept, and was described as early as 1992 by Pelosi *et al*<sup>[5]</sup> who performed a single-puncture laparoscopic appendectomy. In recent years, SILS has been focused upon as a bridge between NOTES and traditional laparoscopic surgery, because NOTES is technically challenging and current instruments need to be further improved. SILS, on the other hand,

enables the application of a wide range of already existing instruments. The main point for reducing the number of incisions is not only the cosmetic advantage but also lowered incision risks, morbidity of bleeding, incisional hernia, and organ damage.

Table 2 provides an overview of comparative features of single-incision laparoscopic cholecystectomy<sup>[6-25]</sup>. Out of 252 reported cases, 14 (5.6%) were converted to standard laparoscopic cholecystectomy. The reasons to convert were difficult dissection in nine cases, bleeding from the cystic artery in two, choledochoscopy for common bile duct exploration in two, and failure in trocar insertion in one case. There were five complications in

252 reported cases: one subcutaneous hematoma, one hepatic injury, one bile leakage, one mesenteric injury, and one injury of the right hepatic duct. Operative times in some series have been reported to be on a par with conventional laparoscopic surgery, but a majority of the procedures are lengthy, which may only be justified in patients who have a special cosmetic interest (Table 2).

The real challenge of SILS is to avoid conflict between the operative instruments and the camera, to maintain the pneumoperitoneum and reduce operative stress. As a result of the limited space with using only a single incision, it is difficult for both the surgeon and the assistant to work in the area. For that reason, we propose that using a semi-flexible endoscopic camera system would make the procedure more comfortable. Especially, the visualization and dissection of Calot's triangle would be easier and safer. Although the use of this semi-flexible endoscopic camera system with a cable connection on the posterior and crossed-over articulating instruments enables the procedure to be performed without interference, the use of crossed-over articulating instruments requires a longer operative time for achievement of careful and precise dissection, and some adjustments in the strategy of exposure are necessary, particularly because less strength is applied to the tissue than with the standard laparoscopic technique.

Some authors have suggested percutaneous puncture of the gallbladder for drainage or introduction of suspension hooks for better visualization of Calot's triangle<sup>[7,18]</sup>. These maneuvers, however, may inadvertently increase the chances of bactobilia and lead to perforation of the gallbladder, which leads to an increased risk of bile peritonitis, particularly in the setting of acute cholecystitis. To avoid these complications, we introduced the mini-loop retractor II to achieve optimal visual exposure. The fundus of the gallbladder was suspended with this retractor, which was only tightened with a looped wire. The use of this retractor enables the surgeon to grasp the gallbladder without injury for visualizing Calot's triangle, without increasing the risk of perforation of the gallbladder.

This report documents the feasibility of single-incision laparoscopic cholecystectomy. The clinical advantages of this approach may eventually require a randomized controlled trial to compare it with conventional laparoscopic cholecystectomy. The major advantage of this method is improved cosmetics, without any visible abdominal scars. Disadvantages of SILS include the conflict between the operative instruments, and the camera and the smaller degree of instrument triangulation compared to that of conventional laparoscopic surgery. Some of these disadvantages may be overcome with the use of the semi-flexible endoscopic camera system and crossed-over articulating instruments. Despite the limitations of SILS, we were able to perform our operation in four cases. All procedures were completed successfully within a reasonable time.

In conclusion, we documented the feasibility of single-incision laparoscopic cholecystectomy. This pro-

cedure is a promising alternative method, with scarless abdominal surgery, for the treatment of some patients with gallbladder disease. Further advantages of single-incision laparoscopic cholecystectomy compared to conventional laparoscopic cholecystectomy will ultimately require a clinical trial.

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## Single port laparoscopic right hemicolectomy with D3 dissection for advanced colon cancer

Sung Il Choi, Kil Yeon Lee, Sun Jin Park, Suk-Hwan Lee

Sung Il Choi, Kil Yeon Lee, Sun Jin Park, Suk-Hwan Lee, Department of Surgery, School of Medicine, Kyung Hee University, #1 Hoegi-dong Dongdaemun-gu, Seoul 130-702, South Korea

Author contributions: Choi SI and Lee KY contributed equally to this work; Lee KY and Lee SH designed research; Park SJ performed research; Choi SI wrote the paper.

Correspondence to: Kil Yeon Lee, MD, Department of Surgery, School of Medicine, Kyung Hee University, #1 Hoegi-dong Dongdaemun-gu, Seoul 130-702, South Korea. [isaac34@korea.com](mailto:isaac34@korea.com)

Telephone: +82-2-9588261 Fax: +82-2-9588277

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### INTRODUCTION

The laparoscopic approach for colorectal surgery has been shown to result in shorter hospital stays, faster return of bowel function, less pain and better cosmetic results than open surgery<sup>[1,2]</sup>. Moreover, the long term recurrence and survival results of laparoscopic surgery for advanced colon cancer are not different from those of conventional surgery<sup>[3,4]</sup>. Therefore, laparoscopic surgery has been considered the best treatment for colon cancer. Natural orifice transluminal endoscopic surgery (NOTES) has recently been developed and introduced for certain procedures in humans. However, there are many technical limitations for the use of NOTES in colon resection and anastomosis. Until now, experimental studies of NOTES for colon surgery have only been performed using animals or cadavers<sup>[5,6]</sup>. Single port laparoscopic surgery (SPLS) has advantages over NOTES in ease of instrument use and operative technique. This is the first report on SPLS performed for a right hemicolectomy in advanced colon cancer with D3 lymph node dissection.

### CASE REPORT

Our patient was a 60-year-old woman who was diagnosed with ascending colon cancer. Colonoscopy revealed an encircling mass in the ascending colon and biopsy demonstrated adenocarcinoma (Figure 1). An abdominopelvic computed tomography scan showed enhanced wall thickening in the ascending colon and no distant metastasis (Figure 2). The patient's body mass index was 25. The patient's consent about single port

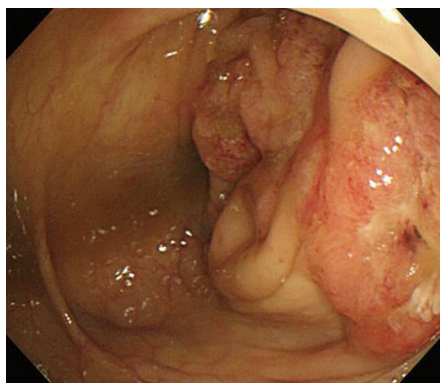
### Abstract

We report the first case of single port laparoscopic right hemicolectomy for advanced colon cancer. An abdominal 3 cm length incision was made *via* the umbilicus. A small wound retractor and a surgical glove were used as a single port. All soft tissue anterior to the superior mesenteric vein was completely removed and D3 lymph node dissection was achieved. The total operative time was 180 min with minimal blood loss (< 50 mL). The size of the tumor was 5 cm × 3 cm and its tumor stage was T3N0. Sixty-nine lymph nodes were harvested and none were positive. We believe that single port surgery for colon cancer is a feasible and safe procedure with surgical results comparable to conventional laparoscopic procedures.

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**Key words:** Single port laparoscopic surgery; Colon cancer; D3 lymph node dissection

**Peer reviewer:** Seon Hahn Kim, MD, Department of Surgery, Korea University Anam Hospital, #126-1, 5-ga, Anam-dong, Sungbook-gu, Seoul 136-705, South Korea



**Figure 1** Encircling mass in the ascending colon visualized during colonoscopy.



**Figure 2** Abdominal CT showed enhanced wall thickening in the ascending colon.

surgery was obtained. A 3 cm abdominal incision was made *via* the umbilicus using the method reported by Casciola *et al*<sup>[7]</sup>. A small wound retractor (ALEXIS wound retractor S, Applied Medical, Santa Margarita, CA, USA) and a surgical glove were used as a single port, as in previous reports<sup>[8,9]</sup> (Figure 3). The surgical technique was similar to laparoscopic right hemicolectomy and involved a medial to lateral approach using a 0 degree 10 mm scope. We used a hook type monopolar suction irrigator and 5 mm standard straight laparoscopic instrument for dissection of lymph nodes and vessels. We successfully removed all lymph nodes and connective tissue around the ileocolic vessels and midcolic vessels. The vessels were divided using Ligasure V (Covidien, Valleylab, Norwalk, CT, USA). All of the soft tissue anterior to the superior mesenteric vein was completely removed and D3 lymph node dissection was achieved (Figure 4A and B). The specimen was put into a plastic bag within the abdominal cavity and was retrieved through the umbilical port without wound extension. Extracorporeal anastomosis was performed using staplers. The total operative time was 180 min with minimal blood loss (< 50 mL). The patient's total hospital stay was 4 d and she was discharged without complications (Figure 5A). The surgical specimen was 27.5 cm in length with 10 cm surgical margins and the tumor size was 5 cm × 3 cm (Figure 5B and C). There were 69 harvested lymph



**Figure 3** Operative photograph of the single port setting with multiple trocars and instruments.

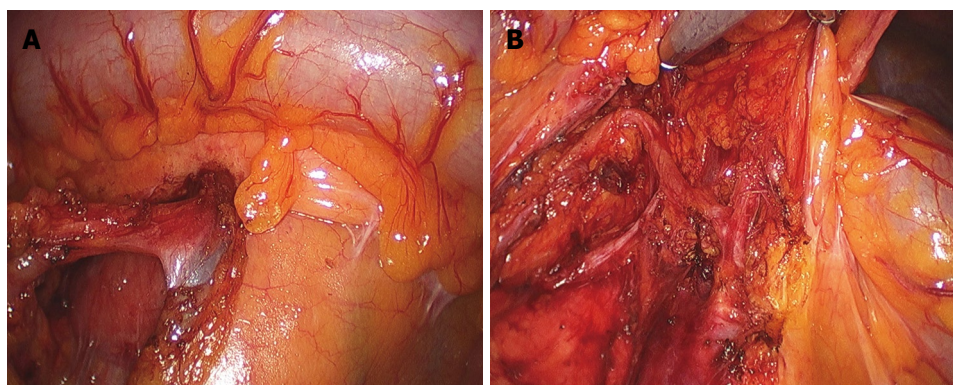
nodes and there were no positive lymph nodes. The pathologic staging of the tumor was T3N0M0.

## DISCUSSION

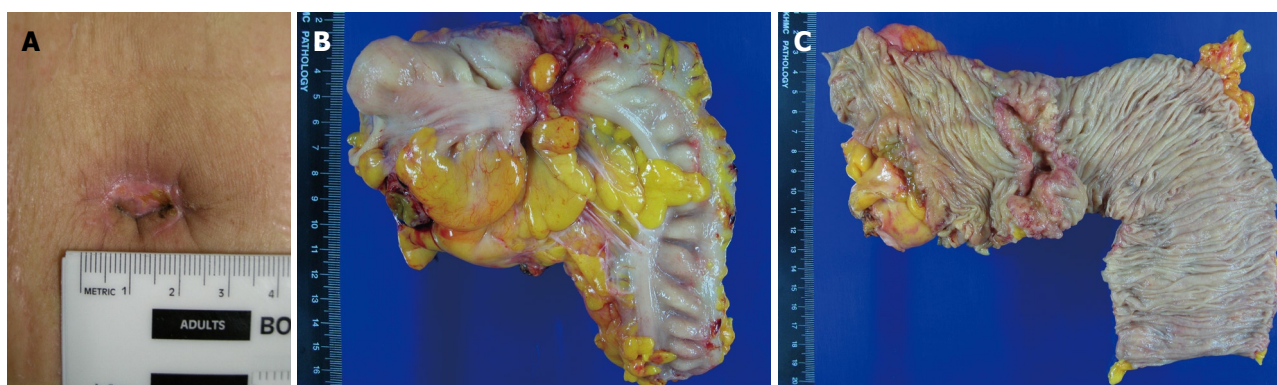
Laparoscopic surgery for advanced colon cancer provides increased patient satisfaction with improvements in postoperative pain, cosmetic results, and recovery<sup>[1-4]</sup>. However, laparoscopic surgery typically requires 5-6 trocars with 5-12 mm-sized incisions for each trocar, with associated risks for trocar site bleeding, herniation of the viscera, wound infection, wound pain and unwanted cosmetic outcomes<sup>[10,11]</sup>. Single port surgery has been reported in nephrectomy, appendectomy, cholecystectomy, adnexal mass resection, and sacrocolpopexy<sup>[12-15]</sup>. Like laparoscopic surgery, SPLS was developed and, gradually, more complex and difficult procedures have been performed. Bucher *et al*<sup>[16]</sup> reported a successful single port access right hemicolectomy operation for a 5 cm sized polyp in the ascending colon. They respected oncologic principles for cancer surgery. The surgical specimen length was 38 cm with a 10 cm surgical margin and 33 lymph nodes were retrieved. Leroy *et al*<sup>[17]</sup> performed a sigmoidectomy using a single port access for a patient with diverticular disease in the sigmoid colon. In this procedure, the surgeons used a specific single port (ASC Triport, Advanced Surgical Concepts, Wicklow, Ireland), magnets and endoscopy for bowel traction. This Triport allows the passage of 1 large-caliber instrument (up to 12 mm) and 2 other instruments of smaller diameter (up to 5 mm each). The surgical specimen was 40 cm long and anastomosis was located 10 cm from the anal margin. Although these reports demonstrated the technical feasibility and safety of single port surgery, it remains a complex and intricate procedure. Single port surgery has limitations with regard to movement range, adequate traction and triangulation for dissection.

We performed single port surgery in an advanced colon cancer patient. Before the single port laparoscopic right hemicolectomy (SPLRH) procedure, we had experience with SPLS in 10 cases of appendectomy and 1 case of colectomy for colon polyps. In those patients, we used a simple single port that was made using a





**Figure 4** Single port laparoscopic right hemicolectomy with D3 node dissection around the superior mesenteric vein (A) and artery (B).



**Figure 5** Surgical wound around the umbilicus (A) and surgical specimen of the resected colon (B and C).

conventional small wound retractor and a surgical glove. We used conventional straight laparoscopic instruments. Surprisingly, SPLRH with conventional instruments was not a difficult procedure. We were able to achieve operating space with adequate forward and backward movements of the scope and instruments. Of note, balanced scope movement according to movement of the laparoscopic instruments is important. Difficulties with scissoring and interference between instruments will probably be overcome with new instruments and scopes in the near future. Limitations which make SPLS difficult to apply across cases include internal and external conflicts between instruments and difficulty achieving traction for triangulation formation. We offer the following technical tips to avoid these limitations. Low tension under the tissue to be dissected is overcome by using cut mode in electrosurgery instead of coagulation mode. To avoid internal conflict between two working laparoscopic instruments, we first established a surgical field to grasp tissue which is not located too close to the area to dissect. To avoid internal conflict between the scope and dissecting instruments, we recommend drawing the scope to the level of the abdominal wound or out of the wound, if it is possible once the right surgical plane could be found in the close view. External conflicts can be categorized into interference between trocars and interference between instruments. Use of a surgical glove is helpful to overcome these conflicts because it is flexible enough to minimize the trocar conflict. Therefore, surgeons can easily avoid trocar

conflict by pushing the trocar forward slightly. SPLS for colon cancer is a challenging procedure and it is essential to develop multiport single access and instruments for single port surgery<sup>[18]</sup>. Recently many commercial ports have been developed. New port systems include the R-Port (ASC, Wicklow, Ireland)<sup>[18]</sup>, the ASC Triport<sup>[17]</sup> and the Pnavel Uni-XTM Single Site Laparoscopic System<sup>[19]</sup> (Pnavel System, Morganville, NJ, USA). New instruments have also been developed specifically to reach curved or articulating structures<sup>[20]</sup>. Camera systems have also been improved. Cadeddu *et al*<sup>[21]</sup> used a magnetically anchored camera system during appendectomy and nephrectomy. The system showed fewer instrument collisions and improved surgical space<sup>[21]</sup>.

Given the evolution of single port surgery, we assume that laparoscopic surgeons will perform more technically difficult procedures including advanced cancer surgery with a single port. This is the first report of single port surgery for colon cancer. We believe that SPLS for colon cancer is an effective, minimally invasive procedure and a bridge to NOTES for colon resection.

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## Controversies about the use of serological markers in diagnosis of inflammatory bowel disease

Qin Xie, Hua-Tian Gan

Qin Xie, Department of Gastroenterology, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China  
Hua-Tian Gan, Department of Geriatrics, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China  
Author contributions: Xie Q and Gan HT contributed equally to this work.

Correspondence to: Hua-Tian Gan, MD, PhD, Department of Geriatric Medicine and Gastroenterology, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China. [ganhuatian@hotmail.com](mailto:ganhuatian@hotmail.com)

Telephone: +86-28-85423253 Fax: +86-28-85423253

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### TO THE EDITOR

We read with great interest the article recently published in *World Journal of Gastroenterology* by Song *et al*<sup>[1]</sup>, presenting that the serum levels of soluble intercellular adhesion molecule-1, D-lactate and diamine oxidase (DAO) can be used as major monitoring indices in diagnosis and treatment of inflammatory bowel disease (IBD). However, some points on designing the study and their conclusion about the markers in diagnosis of IBD may need further considerations.

First, the study was based on the view that the intestinal permeability (IP) is increased in the active phase of IBD so that D-lactate and DAO can enter blood through the dysfunctional barrier. A high serum level of the markers, which reflects the activity of IBD, can reveal the damage to intestinal mucosa. However, several studies have identified a subset of healthy relatives who also have increased IP but no CD<sup>[2-4]</sup>. Moreover, it has been shown that IP increases in remission of IBD, especially in CD<sup>[5]</sup>.

Second, the authors stated that D-lactate could not be well metabolized as mammals have no D-lactate dehydrogenase for its decomposition. Therefore, plasma D-lactate can be used to reveal the damage to intestinal mucosa and permeability alteration in IBD. This conventional opinion is based largely on early experiments and continues to be quoted frequently. It has been reported that D-lactate is indeed metabolized<sup>[6]</sup>. The half-life of oral D-lactate (6.4 mmol/kg) is 21 min in blood of healthy humans while doubling this dosage increases its half-life to 40 min, most likely reflecting the saturation of its metabolism<sup>[7]</sup>. In addition, recent studies have identified D-lactate dehydrogenases in putative human and murine mitochondria<sup>[8,9]</sup>. These findings indicate that the plasma level of D-lactate is not a reliable marker of IBD.

### Abstract

The serological markers are increasingly used in diagnosis of inflammatory bowel disease (IBD). D-lactate and diamine oxidase are new indicators that can be used to reveal the damage to intestinal mucosa and permeability alteration in IBD. Although the two biological markers seem more sensitive, recent clinical trials and animal experiments have shown controversies about the use of them in diagnosis of IBD. Therefore, these markers should be interpreted cautiously and further prospective studies are needed to establish their clinical role in diagnosis of IBD.

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**Key words:** Inflammatory bowel diseases; D-lactate; Diamine oxidase; Intestinal permeability; Diagnosis

**Peer reviewer:** Ashkan Farhadi, MD, MS, FACP, Digestive Disease Center, Bristol Park Medical Group, Orange County, Irvine, CA 92603, United States

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Third, DAO activity in blood, as a diagnostic marker of IBD, should be interpreted cautiously. Actually, serum DAO enzyme activity is changed by several disorders including severe burn, gut injury, diverse enteropathy and abdominal surgery, chemotherapy and kidney injury. Furthermore, gender-related differences in DAO activity with a high inter-individual variability, demonstrate that abnormal serum DAO activity in women is not always associated with a pathological status<sup>[10]</sup>.

Prerequisites for the clinical use of biomarkers in diagnosis of IBD include high sensitivity, specificity and cost-effectiveness. The rapidly expanding markers to the serologic IBD diagnostic algorithm will likely increase their sensitivity. Increased sensitivity, however, can often accompany decreased specificity, which must be carefully assessed and recognized. Further prospective clinical trials are needed to determine the role and importance of such markers in diagnosis of IBD.

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**Taku Aoki, MD**, Division of Hepato-Biliary-Pancreatic and Transplantation Surgery, Department of Surgery, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

**Giuseppe Currò, MD**, University of Messina, Via Panoramica, 30/A, 98168 Messina, Italy

**Peter Draganov, Dr.**, Division Gastroenterology, Hepatology and Nutrition, University of Florida, Gainesville, 1600 SW Archer Road, PO Box 100214, Florida, FL 32610, United States

**Abdellah Essaid, Professor**, Hospital Ibn Sina, Rabat 10100, Morocco

**Piero Marco Fisichella, MD, Assistant Professor** of Surgery, Medical Director, Swallowing Center, Loyola University Medical Center, Department of Surgery, Stritch School of Medicine, 2160 South First Avenue, Room 3226, Maywood, IL 60153, United States

**Jean-Noël Freund, PhD**, INSERM Unit 682, 3 avenue Molière, 67100 Strasbourg, France

**Richard Hu, MD, MSc**, Division of Gastroenterology, Department of Medicine, Olive view-UCLA Medical Center, 14445 Olive View Drive, Los Angeles, CA 91342, United States

**Kalpesh Jani, Dr.**, Consultant GI & Laparoscopic Surgeon, SIGMA Surgery, Baroda, Gujarat 390011, India

**Jonathan D Kaunitz, MD**, Building 114, Suite 217, West Los Angeles VA Medical Center, 11301 Wilshire Boulevard, Los Angeles, California, CA 90073, United States

**John M Mariadason, PhD, Assistant Professor**, Department of Oncology, Albert Einstein College of Medicine, Montefiore Medical Center, Hofheimer Bldg. 413, 111 East 210th Street, Bronx, NY 10467, United States

**Sabine Mihm, Professor**, Department of Gastroenterology, Georg-August-Universität, Robert-Koch-Str.40, Göttingen D-37099, Germany

**James Neuberger, Professor**, Liver Unit, Queen Elizabeth Hospital, Birmingham B15 2TH, United Kingdom

**Bernabe Matias Quesada, MD**, Department of Surgery, Hospital Cosme Argerich, Talcahuano 944 9ªA, Buenos Aires 1013, Argentina

**Tor C Savidge, PhD, Associate Professor**, Department of Gastroenterology & Hepatology, Galveston, Texas, TX 77555, United States

**Mitsuo Shimada, Professor**, Department of Digestive and Pediatric Surgery, Tokushima University, Kuramoto 3-18-15, Tokushima 770-8503, Japan

**Ferenc Sipos, MD, PhD**, Cell Analysis Laboratory, 2nd Department of Internal Medicine, Semmelweis University, Szentkirályi u. 46., Budapest 1088, Hungary

**Kevin J Spring, Dr.**, Conjoint Gastroenterology Laboratory, The Queensland Institute of Medical Research, the Bancroft Centre, rm H07, PO Royal Brisbane Hospital, Herston, QLD 4029, Australia

**Christian D Stone, MD, MPH, Director**, Inflammatory Bowel Disease Program, Assistant Professor of Medicine, Division of Gastroenterology, Washington University School of Medicine, 660 South Euclid Avenue, Campus Box 8124, Saint Louis, MO 63110, United States

**Takato Ueno, Professor**, Research Center for Innovative Cancer Therapy, Kurume University, 67 Asahi-machi, Kurume 830-0011, Japan

**Peer Wille-Jørgensen, Dr.**, Department of Surgery K, Bispebjerg Hospital, Bispebjerg Bakke 23, Copenhagen NV, DK-2400, Denmark

**Tomoharu Yoshizumi, MD, PhD**, Department of Surgery, Saiseikai Fukuoka General Hospital, 1-3-46, Tenjin, Chuou-ku, Fukuoka 810-0001, Japan



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### Events Calendar 2010

January 25-26  
Tamilnadu, India  
International Conference on Medical  
Negligence and Litigation in Medical  
Practice

January 25-29  
Waikoloa, HI, United States  
Selected Topics in Internal Medicine

January 26-27  
Dubai, United Arab Emirates  
2nd Middle East Gastroenterology  
Conference

January 28-30  
Hong Kong, China  
The 1st International Congress on  
Abdominal Obesity

February 11-13  
Fort Lauderdale, FL, United States  
21th Annual International Colorectal  
Disease Symposium

February 26-28  
Carolina, United States  
First Symposium of GI Oncology at  
The Caribbean

March 04-06  
Bethesda, MD, United States  
8th International Symposium on  
Targeted Anticancer Therapies

March 05-07  
Peshawar, Pakistan  
26th Pakistan Society of  
Gastroenterology & Endoscopy  
Meeting

March 09-12  
Brussels, Belgium  
30th International Symposium on  
Intensive Care and Emergency  
Medicine

March 12-14  
Bhubaneswar, India  
18th Annual Meeting of Indian  
National Association for Study of  
the Liver

March 23-26  
Cairo, Egypt  
14th Pan Arab Conference on  
Diabetes PACD14

March 25-28  
Beijing, China  
The 20th Conference of the Asian

Pacific Association for the Study of  
the Liver

March 27-28  
San Diego, California, United States  
25th Annual New Treatments in  
Chronic Liver Disease

April 07-09  
Dubai, United Arab Emirates  
The 6th Emirates Gastroenterology  
and Hepatology Conference, EGHG  
2010

April 14-17  
Landover, Maryland, United States  
12th World Congress of Endoscopic  
Surgery

April 14-18  
Vienna, Austria  
The International Liver Congress™  
2010

April 28-May 01  
Dubrovnik, Croatia  
3rd Central European Congress  
of surgery and the 5th Croatian  
Congress of Surgery

May 01-05  
New Orleans, LA, United States  
Digestive Disease Week Annual  
Meeting

May 06-08  
Munich, Germany  
The Power of Programming:  
International Conference on  
Developmental Origins of Health  
and Disease

May 15-19  
Minneapolis, MN, United States  
American Society of Colon and  
Rectal Surgeons Annual Meeting

June 04-06  
Chicago, IL, United States  
American Society of Clinical  
Oncologists Annual Meeting

June 09-12  
Singapore, Singapore  
13th International Conference on  
Emergency Medicine

June 14  
Kosice, Slovakia  
Gastro-intestinal Models in  
the Research of Probiotics and  
Prebiotics-Scientific Symposium

June 16-19  
Hong Kong, China  
ILTS: International Liver  
Transplantation Society ILTS Annual  
International Congress

June 20-23  
Mannheim, Germany  
16th World Congress for  
Bronchoesophagology-WCBE

June 25-29  
Orlando, FL, United States  
70th ADA Diabetes Scientific  
Sessions

August 28-31  
Boston, Massachusetts, United States  
10th OESO World Congress on  
Diseases of the Oesophagus 2010

September 10-12  
Montreal, Canada  
International Liver Association's  
Fourth Annual Conference

September 11-12  
La Jolla, CA, United States  
New Advances in Inflammatory  
Bowel Disease

September 12-15  
Boston, MA, United States  
ICAAC: Interscience Conference  
on Antimicrobial Agents and  
Chemotherapy Annual Meeting

September 16-18  
Prague, Czech Republic  
Prague Hepatology Meeting 2010

September 23-26  
Prague, Czech Republic  
The 1st World Congress on  
Controversies in Gastroenterology &  
Liver Diseases

October 07-09  
Belgrade, Serbia  
The 7th Biannual International  
Symposium of Society of  
Coloproctology

October 15-20  
San Antonio, TX, United States  
ACG 2010: American College of  
Gastroenterology Annual Scientific  
Meeting

October 23-27  
Barcelona, Spain  
18th United European  
Gastroenterology Week

October 29-November 02  
Boston, Massachusetts, United States  
The Liver Meeting® 2010--AASLD's  
61st Annual Meeting

November 13-14  
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Case-Based Approach to the  
Management of Inflammatory Bowel  
Disease

December 02-04  
San Francisco, CA, United States  
The Medical Management of HIV/  
AIDS





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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 Xiaohua 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

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID: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 1, 274 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. *HRSA 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

#### Author(s) and editor(s)

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek 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

#### 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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### EDITING

Editorial Board of *World Journal of Gastroenterology*, Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China  
Telephone: +86-10-5908-0039  
Fax: +86-10-8538-1893  
E-mail: [wjg@wjgnet.com](mailto:wjg@wjgnet.com)  
<http://www.wjgnet.com>

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Telephone: +86-10-8538-1892  
Fax: +86-10-8538-1893  
E-mail: [baishideng@wjgnet.com](mailto:baishideng@wjgnet.com)  
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Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China  
Telephone: +86-10-8538-1892  
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## Hypoxia, angiogenesis and liver fibrogenesis in the progression of chronic liver diseases

Claudia Paternostro, Ezio David, Erica Novo, Maurizio Parola

Claudia Paternostro, Erica Novo, Maurizio Parola, Department of Experimental Medicine and Oncology, Interuniversity Centre for Hepatic Pathophysiology, University of Torino, Corso Raffaello 30, 10125 Torino, Italy

Ezio David, Department of Pathology, San Giovanni Battista Hospital, 10126 Torino, Italy

Author contributions: Paternostro C, David E, Novo E, and Parola M equally contributed to this paper.

Correspondence to: Maurizio Parola, PhD, Professor, Department of Experimental Medicine and Oncology, Interuniversity Centre for Hepatic Pathophysiology, University of Torino, Corso Raffaello 30, 10125 Torino, Italy. [maurizio.parola@unito.it](mailto:maurizio.parola@unito.it)  
 Telephone: +39-11-6707772 Fax: +39-11-6707753

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### Abstract

Angiogenesis is a dynamic, hypoxia-stimulated and growth factor-dependent process, and is currently referred to as the formation of new vessels from pre-existing blood vessels. Experimental and clinical studies have unequivocally reported that hepatic angiogenesis, irrespective of aetiology, occurs in conditions of chronic liver diseases (CLDs) characterized by perpetuation of cell injury and death, inflammatory response and progressive fibrogenesis. Angiogenesis and related changes in liver vascular architecture, that in turn concur to increase vascular resistance and portal hypertension and to decrease parenchymal perfusion, have been proposed to favour fibrogenic progression of the disease towards the end-point of cirrhosis. Moreover, hepatic angiogenesis has also been proposed to modulate the genesis of portal-systemic shunts and increase splanchnic blood flow, thus potentially affecting complications of cirrhosis. Hepatic angiogenesis is also crucial for the growth and progression of hepatocellular carcinoma. Recent literature has identified a number of cellular and molecular mechanisms governing the cross-talk between angiogenesis and fibrogenesis,

with a specific emphasis on the crucial role of hypoxic conditions and hepatic stellate cells, particularly when activated to the myofibroblast-like pro-fibrogenic phenotype. Experimental anti-angiogenic therapy has been proven to be effective in limiting the progression of CLDs in animal models. From a clinical point of view, anti-angiogenic therapy is currently emerging as a new pharmacologic intervention in patients with advanced fibrosis and cirrhosis.

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**Key words:** Chronic liver diseases; Hepatic myofibroblasts; Hypoxia; Liver angiogenesis; Liver fibrogenesis

**Peer reviewer:** Devanshi Seth, PhD, Senior Scientist, Centenary Institute & Drug Health Services, RPAH & Clinical Senior Lecturer, Clinical School of Medicine, University of Sydney, Camperdown, NSW 2050, Australia

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### INTRODUCTION

Angiogenesis can be envisaged as a dynamic, hypoxia-stimulated and growth factor-dependent ubiquitous process leading to the formation of new vessels from pre-existing blood vessels<sup>[1-5]</sup>, and should be distinguished from other terms that, although related to vessel formation and growth, define significantly different processes such as vasculogenesis, arteriogenesis and collateral vessel growth. Angiogenesis occurs virtually in almost all organs and tissues and is considered a critical step in either physiological conditions or in tissue repair and growth in several pathophysiological conditions<sup>[1-5]</sup>, including chronic liver diseases (CLDs)<sup>[6-8]</sup>.



Where the liver is concerned, physiological and pathological angiogenesis can occur during liver regeneration (after acute liver injury or after partial hepatectomy), in ischemic conditions, during chronic inflammatory and fibrogenic liver diseases as well as in hepatocellular carcinoma and in metastatic liver cancers<sup>[6-8]</sup>. The steps and mechanisms of hepatic angiogenesis mostly overlap with those described in other organs or tissues but a number of liver parenchyma peculiarities are likely to make the overall scenario more complex<sup>[6]</sup>. These include the existence of two different kinds of microvascular structures (portal vessels and liver sinusoids, lined by continuous or fenestrated and discontinuous endothelium, respectively), the expression of a putative liver specific angiopoietin-like peptide defined as ANGPTL3<sup>[9]</sup> and, most relevant, the unique and heterogenous phenotypic profile and functional role of hepatic stellate cells (HSCs) that, although regarded as liver specific pericytes in normal liver, also represent the most relevant pro-fibrogenic cell lineage<sup>[10-13]</sup> in CLDs. HSCs, particularly in their activated and myofibroblast-like phenotype (HSC/MFs), are indeed emerging as cells that may have an active role in modulating angiogenesis that differs from the one attributed to microcapillary pericytes<sup>[14]</sup>. The overall scenario is even more complex if one considers that in CLDs hepatic myofibroblast-like cells (MFs) constitute a heterogenous population of pro-fibrogenic cells. These highly proliferative and contractile cells may also originate from portal (myo) fibroblasts, bone marrow-derived stem cells and, as recently suggested, also from hepatocytes or cholangiocytes through a process of epithelial to mesenchymal transition<sup>[10-13]</sup>. In the following section of this editorial we will try to focus on those relevant features that link angiogenesis to liver fibrogenesis and then the progression of CLDs. The interested reader can refer to more comprehensive reviews - such as articles with more details on the basic principles and mechanisms involved in angiogenesis as well as the analysis of the role of angiogenesis in liver regeneration or hepatocellular carcinoma<sup>[10-14]</sup>.

## HEPATIC ANGIOGENESIS AND ITS RELATIONSHIPS BETWEEN CHRONIC INFLAMMATION AND FIBROGENIC PROGRESSION OF CLDs

CLDs are characterized by reiteration of liver injury due to a number of aetiological conditions, including chronic infection by viral agents [mainly hepatitis B virus (HBV) and hepatitis C virus (HCV)] as well as metabolic, toxic/drug-induced (with alcohol consumption being predominant) and autoimmune causes, resulting in persistent inflammation and progressive fibrogenesis. Chronic activation of the wound healing response is the major driving force for progressive accumulation of

extracellular matrix (ECM) components, eventually leading to liver cirrhosis and hepatic failure. Oxidative stress and redox signalling, derangement of epithelial-mesenchymal interactions or, as recently proposed, the process of epithelial to mesenchymal transition represent additional mechanisms able to sustain fibrogenesis progression towards the final end-point of cirrhosis<sup>[11,12,15-17]</sup>.

Along these lines, cirrhosis should be regarded as an advanced stage of fibrosis characterized by the formation of regenerative nodules of parenchyma, surrounded and separated by fibrotic septa, and associated with significant changes in angio-architecture. The suggestion that angiogenesis may significantly contribute to fibrogenesis and disease progression relies first on the fact that vascular remodelling, irrespective of aetiology, is a common finding in human cirrhotic livers<sup>[11,12,14,18,19]</sup>. Moreover, the formation of fibrotic septa, as well as capillarization of sinusoids, the latter due to early deposition of fibrillar ECM in the space of Disse, can result in an increased resistance to blood flow and oxygen delivery. These are the premises for hypoxia and the transcription of hypoxia-sensitive pro-angiogenic genes, usually modulated by the so-called hypoxia inducible factors (HIFs)<sup>[20-23]</sup>. In addition, it is well known that in CLDs the inflammatory response gains the role of a dynamic state relevant for the progression of fibrogenesis towards the end-point of cirrhosis<sup>[11-13,16]</sup>. Several mediators of the inflammatory response may stimulate other cells in the surrounding microenvironment to express vascular endothelial growth factor (VEGF) and other pro-angiogenic factors as well as to sustain angiogenesis<sup>[24]</sup>. Moreover, cytokines or mediators produced during CLDs such as hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF) and nitric oxide (NO), can play a role in the development of angiogenesis<sup>[6,13,14]</sup>. In particular, one should consider that: (1) neo-vessels themselves can significantly contribute to perpetuation of the inflammatory response by expressing chemokines and adhesion molecules promoting the recruitment of inflammatory cells; (2) angiogenesis, early in the course of a CLD, may contribute to the transition from acute to chronic inflammation<sup>[25]</sup>.

A relevant additional point, as recently proposed<sup>[20]</sup>, is that depending on the specific pattern of fibrosis (post-necrotic or bridging fibrosis, pericellular or perisinusoidal fibrosis, biliary fibrosis or centrilobular fibrosis)<sup>[12,26]</sup>, the extent of neo-angiogenesis, in addition to favouring disease progression, may also represent a key limiting factor for fibrosis reversibility. This is potentially relevant for post-necrotic or bridging fibrosis, a pattern which is mainly seen in patients with advanced fibrosis or cirrhosis by chronic HBV or HCV infection. In this pattern of fibrosis, which is characterized by the formation of bridging septa between portal and central vein areas, angiogenesis, vascular remodelling and altered angio-architecture are particularly impressive.

## **PATHOLOGICAL ANGIOGENESIS AND PRO-ANGIOGENIC CYTOKINES HAVE BEEN DETECTED IN HUMAN CLDs**

Current evidence suggests that angiogenesis and fibrogenesis are detectable and develop in parallel in any clinical condition of CLDs that can progress towards the end-point of cirrhosis, irrespective of aetiology, as well as in the most widely used experimental animal models of CLDs<sup>[6,13,19,20]</sup>.

Where clinical data are concerned, best relationships between angiogenesis and the pattern of fibrosis (i.e. bridging fibrosis)<sup>[12,26]</sup> are usually found during chronic viral infection by either HBV or HCV. This is documented by either the abundant presence of endothelial cells (ECs) and neovessels/capillary structures found in inflamed portal tracts<sup>[6]</sup> or by the over-expression of major pro-angiogenic molecules, including VEGF and Angiopoietin 1 (Ang-1) as well as their related receptors (VEGFR type II, Tie2) and HGF<sup>[27-31]</sup>. In these clinical settings, PDGF, which is released by periportal inflammatory cells as well as by sinusoidal and perisinusoidal cells, may also play a pro-angiogenic role<sup>[32]</sup>. In addition, selected viral proteins may have a multiple pro-angiogenic role like HBV-related X protein<sup>[33]</sup>. This protein has been involved in disruption of inter-endothelial junctions by operating through a src-kinase-dependent signalling pathway, as well as in the up-regulation of inducible nitric oxide synthase (iNOS) through involvement of nuclear factor- $\kappa$ B (NF- $\kappa$ B) transcription factor<sup>[34]</sup> or even by up-regulating membrane-type matrix metalloprotease (MT-MMP) expression, and then MMP-2 activation in hepatocytes<sup>[35]</sup>.

Angiogenesis has also been detected in biopsies from patients affected by either primary biliary cirrhosis (PBC) or autoimmune hepatitis as formation of neovessels by ECs positive for CD-31 and vascular endothelial-cadherin<sup>[6]</sup>. These neo-vessels were located, particularly in PBC, mainly in portal areas in association with inflammatory infiltrate<sup>[6,36]</sup>. Once again, enhanced expression of angiogenic molecules such as VEGF, Ang-1, Ang-2, Tie-2 and endoglin has also been characterized in these PBC patients.

Similar data have been reported for the most widely used experimental animal models of CLDs that have been instrumental in unequivocally documenting that angiogenesis and fibrogenesis develop in parallel during progression towards cirrhosis<sup>[31,37-43]</sup>.

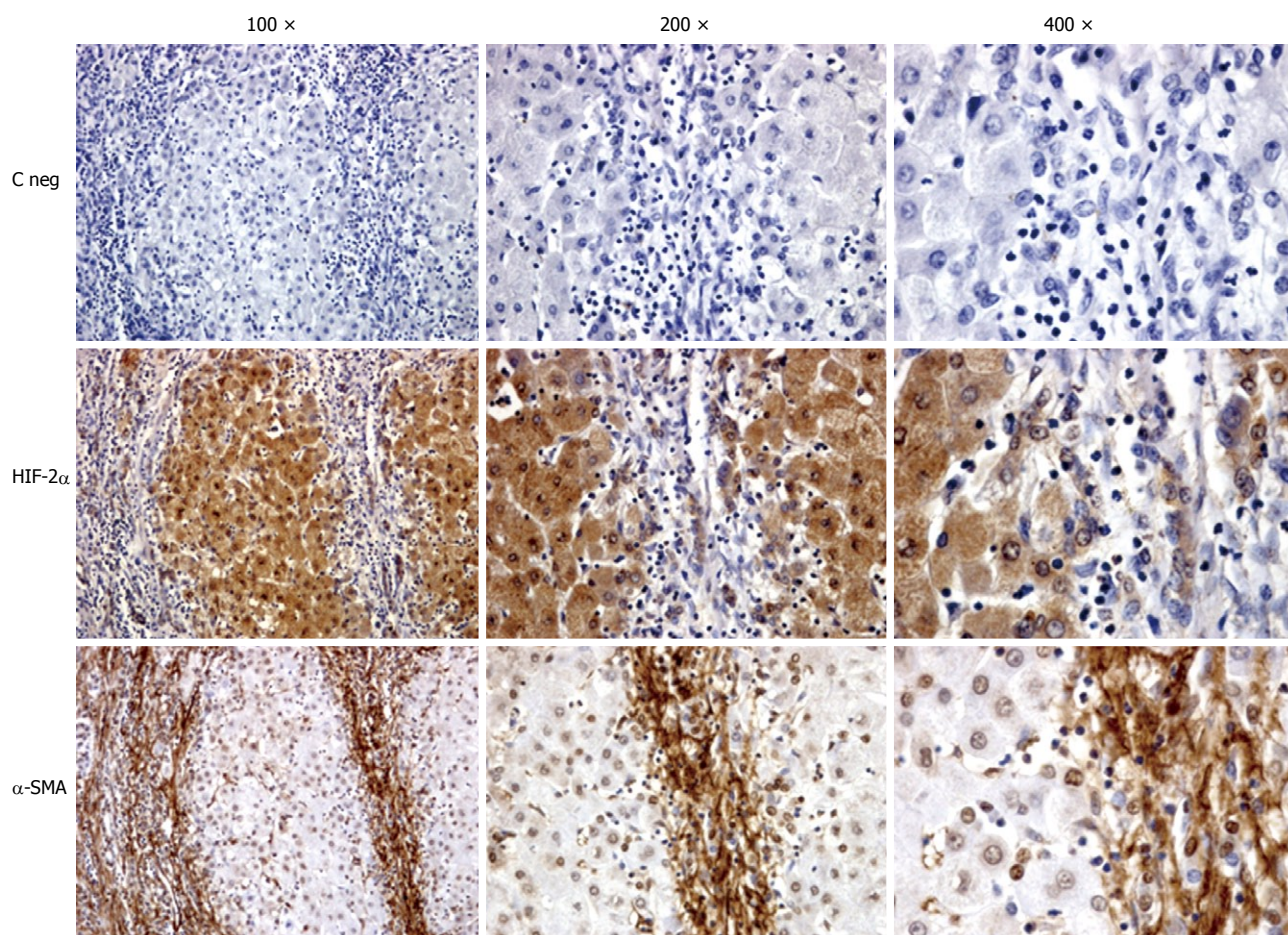
Both human and experimental studies have also outlined that several peptide mediators other than VEGF, Ang-1 and HGF are likely to be involved in hepatic angiogenesis associated with the fibrogenic progression process in CLDs. Unequivocal data have been provided for the pro-angiogenic action of PDGF<sup>[32]</sup> as well as for leptin, an adipocytokine that has been suggested to exert a pro-fibrogenic effect in promoting the development from non-alcoholic fatty liver disease (NAFLD) to non-alcoholic steatohepatitis (NASH)<sup>[40,44]</sup>. Where leptin is concerned, it has been shown that leptin can up-regulate

the expression of VEGF and Ang-1, as well as the pro-inflammatory chemokine monocyte chemoattractant protein 1 or MCP-1<sup>[44]</sup>. The role of angiogenesis in NASH development and fibrosis has been confirmed by a study performed on Zucker rats, animals that naturally develop leptin receptor mutations<sup>[40]</sup>, receiving the steatogenous choline-deficient and amino acid defined (CDAA) diet.

## **THE EMERGING ROLE OF HYPOXIA IN FIBROGENIC CLDs**

Another major message coming mainly from experimental models of CLDs is that VEGF overexpression is strictly associated with hypoxic areas and is mostly limited to hepatocytes as well as to HSC/MFs<sup>[31,37-43]</sup>. This concept fits well with the rational hypothesis that hypoxia is likely to represent the major stimulus for hepatic angiogenesis. The colocalization of hypoxic areas with VEGF overexpression and/or the association between VEGF expression and progression of fibrogenesis was first described in the model of bile duct ligation (BDL)<sup>[37]</sup> and then confirmed in the diethyl-nitrosamine (DEN) model of fibrosis<sup>[38]</sup>, in the model of chronic treatment with CCl<sub>4</sub><sup>[31,39,41]</sup> and in the choline-deficient and amino acid-defined diet rat model of NAFLD evolving into NASH and significant fibrosis<sup>[40]</sup>. Of relevance, a recent study, has outlined in a mechanistic way, the strict relationships between hypoxia, angiogenesis, inflammation and fibrogenesis by using liver conditional HIF-1 $\alpha$ -deficient mice that were subjected to the BDL model of fibrosis<sup>[43]</sup>. In this study, it was clearly shown that the appearance of HIF-1 $\alpha$ -positive hypoxic areas in the liver of BDL mice can take place as early as 3 d after surgery, before the development of detectable signs of fibrosis. In particular, within hypoxic areas HIF-1 $\alpha$  was found to be activated in hepatocytes and Kupffer cells. HIF-1 $\alpha$  -/- conditional mice subjected to BDL were characterized by a very significant decrease in collagen type I and  $\alpha$ -SMA transcripts and protein levels, as well as of transcripts for PDGF-A, PDGF-B, plasminogen activator inhibitor-1 (PAI-1) and fibroblast growth factor 2 (FGF-2) as compared to wild type mice in which the typical scenario of biliary-type fibrosis and cirrhosis was associated with early and sustained up-regulation of HIF-1 $\alpha$ <sup>[43]</sup>. The authors of this study proposed that hypoxic hepatocytes, following activation of HIF-1 $\alpha$  and through the HIF-1 $\alpha$ -dependent release of these growth factors and mediators, may significantly contribute to either initial repair and revascularization of injured parenchyma as well as to fibrosis progression by targeting profibrogenic MFs. In a more recent study from the same group, this hypothesis was further investigated by exposing to hypoxia cultured hepatocytes obtained from normal mice as well as from HIF-1 $\alpha$  or HIF-1 $\beta$  deficient mice<sup>[23]</sup>. The overall scenario which emerged from the latter study indicated that hypoxic hepatocytes, through the involvement of HIF-1 $\alpha$  and HIF-2 $\alpha$ , can express and release PAI-1, VEGF and the vasoactive peptides adrenomedullin-1 (ADM-1)





**Figure 1** Immunohistochemical analysis performed on paraffin liver sections from patients with hepatitis C virus (HCV)-related liver cirrhosis (METAVIR F4). Sections (2  $\mu$ m thick) were incubated with specific antibodies raised against HIF-2 $\alpha$  or  $\alpha$ -SMA that positively stain cells exposed to hypoxia (HIF-2 $\alpha$ ) or myofibroblast-like cells ( $\alpha$ -SMA). Primary antibodies were labelled by using EnVision, HRP-labelled System (DAKO) antibodies and visualized by 3'-diaminobenzidine substrate. Negative controls (C neg) were obtained by replacing the respective primary antibodies by isotype and concentrations matched irrelevant antibody. Original magnification is indicated.

and ADM-2<sup>[23]</sup>. Hepatocytes exposed to hypoxia during CLD progression are then reasonably a relevant source of vasoactive mediators as well as of the master pro-angiogenic cytokine VEGF. This unavoidably underlines the relevance of the cross-talk between hypoxic hepatocytes and surrounding non-parenchymal cells like sinusoidal endothelial cells (SECs) and, as we will see later in this editorial, activated MFs. Indeed, VEGF has a well known major pro-angiogenic role by increasing vascular permeability as well as triggering endothelial cell proliferation and regulating neo-vessel lumen diameter<sup>[1-8,14,19]</sup>.

Along these lines, it is worth mentioning that in the rat model of chronic administration of CCl<sub>4</sub>, hypoxic hepatocytes have also been shown to contribute to the expression of the master profibrogenic cytokine transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1)<sup>[45]</sup>. This contribution has been reported to be relevant mainly in an advanced stage of fibrosis or cirrhosis, whereas TGF- $\beta$ 1 is mainly produced by MFs and activated macrophages during early fibrosis and middle stage fibrogenic progression<sup>[45]</sup>.

A strict association between hypoxia and liver fibrogenesis can also be easily appreciated in the liver of human HCV cirrhotic patients by detecting HIF-2 $\alpha$  and  $\alpha$ -SMA

in serial sections (Figure 1). HIF-2 $\alpha$ -positive staining in these human specimens is usually detectable in hepatocytes of pseudolobules as well as in some MFs at the interface between fibrotic septa and liver parenchyma or even within pseudolobules. Moreover, it is also evident that  $\alpha$ -SMA-positive MFs are in close contact with HIF-2 $\alpha$  positive hepatocytes. Along these lines, it has been suggested that hypoxia may result in VEGF- and Angiopoietin 1-dependent increased migration and chemotaxis of HSC/MFs, contributing to their recruitment towards the site of injury and alignment with both nascent and established fibrotic septa, a relevant pro-fibrogenic feature<sup>[31]</sup>.

Hypoxia has also been suggested to result in the down-regulation of HGF expression by HSCs and in the inhibition of c-met expression by hepatocytes<sup>[46]</sup>, both events being able to significantly contribute to depressed liver regeneration during chronic liver injury.

## THE ROLE OF PROFIBROGENIC CELLS IN LIVER ANGIOGENESIS

Hepatic stellate cells, due to their strategic location in the space of Disse and intimate contact with sinusoidal ECs,

have been described to behave as liver specific pericytes and then to play a role in physiological angiogenesis<sup>[10]</sup>. Recent literature data suggest that HSC/MFs in CLDs are likely to represent a hypoxia-sensitive and cyto- and chemokine-modulated cellular crossroad between necro-inflammation, pathological angiogenesis and fibrogenesis. The latter statement is strongly suggested, in addition to exhaustive literature that has characterized the pro-fibrogenic and pro-inflammatory role of these cells, by a series of studies that outlined the following major concepts: (1) HSC and HSC/MFs can behave as pro-angiogenic cells able to react to conditions of hypoxia by up-regulating transcription and synthesis of VEGF, Ang-1 and their related receptors VEGFR-2 and Tie2<sup>[31,44,47,48]</sup>; the same behaviour has also been described for human HSC/MFs in which leptin was able to trigger a ERK1/2 and PI3-K-dependent nuclear translocation of HIF-1 $\alpha$ <sup>[44]</sup>; (2) HSC/MFs also represent a cellular “target” for the action of VEGF and Angiopoietin 1; VEGF has been reported to be able to trigger HSC/MFs proliferation<sup>[39,49,50]</sup>, increase deposition of ECM components<sup>[38,39,50]</sup>, as well as increase migration and chemotaxis<sup>[31]</sup>.

This is a scenario that is likely to be relevant in the progression of a CLD, as shown recently by *in vivo* morphological data obtained in human and rat fibrotic/cirrhotic livers<sup>[31]</sup>.  $\alpha$ -SMA-positive MFs, able to express concomitantly VEGF, Ang-1 or the related receptors VEGFR-2 and Tie-2, are found at the leading edge of tiny and incomplete developing septa, but not in larger bridging septa. This distribution may reflect the existence of two different phases of the angiogenic process during CLDs: an early phase, occurring in developing septa, in which fibrogenesis and angiogenesis may be driven/modulated by HSC/MFs, and a later phase occurring in larger and more mature fibrotic septa where the chronic wound healing is less active and fibrogenic transformation more established. In such a late setting, pro-angiogenic factors are expressed only by endothelial cells, a scenario that is likely to favour stabilization of the newly formed vessels.

A very recent and elegant experimental study<sup>[51]</sup> has outlined another putative pro-angiogenic mechanism, that may have a role in vascular remodelling in cirrhosis. This mechanism is related to the action of so-called microparticles Hedgehog (Hh) ligands, which are known to be released during embryogenesis and to activate Hh signalling in endothelial cells. In this study, the authors showed that cholangiocytes and HSC/MFs can produce and then release, mainly in response to PDGF, Hh ligands in microparticles and that this event is relevant under conditions leading to experimental biliary cirrhosis (BDL model). The authors propose the following scenario: (1) in normal conditions the action of the low amount of Hh ligand released by rare immature ductular-type progenitors is counteracted by expression of Hh interacting protein (HIP) expressed by either quiescent HSC or fenestrated SEC; (2) under conditions of chronic injury, HIP expression is repressed and activation of

ductular-type progenitor cells may result in PDGF-BB up-regulation and release; this, in turn, is likely to lead HSC/MFs and ductular cells to produce Hh ligands. Hh ligands, apart from promoting proliferation and survival of both cholangiocytes and HSC/MFs, may also promote changes in SEC gene expression resulting in capillarization of sinusoids and the release of vasoactive factors such as nitric oxide, then contributing to vascular remodelling in cirrhosis<sup>[51]</sup>.

## **PATHOLOGICAL ANGIOGENESIS AS A POTENTIAL THERAPEUTIC TARGET IN CLDs**

The analysis of the data and concepts presented in the previous sections, concerning the proposed relationships between angiogenesis, chronic wound healing and fibrogenesis and then disease progression, unavoidably leads to the following theoretical clinical goals: (1) detection of selected pro-angiogenic molecules (i.e. in serum or plasma) may serve as a non-invasive way to monitor both disease progression as well as the response to therapy; (2) anti-angiogenic therapy may be an effective tool for blocking or slowing down fibrogenic progression of CLDs.

We are indeed far from the first goal, and at present just a single study performed on 36 chronic HCV patients (*vs* 15 healthy controls) has tried to correlate circulating levels of molecules related to angiogenesis, disease progression and efficacy of standard pegylated interferon  $\alpha$ -2b (IFN- $\alpha$ 2b) plus ribavirin therapy. VEGF, Ang-2 and soluble Tie-2 (sTie-2) were determined in the serum before and after therapy and authors reported increased levels of VEGF and Ang-2 that were significantly decreased after therapy in these patients<sup>[52]</sup>.

Where the efficacy of the angiogenic therapy is concerned, experimental data unequivocally indicate that anti-angiogenic therapy is indeed effective in preventing progressive fibrogenesis. Pioneering studies employed anti-angiogenic molecules or drugs like the semi-synthetic analogue of fumagillin TNP-470<sup>[53]</sup> or antibodies able to neutralize either VEGFR-1 (Flt-1) and/or VEGFR-2 (Flk-1)<sup>[39]</sup>, both conditions being able to significantly inhibit angiogenesis, the number of  $\alpha$ -SMA-positive cells and the development of fibrosis. The latter study also showed the *in vivo* predominance of VEGF interaction with VEGFR-2 to mediate angiogenesis during chronic liver injury. Neutralizing antibodies against VEGFR-2 were employed in other relevant studies, performed on a model of portal hypertensive rats, where VEGF expression and related angiogenesis were correlated to the development of porto-systemic collateral vessels and hyperdynamic splanchnic circulation<sup>[54,55]</sup>. These data suggest that the increase in portal blood flow, which is an important contributor to portal hypertension, depends not only on vasodilation, but also on the enlargement of the splanchnic vascular tree caused by angiogenesis.

More recently, positive results have been obtained



in the chronic CCl<sub>4</sub> rat model of CLD by employing Sunitinib, a tyrosine kinase receptor inhibitor able to target VEGF and PDGF receptors<sup>[41]</sup>. The treatment of cirrhotic animals with Sunitinib resulted in a significant decrease in hepatic vascular density, inflammatory infiltrate, abundance of  $\alpha$ -SMA-positive mesenchymal cells, ECM deposition and even portal pressure.

Positive results have been also reported in another recent study in which mice undergoing BDL or chronic CCl<sub>4</sub> treatment received an adenovirus expressing soluble Tie-2 (AdsTie-2), the receptor for Ang-1, resulting in the blocking of Ang-1 signalling and in a significant prevention of both angiogenesis and fibrosis<sup>[42]</sup>.

A final and very recent experimental study reported a beneficial effect of Sorafenib<sup>[56]</sup>, a receptor tyrosine kinase inhibitor already approved for the treatment of hepatocellular carcinoma<sup>[57,58]</sup>, one of the most common complications of liver cirrhosis. In this study, oral administration of Sorafenib in rats with portal hypertension and cirrhosis (once a day for 2 wk) resulted in the inhibition of VEGF, PDGF and Raf kinase signalling; this, in turn, resulted in an approximately 80% decrease in splanchnic neovascularization and a very significant attenuation of hyperdynamic splanchnic and systemic circulations, as well as a significant decrease in the extent of portosystemic collaterals. Of relevance, Sorafenib treatment in cirrhotic rats also led to a 25% reduction in portal pressure and to a relevant improvement in liver injury, inflammation, fibrosis and angiogenesis.

The latter study has raised, in a more compelling way, the obvious question of whether anti-angiogenic therapy has an adequate rationale to be seriously considered for therapy in patients with cirrhosis and portal hypertension. As nicely pointed out by Shah and Bruix<sup>[59]</sup>, any future clinical trial employing Sorafenib in cirrhotic patients should assess a number of critical issues, the first being the optimal dosage to be used which may be theoretically lower than the dosage used in patients with hepatocellular carcinoma (HCC). The adverse effects of Sorafenib may represent a relevant concern: it is already known that more than 40% of HCC patients are forced to interrupt treatment<sup>[57,58]</sup>, and it has been shown that a major complication of angiogenic treatments employing Bevacizumab and Sunitinib in HCC patients is variceal bleeding<sup>[60,61]</sup>. Although available data indicate that Sorafenib may be relatively safe in this complication, caution is in any case necessary because it has been authoritatively suggested that an intense anti-angiogenic effect may lead to significant damage of the vasa vasorum needed to maintain the structure of varices<sup>[59]</sup>.

Another note of caution has recently been provided by an experimental study performed in order to assess the anti-fibrotic potential of the inhibition of the vitronectin receptor integrin  $\alpha$ v $\beta$ 3, which has been shown to both promote angiogenesis by mediating migration and proliferation of SECs as well as to drive fibrogenic activation of HSCs<sup>[62]</sup>. These authors employed the specific inhibitor of integrin  $\alpha$ v $\beta$ 3, Cilengitide which was administered orally in two different animal models of liver fibrosis, BDL and chronic administration of

thioacetamide (TAA). The relevant point was that this treatment was very effective in decreasing the overall formation of neo-vessels in both portal areas of BDL and septal areas of TAA fibrotic rats. Unfortunately, despite the anti-fibrogenic *in vitro* effect exerted by Cilengitide on cultured HSC/MFs, *in vivo* treatment with this inhibitor was associated in both models with a significant increase in liver collagen deposition and up-regulation of other profibrogenic genes and of matrix metalloproteinase-13, that is an overall worsening of liver fibrosis, with no relevant effect on inflammatory response<sup>[62]</sup>.

The use of anti-angiogenic drugs and, in particular, those drugs which have already been approved for the treatment of HCC, may then represent an attractive alternative therapeutic tool to prevent or significantly slow down fibrosis progression towards cirrhosis, which also represents the main risk factor for liver cancer development, as well as the development of portal hypertension and its complications. However, a tempered final message is that angiogenesis inhibitors should be used with caution and carefully balanced in these patients, bearing in mind that angiogenesis unavoidably is a relevant event for wound healing and excessive blocking of angiogenesis may not represent the desired therapeutic objective. Clinical trials with an appropriate design and primary end-points are needed.

## CONCLUSION

Hepatic angiogenesis has been unequivocally described in CLDs, irrespective of aetiology, and in the most reliable experimental models of liver fibrosis and cirrhosis. Angiogenesis and related changes in angio-architecture have been proposed to favour fibrogenic progression of the disease towards the end-point of cirrhosis. Moreover, in CLDs these changes are believed to be involved in the increase of vascular resistance and portal hypertension as well as in the decrease of parenchymal perfusion. At the same time, hepatic angiogenesis has been proposed to modulate the genesis of portal-systemic shunts and increase splanchnic blood flow, potentially affecting complications of cirrhosis. Hypoxia and HIFs-related cellular responses are emerging as crucial in the overall scenario of CLD progression. Several cellular and molecular mechanisms have been identified which regulate the cross-talk between angiogenesis and fibrogenesis as well as between the different hepatic cell populations. In this scenario a major role is played by hypoxic hepatocytes, sinusoidal endothelial cells as well as hepatic MFs. Where MFs are concerned, whatever their origin, they are currently believed to represent a crucial cellular cross-road at the intersection between inflammation, angiogenesis and fibrogenesis. Indeed, these profibrogenic and pro-inflammatory cells also represent a cellular target for the action of pro-angiogenic cytokines as well as an effective source of VEGF and Ang-1.

Finally, experimental anti-angiogenic therapy has proven to be very effective in limiting the fibrogenic progression of animal models of CLDs. The use of

anti-angiogenic drugs, particularly of those that have already been approved for HCC therapy like Sorafenib, may then represent a rationale therapeutic option to limit the progression of human CLDs towards cirrhosis and its complications, including the development of HCC.

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## Hepatic tight junctions: From viral entry to cancer metastasis

Nikki P Lee, John M Luk

Nikki P Lee, Department of Surgery, Queen Mary Hospital, Hong Kong, China

John M Luk, Department of Pharmacology and Department of Surgery, National University Health System, National University of Singapore, Singapore 117597, Singapore

**Author contributions:** Lee NP collected data, prepared the figures and wrote the paper; Luk JM initiated the review, wrote, commented on and revised the paper.

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**Correspondence to:** John M Luk, Professor, Department of Pharmacology and Department of Surgery, National University Health System, National University of Singapore, Singapore 117597, Singapore. [jmluk@nus.edu.sg](mailto:jmluk@nus.edu.sg)

Telephone: +65-65164516 Fax: +65-68737690

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### Abstract

The tight junction (TJ) is a critical cellular component for maintenance of tissue integrity, cellular interactions and cell-cell communications, and physiologically functions as the "great wall" against external agents and the surrounding hostile environment. During the host-pathogen evolution, viruses somehow found the key to unlock the gate for their entry into cells and to exploit and exhaust the host cells. In the liver, an array of TJ molecules is localized along the bile canaliculi forming the blood-biliary barrier, where they play pivotal roles in paracellular permeability, bile secretion, and cell polarity. In pathology, certain hepatic TJ molecules mediate virus entry causing hepatitis infection; deregulation and functional abnormality of the TJ have also been implicated in triggering liver cancer development and metastasis. All these findings shed new insights on the understanding of hepatic TJs in the development of liver disease and provide new clues for potential intervention.

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**Key words:** Tight junctions; Hepatocytes; Blood-biliary

### INTRODUCTION

The liver is the core metabolic center in the mammalian body and is responsible for major physiological functions such as carbohydrate/amino acid/fatty acid metabolism, bile secretion, and detoxification. The cellular composition of the liver comprises specialized cell types - hepatocytes and nonparenchymal cells. In a normal adult liver, hepatocytes are epithelial cells and alone account for 60%-70% of the total cell mass. Hepatocytes adopt a polarized architecture, resulting in the formation of numerous cell plates in the liver. Because of the highly vascularized nature of the liver, this hepatic parenchyma is infiltrated by an extensive microcirculatory network<sup>[1,2]</sup>. In order to maintain this anatomical organization, hepatocytes are equipped basically with a vast variety of junctions, such as anchoring junctions, tight junctions (TJs), and gap junctions (GJs)<sup>[3-5]</sup>. These junctions are situated at the surface of the hepatocytes so as to mediate cell-cell contact and communication. This editorial focuses on the hepatocyte TJs - as the "cements" of the building block and the "door" for entry of hepatitis viruses. Deregulation of TJ expression and function dismantles



the architecture of the hepatic parenchyma and causes liver diseases and cancer.

## TJ IN THE LIVER: FROM STRUCTURAL ARCHITECTURE TO SIGNALING NETWORK

In the liver, TJs can be found in 2 places, associating with either hepatocytes or bile duct epithelial cells (cholangiocytes). Those associated with the former cell type are alternatively called the blood-biliary barrier (BBB). Here we review the hepatocyte-associated TJ that concentrates at the specialized location surrounding the bile canaliculi. In addition to modulating paracellular passage of small molecules and ions, this BBB functions to keep bile in the bile canaliculi and apart from the blood circulation. TJ in the liver also segregates the apical surface from the basolateral surface of the hepatocytes, thereby maintaining cell polarity<sup>[4]</sup>. Having the same TJ components as in other epithelia and endothelia, the TJ in the liver is also composed of claudins, occludin, junctional adhesion molecules (JAMs), and others such as coxsackievirus and adenovirus receptor (CAR)<sup>[6-11]</sup>.

Claudins constitute the largest TJ family; 24 claudins have been found in mammals and at least 7 of these, namely claudin-1, -2, -3, -4, -5, -7, and -10, have been studied in the liver. Most claudins are small molecules having molecular weights of approximate 22-27 kDa. They are tetra-span molecules with the amino-terminus and carboxyl-terminus in the cell cytoplasm, and they possess 2 extracellular loops and one intracellular loop. For sealing the intercellular gap, claudin needs to interact with other claudins in the adjacent cell through its extracellular loops<sup>[12,13]</sup>. Occludin is the first studied TJ integral molecule. It has similar structural features to that of claudin, being a molecule with 4 transmembrane domains and utilizes a similar binding mechanism to that of claudin. However, it differs from claudin in its large molecular weight of 65 kDa<sup>[11,14]</sup>. Another variant of occludin, occludin 1B, has been identified and it differs from occludin in having an extended amino-terminus. Both of them are found in mouse livers<sup>[15]</sup>.

JAM is a TJ molecule which has gained much attention recently. It is a single-pass membrane protein with its amino-terminus in the extracellular region and carboxyl-terminus in the cytoplasm. As the component of a barrier, a single JAM molecule needs to couple with another JAM in an adjacent cell. At least 4 members of JAM have been identified and JAM-1, JAM-2, and JAM-3 have been found in mouse livers<sup>[10,11,16]</sup>.

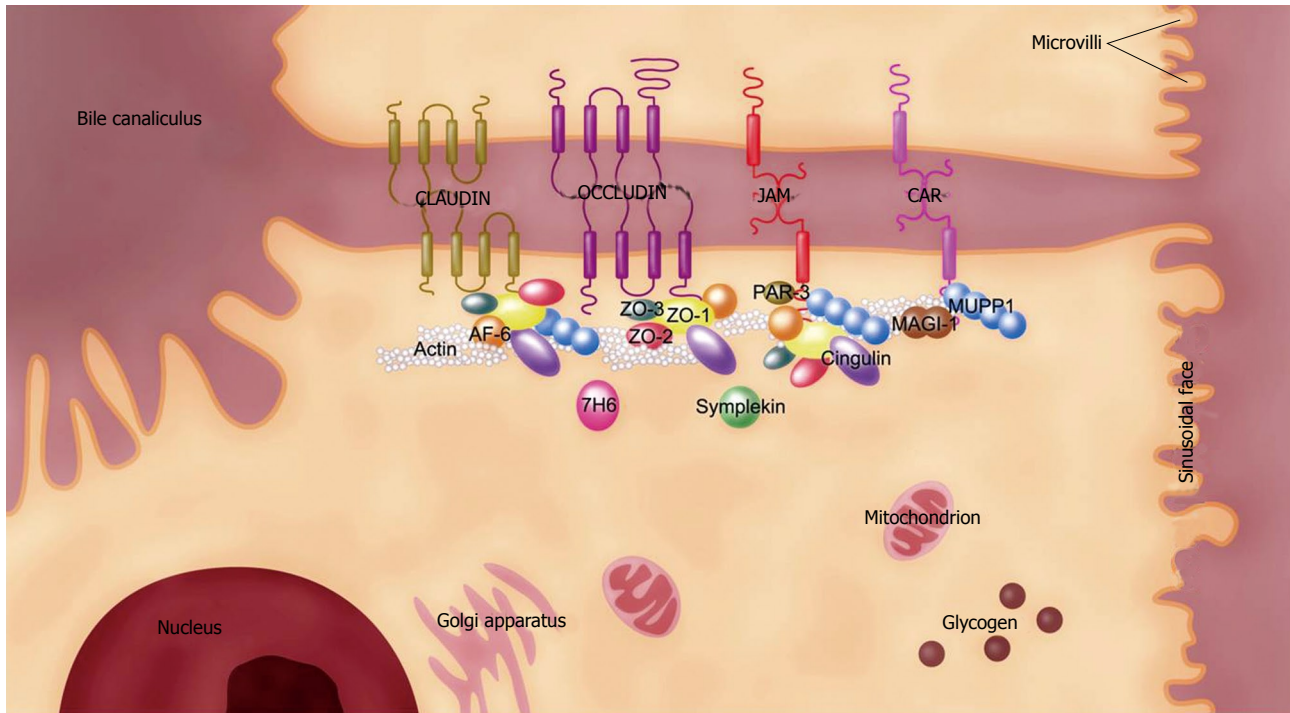
CAR was known originally as a new class of viral receptor, belonging to the immunoglobulin-like family and further studies demonstrated that CAR also had TJ functions<sup>[17]</sup>. Of the 3 isoforms of CAR (i.e. CAR-1, CAR-2, and CAR-3), only CAR-2 has been positively identified in hepatocytes and both CAR-1 and CAR-2 are associated with cholangiocytes in mouse livers<sup>[7]</sup>. As in other organs, these integral TJ proteins interact with

various scaffolding proteins to ensure structural integrity.

A plethora of adaptors and peripheral proteins are known to be present and associated with claudins, occludin, JAMs, and CARs. Adaptors such as zonula occludens-1 (ZO-1) act as a bridge linking the integral proteins to the underlying actin filament<sup>[18-20]</sup>. Cingulin, symplekin, and MAGI-1 (membrane-associated guanylate kinase inverted-1) are other peripheral proteins at TJs, and some of them have also been demonstrated in the liver<sup>[3,21-24]</sup>. By this way, a high degree of structural architecture is established at the TJ strand guarding the selective permeability barrier in the liver<sup>[25]</sup> (Figure 1).

Apart from its barrier functions, recent studies have also elucidated the other roles of the TJ as a core component in the signaling network, in particular for those junctional complexes concentrating at the BBB. Accumulating evidence suggests that the junction does not function alone on the plasma membrane, but different junctions can interact with each other either directly or indirectly. Studies performed in different systems demonstrated a disruption of one junction type could lead to loss or gain of function of another junction type, emphasizing the significance of inter-junctional crosstalk<sup>[26-28]</sup>. Epithelial cells, including hepatic cells, adopt this kind of junction-junction regulation. It is noted that enforced expression of connexin 32 into mouse hepatocytes derived from connexin 32-deficient mice results in TJ formation, accompanied by induced expression of occludin, claudin-1 and ZO-1, thereby leading to establishment of cell polarity<sup>[29]</sup>. In addition, using the same experiment setup induced the expressions of another junction protein MAGI-1 at the TJ in connexin 32 transfectants<sup>[30]</sup>. These findings unequivocally demonstrate the presence of a macrocomplex in the liver composed of at least a TJ and GJ<sup>[4]</sup>. Solid evidence from other studies also suggested the possible involvement of the TJ in manipulating other junctions such as the adherens junction (AJ). For instance, an abnormality of JAM-1 in hepatoma HepG2 cells induced the production of an AJ protein E-cadherin<sup>[31]</sup>. Our current understanding is that ZO-1 acts as the moderator in coordinating the cellular dynamics of various associated junctions and maintains the structural functionality of this multi-junctional network<sup>[32-34]</sup>.

Several cellular proteins, such as protein kinases and phosphatases, are some of the major regulators of junctions. Since most of them have numerous substrates, events of phosphorylation or dephosphorylation can modulate the status of components related to certain junctions. The p38 mitogen-activated protein kinase (MAPK) is a serine/threonine kinase that phosphorylates a handful of substrates including those associated with TJs<sup>[35]</sup>. Treatment with SB203580, a p38 MAPK inhibitor, led to strengthening of the TJ with a concomitant increase in claudin-1 in rat livers after partial hepatectomy<sup>[36]</sup>. Other events triggered by cytokines and growth factors are also involved in the regulation of TJ dynamics<sup>[37,38]</sup>. For instance, incubation of rodent hepatocytes with a multifunctional cytokine oncostatin M triggered the



**Figure 1 Molecular structure of the TJ in the mammalian liver.** The TJ in the liver is associated with hepatocytes and bile duct cells. TJ location around the bile canaliculus between 2 adjacent hepatocytes is shown. For simplicity, the molecular structure depicted in this figure represents the TJ molecules found in the mammalian liver. Claudin, occludin, JAM, and CAR are 4 core units for constituting TJ by uniting a panel of peripheral proteins like ZO-1 to form multiprotein complexes. TJ molecules display differential localizations in the mammalian liver, such that some of them like human symplekin and mouse CAR-2 are associated with both hepatocytes and bile duct cells while others, such as mouse CAR-1, are only found in the latter cell type. CAR: Coxsackievirus and adenovirus receptor; JAM: Junctional adhesion molecule; MAGI-1: Membrane-associated guanylate kinase inverted-1; MUPP1: Multiple PDZ domain protein-1; PAR-3: Partitioning defective 3 homolog; TJ: Tight junction; ZO: Zonula occludens.

production of claudin-2 and subsequently strengthened the TJ barrier<sup>[39]</sup>. Further, the transforming growth factor- $\beta$  (TGF- $\beta$ ) could reduce the production of claudin-1 and weaken the barrier function in rat hepatocytes<sup>[40]</sup>.

## TJ AND LIVER-RELATED DISEASES

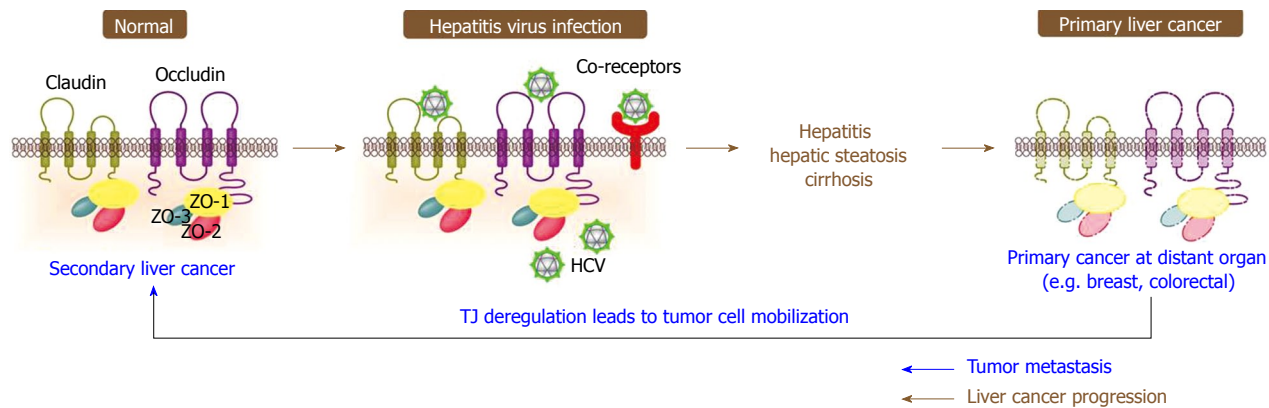
### Hepatitis

Hepatitis is an infection of the liver caused commonly by hepatitis viruses such as hepatitis B and C. At least 7 hepatitis viruses are known today and new species are being identified<sup>[41]</sup>. Hepatitis C virus (HCV) is the best studied for its ability to bind to TJ molecules on hepatocytes, and these molecules act as co-receptors for HCV entry<sup>[42]</sup>. The concept of junction proteins mediating viral entry is not restricted to the hepatitis virus, but is apparent for several other viruses including the adenovirus and coxsackievirus<sup>[43]</sup>. For the scenario of HCV infection, occludin and claudin-1 have been determined to be 2 key molecules for HCV entry<sup>[44,45]</sup>. Some reports also demonstrated other molecules including CD81 and human scavenger receptor class B member 1 (SR-BI) as co-receptors for HCV entry<sup>[46,47]</sup> and the expression levels of some of these receptors define the viral entry rate<sup>[48]</sup>. Cooperation between these receptors and TJ molecules is essential for viral entry into hepatocytes. Several studies provided further evidence indicating that the events occurred in the hepatocytes

after HCV infection. Hepatoma HuH-7 cells, having genomic replicons of HCV, could alter TJ dynamics, such that a disarrangement of TJ components was found and retention of occludin in the endoplasmic reticulum was noted<sup>[49]</sup>. Internalization of HCV is accompanied by an induced synthesis of fatty acid synthase, which is an enzyme responsible for fatty acid synthesis, and this event is associated with the production of claudin-1, but not CD81, in hepatoma HuH-7 cells<sup>[50]</sup>. This can partly explain why HCV infection frequently leads to steatosis, a fatty liver-related disease. In addition to fatty acid synthase, protein kinase A has an important role in HCV infection, since an aberration in protein kinase A function in hepatoma HuH-7 cells led to a disorganization of claudin-1 and reduced the infection susceptibility<sup>[51]</sup>. Together, these findings suggest an explicit role of TJ molecules, especially claudin-1 and occludin, in mediating HCV infection (Figure 2).

### Liver cancer

Liver cancer or hepatocellular carcinoma (HCC) is one of the most aggressive liver malignancies worldwide. The development of HCC is a complex process that is not totally established even after several decades of research. In most cases, HCC results from a pre-neoplastic inflammation of the diseased liver and is the end stage of a progressive worsening of liver conditions originating from hepatitis or cirrhotic livers, encompassing 3 phases



**Figure 2** The TJ at different stages of liver cancer progression and distant liver metastasis. During viral infection, HCV binds onto several TJ molecules (claudin-1 and occludin) and co-receptors (CD81 and SR-BI) on hepatocytes before its internalization. This event leads to hepatic steatosis and/or cirrhosis before the subsequent development of primary liver cancer that is associated with TJ deregulation. For metastatic liver cancer originating from the intrahepatic site or distant organs such as breast and colorectum, there is an initial loss of TJ molecules at the primary tumor site and a subsequent gain of these molecules in the liver with tumor cell colonization. HCV: Hepatitis C virus; SR-BI: Scavenger receptor class B member-I.

Table 1 Clinical significance of TJ molecules in human HCC			
Molecules	Expression in HCC <sup>1</sup>	Clinical correlations	Ref.
CAR	Reduced	Poor tumor differentiation	[64]
Claudin-1	Reduced	Poor tumor differentiation, tumor invasion, poor survival	[63]
Claudin-10	Higher	Tumor recurrence	[67]
Occludin	Reduced	-	[9]
Symplekin	Reduced	Poor tumor differentiation	[3]
ZO-1	Reduced	-	[9]

<sup>1</sup>Relative expression of TJ molecules in HCC tissues compared to normal or adjacent non-tumor tissues. TJ: Tight junction; HCC: Hepatocellular carcinoma; CAR: Coxsackievirus and adenovirus receptor.

of development - molecular, preclinical, and clinical<sup>[52]</sup>. At the molecular level, this malignant transformation of the liver is accompanied by a stepwise change in genetic and proteomic information, which can be readily revealed using laboratory technologies including gene microarray and gel- or non-gel-based proteomics profiling, coupled with mass spectrometry<sup>[53-55]</sup>. As a result of rapid developments in molecular and profiling techniques, the gene, protein, and microRNA data related to HCC are gradually being decoded<sup>[56]</sup>. A handful of molecules, such as heat shock proteins and cadherins, and different pathways, such as Wnt and TGF- $\beta$  pathways, have been determined to be HCC-related<sup>[56-60]</sup>. The newly derived information assists the construction of the molecular network of HCC and enhances our knowledge of this cancer.

**Primary tumors in the liver**

Tumor nodules originating in the liver are generally termed as primary tumors. During hepatocarcinogenesis, the liver usually undergoes several phases of transition from pre-neoplasia, dysplasia, to neoplasia<sup>[61]</sup>. Several TJ molecules are regarded as HCC biomarkers<sup>[55,62]</sup>. An endogenous expression of certain claudins is found in the normal adult liver and their expression is attenuated when HCC develops. It is noted that claudin-1 has

reduced expression in cancerous liver when compared to its healthy counterpart<sup>[63]</sup>. A general reduction in the levels of occludin, ZO-1, and CAR has been found in HCC when compared to normal liver<sup>[9,64]</sup>. Also, there is a gradual decrease in the level of 7H6 TJ-associated antigen in rats during hepatocarcinogenesis<sup>[65]</sup>. Apart from these molecules, other TJ molecules, such as JAM and cingulin, are present in hepatic cells<sup>[31,66]</sup>, but not all of these are associated with HCC. Those claudins with a high expression in normal liver have a role in liver physiology by maintaining a functional TJ barrier, whereas those claudins with elevated expression in the cancerous liver are likely to be involved in tumor formation. Sometimes these biomarkers not only indicate the advent of tumors, but can also be prognostic in nature (Table 1). A loss of claudin-1 expression in resected HCC indicates poor differentiation and high invasiveness of the tumor, and is associated with poor outcomes of patients<sup>[63]</sup>. Similarly for other TJ molecules, a reduced CAR expression in resected livers is correlated with poor differentiation of HCC<sup>[64]</sup>. However, Cheung *et al*<sup>[67]</sup> linked the high expression of claudin-10 in HCC with the high incidence of postoperative tumor recurrence in patients.

With regard to biological significance, these biomarkers usually demonstrate gain- or loss-of-function in HCC. For instance, the expression of claudin-1 is associated preferentially with the fetal cell type of human hepatoblastoma, but not the highly proliferating embryonal cell type, with expression of proliferating cell nuclear antigen (PCNA) and Ki-67, suggesting its expression is negatively correlated with rapid cell growth and division<sup>[68]</sup>. This anti-proliferative behavior of hepatic claudin-1 is further supported by a study showing a loss of claudin-1 associated with tumor aggressiveness<sup>[63]</sup>. An overexpression of claudin-10 is linked to poor outcome of HCC patients after hepatic resection. To prove the tumorigenic features of this molecule, an overexpression experiment was performed in claudin-10-deficient Hep3B hepatoma cells and an induction of tumor phenotypes was observed. In the reciprocal experiment



using RNA interference (RNAi) to silence claudin-10 in HLE hepatoma cells with a high level of claudin-10, an alleviation of tumorigenic potential accompanied by reduced cell invasion was found<sup>[69]</sup>. By these approaches, molecules can be studied for their tumorigenic properties. Besides their intrinsic tumorigenic properties being the subject of intense interest in research, TJ molecules have also been studied with regard to their associated pathways, some of which have been unfolded successfully. Borlak *et al.*<sup>[70]</sup> utilized an epidermal growth factor-induced HCC mouse model showing a positive effect of this growth factor in inducing claudin-7 in small HCCs. Also, vascular endothelial growth factor (VEGF)-treated HepG2 hepatoma cells had disruptive TJs accompanied by reduced occludin expression, suggesting VEGF as one factor triggering the spread of tumor cells into the normal liver parenchyma<sup>[71]</sup>. All these findings implicate the direct involvement of TJ molecules in the presentation of tumor phenotypes and the tumor-related signaling pathways, suggesting their interference may counteract the process of HCC. Therefore, TJ molecules may be another class of therapeutic target for HCC.

### Metastatic tumors in liver

Metastatic tumors initiated elsewhere in the body may spread to and colonize the liver. Angiogenesis is a prerequisite process for tumor metastasis, enabling the migration of tumor cells through the circulatory system of the body from one site to the other<sup>[72]</sup>. A number of factors such as growth factors and chemokines are important in triggering this event<sup>[73,74]</sup>. For tumor cells to metastasize, loss of TJ function is usually observed in cancer cells prior to this process<sup>[75]</sup>. Of the TJ molecules, claudin-7, ZO-1, and other emerging ones are associated with tumor metastasis. Thus, a decrease of claudin-7 in tumor tissues is associated with tumor metastasis in patients with breast carcinoma<sup>[76]</sup>. This finding is further validated by a separate gene microarray study, in which breast cancer metastasis to the liver is associated with a reduced expression of a panel of TJ molecules including claudin-4 and ZO-1, in addition to claudin-7<sup>[77]</sup>. For lung cancer, overexpression of claudin-1 in human lung adenocarcinoma cells reduced the metastatic potentials of tumor cells<sup>[78]</sup>. Apart from these 2 cancer types, elimination of claudin-7 is frequently observed in colorectal cancer, and is clinically related to the event of tumor cell invasion into the blood circulation and the eventual development of tumor masses in the liver<sup>[79]</sup>. In addition, malfunction of ZO-1 is observed after its phosphorylation, which also induces the migration of colorectal tumor cells into the liver<sup>[80]</sup>. Interestingly, there is a restoration of the expression of TJ molecules in tumor cells after metastasis to the liver from a distant organ. It is noted that a re-expression of ZO-1 is observed in colorectal cancer cells after metastasis to the liver when compared to those developing metastatic potential at the primary cancer site<sup>[80]</sup>. Similar re-expression of claudins such as claudin-1 and claudin-4 in liver-residing colorectal cancer cells is reported in another study examining colorectal tumor

metastasis<sup>[81]</sup>. Therefore, it is clear that loss of TJ function is a key factor triggering the induction of metastatic potential in tumor cells to other sites including the liver, while a restoration of its function is needed for tumor cells to colonize in the liver.

## CONCLUSION

In the liver, TJ is found to be associated with bile duct cells and hepatocytes. As hepatocytes are the most predominant cell type in the liver and the most studied in liver diseases, we focused our discussion on the role of hepatocyte-associated TJ molecules in hepatitis infection and liver cancer. TJs found in hepatocytes are also known as the BBB, keeping the bile in the bile canaliculi away from the blood circulation. Emerging evidence has further demonstrated the direct involvement of TJ molecules as co-receptors for HCV. On the other hand, accumulating evidence support the notion that deregulation of TJ molecules is frequently associated with increased incidence of HCC and poor prognosis of patients, signifying their putative use as biomarkers for diagnosis and prognosis of this liver malignancy. Molecules with induced expression in HCC are predicted to be tumor-inducing, while those with reduced expression are likely to be anti-tumor molecules. Proof-of-principle studies by means of RNAi or overexpression should enhance our knowledge of the roles of specific TJ molecules in liver diseases. Based on previous findings of TJ molecules as viral receptors, it is highly possible that a blocking peptide or antibody can be developed to prevent the binding of the viral particles to the TJ receptors on hepatocytes. This can open a new study area for therapeutic targeting of the TJ. Those TJ molecules with tumor-inducing properties are potential therapeutic targets for HCC. More in-depth studies should be performed to find antagonists to inhibit the functions of these molecules or to block their specific signaling pathways. With regard to advancement of HCC diagnosis, novel TJ molecules may be useful as HCC biomarkers. Further studies should be performed to investigate whether these biomarkers are eligible as supplemental criteria to diagnose HCC in patients with low levels of serum alpha-fetoprotein who constitute up to one-third of HCC cases. Therefore, study of the TJ in the liver can increase the clinical usefulness of this molecule in liver diseases.

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Hugh James Freeman, MD, CM, FRCPC, FACP, Series Editor

## Update on collagenous sprue

Hugh James Freeman

Hugh James Freeman, Department of Medicine, University of British Columbia Hospital, 2211 Wesbrook Mall, Vancouver, BC, V6T 1W5, Canada

Author contributions: Freeman HJ contributed all to this paper. Correspondence to: Dr. Hugh James Freeman, MD, CM, FRCPC, FACP, Department of Medicine, University of British Columbia Hospital, 2211 Wesbrook Mall, Vancouver, BC, V6T 1W5, Canada. [hugfree@shaw.ca](mailto:hugfree@shaw.ca)

Telephone: +1-604-8227216 Fax: +1-604-8227236

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### Abstract

Collagenous sprue has traditionally been defined as a small intestinal mucosal disorder characterized by persistent diarrhea, severe malabsorption with multiple nutrient deficiencies and progressive weight loss. Pathologically, a severe to variably severe "flattened" mucosal biopsy lesion with distinctive sub-epithelial deposits in the lamina propria region is detected. Histochemical stains and ultrastructural studies have confirmed that these deposits contain collagens. Often, an initial diagnosis of celiac disease is considered but no continued response to treatment with a gluten-free diet occurs. Recent reports indicate an intimate relationship between collagenous sprue and celiac disease, sometimes with concomitant T-cell enteropathy. In addition, permanent disappearance of these deposits after resection of a localized colon cancer suggested that this disorder could actually represent a paraneoplastic morphologic marker of an occult malignancy. Studies showing either gastric or colonic involvement (or both) with this unusual collagenous inflammatory mucosal process may also reflect a far more extensive and heterogeneous process than previously appreciated.

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**Key words:** Collagenous sprue; Celiac disease; Lymphoma; Paraneoplastic disease

### INTRODUCTION

In 1970, Weinstein *et al*<sup>[1]</sup> reported on a small intestinal mucosal biopsy lesion from a 51-year-old female initially thought to have celiac disease as histopathological changes included severely flattened villi. However, a long-term response to a gluten-free diet failed to develop. Subsequently, routine hematoxylin-eosin stained biopsies showed a prominent band-like deposit of sub-epithelial hyaline material in the lamina propria region of the small bowel. The deposit had the histochemical features of collagen and ultrastructural studies confirmed an electron-dense material with the typical 640 Å axial periodicity of collagen fibers. Her symptoms transiently improved with corticosteroids, but she then developed worsening diarrhea, severe malabsorption and progressive weight loss. Post-mortem examination showed very extensive pathologic changes in the proximal small intestine with sub-epithelial eosinophilic hyaline deposits of varying thickness. Short segments of normal mucosa were present in the distal small intestine. Two earlier reports by Schein<sup>[2]</sup> in 1947 and Hourihane<sup>[3]</sup> in 1963 may have reflected the same biopsy lesions (although in the latter, ileal involvement was also present).

Collagenous sprue was thought to be a "new" form of malabsorptive disorder with the specific clinical and pathological features: (1) persistent diarrhea with pan-malabsorption causing nutrient deficiencies and progressive weight loss; (2) a biopsy lesion included a unique



morphologic marker, a sub-epithelial band-like deposit with histochemical and ultrastructural features of collagen; (3) other pathologic changes of untreated celiac disease were present, but not responsive to a gluten-free diet; and (4) diffuse and patchy mucosal changes of variable severity, localized mainly in the proximal small intestine.

## OTHER CAUSES OF SEVERE “FLAT” BIOPSY LESION

Traditionally, the diagnosis of celiac disease (or gluten-sensitive enteropathy) has been established pathologically and depended on two sequential criteria: first, documentation of the typical histopathologic features of untreated disease in small bowel biopsies, and, second, a response to a gluten-free diet. Otherwise, celiac disease, even if present, cannot be diagnosed with certainty. In some cases, a “flattened” biopsy appearance may be present, but a gluten-free diet response has not been documented. This may require months to years<sup>[4]</sup>. Some investigators have loosely labeled these cases as refractory celiac disease, but this label should be reserved for those who show an initial (and documented) response to a gluten-free diet followed by later development of recurrent symptoms and biopsy changes. The most commonly reported causes for recurrent symptoms and biopsy changes include poor dietary compliance or inadvertent ingestion of a ubiquitous gluten-containing food (e.g. pill capsules, communion wafers). In these cases, removal of the offending gluten should be sufficient to resolve symptoms and biopsy changes. A second or superimposed cause (e.g. infection, folate or zinc deficiency) could also develop. In addition, another entirely separate cause for a “flat” biopsy lesion could be present<sup>[5]</sup>, as the initial true diagnosis (e.g. Crohn’s disease in duodenum without mucosal granulomas) may have been missed<sup>[6]</sup> or an associated or complicating disease (e.g. collagenous colitis, lymphoma) could have developed. In these patients, symptoms and biopsy changes may be improved with specific treatment, but not with a gluten-free diet. Finally, another “wastebasket” group with a “flat” biopsy appearance that has never been responsive to a gluten-free diet may be present, so-called sprue-like intestinal disease or unclassified sprue<sup>[7]</sup>.

## RELATIONSHIP WITH CELIAC DISEASE

Collagenous sprue has a “flat” biopsy appearance, like untreated celiac disease, but fails to show a persistent response to a gluten-free diet. In addition, collagenous sprue is characterized by the appearance of distinctive subepithelial collagen deposits. Some believed that this histopathological change might simply represent a prognostic pathologic marker for a poor outcome in celiac disease<sup>[8]</sup>. Others, however, viewed collagenous sprue as a new and previously unrecognized small bowel disorder<sup>[9]</sup>. Later reports have also described

further elements between celiac disease and collagenous sprue. Common clinical features include hyposplenism and positive endomysial antibodies that have been documented in both entities<sup>[10]</sup>. In collagenous sprue, similar complications recorded in celiac disease may also occur, including both T-cell and B-cell lymphomas<sup>[11,12]</sup>.

## NATURAL HISTORY AND LOCALIZATION

Collagen deposits may also be present in the colon (i.e. collagenous colitis) or even stomach (i.e. collagenous gastritis)<sup>[13]</sup>. An associated inflammatory process in either colonic or gastric mucosa, or both, is also present, usually with epithelial lymphocytosis. Interestingly, collagenous or lymphocytic colitis as well as collagenous or lymphocytic gastritis are all associated with biopsy-defined celiac disease<sup>[13-15]</sup>. These pathological changes also suggest that a far more extensive pathologic process may occur elsewhere in the gastrointestinal tract with collagenous sprue.

Previously published reports noted that the natural history of collagenous sprue was characterized by worsening malabsorption with an inevitably fatal outcome. In most patients, diarrhea and progressive weight loss occurred, and rarely, severe abdominal pain, sometimes with an associated vasculitis, was recorded<sup>[16]</sup>. However, more recently, independent reports with extensive biopsy studies have documented complete resolution of the lesion for prolonged periods after corticosteroid treatment<sup>[17,18]</sup> suggesting that the lesion may be reversed, at least temporarily, for extended periods, even years. Immunosuppressants have also been used in some cases.

## DISEASE HETEROGENEITY

The etiology and pathogenesis of these collagenous deposits are not known, however, different causes could be responsible. In addition to celiac disease, collagenous sprue has not only been complicated with T-cell lymphoma<sup>[12]</sup>, but associated with its co-occurrence<sup>[19]</sup>. Finally, collagen deposits in both small and large intestines were detected with an apparently coincidental, but localized, colon cancer<sup>[20]</sup>. Later, clinical and histopathological changes were resolved after the cancer was resected, suggesting that these collagen deposits could represent a paraneoplastic morphologic marker of occult malignant disease.

## FUTURE DIRECTIONS

Recent reports suggest that collagenous sprue may be more heterogeneous than previously appreciated. This has been reflected in frequently associated, but variable collagenous mucosal inflammatory changes elsewhere in the gastrointestinal tract, differential responses to treatment, particularly with steroids, and its association with other conditions, including malignant disease as a possible paraneoplastic morphologic marker.



Treatment of this condition remains an empirical exercise. Steroids and/or immunosuppressant agents have been used, but, to date, this approach has resulted in only rare success.

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## Pulmonary involvement and allergic disorders in inflammatory bowel disease

Nikolaos E Tzanakis, Ioanna G Tsiligianni, Nikolaos M Siafakas

Nikolaos E Tzanakis, Department of Social Medicine & Thoracic Medicine, Medical School University of Crete, Crete 71110, Greece

Ioanna G Tsiligianni, General Practitioner, Agia Barvara Health Care Center, Heraklion, Crete 70003, Greece

Nikolaos M Siafakas, Department of Thoracic Medicine, Medical School University of Crete, Crete 71110, Greece

**Author contributions:** Tzanakis NE designed the study; Tzanakis NE and Tsiligianni IG performed the search strategy for identification of studies and wrote the article; Siafakas NM reviewed the article and gave relevant suggestions.

**Correspondence to:** Ioanna G Tsiligianni, MD, PhD, MPH, General Practitioner, Agia Barbara Health Care Centre, Heraklion, Crete 70003, Greece. [pdkapa@yahoo.gr](mailto:pdkapa@yahoo.gr)

**Telephone:** +30-2810-392855 **Fax:** +30-2810-542650

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Health Sciences, Avd del Conocimiento s/n, 18100, Armilla, Granada, Spain

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### INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are the two major forms of chronic relapsing inflammatory bowel disease (IBD). Apart from overlapping epidemiological, clinical, radiological, endoscopic and histological characteristics<sup>[1,2]</sup> between UC and CD, there are clear differences in the extent of inflammation in the gastrointestinal tract and in several immunological parameters<sup>[3-5]</sup> suggesting that they are distinct disease processes. The pathogenesis of IBD seems to be more complex than one single cause and probably involves an interaction between genetic predisposing factors<sup>[6-8]</sup>, exogenous and endogenous triggers<sup>[3,9-14]</sup>, and modifying factors<sup>[3,15,16]</sup>. The outcome of these interactions is a spontaneously relapsing and remitting inflammatory process in intestinal mucosa associated with recruitment and activation of lymphocytes, macrophages and other inflammatory cells<sup>[3,17-20]</sup>.

Extraintestinal and systemic manifestations occur frequently in patients with IBD<sup>[20-25]</sup>. These various disease states can be diagnosed before, concomitant with, or after the diagnosis of a specific type of IBD. Two large case studies have demonstrated that between 25% and 36% of patients with either type of idiopathic IBD will have at least one such associated disease<sup>[22-23]</sup>. Yamamoto-Furusho *et al*<sup>[8]</sup> found that extraintestinal manifestations were present in 41.5% of 848 cases with UC. More than 100 systemic complications involving almost every organ system in the body have been described<sup>[26]</sup>. The

### Abstract

Inflammatory bowel disease (IBD) has been associated with either clinical or subclinical airway and parenchymal lung involvement and interstitial lung complications. Several studies have reported that atopy has a high prevalence in IBD patients. Overlapping allergic disorders seem to be present in both the respiratory and gastrointestinal systems. The purpose of this review is to update clinicians on recent available literature and to discuss the need for a highly suspicious approach by clinicians.

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**Key words:** Atopy; Inflammatory bowel disease; Pulmonary involvement

**Peer reviewers:** Dr. Zoran Krivokapic, Professor, MD, FRCS, Institute for Digestive Disease, First Surgical Clinic, Clinical Center of Serbia, 6, Dr Koste Todorovica, Belgrade, 11000, Serbia; Javier Martin, MD, PhD, Department of Immunology, Institute of Parasitology and Biomedicine López-Neyra, P.T.

spectrum, the frequency and the temporal relation of the complications have led to the hypothesis that IBD is a systemic disorder<sup>[22-23,26]</sup>.

IBD is associated with a wide variety of extraintestinal lesions in many organs and over some decades the pattern has changed and the lung is regarded as one of these affected organs<sup>[21,26-31]</sup>. In recent years a few hundred cases with pulmonary involvement and IBD have been reported and in that way pulmonary involvement has been proved to be common. A growing number of studies in the literature have reported either clinical or latent pulmonary involvement in patients with IBD<sup>[29,31-34]</sup>. In this review we will focus on the extraintestinal manifestations that are associated with lungs and airways. In an attempt to classify the reported lung manifestations in patients with IBD in a more useful way, the manifestations were distinguished as follows: (1) involvement of airways; (2) pulmonary function testing abnormalities; and (3) diffuse or localized interstitial lung complications caused by either disease or treatment received. Finally, studies regarding the relationship between allergy and IBD will be discussed in detail.

## IBD AND AIRWAYS

The manifestations of IBD in airways include, chronic bronchial suppuration particularly in patients with UC<sup>[31,34-36]</sup>, bronchiectasis<sup>[32,37-40]</sup>, localized obstruction of upper airways<sup>[41]</sup>, bronchiolitis obliterans organizing pneumonia<sup>[32,42,43]</sup>, granulomatous bronchiolitis<sup>[44]</sup>, tracheobronchitis<sup>[45,46]</sup>, bronchiolitis obliterans<sup>[47]</sup>, tracheobronchial stenosis<sup>[48]</sup> and diffuse obstructive disease<sup>[34,49]</sup>. Obstructive disease was not confirmed in some studies<sup>[50]</sup>. An increased risk of both UC and CD in chronic obstructive pulmonary disease (COPD) patients has been reported in some studies, focusing attention on the association between airway diseases (AD) and IBD<sup>[51]</sup>. We have reported a small airway dysfunction, detected by density dependence methods, in patients with IBD<sup>[52]</sup>. In the study by Louis *et al*<sup>[53]</sup>, patients with IBD, free of pulmonary symptoms, independently of the presence of atopy, showed bronchial hyperresponsiveness. This interesting finding could lead to the hypothesis that local mucosal inflammation in the intestine is responsible for the mild airway inflammation and not atopy. This hypothesis is not new. Basal cell hyperplasia, membrane thickening and submucosal inflammation have been reported in patients with UC and bronchial suppuration<sup>[36]</sup>. CD may affect the oral cavity and the colon<sup>[26,54,55]</sup> while both UC<sup>[34,41]</sup> and CD<sup>[56]</sup> have been reported to involve the larynx. There are also some morphological and developmental similarities between colonic and bronchial epithelium. Both are derived from the primitive gut, whereas the lungs arise from the laryngo-tracheal bud. Both are composed of columnar epithelium with goblet cells and submucosal mucous glands. Furthermore, there is increasing evidence that an immune system specific to the gastrointestinal tract common to all mucosal surfaces exists<sup>[57]</sup>, in which lymphocytes are sensitized to antigens at one mucosal site and by circulation are localized and produce inflammation in other mucosal surfaces<sup>[53,58-61]</sup>.

## PULMONARY FUNCTION TESTING ABNORMALITIES

Previous reports concerning pulmonary function abnormalities in patients with IBD are conflicting. In some studies no differences in pulmonary function tests (PFTs) between patients with IBD and the control group were found<sup>[49,62]</sup>. In the study by Neilly *et al*<sup>[49]</sup> airway obstruction was the most common finding affecting patients with CD (45%). However, the indices of airway obstruction were not significantly different from those obtained in age-, sex- and smoking-matched controls. As discussed above, Louis *et al*<sup>[53]</sup> reported an increased bronchial responsiveness in IBD patients, while the baseline lung function tests were within the normal range. In the study by Mohamed-Hussein *et al*<sup>[63]</sup>, fifteen out of 26 patients with UC had an important impairment in PFTs. In the study by Herrlinger *et al*<sup>[64]</sup>, the impairment in PFTs was more pronounced in IBD patients with active disease than in those with inactive disease.

Pulmonary diffusion capacity (TLCO) is often impaired in IBD patients. Heatley *et al*<sup>[65]</sup> found an increased prevalence of TLCO impairment in 25% of patients with CD. Reduction of TLCO in patients with IBD has been reported in various studies<sup>[61,64,66-69]</sup>. Eade *et al*<sup>[66]</sup> and Bonniere *et al*<sup>[59]</sup> found that the reduced TLCO or other PFTs parameters were not correlated with the location and severity of IBD or with the concurrent medication mode<sup>[59,66]</sup>. We examined 132 patients, 47 (17 female, 30 male) with CD and 85 (35 female, 50 male) mean age 40 years with UC. The main finding of our study was a high prevalence of impaired TLCO in patients with CD and UC suggesting involvement of the lung parenchyma<sup>[70]</sup>. All other PFTs parameters were abnormal in a high percentage of patients, however, they did not show statistically significant differences from those in the control group. Our data suggest that the impairment of TLCO was statistically significantly higher in patients with exacerbation of disease than in remission<sup>[70]</sup>. This finding is in accordance with other studies<sup>[61,65,68-70]</sup> which reported a higher prevalence of impaired TLCO among patients with active IBD disease as compared to patients in remission. In contrast, Douglas *et al*<sup>[71]</sup> reported a reduced gas transfer factor in 16% of 44 patients with IBD but these abnormalities were not related to disease activity. The reduction in gas transfer factor indicates damage to lung parenchyma. The nature of this lung involvement remains debatable. However, some explanations will be discussed in the following section concerning the relationship between IBD and interstitial lung complications.

## INTERSTITIAL LUNG COMPLICATIONS

Interstitial lung involvement has been reported to accompany both clinical IBD entities, UC<sup>[72-78]</sup> and CD<sup>[79-83]</sup>. The interstitial lung infiltrates have been proven histologically to be either pulmonary vasculitis<sup>[76,77,84]</sup> or more often granulomatous disease<sup>[74,79,80,82,83,85,86]</sup>.

Table 1 Studies on the relationship between IBD and atopic features

Group	Protocol	Atopy history	Skin prick tests	IgE	Ref.
CD: 11 UC: 19 Normals: 16	Skin prick tests SIgE	Allergic symptoms were more prevalent in IBD <i>vs</i> controls $P < 0.007$ (in UC $P < 0.004$ )	IBD <i>vs</i> control $P < 0.02$	No statistically significant differences	[109]
UC: 14 CD: 20 Controls: 72	Atopy history IgA, IgG, IgM IgE	-	-	Increased IgG, IgM and IgE in patients <i>vs</i> controls ( $P < 0.01$ )	[114]
UC: 300 CD: 200 Controls: 254	Questionnaire	Asthma, hay fever, allergic rhinitis; UC <i>vs</i> Controls: $P < 0.02$ ; CD <i>vs</i> Controls: NS; Eczema-Any atopy-Family history; Both UC & CD <i>vs</i> controls: $P < 0.001$	-	-	[117]
UC: 39 CD: 35 Healthy: 37	Skin prick tests to various common allergens	23.1% in UC; 22.9% in CD; 21.4% in disease controls; 20% among healthy subjects	14/39 in UC and 12/35 in CD in food allergens ( $P < 0.001$ )	No differences	[119]
UC: 39 CD: 19 Normals: 20	Skin prick tests to milk proteins	Positive: 15.7% of UC and 13.3% of CD; Significant difference between patients and healthy subjects	No differences	No differences	[120]
UC: 63 CD: 59 Controls: 103	Skin prick tests to various common allergens	No difference between patients and healthy subjects	No difference between patients and healthy subjects	No difference between patients and healthy subjects	[121]
CD: 308 Normals: 930	Questionnaire	Atopic disease was more common in CD <i>vs</i> normal ( $P = 0.001$ ); Atopic eczema was twice as common in CD <i>vs</i> normal ( $P = 0.001$ )	-	-	[124]
UC: 50 Healthy: 50	Skin prick/patch tests to airborne, food, contact allergens SIgE Atopy history and family history	Allergic symptoms were more prevalent in UC and first degree relatives than in controls ( $P < 0.0001$ , $P = 0.008$ )	UC <i>vs</i> controls; Immediate type hypersensitivity $P = 0.01$ ; Delayed type hypersensitivity $P = 0.03$	IgE levels were higher in UC than in controls $P = 0.02$	[125]

IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis.

Treatment with corticosteroids<sup>[75,78]</sup> or with appropriate medication such as sulfasalazine or mesalamine for the basic gastrointestinal disease<sup>[74]</sup> appeared to be satisfactory for both diseases. Pneumonitis, in contrast, due either to sulphasalazine or mesalamine is a well-recognized adverse drug reaction in these patients<sup>[50,87-95]</sup>.

The above observations and the histological similarities between CD and sarcoidosis in particular, have led several groups to investigate the number and types of cells recovered by bronchoalveolar lavage (BAL)<sup>[58,59,96]</sup>. An increased percentage of alveolar lymphocytes was reported in the study by Wallaert *et al*<sup>[58]</sup> in patients with CD. In the same study, a correlation between BAL differential cell count and PFTs abnormalities, drug treatment or CD site and activity was not reported<sup>[58]</sup>. The same group reported an increased level of IgG and IgM in BAL recovered from patients with alveolitis but not in those with normal BAL<sup>[97]</sup>. This observation of subclinical alveolitis was confirmed in the study by Bonniere and associates in 22 patients with CD<sup>[59]</sup>. In the same study, a significant increase in superoxide anion production by alveolar macrophages related to spontaneous activation and alteration of pulmonary function was observed<sup>[59,98]</sup>. Bartholo *et al*<sup>[99]</sup> found lymphocytosis in induced sputum of patients with CD even without pulmonary symptoms. Raj *et al*<sup>[100]</sup> reported a trend for higher lymphocyte counts in the sputum of patients with CD compared with UC. Smićan *et al*<sup>[96]</sup> reported lymphocytosis alveolitis in patients with CD but not in patients with other inflammatory bowel

disorders including UC. In the same study, an increase in the CD4 lymphocyte subset (increased ratio of CD4/CD8) was found in patients with an active stage of CD similar to patients with sarcoidosis<sup>[96]</sup>. Yamaguchi *et al*<sup>[101]</sup> reported increased BAL lymphocytes with an elevated CD4/CD8 ratio and enhanced expression of CD2 antigen in lung T cells in 8 patients with CD. Ussov *et al*<sup>[102]</sup> found a significant increase in the pulmonary vascular granulocyte pool in patients with CD. The meaning of this subclinical alveolitis and alterations in lung parenchyma is unclear. A subclinical inflammatory alveolitis as assessed by BAL cell analysis may be present in asymptomatic patients with immunological systemic disorders and with normal chest X-ray<sup>[103]</sup>. The fact that pulmonary involvement is not as common during extrathoracic granulomatosis as CD, whereas subclinical alveolitis is frequent, suggests that the lung possibly downregulates, in some way, alveolar inflammation due to the systemic immune disorder. The alveolitis observed in IBD patients does not necessarily precede the development of pulmonary granulomatosis and fibrosis<sup>[97,103]</sup>. Increased pulmonary permeability to diethylenetriaminepenta-acetate radiolabelled with 99m-technetium (<sup>99m</sup>Tc-DTPA) related to abnormal BAL findings has also been reported in patients with CD<sup>[104]</sup>. The reduction in diffusing capacity of the lungs (DLCO) is common and early manifestations of interstitial lung diseases<sup>[64,68,97]</sup> and latent lymphocytosis alveolitis could explain, in part, the reduction in DLCO observed in patients with CD<sup>[60]</sup>.



## ATOPY AND IBD

The gastrointestinal tract comes into direct contact with a great variety of foreign substances and under certain conditions these may act as antigens causing allergic reactions<sup>[105]</sup>. On the other hand, atopic subjects are possibly susceptible to several inhalants or food allergens<sup>[106]</sup>, while clinical features of atopic disorders include many organs among them both the pulmonary and gastrointestinal systems. Hippocrates reported that milk could cause gastric upset and urticaria and was probably the first to relate general atopy with gastrointestinal allergy. Hammer *et al*<sup>[107]</sup> found an increased prevalence of all atopic features. Asthma was also documented as being highly prevalent in a large study by Bernstein *et al*<sup>[108]</sup>. Studies on the relation between IBD and atopy are listed in Table 1. Ceyhan *et al*<sup>[109]</sup> reported that allergic symptoms and skin prick test positivity were more common in IBD patients (Table 1). Fireman *et al*<sup>[110]</sup> reported a higher percentage of eosinophils in induced sputum in patients with UC. Several studies have tried to investigate the attractive hypothesis that IBD, in particular UC, may be an allergic response to food<sup>[111,112]</sup> especially in individuals susceptible to various allergens. This hypothesis is supported by certain evidence that eosinophils and eosinophil-derived mediators contribute to the histopathology and pathophysiology of IBD<sup>[19,113-116]</sup>. Most studies confirmed the observation that atopic features are more frequent in patients with IBD than in the general population<sup>[114,117-120]</sup> (Table 1). This may be an explanation for the overlapping allergic disorders in both the respiratory and gastrointestinal systems. However, the frequency of bronchial hyperresponsiveness was significantly higher in IBD patients than in normal subjects (41% *vs* 5%), even when non-atopic subjects were considered<sup>[53]</sup>. This finding is consistent with the hypothesis that another immune system common to both exists and may be responsible for the inflammation in both systems<sup>[36]</sup>. Only one study by Troncone *et al*<sup>[121]</sup> showed that there was no correlation between atopy and IBD.

Engkilde *et al*<sup>[122]</sup> found an inverse association between a contact allergy and IBD. In this study although there was a chronic contact allergic dermatitis which was considered by the authors to have a Th2 profile, contact allergy has a Th1 profile. Engkilde *et al*<sup>[122]</sup> suggested that this may be due to shared genetic factors, common environmental determinants or skewness of the immune system. Medoff *et al*<sup>[123]</sup> suggested that T cell trafficking takes place in peripheral tissue in allergic asthma. It is suggested that this trafficking may involve several interactions between innate immune cells and T cells<sup>[123]</sup>. Several explanations for this phenomenon have been given over the years, however, no definite conclusions have been reached. Hammer *et al*<sup>[107]</sup> suggested a genetic predisposition, Myrelid *et al*<sup>[124]</sup> implicated TNF mast cells and D'Arienzo *et al*<sup>[125]</sup> suggested a Th2 or Th1 helper response. The mechanisms of atopy in IBD merit further investigation.

## CONCLUSION

Three patterns of pulmonary involvement have been reported to accompany IBD: (1) airway disease including large airway stenosis, chronic bronchitis, small airway dysfunction, severe bronchial suppuration and bronchiectasis; (2) parenchymal lung involvement either as subclinical lymphocytic alveolitis or several types of pulmonary infiltrate such as granulomatous bronchiolitis and bronchiolitis obliterans; and (3) a reduction in the diffusing capacity of the lung is a well established abnormality of pulmonary function testing in some patients with IBD.

We propose that patients suffering from IBD should undergo pulmonary evaluation which should include physical examination, chest X-ray and pulmonary function testing with DLCO measurement. This pulmonary evaluation may be useful in detecting subclinical or clinical pulmonary involvement in IBD patients or as a baseline evaluation. In clinical cases with pulmonary manifestations, inhaled or systemically administered steroids appear to be an effective treatment. With regard to atopy, routine investigations should be considered, at least in patients with IBD who also present with airway dysfunction.

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## Heparanase and hepatocellular carcinoma: Promoter or inhibitor?

Shuo Dong, Xiong-Zhi Wu

Shuo Dong, Xiong-Zhi Wu, Zhong-Shan-Men In-patient Department, Tianjin Medical University Cancer Institute and Hospital, Tianjin 300171, China

**Author contributions:** Dong S conducted the literature review and wrote the manuscript; Wu XZ provided guidance throughout the preparation of this manuscript, reviewed the text and made significant revisions to drafts of this manuscript.

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**Correspondence to:** Dr. Xiong-Zhi Wu, Zhong-Shan-Men In-patient Department, Tianjin Medical University Cancer Institute and Hospital, Guang-Lin Road, He-Dong District, Tianjin 300171, China. [ilwxz@163.com](mailto:ilwxz@163.com)

**Telephone:** +86-22-23921723 **Fax:** +86-22-23537796

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### Abstract

Heparan sulphate proteoglycans (HSPGs) consist of a core protein and several heparan sulphate (HS) side chains covalently linked. HS also binds a great deal of growth factors, chemokines, cytokines and enzymes to the extracellular matrix and cell surface. Heparanase can specially cleave HS side chains from HSPGs. There are a lot of conflicting reports about the role of heparanase in hepatocellular carcinoma (HCC). Heparanase is involved in hepatitis B virus infection and hepatitis C virus infection, the activation of signal pathways, metastasis and apoptosis of HCC. Heparanase is synthesized as an inactive precursor within late endosomes and lysosomes. Then heparanase undergoes proteolytic cleavage to form an active enzyme in lysosomes. Active heparanase translocates to the nucleus, cell surface or extracellular matrix. Different locations of heparanase may exert different activities on tumor progression. Furthermore, enzymatic activities and non-enzymatic activities of heparanase may play different roles during HCC development. The expression level of heparanase may also contribute to the discrepant effects of heparanase. Growth promoting as well as

growth inhibiting sequences are contained within the tumor cell surface heparan sulfate. Degrading different HSPGs by heparanase may play different roles in HCC. Systemic studies examining the processing, expression, localization and function of heparanase should shed a light on the role of heparanase in HCC.

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**Key words:** Apoptosis; Heparanase; Heparan sulphate; Hepatocellular carcinoma; Infection; Metastasis

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### INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. Extracellular matrix (ECM) remodeling plays an important role in the development of HCC<sup>[1]</sup>.

Heparan sulphate proteoglycans (HSPGs), one of the main components of ECM, are abundant macromolecules associated with the cell surface and ECM of a wide range of cells of vertebrate and invertebrate tissues. The basic HSPG structure consists of a core protein and several heparan sulphate (HS) side chains covalently linked. Extracellular HSPGs can maintain the ECM self assembly and integrity with other macromolecules, while cell surface HSPGs may act as co-receptor for several signal pathway molecules. In fact, HS chains also bind a great deal of growth factors, chemokines, cytokines and enzymes to the ECM and cell surface. HSPGs can thus influence a

number of normal and pathological processes, among which are tissue repair, inflammation, tumor growth and metastasis, and angiogenesis<sup>[2,3]</sup>.

Recent discoveries indicated that HSPGs localized within the tumor microenvironment can be attacked by enzymes that alter proteoglycan structure resulting in dramatic effects on tumor growth and metastasis<sup>[4,5]</sup>. Heparanase, an endoglycosidase, can specially cleave HS side chains from HSPGs and release a multitude of bioactive molecules. Then, the generated HS fragments and released bioactive mediators could facilitate tumor metastasis cooperatively. In addition, heparanase also exhibits non-enzymatic activities, including cell adhesion and survival, upregulation of vascular endothelial growth factor (VEGF) and tissue factor, induction of signal transduction, and enhancement of certain HSPG shedding from the tumor cell surface<sup>[6-16]</sup>.

A large body of evidence suggest that the expression of heparanase in the tumor closely relates with the potential for tumor invasion, angiogenesis and metastasis in most tumors examined<sup>[7-10]</sup>. However, there are a lot of conflicting reports about the relationship between heparanase and HCC. It is timely to review the literature to evaluate the arguments for and against the possible roles of heparanase in HCC.

## HEPARANASE AND HEPATITIS B VIRUS (HBV) AND HEPATITIS C VIRUS (HCV) INFECTION

Cell surface heparan sulfate mediates entry and initiation of infection of HBV and HCV, the most important pathogenic factors for HCC. Proper structure and sulfation levels of heparan sulfate are prerequisite for this mediation<sup>[17-23]</sup>. Heparanase might inhibit HS-mediated HCV and HBV entry and the initiation of infection<sup>[18,21]</sup>. Degradation of cell surface heparan sulfate by pretreatment with heparanases resulted in a marked reduction of HCV envelope glycoprotein E2 binding to HepG2 cells<sup>[18]</sup>. Treatment of Namalwa B cells and human erythroleukemia K562 cells with heparinase I also reduced the cellular binding of HBV nucleocapsids<sup>[21]</sup>. However, HCV E2 bound to target cells *via* putative receptors in a noncompetitive manner. Incomplete inhibition of heparan sulfate might lead to a partial E2 blockade and evasion of the host immune response<sup>[23]</sup>. El-Assal *et al*<sup>[24]</sup> reported that heparanase expression was significantly higher in HCV-related HCC compared with that in HCV-negative patients. It is possible to assume that HCV enhances heparanase expression that may be involved in the HCV-related pathological and malignant changes.

## HEPARANASE EXPRESSION IN LIVER DISEASES

A biphasic pattern of heparanase expression is also significantly observed in rat liver following partial hepatectomy, peaking at 12 h and 96-168 h and decreasing

at 360 h post-surgery<sup>[25]</sup>. Elevated heparanase levels are noted in the early stages of thioacetamide induced rat liver fibrosis, with no further increase evident in rats exhibiting higher fibrotic grades<sup>[25]</sup>. Reduction or no significant difference in heparanase expression levels are found in liver fibrosis or cirrhosis samples resected from human patients<sup>[24,26-31]</sup>.

There are conflicting reports about the expression level of heparanase in HCC. Examining HCC patients' specimens by reverse transcriptase-polymerase chain reaction (RT-PCR) or Real-Time Quantitative RT-PCR, *in situ* hybridization, Western blotting, immunohistochemistry and tissue microarrays (TMAs), five out of the seven studies reported that heparanase was over-expressed in HCC<sup>[24,28-31]</sup>. However, two studies indicated that the expression level of heparanase was lower than that in adjacent noncancerous tissue<sup>[26,27]</sup> (Table 1).

## HEPARANASE AND HCC

### Heparanase and metastasis of HCC

Metastasis is a sequential process including breaking off from the primary tumor, traveling through the bloodstream and stopping at a distant site. Heparanase enhances HCC metastasis by degrading ECM and releasing ECM-resident growth factors and angiogenic factors. Furthermore, non-enzymatic activities of heparanase, such as promoting cell adhesion, might also play a role in HCC metastasis<sup>[6-16]</sup>.

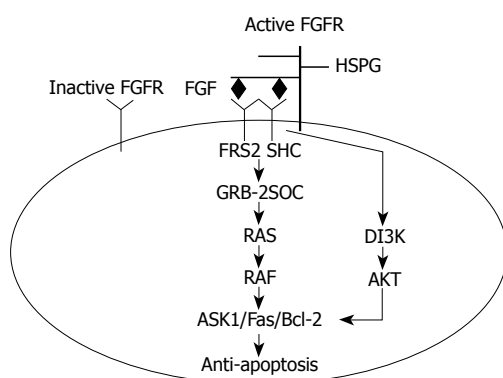
Hepatoma heparanase was first purified from a human hepatoma cell line Sk-hep-1 in 1998<sup>[32]</sup>. El-Assal *et al*<sup>[24]</sup> reported that expression of heparanase mRNA was significantly correlated with larger tumor size, potential for tumor invasion and tumor microvessel density. Many research studies also support the concept that heparanase expression closely relates with metastasis and recurrence of HCC, tumor differentiation and tumor stage<sup>[28-31]</sup>. More recently, some researchers reported that down-regulating heparanase expression either by antisense oligodeoxynucleotide or by RNA interference could significantly inhibit the invasiveness, metastasis, and angiogenesis of human HCC SMMC7721 cells both *in vitro* and *in vivo*<sup>[33]</sup>. Yang *et al*<sup>[34]</sup> reported that two polypeptide antibodies, anti-MAP1 (multiple antigenic peptides)- and anti-MAP2-antibody, can effectively inhibit the heparanase activity of HCCLM6 human hepatocellular carcinoma cells *in vitro* and influence their invasive ability. Recently, PI-88, an heparanase inhibitor, showed preliminary efficacy as an adjunct therapy for post-operative HCC<sup>[35]</sup>. Glycosaminoglycan mimetics may also compete with cellular heparan sulfate chains for the binding to CXC-chemokine Stromal cell-Derived Factor-1 (SDF-1)/CXCL12 and may affect heparanase expression, leading to inhibition of SDF-1/CXCL12-mediated migration and invasion of the Huh7 human hepatoma cells<sup>[36]</sup>.

However, Ikeguchi *et al*<sup>[26]</sup> reported that heparanase mRNA in HCC was significantly lower than that of noncancerous liver tissue and heparanase expression did not correlate with tumor differentiation, tumor stage, or patient prognosis. In another study conducted by Ikeguchi's group, the expression level of heparanase was low in HCC

Table 1 Studies examining the pro-metastatic role of heparanase in HCC

Studies	No.	Methods	Positive rate of HCC tissue	Correlation between heparanase expression and HCC progression
El-Assal <i>et al.</i> <sup>[24]</sup> , 2001	55 <sup>1</sup>	RT-PCR	47%	Significant positive correlation
Ikeguchi <i>et al.</i> <sup>[26]</sup> , 2002	50 <sup>2</sup>	QRT-PCR	< adjacent noncancerous tissue	No significant correlation
Ikeguchi <i>et al.</i> <sup>[27]</sup> , 2003	48 <sup>2</sup>	QRT-PCR	< adjacent noncancerous tissue	Significant negative correlation
Xiao <i>et al.</i> <sup>[28]</sup> , 2003	11 <sup>3</sup>	QRT-PCR, WB, ISH, IHC	> normal and cirrhosis tissue	Significant positive correlation
Chen <i>et al.</i> <sup>[30]</sup> , 2004	33 <sup>2</sup>	RT-PCR	48.5%, > adjacent tissue	Significant positive correlation
Liu <i>et al.</i> <sup>[31]</sup> , 2005	33 <sup>4</sup>	RT-PCR	48.5%, > paracancerous and normal tissue	Significant positive correlation
Chen <i>et al.</i> <sup>[29]</sup> , 2008	120 <sup>5</sup>	IHC in TMAs	45.83%, > adjacent tumor tissue, cirrhosis, and normal liver tissue	Significant positive correlation

<sup>1</sup>55 HCC tissue samples; <sup>2</sup>Both HCC tissue samples and non-cancerous liver samples were obtained from the same patients; <sup>3</sup>16 normal liver tissue samples, 14 liver cirrhosis tissue samples and 11 HCC tissue samples; <sup>4</sup>HCC tissue samples and paracancerous tissue samples were obtained from 33 HCC patients; paracancerous tissues of 9 cases of benign liver tumor were used as normal controls; <sup>5</sup>48 cases of adjacent HCC liver, 62 cases of cirrhosis, and 23 cases of normal liver tissues. HCC: Hepatocellular carcinoma; RT-PCR: Reverse transcriptase-polymerase chain reaction; QRT-PCR: Real-time quantitative RT-PCR; WB: Western blotting; ISH: *In situ* hybridization; IHC: Immunohistochemistry; TMAs: Tissue microarrays.



**Figure 1** Heparan sulphate proteoglycan (HSPG) and fibroblast growth factor (FGF)-induced signal transduction. Basic FGF (bFGF) enhances tumor progression by protecting tumor cells from apoptosis. Cell surface HSPGs could act as a co-receptor for formation of a bFGF high-affinity receptor complex. The alteration of cell surface HSPGs resulting from heparanase might down-regulate HSPG-mediated bFGF-induced signal pathway, resulting in apoptosis of tumor cells.

and a high expression level of heparanase was associated with better disease-free 5-year survival rate<sup>[27]</sup>. Ogawa *et al.*<sup>[37]</sup> established rat HCC cell lines with a high metastatic potential and found that one cell line, showing high levels of lung metastasis when injected subcutaneously in nude mice, exhibited decreased heparanase mRNA expression compared with other cell lines.

In a study of fibroblasts transfected with various oncogenes, one cell line exhibiting a metastatic phenotype was not found to have a significant increase in heparanase activities, though another one having the highest metastatic potential was shown to contain the greatest heparanase activity<sup>[38]</sup>. The hypothesis is that high heterogeneity of HCC might contribute to such discrepancy. Growth promoting as well as growth inhibiting sequences are contained within the tumor cell surface heparan sulfate<sup>[39]</sup>. Degrading different HSPGs by heparanase may play different roles in the complex process of metastasis.

### Heparanase and apoptosis of HCC

The HS side chains of HSPGs could bind a multitude

of growth factors, chemokines, cytokines and enzymes in ECM and cell surface, such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor and hepatocyte growth factor. The cleaving of HSPGs by heparanase could release of HS-bound growth factors and exhibit complicated effects<sup>[7-10]</sup>. bFGF might enhance endothelial cell and tumor cell proliferation, contributing to HCC progression<sup>[40,41]</sup>. El-Assal *et al.*<sup>[24]</sup> reported that bFGF and heparanase co-expressed in HCC patients' specimen and this co-expression was associated with higher tumor microvessel density than that in specimens with expression of either factor alone.

Heparanase is involved in the activation of several signal pathways including bFGF-induced signal transduction<sup>[42-47]</sup> (Figure 1). In an *in vitro* study of melanoma cells, heparanase seemed necessary for phosphorylation of extracellular signal-related kinase (ERK) or focal adhesion kinase (FAK) in response to bFGF<sup>[46]</sup>. Kato *et al.*<sup>[47]</sup> reported that in postmastectomy wound fluids, syndecan-1 was converted from an inhibitor to an activator of bFGF by the degrading activities of heparanase. The release of bFGF and HS degrading fragments by heparanase might promote bFGF-receptor binding and activation<sup>[42-47]</sup>.

Heparanase expression closely related with apoptosis of several tumor cells, including HCC cells<sup>[27,48,49]</sup>. Ikeguchi *et al.*<sup>[27]</sup> found a significant positive correlation between heparanase mRNA expression levels and the percentages of apoptotic hepatocytes in liver tissues. In addition to mitogenic effects, bFGF also could enhance some tumor progression by protecting tumor cells from apoptosis<sup>[50-56]</sup>. Targeting bFGF by neutralizing antibody or antisense oligonucleotides could result in apoptosis of some tumor cells<sup>[57-59]</sup>. Cell surface HSPGs could not only act as co-receptors for formation of bFGF high-affinity receptor complexes, but could also function directly as receptors for bFGF-induced signal transduction, depending on core protein or HS specific manner<sup>[60-62]</sup>. One possibility is that the alteration of cell surface HSPGs resulting from heparanase might down-regulate HSPG-mediated bFGF-induced signal pathways, resulting in apoptosis of tumor cells.



### Location of heparanase in HCC

Human heparanase is synthesized as a 65 kDa inactive precursor within late endosomes and lysosomes. Then heparanase undergoes proteolytic cleavage, yielding 8 and 50 kDa protein subunits that heterodimerize to form an active enzyme in lysosome. Active heparanase translocates to the nucleus, cell surface or ECM<sup>[6,63]</sup>. Different locations of heparanase may exert different activities. Cell surface expression and secretion of heparanase in EB mouse lymphoma cells markedly promotes tumor angiogenesis and metastasis compared with intracellular enzyme<sup>[64]</sup>. However, nucleus heparanase induces differentiation of some tumor cells, such as esophageal cancer cells, mammary cancer cells and leukemic cells. Furthermore, a nuclear location of heparanase represents a better prognosis in tumor patients than its cytoplasmic location<sup>[65-70]</sup>.

During liver regeneration, the location of heparanase exhibits a dramatic alteration from cytoplasm to cell surface in a time-dependant manner<sup>[25]</sup>. Xiao *et al.*<sup>[28]</sup> and Chen *et al.*<sup>[29]</sup> reported that high heparanase expression in HCC was localized within the cytoplasm of tumor cells and there was a significant correlation between the expression level of heparanase mRNA and tumor stage. Does the role of heparanase in HCC depend on its location?

### CONCLUSION

There are a lot of conflicting reports about the role of heparanase in HCC. Several questions are intriguing and shouldn't be ignored. (1) In a human glioma cell xenograft tumor model, moderate heparanase expression levels significantly enhanced tumor development, whereas high heparanase expression levels inhibited tumor growth<sup>[71]</sup>. Another study also showed that extensive heparanase inhibited bFGF binding in human metastatic melanoma 70W cells, while treatment of 70W cells with low heparanase concentrations enhanced bFGF binding<sup>[46]</sup>. Does the effect of heparanase depend on its expression level in HCC? (2) During the course of colon adenoma-carcinoma progression, active heparanase increases in the early stage, while latent heparanase predominantly increases in the late stage. The possibility was that enzymatic activities and non-enzymatic activities of heparanase have different roles in the early and late stages of colon cancer development<sup>[72]</sup>. Do enzymatic activities and non-enzymatic activities of heparanase play different roles during HCC development? (3) HSPGs may have promoting or inhibiting activities depending on the core protein and localization<sup>[73]</sup>. For example, Glypican-3 and syndecan-1 might act as promoter and inhibitor during the development of HCC, respectively<sup>[74,75]</sup>. Degrading different HSPGs by heparanase may play different roles in HCC; and (4) Researchers have already observed that heparan sulfates could occur in hepatic nucleus and hypothesized that alteration of heparan sulfates detected in HCC might be involved in HS-related gene expression<sup>[76-81]</sup>. For example, DNA topoisomerase I activity is modulated by heparan sulfates present in normal liver cells but is markedly reduced or absent in their transformed counterparts<sup>[80]</sup>. In-

terestingly, active heparanase also could translocate to the nucleus and degrade nuclear HS<sup>[65-70,81]</sup>. Is heparanase the criminal for the lack of biologically active HS in HCC? Do the effects of heparanase depend on its location in HCC? Systemic studies examining the processing, expression, localization and function of heparanase should shed a light on the role of heparanase in HCC.

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## ***Helicobacter pylori* and EBV in gastric carcinomas: Methylation status and microsatellite instability**

Adriana Camargo Ferrasi, Nídia Alice Pinheiro, Silvia Helena Barem Rabenhorst, Otávia Luisa Caballero, Maria Aparecida Marchesan Rodrigues, Fabrício de Carvalho, Celso Vieira de Souza Leite, Marcia Valéria Pitombeira Ferreira, Marcos Aurélio Pessoa Barros, Maria Inês de Moura Campos Pardini

Adriana Camargo Ferrasi, Nídia Alice Pinheiro, Maria Inês de Moura Campos Pardini, Blood Transfusion Center, Botucatu Medical School, UNESP - Sao Paulo State University, 18618-970 Botucatu-SP, Brazil

Silvia Helena Barem Rabenhorst, Marcia Valéria Pitombeira Ferreira, Marcos Aurélio Pessoa Barros, Department of Pathology - Federal University of Ceara, 60430-160 Fortaleza-CE, Brazil

Otávia Luisa Caballero, Ludwig Institute of Cancer Research, New York, NY 10065, United States

Maria Aparecida Marchesan Rodrigues, Department of Pathology, Medical School, UNESP - Sao Paulo State University, 18618-970 Botucatu-SP, Brazil

Fabrício de Carvalho, Ludwig Institute of Cancer Research, Sao Paulo-Brazil, 01323-903 Sao Paulo-SP, Brazil

Celso Vieira de Souza Leite, Department of Gastroenterology Surgery, Medical School, UNESP - Sao Paulo State University, 18618-970 Botucatu-SP, Brazil

**Author contributions:** Ferrasi AC, Pinheiro NA and Pardini MIMC designed the research; Ferrasi AC performed the research; Carvalho F and Caballero OL contributed new reagents and analytical tools; Ferrasi AC, Pinheiro NA, Pardini MIMC and Rabenhorst SHB analyzed the data; Barros MAP and Souza Leite CV provided specimens; Ferreira MVP and Rodrigues MAM contributed to the histopathological analyses.

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**Correspondence to:** Maria Inês de Moura Campos Pardini, PhD, Blood Transfusion Center, Botucatu Medical School, UNESP - Sao Paulo State University, Distrito de Rubião Júnior, s/n, 18618-970 Botucatu-SP, Brazil. [inespardini@gmail.com](mailto:inespardini@gmail.com)

Telephone: +55-14-38116041 Fax: +55-14-38116041

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*cagA*<sup>+</sup> and Epstein Barr virus (EBV) infections in gastric adenocarcinomas.

**METHODS:** Methylation-specific PCR (MSP) assay was performed in 89 primary gastric carcinomas (intestinal and diffuse types). Microsatellite instability (MSI) analysis was performed using the BAT26 primer set and PCR products were analyzed with the ABI PRISM 3100 Genetic Analyzer using Genescan 3.7 software (Applied Biosystems). Detection of *H. pylori* and genotyping were performed by PCR, using specific primers for *ureaseC* and *cagA* genes. The presence of EBV was assessed by *in situ* hybridization. Statistical analyses were performed using the  $\chi^2$  or Fisher's exact test.

**RESULTS:** The most frequent hypermethylated gene was *COX-2* (63.5%) followed by *DAPK* (55.7%), *CDH1* (51%), *CDKN2A* (36%) and *hMLH1* (30.3%). Intestinal and diffuse adenocarcinomas showed different methylation profiles and there was an association between methylation of *E-CDH1* and *H. pylori-cagA*<sup>+</sup> in the intestinal adenocarcinoma type. MSI was correlated with *hMLH1* methylation. There was an inverse correlation between *DAPK* hypermethylation and MSI.

**CONCLUSION:** We found a strong association between *CDH1* methylation and *H. pylori-cagA*<sup>+</sup> in intestinal-type gastric cancer, association of MSI and better prognosis and an heterogeneous *COX-2* overexpression.

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**Key words:** Gastric cancer; Methylation; Microsatellite instability; *Helicobacter pylori*; Epstein Barr virus

**Peer reviewer:** Ulrike S Stein, PhD, Assistant Professor, Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Straße 10, 13125 Berlin, Germany

### **Abstract**

**AIM:** To verify the methylation status of *CDH1*, *DAPK*, *COX2*, *hMLH1* and *CDKN2A* genes and to evaluate their association with *Helicobacter pylori* (*H. pylori*)-



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## INTRODUCTION

Gastric cancer (GC), one of the most common cancer types, is associated with high mortality rates<sup>[1,2]</sup>. The prognosis of GC remains poor, especially when the diagnosis is undertaken at advanced stages<sup>[3]</sup>. Thus, studies to elucidate the mechanisms acting in gastric carcinogenesis and which search for possible markers to assist in both earlier diagnosis and therapeutic approaches are relevant.

Gastric adenocarcinomas can be divided into intestinal or diffuse histological types. Environmental factors appear to be related to the intestinal type, which may play a role in carcinogenesis characterized by precursor lesions of gastric mucosa, followed by intestinal metaplasia that can lead to dysplasia and GC. In contrast, in the diffuse carcinoma no precursor lesions have been identified to date<sup>[4]</sup>.

Many studies have identified the transcriptional silencing by DNA methylation as a mechanism responsible for tumor suppressor inactivation. Methylation of promoter CpG islands leads to DNA structural changes and, consequently, gene inactivation<sup>[5]</sup>. Several cancers, including gastric tumors, show methylation of multiple genes including *CDH1*, *DAPK*, *COX2*, *hMLH1* and *CDKN2A*<sup>[6,7]</sup>.

Microsatellite instability (MSI) reflects an erroneous form of DNA replication in repetitive microsatellite sequences and has been considered a hallmark of mismatch repair gene inactivation. MSI has been associated with less aggressive tumor behavior and favorable prognosis in sporadic colorectal cancer<sup>[8-10]</sup>. MSI status has been determined by means of BAT26 mononucleotide repeats because this marker is quasi-monomorphic in normal DNA and has shown high sensitivity and specificity in the identification of MSI phenotype<sup>[11]</sup>.

*Helicobacter pylori* (*H. pylori*), carcinogen class I<sup>[12]</sup>, colonizes the gastric epithelium and causes a severe inflammatory reaction that depends on factors including host genetic susceptibility, immune response, age at the time of initial infection, and environmental and virulence factors such as cytotoxin-associated gene A (*cagA*)<sup>[13-15]</sup>. The complex interactions among the different types of *H. pylori*, inflammation and genetic features of the host could promote a cascade of morphological events leading to GC<sup>[16]</sup>.

Apart from the accepted role of *H. pylori* in the pathogenesis of GC, the Epstein Barr virus (EBV) has been associated with gastric carcinoma in at least 10% of cases<sup>[17]</sup>. Countries with the highest incidences are Japan (19.3%) and Germany (18%)<sup>[17,18]</sup>. In Brazil, frequencies of EBV infec-

tion ranging between 8% and 11% have been described<sup>[19,20]</sup>.

Some studies have linked DNA hypermethylation with *H. pylori-cagA*<sup>+</sup> and EBV infection but these data are not conclusive and the studies did not examine both agents at the same time. By examining 89 primary gastric carcinomas, the present study verifies MSI frequency and the methylation status of the *CDH1*, *DAPK*, *COX2*, *hMLH1* and *CDKN2A* genes and evaluates their association with *H. pylori* (*cagA*<sup>+</sup> and *cagA*) and EBV infections and also with clinicopathological features of gastric carcinomas.

## MATERIALS AND METHODS

### Samples

Eighty-nine gastric adenocarcinomas and their corresponding adjacent normal tissue were obtained surgically from Brazilian patients at the Federal University of Ceara State, the Clinical Hospital at the UNESP Medical School in Botucatu, Sao Paulo State and the Amaral Carvalho Hospital, and immediately frozen in liquid nitrogen until micro-dissection and DNA extraction. The Research Ethics Committees of the respective institutions approved this study and each subject signed an informed consent form before tissue was obtained. Histopathological analyses determined that the tumor specimens consisted mainly (> 80%) of tumor tissues and that the adjacent tissue was free of tumor cells. The histological classification was made according to the Laurén classification system<sup>[4]</sup> and the tumors were staged according to the TNM criteria<sup>[21]</sup>. DNA was extracted using standard methods<sup>[22]</sup>.

### Bisulfite modification and methylation-specific PCR (MSP)

DNA from both tumoral and normal tissues was subjected to treatment with sodium bisulfite as described by Herman *et al.*<sup>[23]</sup>. The modified DNA was amplified with primers specific for either the methylated or unmethylated sequences of *hMLH1*, *COX2*, *DAPK*, *CDKN2A* and *CDH1* (Table 1). PCR was individually performed in 25 µL reaction volumes, containing 1 × Platinum Taq buffer, 1.5 mmol/L MgCl<sub>2</sub>, 0.2 mmol/L of each dNTP, 0.4 µmol/L of each primer set, 1 U of Platinum Taq DNA Polymerase (Invitrogen) and 1 µL of treated DNA. DNA methylated *in vitro* by *Sss-I* methylase (New England Biolabs) was used as a positive control, and water and DNA from peripheral lymphocytes of healthy donors were used as negative controls. PCR products were separated on silver-staining 6% non-denaturing polyacrylamide gels<sup>[22]</sup>.

### Bisulfite sequencing analysis

To confirm reaction specificity, MSP-PCR products from each gene analyzed were cloned with TOPO TA Cloning Kit (Invitrogen) and sequenced using the ABI PRISM® BigDye Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems) and ABI Prism 3100 DNA Sequencer (Applied Biosystems).



Table 1 Primer Sequences and PCR conditions for methylation-specific PCR (MSP) analysis

Gene	Primer (5'-3') forward	Primer (5'-3') reverse	Size (bp)	T (°C)	Ref.
COX2	M TTAGATACGGCGGCGCGGC	TCITTTACCCGAACGCTTCCG	161	68	[24]
	U ATAGATTAGATATGGTGGTGGTGGT	CACAATCTTTACCCAAACACTTCCA	171	67	
DAPK	M GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCGA	98	65	[25]
	U GGAGGATAGTTGGATTGAGTTAATGTT	CAAAATCCCTCCCAAACACCAA	116	65	
CDH1	M TTAGGTTAGAGGGTTATCGCGT	TAACATAAAATTCACCTACCGAC	115	61	[26]
	U TAAATTTAGGTTAGAGGGTTATTGT	CACAACCAATCAACAACACA	97	59	
hMLH1	M TATATCGTTCGTAGTATTCGIGT	TCCGACCCGAATAAACCCAA	153	65	[26]
	U TTTTGATGTAGATGTTTATTAGGGTGT	ACCACCTCATCATAACTACCCACA	124	63	
CDKN2A	M TTATTAGAGGGTGGGGCGGATCGC	GACCCGAACCGCGACCGTAA	150	70	[21]
	U TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCAACCATAA	151	70	

### Microsatellite instability analysis

MSI analysis was performed using the BAT26 primer set (5'-TGACTACTTTTGACTTCAGCC-3', sense and 5'-AACCATTCAACATTTTAACCC-3', antisense). Sense primer was labeled with 6-FAM. PCR was performed in a final volume of 25 µL containing 1 × PCR buffer, 3.0 mmol/L MgCl<sub>2</sub>, 0.2 µmol/L dNTPs, 0.4 µmol/L of each primer, 2 U of Platinum Taq DNA Polymerase (Invitrogen) and 50 ng of DNA. The thermal conditions were 94°C/5 min followed by 40 cycles (94°C/1 min, 50°C/1 min and 72°C/1 min) and a final extension at 72°C/7 min. The dye-labeled PCR products were analyzed with ABI PRISM 3100 Genetic Analyzer using Genescan 3.7 software (Applied Biosystems). Both tumoral and normal samples were analyzed. Negative (SW480 cells) and positive (HCT116 cells) controls for MSI had been included in all the analyses. Deletions or insertions of at least 4 bp were required to satisfy the definition of instability<sup>[27]</sup>. All cases were repeated twice.

### *H. pylori* and *CagA* detection

Detection of *H. pylori* in gastric samples was performed for PCR amplification with primers specific to *H. pylori ureaseC* gene. The primer sequence used (5'-AAGCTTT-TAGGGGTGTTAGGGGT'TT-3', sense and 5'-CT-TACTTTCTAACACTAACGC-3', antisense)<sup>[28]</sup> amplifies a 294 bp fragment. To detect *cagA*, the primer set 5'-ATAATGCTAAATTAGACAACTTGAGCGA-3' (sense) and 5'-TTAGAATAATCAACAAACATAACG CCAAT-3' (antisense)<sup>[29]</sup> was utilized to amplify a 297 bp fragment. Each primer set (*ureaseC* and *cagA*) was used in an independent PCR reaction in a final volume of 25 µL containing 1 × PCR buffer [20 mmol/L Tris-HCl (pH 8.4) and 50 mmol/L KCl], 1.5 mmol/L MgCl<sub>2</sub>, 0.2 mmol/L of each dNTP, 0.4 µmol/L of each primer set, 1 U of Platinum Taq DNA Polymerase (Invitrogen) and 100 ng of DNA, under the following conditions: for *ureaseC* PCR, initial denaturation at 94°C/3 min followed by 35 cycles of denaturation at 94°C/30 s, annealing at 58°C/30 s and extension at 72°C/2 min and final extension at 72°C/5 min. The *cagA* PCR included an initial denaturation at 94°C/3 min followed by 40 cycles of denaturation at 94°C/30 s, annealing at 58°C/45 s and extension at 72°C/2 min and final extension at 72°C/5 min. Both tumoral and normal samples were analyzed. Negative and

positive controls were assayed in each run. PCR products were separated by silver-stained 6% non-denaturing polyacrylamide gel electrophoresis<sup>[22]</sup>.

### EBER1 *in situ* hybridization

The presence of EBV was assessed by RNA *in situ* hybridization reaction with a 30 bp biotinylated probe (5'-AGACACCGTCCTCACCACCCGGGACTTG TA-3') complementary to the RNA *EBER1*. EBV transcript was shown in high amounts in the nuclei of latently infected cells. Signal amplification was employed with anti-biotin antibody (clone BK, mouse, dilution 1:20; DakoCytomation®) and biotinylated anti-immunoglobulin antibody (polyclonal, rabbit, dilution 1:100; DakoCytomation®). The reaction was detected with the streptavidin-biotinperoxidase complex (DakoCytomation®) and diaminobenzidine chromogen (DakoCytomation®). The slides were counterstained with Harris's hematoxylin. A case of nasopharyngeal carcinoma was used as positive control.

### Statistical analysis

For statistical analysis the  $\chi^2$  test or Fisher's exact test was used. *P* values ≤ 0.05 were considered statistically significant.

## RESULTS

### Patients and tumor characteristics

The clinicopathological and epidemiological features of patients are shown in Table 2.

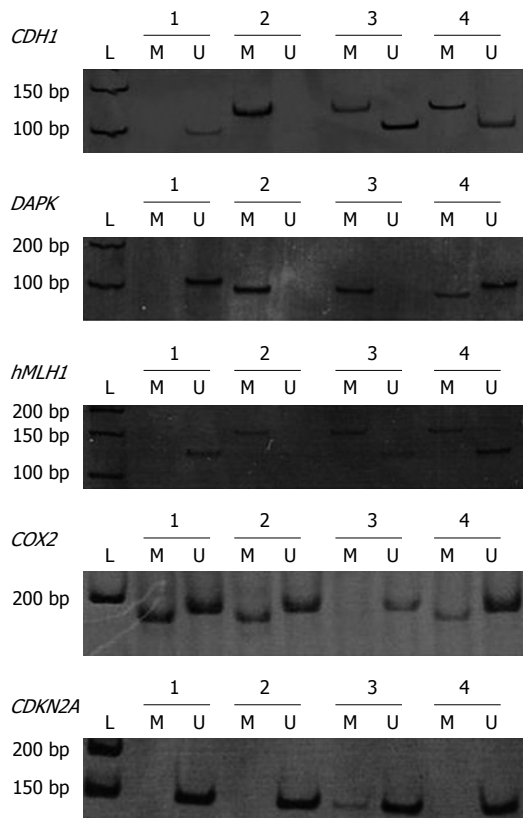
### Methylation status

Of the five genes analyzed, the *COX2* gene was the one most frequently hypermethylated (63.5%) followed by *DAPK* (55.7%), *CDH1* (51%), *CDKN2A* (36%) and *hMLH1* (30.3%). Figure 1 displays representative examples of the MSP products.

In the diffuse adenocarcinoma cases, methylation of *CDH1*, *COX2* and *CDKN2A* presented higher frequencies in early stage tumors (0- I) with a tendency to decrease along with the tumor grades (mainly *CDH1* and *COX2*). On the other hand, in the intestinal type, the methylation status of *CDH1*, *COX2*, *hMLH1* and *CDKN2A* tended to increase from the earliest stages

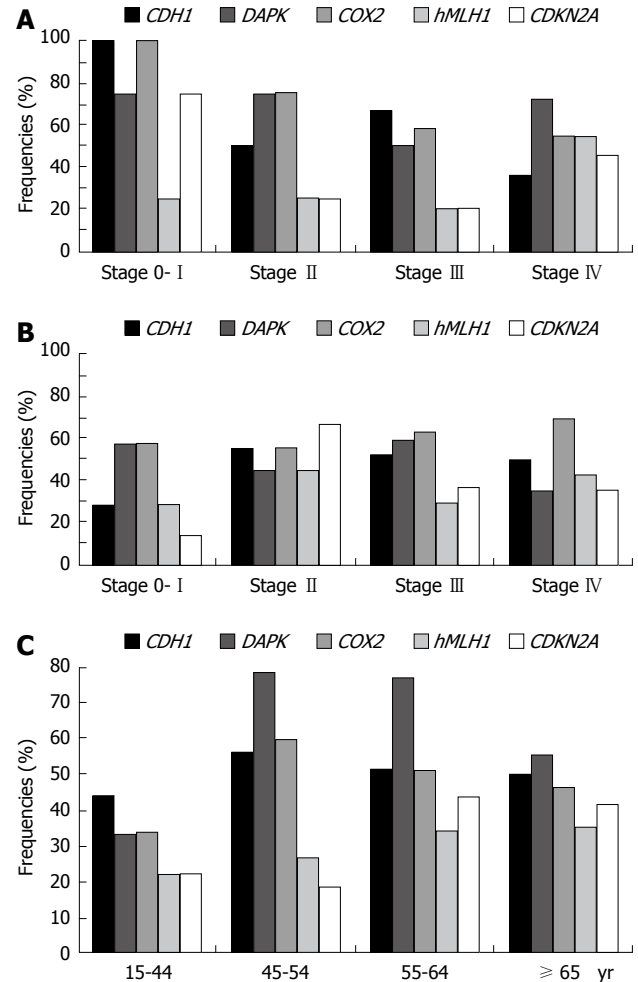
**Table 2** Clinicopathological and epidemiological features of patients in the four tumor stages *n* (%)

Variables	Patients	Tumor stage			
		0- I	II	III	IV
Age (yr)					
≤ 50	19	3 (15.8)	2 (10.5)	9 (47.4)	5 (26.3)
> 50	70	8 (11.4)	11 (15.7)	30 (42.9)	21 (30)
Gender					
Male	60	8 (13.3)	8 (13.3)	27 (45)	17 (28.3)
Female	29	3 (10.3)	5 (17.2)	12 (41.4)	9 (31)
Anatomic subsite					
Proximal (Cardia)	13	1 (7.7)	1 (7.7)	5 (38.5)	6 (46.1)
Distal (Antrum/body)	46	2 (4.3)	9 (19.6)	21 (45.7)	14 (30.4)
Mixed	3	0 (0)	1 (33.3)	2 (66.7)	0 (0)
Laurén type					
Diffuse	31	4 (12.9)	4 (12.9)	12 (38.7)	11 (35.5)
Intestinal	57	7 (12.3)	9 (15.8)	27 (47.4)	14 (24.6)
Mixed	1	0 (0)	0 (0)	0 (0)	1 (100)

**Figure 1** Example of results from methylation-specific PCR analysis performed in tumoral samples from patients with gastric cancer. The studied gene is given on the right of the each panel. Lane U: Amplified product with primers recognizing unmethylated sequences; Lane M: Amplified product with primers recognizing methylated sequences; L: Ladder 50 bp.

(0- I) to advanced stages (II-IV) ( $P < 0.001$ , Figure 2A and B). The methylation status tended to increase with age; the highest frequency of cases with promoter methylation was found in patients between 45 and 64 years old. Patients aged more than 50 years had a higher frequency of methylation in *CDKN2A* ( $P = 0.035$ ).

Table 3 summarizes overall results of the correlation

**Figure 2** Frequencies of methylation at *CDH1*, *DAPK*, *hMLH1*, *COX2* and *CDKN2A* genes in gastric carcinomas. A: Frequencies of methylation at *CDH1*, *DAPK*, *hMLH1*, *COX2* and *CDKN2A* distributed according to tumor stages in diffuse adenocarcinoma; B: Frequencies of methylation at *CDH1*, *DAPK*, *hMLH1*, *COX2* and *CDKN2A* distributed according to tumor stages in intestinal adenocarcinoma; C: Frequencies of methylation at *CDH1*, *DAPK*, *hMLH1*, *COX2* and *CDKN2A* distributed with regard to age groups.

between the promoter methylation frequency of each gene and relevant clinicopathological parameters.

### Microsatellite instability analysis

MSI was observed in 17.2% of GCs. Most of the patients with MSI tumors (86.7%) had an advanced form of the disease and 66.7% of them shown lymph node metastasis. Intestinal and diffuse adenocarcinomas had similar MSI frequencies. There was a significant correlation between MSI and *hMLH1* methylation ( $P = 0.001$ ). Conversely, a significant inverse correlation was demonstrated between *DAPK* methylation and MSI ( $P = 0.012$ ).

### Detection of *H. pylori*

The presence of *H. pylori* was identified in 98% of GC patients, of whom 63.2% were *cagA*<sup>+</sup>. The frequency of *H. pylori-cagA*<sup>+</sup> infection was similar between intestinal and diffuse adenocarcinomas (64.5% *vs* 59.6%, respectively). In the intestinal type, *CDH1* methylation was more frequent in *H. pylori-cagA*<sup>+</sup> cases (55.8%) than in

**Table 3** Associations between gene methylation status and clinicopathological features, age, *H. pylori*, MSI and EBV *n* (%)

	Total	Methylated genes									
		<i>CDH1</i>	<i>P</i> -value	DAP-kinase	<i>P</i> -value	<i>COX2</i>	<i>P</i> -value	<i>hMLH1</i>	<i>P</i> -value	<i>CDKN2A</i>	<i>P</i> -value
Mean age (yr)		60.2		59.9		60.9		63.1		62.5	
Age group (yr)											
≤ 50	19	10 (52.6)	0.883	8 (44.4)	0.282	9 (50)	0.179	6 (33.3)	0.903	3 (15.8)	0.035 <sup>1</sup>
> 50	69	35 (50.7)		41 (58.6)		45 (67.2)		21 (31.8)		29 (42)	
Gender											
Male	59	30 (50.8)	0.938	33 (55.9)	0.946	34 (59.6)	0.289	21 (35)	0.787	21 (35.6)	0.830
Female	29	15 (51.7)		16 (55.2)		20 (71.4)		11 (37.9)		11 (37.9)	
Nodal metastases											
Absent	17	8 (47.1)	0.708	9 (50)	0.586	11 (64.7)	0.910	6 (33.3)	0.903	6 (33.3)	0.764
Present	71	37 (52.1)		40 (57.1)		43 (63.2)		21 (31.8)		26 (37.1)	
Tumor site											
Cardia	13	8 (61.5)	0.855	9 (69.2)	0.427	10 (83.3)	0.228	4 (30.8)	0.304	8 (61.5)	0.266
Antrum	38	21 (55.3)		20 (52.6)		21 (56.8)		14 (37.8)		14 (35.9)	
Body	10	5 (50)		7 (70)		7 (70)		1 (11.1)		4 (40)	
Mixed	3	3 (100)		2 (66.7)		3 (100)		0		0	
Lauren											
Diffuse	30	17 (56.7)	0.503	10 (33.3)	0.628	19 (63.3)	0.973	8 (27.6)	0.481	10 (33.3)	0.628
Intestinal	57	28 (49.1)		22 (38.6)		34 (63)		19 (35.2)		22 (38.6)	
Mixed	1	0 (0.0)		0		1 (100)		0		0	
<i>H. pylori</i>											
<i>CagA</i> <sup>+</sup>	55	31 (56.4)	0.261	30 (54.5)	0.782	35 (66)	0.536	15 (28.8)	0.41	22 (40)	0.468
<i>CagA</i> <sup>-</sup>	34	15 (44.1)		19 (57.6)		19 (59.4)		12 (37.5)		11 (32.4)	
MS status											
MSI	14	5 (35.7)	0.174	4 (26.7)	0.012 <sup>1</sup>	8 (53.3)	0.349	10 (66.7)	0.001 <sup>1</sup>	4 (26.7)	0.405
MSS	72	40 (55.6)		44 (62)		45 (66.2)		16 (23.5)		27 (38)	
EBV											
Positive	5	1 (20)	0.101	4 (80)	0.346	4 (80)	0.694	0	0.179	3 (60)	0.462
Negative	48	28 (58.3)		28 (58.3)		33 (71.7)		15 (31.9)		21 (42.9)	

<sup>1</sup>Statistically significant result.

*H. pylori-cagA*<sup>-</sup> cases (39.1%), a phenomenon not observed in the diffuse type. Also, *cagA*<sup>+</sup> among the intestinal cases displayed a higher frequency of *hMLH1* methylation than among diffuse *cagA*<sup>+</sup> cases (31.8% *vs* 15.8%, respectively). On the other hand, methylation of *DAPK* and *COX2* did not vary when the samples were grouped by histotype and *cagA* status. With regard to MSI and the presence of *cagA*, MSI was inversely correlated with the *cagA* gene (*P* = 0.012), as shown in Table 3.

### Detection of EBV

Fifty-four tumors were analyzed for EBV, of which 5 (9.3%) specimens were EBV-positive (EBV<sup>+</sup>). According to histological classification, 4 patients presented intestinal type adenocarcinoma and one was diffuse. All EBV<sup>+</sup> cases were advanced grade (III and IV stages) and presented lymph node metastases. Two cases were EBV/*H. pylori*. The EBV<sup>+</sup> cases were found to be associated with *H. pylori*, but only one was *H. pylori-cagA*<sup>+</sup>. Although the number of EBV<sup>+</sup> cases was small, most of them displayed *DAPK*, *COX2* and *CDKN2A* methylation.

## DISCUSSION

Our results show that methylation status tends to increase with age. This finding corroborates the fact that GC occurs at a higher frequency in older individuals<sup>[1,2]</sup>.

Moreover, previous studies showed that the age-related phenomenon of methylation of some tumor-suppressor and tumor-related genes can also be present in various non-neoplastic tissues, suggesting an association between age-related methylation and GC development<sup>[30-32]</sup>.

In this study, we demonstrate that 84.3% of GCs in our sample present methylation for about one in five of the genes analyzed. *COX2* was the gene found to be most commonly hypermethylated (63.5%) followed by *DAPK* (55.7%), *CDH1* (51%), *CDKN2A* (36%) and *hMLH1* (30.3%). An interesting observation in this study was related to the difference in methylation profiles between diffuse and intestinal adenocarcinoma types: in diffuse cases, the global methylation status, especially of *CDH1*, *COX2* and *CDKN2A*, has the highest frequency in early stage tumors (0- I) with a tendency to decrease along with tumor grades; while in the intestinal-type, the methylation status for *CDH1*, *COX2*, *hMLH1* and *CDKN2A* tended to increase from the earliest (0-I) to advanced stages (II-IV), as shown in Figure 2A and B. *CDH1* methylation was more frequent in the diffuse histotype. In fact, a vast difference was verified in stage I tumors where all diffuse-type tumors presented *CDH1* promoter methylation (100%) compared with only a small fraction (28.6%) in the intestinal-type, suggesting that *CDH1* methylation is an early event occurring in diffuse-type GCs. Since *CDH1* plays a fundamental role in maintaining cell differentiation,

polarity and normal tissue architecture<sup>[33]</sup>, the diffusely-growing and low cell cohesion characteristics of diffuse-type GC could be a function of *CDH1* down-regulation. Differences in the clinicopathological features between the intestinal and diffuse GC histological subtypes may be determined by different pathogenic processes<sup>[16,34]</sup>. The data presented in this study show that methylation in the same crucial genes could be an important pathway for the development of diffuse types. Identification of epigenetic differences between these two pathways could be of great importance in understanding gastric carcinogenesis and useful in the delineation of new therapeutic strategies.

The mechanisms that are implicated in *CDH1* promoter methylation are yet to be identified. The role of *H. pylori* in the regulation of *CDH1* expression has been described in recent studies showing that after *H. pylori* eradication, *CDH1* methylation is decreased<sup>[35,36]</sup>. In our study, we observed that in the intestinal adenocarcinoma cases, methylation in the *CDH1* promoter was more frequent in the group *H. pylori-cagA*<sup>+</sup> (55.8%) than in those with *H. pylori-cagA*<sup>-</sup> (39.1%), indicating that *H. pylori-cagA*<sup>+</sup> may be involved in *CDH1* methylation in these tumors.

*DAPK* methylation has been shown to be associated with aggressive and metastatic phenotypes in some human cancers<sup>[25,37]</sup>. In the present study, we found a substantial frequency of *DAPK* methylation and observed that positive lymph node cases showed a slightly higher frequency of *DAPK* methylation than unmethylated cases (57.1% *vs* 42.9%). An important finding in this study was the inverse correlation observed between *DAPK* methylation and MSI. Although the mechanisms linking MSI to *DAPK* methylation are not known, this finding may provide a clue towards a better understanding of the association between MSI and better prognosis, since *DAPK* participates in the positive control of apoptosis.

Similar to previously reported results<sup>[38]</sup>, 36.4% of the cases displayed hypermethylated *CDKN2A*. The EBV seems to play an important role in *CDKN2A* methylation<sup>[38,39]</sup>. However, the fact that, in this study, 60% of the EBV<sup>+</sup> cases showed methylated *CDKN2A* should be interpreted cautiously because of the low number of such cases. Methylation in *CDKN2A* was more frequent in patients over 50 years of age ( $P = 0.035$ ), and was also present in some samples of non-neoplastic gastric epithelia (data not shown) suggesting a link between aging and cancer *via* an increase in methylation. However, it is noteworthy that younger patients (< 50 years) who did not present *CDKN2A* methylation still developed GC, which suggests that other factors may account for the gastric carcinogenesis in these patients. In order to better evaluate in our population the correlation between *CDKN2A* and EBV it will be necessary to increase the number of tumors analyzed for EBV.

Most of the gastric tumors in our sample (63.5%) exhibit aberrant methylation of *COX2*. The correlation between *COX2* methylation and gene downregulation has been well documented in the literature<sup>[40]</sup> although overexpression of *COX2* has also been reported in GCs and some precancerous tissues<sup>[41]</sup>. *COX2* overexpression

is associated with enhanced proliferation, angiogenesis, resistance to apoptosis and tumorigenesis<sup>[41]</sup>. Despite the apparent selective advantage given by *COX2* overexpression, the results from our research group and others<sup>[42]</sup> suggest that *COX2* overexpression may not be essential in all cases of gastric tumorigenesis. Recent studies have documented that *H. pylori*-induced inflammation is linked to *COX2* overexpression<sup>[43]</sup> and these findings led us to investigate whether *cagA* presence was related to the methylation status of *COX2*. In the present study, no significant correlation between *cagA* and *COX2* methylation status was found. Thus, *cagA* presence appears not to be the only mechanism involved in the control of *COX2* expression in *H. pylori*<sup>+</sup> gastric cells.

Methylation of promoter regions is reported to be the main cause of inactivation of *hMLH1*<sup>[44]</sup>. The present study revealed a significant relationship between *hMLH1* methylation and MSI ( $P = 0.001$ ), corroborating findings from other studies<sup>[44,45]</sup>. In fact, methylation of this mismatch repair gene has been shown to play a major role in MSI in several cancers. The data from MSI analyses found in our study (17.2%) corroborated other studies in the literature which demonstrated MSI ranging from 13% to 39%<sup>[46]</sup>. The association between MSI and clinicopathological characteristics of GC remains unknown. While some studies reported that MSI gastric tumors are associated with distal tumor location, intestinal histotype, fewer lymph node metastases and better prognosis<sup>[46,47]</sup>, others have found the absence of an association among these parameters<sup>[48]</sup>. In our data, 33.3% of MSI patients had no positive lymph nodes (N0) *vs* 18.1% for MSS (stable) patients, suggesting a tendency for a better prognosis. Previous studies detected that MSI frequency in *H. pylori*-positive groups was significantly higher than in *H. pylori*-negative ones<sup>[49-51]</sup> indicating that this agent may play an important role in genetic stability. Several studies have suggested that the virulence attributed to the *H. pylori-cagA*<sup>+</sup> strain is associated with a severe inflammatory response. It is known that in the gastric mucosa, reactive oxygen species are released as a result of *H. pylori* infection<sup>[51]</sup> and that the expression of DNA mismatch repair proteins in mismatch-competent cells might be transiently suppressed in the presence of oxidative stress<sup>[52]</sup>. In the present study, when the cases were divided into two groups (*H. pylori-cagA*<sup>+</sup> and *H. pylori-cagA*<sup>-</sup>) we observed that, in contrast to our expectations, MSI was inversely correlated with *cagA* ( $P = 0.012$ ), suggesting that, apart from *cagA*, other factors may contribute to MSI occurrence. These results require further exploration with larger numbers of cases.

In conclusion, our results confirm that methylation is an early epigenetic event in the molecular pathogenesis of GC. The methylation pattern of the genes studied suggests that gastric tumorigenesis can occur through different pathways. It appears that in diffuse adenocarcinoma tumors, methylation can be an early and crucial event in enabling tumorigenesis, where methylation in *CDH1* assumes an important role and that MSI is associated with *hMLH1* methylation, although this event is infrequent. The inverse association discovered between *DAPK* methylation



and MSI provides new data for elucidating the mechanisms involved in the association of MSI and better prognosis. Analysis of a larger number of patients is necessary to confirm our findings and to ascertain the significance of the association between promoter methylation, MSI and the presence of infectious agents in gastric carcinogenesis. We observed that *H. pylori-cagA*<sup>+</sup> may be involved in the methylation process of *CDH1* in intestinal adenocarcinoma. Microsatellite instability was inversely correlated with the *cagA* gene, suggesting that other factors may contribute to MSI occurrence. COX-2 overexpression does not occur in all GC cases.

## COMMENTS

### Background

Gastric cancer (GC) is one of the most common cancer types and it is associated with high mortality rates. The prognosis of this disease remains poor, especially when the diagnosis is undertaken at advanced stages. The most common GC is adenocarcinoma divided into two types, intestinal and diffuse, and these differ substantially in epidemiology, pathogenesis, genetic profile and prognostic features. *Helicobacter pylori* (*H. pylori*) is one of the more important etiological factors in GC. The *cagA* gene codes for one of the major *H. pylori* virulence proteins. Bacterial strains that have the *cagA* gene are associated with an ability to cause a stronger inflammatory response in the stomach and patients are at a greater risk of developing peptic ulcers or stomach cancer. The second infectious agent found to be associated with a subset of GCs is the Epstein Barr virus (EBV). DNA methylation is an epigenetic modification found in physiological events, however, when it is aberrant it can be associated with inactivation of tumor suppressor genes. Microsatellite Instability (MSI) reflects an erroneous form of DNA replication in repetitive microsatellite sequences and has been considered a hallmark of DNA mismatch repair gene inactivation and therefore consequently leads to genetic instability.

### Research frontiers

Some studies have linked DNA hypermethylation and MSI with *H. pylori-cagA*<sup>+</sup> and EBV infection but these data are not conclusive and the studies did not look at both agents at the same time. Therefore it is very important to analyze the same cases for both etiological factors and correlate them with MSI genetic and epigenetic alterations as well as with clinical and epidemiological data.

### Innovations and breakthroughs

The present study suggests that intestinal-type and diffuse-type GC show different methylation profiles in the genes analyzed and a strong association was found between methylation of *CDH1* and *H. pylori-cagA*<sup>+</sup> in the intestinal-type GC. In addition, a very significant inverse correlation was found between methylation of *DAPK* gene (*DAPK* has a pivotal role in regulation of apoptosis and survival in cells) and MSI, providing new evidence for the association of MSI and better prognosis.

### Applications

The data presented in this article represent important information about methylation profiles for intestinal-type and diffuse-type GC. Results show the association between *H. pylori-cagA*<sup>+</sup> and methylation in an important gene involved in metastasis (*CDH1*) in addition to showing the inverse association between *DAPK* methylation and MSI, providing new data for elucidating the mechanisms involved in the association of MSI and better prognosis. This will add to the available body of knowledge about gastric carcinogenesis and aid in future research into this important disease.

### Peer review

The manuscript by Ferrasi *et al* describes the methylation status and MSI frequency in the context of *H. pylori* and EBV infections in GC. This topic is of scientific and of clinical interest.

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## ***Helicobacter pylori* HopE and HopV porins present scarce expression among clinical isolates**

Maritza Lienlaf, Juan Pablo Morales, María Inés Díaz, Rodrigo Díaz, Elsa Bruce, Freddy Siegel, Gloria León, Paul R Harris, Alejandro Venegas

Maritza Lienlaf, Juan Pablo Morales, María Inés Díaz, Rodrigo Díaz, Elsa Bruce, Alejandro Venegas, Microbial Pathogenesis and Vaccine Biotechnology Laboratory, Department of Molecular Genetics and Microbiology, Faculty of Biological Sciences, Pontificia Universidad Católica de Chile, Alameda 340, Santiago 651-3492, Chile

Freddy Siegel, Gastroenterology Unit, Faculty of Medicine, Universidad Austral de Chile, Independencia 641, Valdivia 511-0566, Chile

Gloria León, Institute of Biochemistry, Faculty of Sciences, Universidad Austral de Chile, Independencia 641, Valdivia 511-0566, Chile

Paul R Harris, Department of Pediatrics, School of Medicine, Pontificia Universidad Católica de Chile, Lira 85, Santiago 833-0074, Chile

**Author contributions:** Lienlaf M and Morales JP performed most of the cloning and immunoblotting experiments; Díaz MI was in charge of strains stock and growth; Díaz R and Bruce E collected biopsies and isolated strains; Harris PR, Siegel F and León G provided the collection of human material and were involved in editing the manuscript; Venegas A designed the study, provided financial support for this work and wrote the manuscript.

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**Correspondence to:** Dr. Alejandro Venegas, Microbial Pathogenesis and Vaccine Biotechnology Laboratory, Department of Molecular Genetics and Microbiology, Faculty of Biological Sciences, Pontificia Universidad Católica de Chile, Alameda 340, Santiago 651-3492, Chile. [avenegas@bio.puc.cl](mailto:avenegas@bio.puc.cl)

Telephone: +56-2-6862661 Fax: +56-2-2225515

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**METHODS:** *H. pylori* *hopE* and *hopV* genes derived from strain CHCTX-1 were cloned by polymerase chain reaction (PCR), sequenced and expressed in *Escherichia coli* AD494 (DE3). Gel-purified porins were used to prepare polyclonal antibodies. The presence of both genes was tested by PCR in a collection of *H. pylori* clinical isolates and their expression was detected in lysates by immunoblotting. Immune responses against HopE, HopV and other *H. pylori* antigens in sera from infected and non-infected patients were tested by Western blotting using these sera as first antibody on recombinant *H. pylori* antigens.

**RESULTS:** PCR and Western blotting assays revealed that 60 and 82 out of 130 Chilean isolates carried *hopE* and *hopV* genes, respectively, but only 16 and 9, respectively, expressed these porins. IgG serum immunoreactivity evaluation of 69 *H. pylori*-infected patients revealed that HopE and HopV were infrequently recognized (8.7% and 10.1% respectively) compared to *H. pylori* VacA (68.1%) and CagA (59.5%) antigens. Similar values were detected for IgA serum immunoreactivity against HopE (11.6%) and HopV (10.5%) although lower values for VacA (42%) and CagA (17.4%) were obtained when compared to the IgG response.

**CONCLUSION:** A scarce expression of HopE and HopV among Chilean isolates was found, in agreement with the infrequent seroconversion against these antigens when tested in infected Chilean patients.

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**Key words:** *Helicobacter pylori*; Gene expression; HopE and HopV porins; Antigens; Immune response

**Peer reviewers:** Mario M D'Elios, Professor, University of Florence, viale Morgagni 85, Florence 50134, Italy; Andrew Day, Assistant Professor, University of Otago, Christchurch Hospital, Christchurch 8140, New Zealand

### **Abstract**

**AIM:** To evaluate how widely *Helicobacter pylori* (*H. pylori*) HopE and HopV porins are expressed among Chilean isolates and how seroprevalent they are among infected patients in Chile.



Lienlaf M, Morales JP, Díaz MI, Díaz R, Bruce E, Siegel F, León G, Harris PR, Venegas A. *Helicobacter pylori* HopE and HopV porins present scarce expression among clinical isolates. *World J Gastroenterol* 2010; 16(3): 320-329 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i3/320.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i3.320>

## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) are Gram-negative, micro-aerophilic, spiral-shaped bacteria isolated from human gastric biopsies in 1983<sup>[1]</sup>. In order to survive in this aggressive environment, *H. pylori* are able to neutralize their close surrounding space by production of urease, which catalyzes the conversion of urea into ammonium and CO<sub>2</sub>, raising pH close to neutral. In addition, to colonize the epithelium, this bacterium is able to bind to the epithelial cell surface, partially avoiding its removal by natural peristalsis or mucus renewal. These characteristics allow *H. pylori* to persist for decades.

*H. pylori* infection affects one half of the world population, roughly 73% in Chile<sup>[2]</sup>, with higher prevalence as age increases. After several years of chronic gastric infection, approximately 10%-15% of infected patients develop severe gastrointestinal diseases such as chronic gastritis, peptic ulcer and gastric carcinoma<sup>[3,4]</sup>. In Chile, 5% of the infected population develops gastric cancer<sup>[2]</sup> and this malignancy is the second cause of death by cancer in the country.

*H. pylori* carries various virulence factors, and some may have potential as vaccine antigens. These factors may be grouped as: (1) colonization factors, which allow bacterial residence; (2) persistence factors which enable bacteria to accomplish an effective and lasting survival; and (3) disease inducing factors which cause adverse pathological effects on the gastric mucosa<sup>[5]</sup>.

Based on a bioinformatics analysis of the *H. pylori* genome, a family of outer membrane proteins (OMPs) composed of 33 members has been identified<sup>[6]</sup>. These proteins are assembled into the outer membrane exposing, on the bacterial surface, small peptide loops which may act as epitopes to induce an immune response. This feature may be useful when selecting appropriate antigens for vaccine design. All these members contain an N-terminal signal peptide (processed by signal peptidase type I or II) that allows these proteins to cross the inner membrane on their way towards the outer membrane. The *H. pylori* OMPs form 2 families: the Hop members (21 proteins) and the Hor members (12 proteins). Hor proteins lack a characteristic N-terminal Hop motif<sup>[7]</sup>. Hop proteins have structural homology with the *Escherichia coli* (*E. coli*) outer membrane protein F (OmpF) porin<sup>[8]</sup>. Currently, 5 *H. pylori* Hop members (HopA, HopB, HopC, HopD and HopE) from strain 26695 have been characterized as porins using planar bilayer techniques<sup>[7,9]</sup> and some also behave as adhesins<sup>[10,11]</sup>. These properties make them attractive

candidates as vaccine antigens. In fact, other bacterial porins from *Salmonella*, *Pseudomonas*, *Chlamydia* and *Neisseria*, have been found to be strong immunogens<sup>[12-15]</sup>. However, in the case of *H. pylori*, it has been suggested that not all the genes encoding OMPs may be functional at a given time. Some of these genes are under a control mechanism that operates by strand slippage during DNA replication or DNA repair. DNA polymerase slippage may easily add or remove nucleotides when DNA synthesis occurs in front of a homopolymeric tract or dinucleotide repeats at the template strand (i.e. *polyG* or *polyCA* gene segments) causing mutations either at the promoter or at the coding region. This type of mutation may turn off or on some *hop* genes that may include these polynucleotide features. For instance, the *hopC* gene has been reported to carry a *polyT* tract (13 Ts in length) near the 5' end but *hopA*, *hopB* and *hopE* do not have such long *polyT* tracts either at their coding regions or 5' upstream at the promoter regions. Gene switching will produce a change in the antigenic bacterial surface, a strategy that will distract the host immune system. For this reason, whether any *H. pylori* OMP would be considered as a vaccine antigen, *omp* genes containing long homopolymeric tracts or dinucleotide repeats should be avoided.

Regarding HopV and HopW, genetic heterogeneity in orthologous members of the Hop family among *H. pylori* strains has been described<sup>[16]</sup>. These new members were defined as part of the HopA/HopE family, because of their homology at the N-terminal sequence and the presence of 7 homologous domains in the C-terminus region. Regarding functional aspects, HopV and HopW have pore sizes similar to that of the *E. coli* OmpF porin<sup>[16]</sup> and HopE has been defined as the homolog to the *E. coli* OmpF porin<sup>[17]</sup>.

Since the use of porins as antigens has been reported as successful<sup>[12-15]</sup>, we decided to evaluate members of the *H. pylori* Hop family as putative antigen candidates for vaccine development by determining how widely they are expressed among Chilean *H. pylori* isolates and how often Chilean patients develop antibodies against them. A brief bioinformatic survey indicated that some genes of the Hop family had homopolymeric tracts or dinucleotide repeats in their coding sequences and promoter regions, with potential capability to promote strand slippage which may affect stability of gene expression<sup>[18]</sup>. Considering this aspect, only porin genes having single homopolymeric tracts or dinucleotide repeats no longer than 6 bases in their coding sequences were chosen as source of putative useful antigens for a vaccine. For this reason, among several OMP genes, only *hopE* and *hopV* sequences were selected for the present study.

## MATERIALS AND METHODS

### Bacterial strains and plasmid vectors

*E. coli* DH5α was used for polymerase chain reaction



Table 1 Primer sequences used for amplification and sequencing of *H. pylori* *hopE* and *hopV* genes

Name	5'→3' sequence	Restriction sites <sup>1</sup>
HopV1 <sup>2</sup>	GGGCCCATATGCTCAATTTATGACAAAGAAGAAAAATAGAATGC	<i>Nde</i> I
HopV2 <sup>2</sup>	GGATCCCATGGTTAAAAATCCCTCAAGTAAGTACTGATTG	<i>Bam</i> HI
HopE1 <sup>2</sup>	GGCGCCATGGAAATTTATGAAAAAGTTTGTAGCTTTAGG	<i>Nco</i> I
HopE2 <sup>2</sup>	CGCGAAGCTTTTAAAAAGTGTAGTTATACCCTAAATAAAG	<i>Hind</i> III
HopE11 <sup>3</sup>	GCAAGTGGTTTGGTTTATAGAG	-
HopE22 <sup>3</sup>	ACCATATCCAAGTGGATTTT	-
HopV11 <sup>3</sup>	GGCGTGGGGTTAGATACGCTG	-
HopV22 <sup>3</sup>	ACCATGTTTTCTTTATTAC	-
HopVint <sup>3</sup>	ATGCGTTATTATGGGTTTTTGACT	-
pETT7d <sup>4</sup>	TAATACGACTCACTATAGGG	-
pETT7r <sup>4</sup>	GCTAGTTATTGCTCAGCGG	-

<sup>1</sup>The restriction sites included in primer sequences and used for ligation to plasmid vectors are shown underlined;

<sup>2</sup>External primers used to clone porin genes; <sup>3</sup>Internal primers used for gene sequencing and also for confirmatory PCR reactions for those cases in which primers derived from 5' and 3' gene ends failed to raise PCR products; <sup>4</sup>Vector primers for 5' and 3' end gene sequencing.

(PCR) cloning, and *E. coli* BL21 (DE3), JM109 (DE3) and AD494(DE3)pLysS as host for porin expression assays. For cloning of PCR fragments plasmid pGEM-T Easy from Promega was used. For expression studies pET21a and pET21d (Novagen) were selected. *H. pylori* Chilean strain CHCTX-1 was used as DNA source for gene amplifications<sup>[19]</sup>. A collection of 130 *H. pylori* strains isolated from infected patients living in different Chilean cities was already available<sup>[20]</sup>.

### Bacterial cultures

*E. coli* cells were grown overnight in LB media at 37°C with shaking. *H. pylori* strains were grown under 50 mL/L CO<sub>2</sub> and 80% humidity in *Brucella* agar plates enriched with 5% horse blood cells and grown at 37°C for 2-3 d. *E. coli* strains were kept for short periods in LB plates at 4°C. Bacterial strains containing 14% glycerol were stored frozen for longer periods at -70°C.

### Plasmid purification, DNA manipulation and bacterial transformation

Plasmids were usually detected by the “one step” method<sup>[21]</sup>, and purified by alkaline lysis method<sup>[22]</sup>. Restriction digestions, DNA ligations and plasmid dephosphorylations were done according to standard procedures<sup>[22]</sup>. Electroporation in 0.2 cm electrode separation cuvettes was performed as previously described<sup>[23]</sup>, in a Gene Pulser™ apparatus. Electrocompetent cells were prepared according to described protocols<sup>[23]</sup> with a yield of  $1 \times 10^9$  to  $1 \times 10^{10}$  cells/mL.

### PCR assays

Primers corresponding to the 5' and 3' ends as well as the internal sequences of the *hopE* and *hopV* genes (Table 1) were designed based on *H. pylori* 26 695 GenBank sequences. As templates, chromosomal DNA from the CHCTX-1 strain<sup>[19]</sup> and from clinical isolates was prepared according to described procedures<sup>[24]</sup>. PCR reactions were carried out in a BioRad Mastercycler II thermocycler, with *Pfu* polymerase (Stratagene, CA,

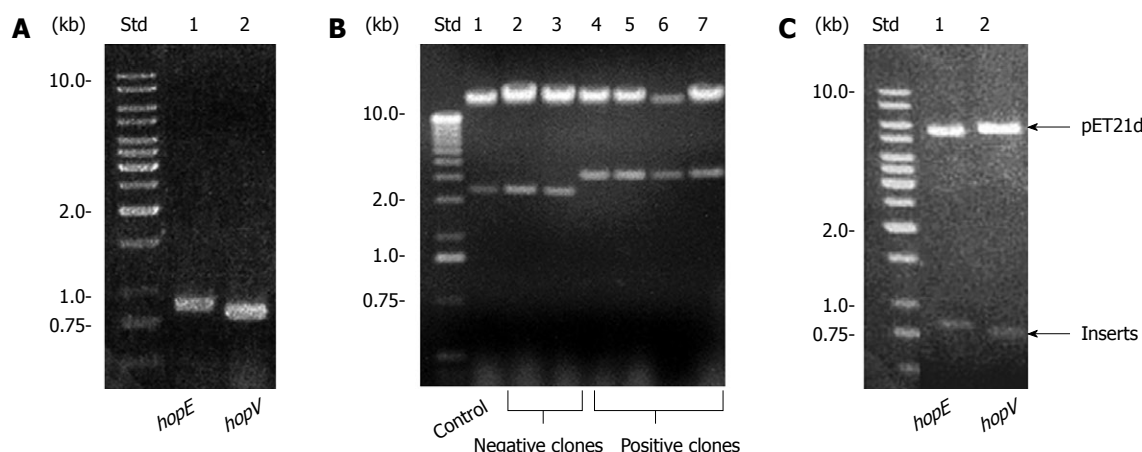
USA) or *Taq* polymerase (Promega, Madison WI, USA). Assays were done in 25 µL final volume following the manufacturer's instructions. Gene amplicons were detected by 1% agarose gel electrophoresis. Other conditions were as previously described<sup>[25]</sup>. *VacA* alleles were determined by using primers and assay conditions described by Atherton *et al*<sup>[26]</sup>.

### Polyclonal antibodies against *H. pylori* OMPs

According to standard procedures<sup>[27]</sup>, anti-HopE and anti-HopV rabbit antibodies were prepared. Proteins were obtained from *E. coli* clones expressing the *H. pylori* porins after separation by SDS-PAGE and purification from gel slices by electroelution as previously described<sup>[28]</sup>. Pathogen-free New Zealand adult female rabbits (approximately 1400 g in weight) were immunized with 250 µg of each porin dissolved in 2 mL of Tris-glycine buffer mixed (1:1) with complete Freund's adjuvant. Two animals were used for each porin inoculation and 100 µL aliquots were applied subcutaneously in the back. This was followed by 3 boosters every 15 d.

### SDS-PAGE and Western blotting

Lysates from clones expressing HopE and HopV were separated by polyacrylamide gels (12% or 15%) and run in minichambers according to Laemmli<sup>[29]</sup>. Western blotting were done as previously described<sup>[30]</sup>. As first antibody, patient serum (1:100 dilution) or rabbit anti-porin antibodies against HopE or HopV (1:1000 dilution) were used. As a second antibody for patient assays, goat anti-human IgA or anti-human IgG conjugated to peroxidase, were incubated (1:1000) overnight at 4°C. For anti-porin rabbit sera, a goat anti-rabbit peroxidase-conjugated antibody (1:1000) second antibody was used. To reduce cross reactions, rabbit antisera and human antisera were adsorbed with sonicated lysates from *E. coli* AD494(DE3)pLysS/pET21d and BL21 (DE3), respectively. Human sera immunoblotting were done with lysates expressing HopE or HopV porins and products



**Figure 1** Cloning of *H. pylori* *hopE* and *hopV* genes. A: Polymerase chain reaction (PCR) amplification of *hopE* and *hopV* genes. Amplicons and plasmids were separated by 1% agarose gel electrophoresis. Lane Std: 1 kb DNA ladder standard; B: Detection of plasmids carrying the *hopE* gene. Lane 1: Strain AD494(DE3)pLysS with pET21d as control. Lanes 2, 3: Negative clones, lanes 4-7 plasmids carrying the *hopE* gene; C: Release of inserts carrying the *hopE* and *hopV* genes after *Nco* I / *Hind* III and *Nde* I / *Bam* H I digestions respectively. Lane Std: 1 kb DNA ladder standard (Fermentas).

derived from expression of *cagA* and *vacA* gene fragments cloned from strain CHCTX-1<sup>[19]</sup>.

### Patient sera

A sera panel from 69 infected patients (63 with gastritis, 6 with ulcers) recruited from the Universidad Católica de Chile Medical Center in Santiago, with signed consent, was available. Each patient's infected condition was defined by endoscopy, positive urease rapid test and detection of hematoxylin/eosin-stained curved bacteria on gastric tissue biopsies. Also, 8 non-infected patients were included in this study. The local ethics committee approved the protocol for this study.

### Immunoprecipitation of IgG from patient sera

In order to obtain a cleaner IgA reaction in Western blotting assays using patient serum antibodies, protein G-plus-Agarose (Santa Cruz Biotechnology, catalogue #sc-2002) was utilized to first remove IgG from serum by immunoprecipitation. One hundred microliters of each serum without pre-adsorption treatment were incubated overnight with 200  $\mu$ L of protein G-plus-agarose at 4°C with mild shaking. After sedimentation for 5 min at 2500 r/min and 4°C, the supernatant of each sample was used as a source of IgG-free serum.

### DNA sequencing

DNA samples were previously purified by a commercial kit, and sequenced at our Sequencing Core Facility. T7 and internal primers for DNA sequencing are listed in Table 1.

## RESULTS

### Cloning of porin genes derived from a Chilean *H. pylori* strain as putative antigens

Selection for cloning and expression studies of the *hopE* and *hopV* genes were based on known gene

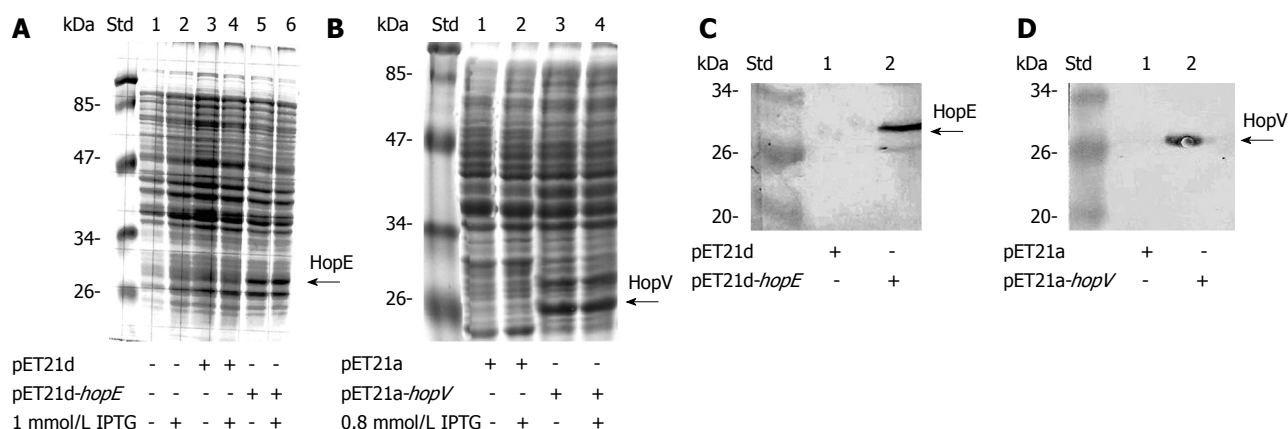
sequences from strain *H. pylori* 26 695. Since our study was focused on antigens obtained from local strains, *H. pylori* CHCTX-1 strain, a clinical isolate obtained from a Chilean patient<sup>[19]</sup> was selected as the DNA source for gene cloning in this study.

Cloning of HopE and HopV porin genes, including their signal peptide regions, was done by PCR. Primers and assay conditions are described in Table 1 and Methods, respectively. Amplicons from *hopE*, and *hopV* genes were separated in a 1% agarose gel (Figure 1A), purified and treated with Taq polymerase and dATP to be ligated to pGEM-T. Recombinant plasmids were detected by insert release after *Eco*R I digestion and separation in 1% agarose gel electrophoresis. The *hopE* and *hopV* cloned inserts were subjected to *Nco* I - *Hind* III and *Nde* I - *Bam* HI double digestions and ligated to pET21d and pET21a, respectively. As expected, fragments with sizes corresponding to these genes were observed. For expression purposes, plasmids were transferred to *E. coli* AD494(DE3)pLysS cells and visualized by the "one step method"<sup>[21]</sup>. Some clones containing plasmids with the *hopE* gene are displayed in Figure 1B. Purified plasmids were used for restriction digestions and also as DNA templates for PCR gene detection. *Nco* I - *Hind* III and *Nde* I - *Bam* HI double digestions of plasmid DNA isolated from single clones were analyzed by agarose gel electrophoresis, and released inserts with sizes close to the expected ones (819 bp for *hopE* and 735 bp for *hopV*) were observed (Figure 1C).

### Expression of *H. pylori* HopE and HopV porin genes in *E. coli*

*E. coli* AD494(DE3)pLysS was able to express detectable amounts of HopE and HopV porins, as seen after SDS-PAGE and Coomassie blue staining (Figure 2A and B) and Western blotting assays (Figure 2C and D).

Expression conditions were optimized by 5 h induction with 1 mmol/L isopropyl  $\beta$ -D-thiogalactoside



**Figure 2** Expression of *H. pylori* *hopE* and *hopV* genes in *Escherichia coli* AD494(DE3)pLysS. Bacterial lysates were separated in 12%-15% PAGE-SDS gel, stained with Coomassie blue (A and B) or analyzed by Western blotting (C and D). Conditions for HopE expression are indicated below the figure (A, lanes 5 and 6; C, lane 2). Conditions for HopV expression are indicated under the figure (B, lanes 3 and 4; D, lane 2). Arrows indicate electrophoretic migration of these proteins. Lane Std: Prestained molecular weight marker in kDa (Fermentas).

(IPTG) for *hopE* and 0.8 mmol/L for *hopV* on previously saturated cultures. Protein sizes of 32 kDa for HopE and 28 kDa for HopV were observed. These porins displayed a certain amount of expression without IPTG induction, partially explained by the fact that the induction procedure was done on saturated cultures.

#### Sequence analysis of *H. pylori* *hopE* and *hopV* genes

The *hopE* (clone 1) and *hopV* (clone 13) gene sequences from the CHCTX-1 strain were obtained using external (T7 promoter and T7 terminator) as well as internal primers (Table 1) as described in Methods. Both *hopE* and *hopV* sequences were deposited at GenBank (accession numbers #EF635415 and #EF635416, respectively). As expected, these genes did not contain nonsense or frameshift mutations at their coding regions. Also, neither homopolymeric nor dinucleotide tracts longer than 6 nucleotides were found.

#### Detection of *hopE* and *hopV* genes in Chilean clinical isolates and their expression

From a collection of 240 clinical strains previously isolated<sup>[20]</sup>, we selected 130 colonies (1 to 5 isolates per patient) as representatives from 6 Chilean cities: Iquique (IQ) in the North, Valparaíso (VA) and Santiago (SA) in the central region, Los Angeles (LA) and Valdivia (VL) in the South, and Punta Arenas (PA), the Southernmost city, to evaluate the distribution of strains carrying *hopE* and *hopV* genes and their expression throughout the country.

Detection of the genes was done by standard PCR. The amplicons were almost identical in size to those expected for *hopE* and *hopV* genes from strain 26695. Representative groups of isolates carrying *hopE* and *hopV* genes are shown in Figure 3A and B, respectively.

The *hopE* and *hopV* genes were detected in 46.9% (61 out of 130 strains) and 63.1% (82 out of 130) of the studied strains, respectively, and 40% (52 out of 130) of the strains revealed the presence of both genes simultaneously (Table 2). Among different cities,

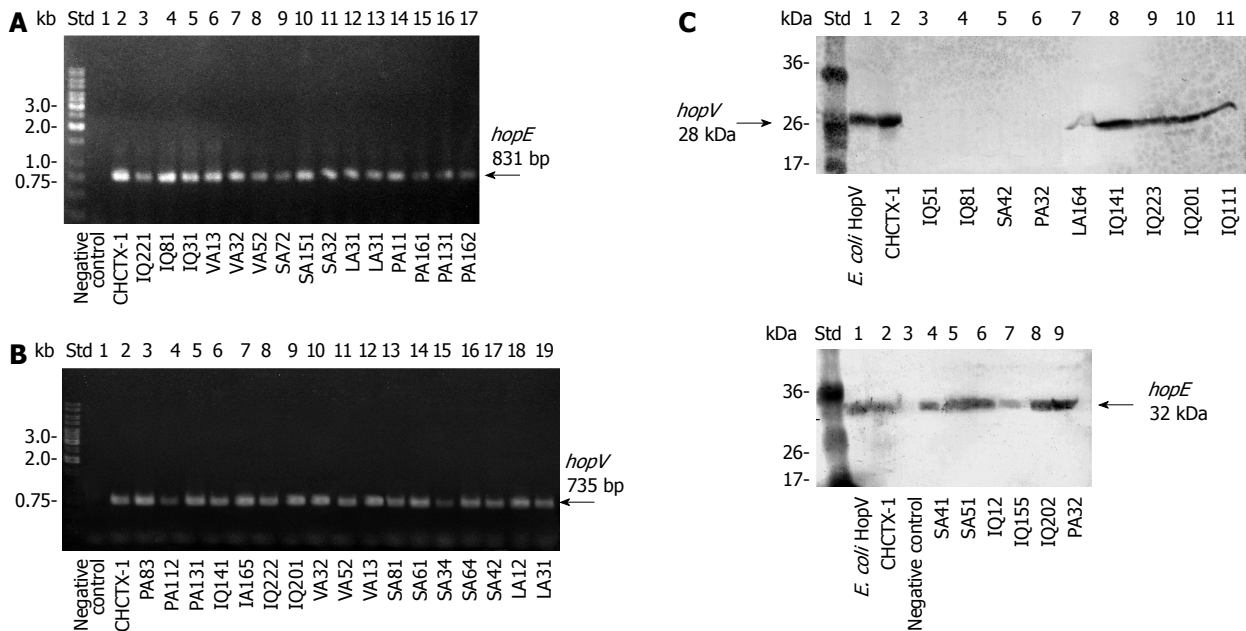
*hopE* and *hopV* gene contents varied between 30% and 69%. Curiously, *hopV* was frequently detected (69.2%) in strains from infected patients living in PA, the southernmost city. Patients from VL (mostly descendants from ancient aborigines) carried strains with a lower content (42.8%) of *hopV* gene. All PCR reactions were done at least twice using a pair of primers which bound to the gene ends. For those cases with negative amplification, additional assays using 2 primer combinations, including in each pair of primers one of the internal primers (Table 1), were performed. In most cases negative PCR reactions were confirmed and just a few strains showed positive PCR amplification only with pairs including internal primers, indicating that our initial estimation about the reduced presence of these genes in Chilean isolates was valid.

The positive results of HopE and HopV Western blotting expression assays in these isolates revealed no protein size variation, and selected results are displayed in Figure 3C.

Regarding porin expression, results showed that only 13.1% of the 130 strains expressed HopE and 6.9% expressed HopV. Altogether, 83.8% (109 out of 130 strains) did not express HopE or HopV porins either because of a lack of these genes, random inactivating gene mutations or gene silencing by the strand slippage mechanism (Table 2).

#### Recognition of HopE and HopV porins by sera from infected patients

In order to evaluate the capability of sera from Chilean *H. pylori*-infected patients to recognize recombinant *H. pylori* HopE and HopV porins, sera from 69 infected and 8 non-infected patients were tested. IgG and IgA serum antibodies against HopE and HopV antigens expressed as recombinant proteins in *E. coli* clones were tested using Western blotting assays on these bacterial lysates. VacA and CagA expressed similarly were used as immunodominant controls. The number of infected



**Figure 3** Detection of *hopE* and *hopV* genes and their expression in *H. pylori* clinical isolates from different Chilean cities. Amplicons were separated in 1% agarose gels. A: PCR detection of the *hopE* gene; B: PCR detection of the *hopV* gene. Arrows indicate migration of the respective gene fragments. Lane Std: 1 kb DNA ladder standard (Fermentas); Lane Std2: Lambda DNA/HindIII marker (Fermentas); C: Expression of HopV and HopE porins in *H. pylori* Chilean strains separated by 12% SDS-PAGE gels and detected by Western blots with respective polyclonal antibodies. Clinical isolates are indicated under the respective lanes. Std: Prestained molecular weight marker (Fermentas). The CHCTX-1 strain was included as a positive control.

**Table 2** Number of strains presenting genotypes and corresponding phenotypes indicating the presence of *hopE* (E) and *hopV* (V) genes and their expression in 130 *H. pylori* strains isolated from infected patients from 6 Chilean cities

Genotypes <sup>1</sup> (E/V)	No. of strains	Phenotypes <sup>2</sup> (E/V)	No. of strains	No. of strains with different E/V phenotypes per city <sup>3</sup>					
				IQ	VA	SA	LA	VL	PA
(+/+)	52	(+/+)	5	4	-	1	-	-	-
		(+/-)	12	4	-	5	1	-	2
		(-/+)	-	-	-	-	-	-	-
		(-/-)	35	15	3	3	1	7	6
(+/-)	9	(+/-)	-	-	-	-	-	-	-
		(-/-)	9	2	-	-	-	5	2
(-/+)	30	(-/+)	4	1	-	2	-	-	1
		(-/-)	26	11	-	4	-	2	9
(-/-)	39	(-/-)	39	11	2	11	2	7	6
Totals	130	Totals	130	48	5	26	4	21	26

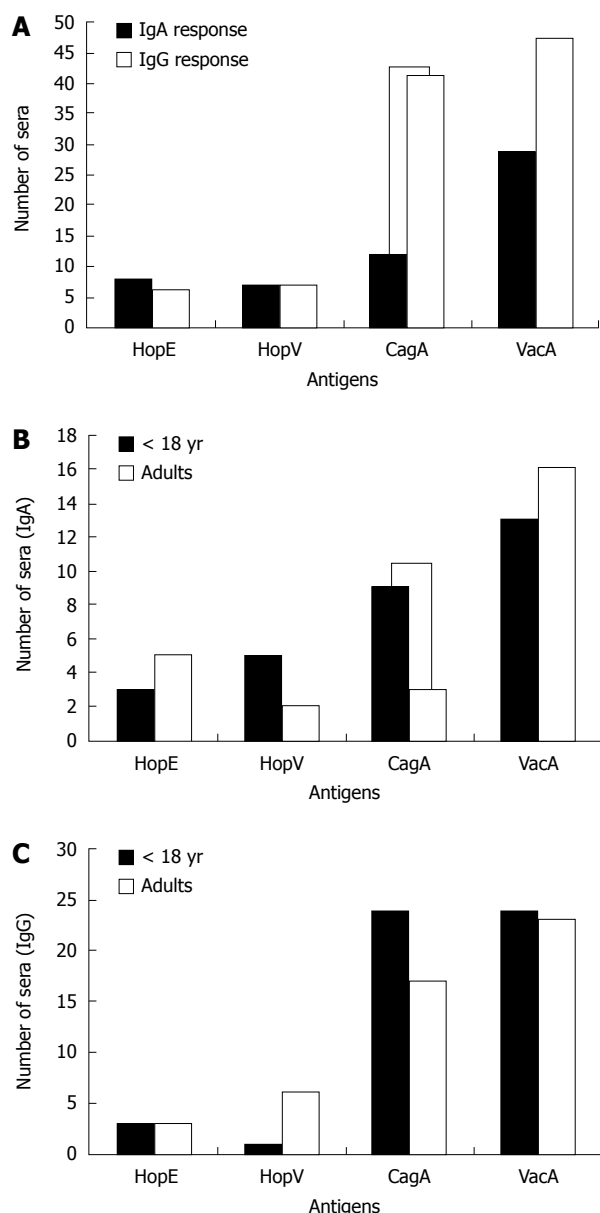
<sup>1</sup>Presence of *hopE* (E) and/or *hopV* (V) genes in Chilean *H. pylori* isolates determined by PCR amplifications as described in Methods using purified DNA templates from single colonies collected from 69 patients of the indicated cities. (+): gene presence; (-): no detection; <sup>2</sup>HopE and/or HopV expression assayed by Western blotting (see Methods). (+): detection; (-): no detection; <sup>3</sup>Total number of strains with the assigned phenotype per city. IQ: Iquique; VA: Valparaíso; SA: Santiago; LA: Los Angeles; VL: Valdivia; PA: Punta Arenas. The number of patients per city was IQ = 21, VA = 4, SA = 12, LA = 3, VL = 5, PA = 15. Number of strains isolated per patient ranged between 1 and 5.

patient sera able to recognize these antigens are shown in Figure 4A. It was found that, as expected, IgG human antibodies more frequently recognized VacA (68.1% or 47 out of 69) and CagA (59.4%), but rarely recognized HopE (8.7%) and HopV (10.1%) porins. A similar distribution for HopE (11.6%) and HopV (10.5%), but lower distribution values for VacA (42%) and CagA (17.4%) were found for IgA antibodies. The lower number of anti-HopE and anti-HopV reactive sera can be explained by the low proportion of *H. pylori* strains able to express these genes, being 13.1% (17/130) for

HopE and 6.9% (9/130) for HopV (Table 2). Taken together, these results strongly suggest that *H. pylori* possesses a mechanism to switch on/off these OMP genes as a strategy to evade the host immune response.

In addition, considering that the immune response in children<sup>[31,32]</sup> could be quite different from that in adults<sup>[33]</sup>, the IgA (Figure 4B) and IgG (Figure 4C) immune responses of the infected patients were plotted for 2 age groups: under 18 years of age and adults. It was noted that the number of sera with IgA and IgG responses against CagA antigen was significantly higher





**Figure 4** Frequency of recognition of 4 *H. pylori* antigens by human sera. Bars represent number of immunoreactive human sera from a panel of 69 *H. pylori*-infected patients showing IgG- and IgA-type immunoreactivity tested by Western blotting assays on *E. coli* lysates expressing separately HopE, HopV, CagA and VacA cloned antigens. A: Frequency of IgA- and IgG-type immune responses (as number of sera) which reacted with lysates containing one of these antigens; B: IgA response data taken from panel A, with patients separated by age into 2 groups: under 18 years old and adults; C: IgG response data taken from panel A, separated by age as above. As negative controls, sera from 8 non-infected patients did not display any immune response when tested with these antigens (not shown). Fisher's test was used for statistical analysis, and significance lower than 0.05 is indicated.

in patients under 18 than in adults. In contrast, IgA responses against HopE and VacA and the IgG response against HopV seemed to be more frequent in adults than in children.

## DISCUSSION

*H. pylori* colonizes the human gastric epithelium in half

of the world's population and induces strong serological and inflammatory responses in the host which persist during the entire life of the subject, rendering the host unable to eradicate infection. Knowledge of the most frequently recognized antigens in the infected population may contribute to an understanding of the bacterium survival strategy. In addition, this could also help to select appropriate antigens for vaccine design. The most extensively studied *H. pylori* virulence factors as potential vaccine antigens are urease subunits<sup>[34,35]</sup>, VacA and CagA<sup>[36]</sup>, *H. pylori* adhesin A<sup>[37]</sup> and neutrophil-activating protein<sup>[38,39]</sup>. However, results indicating reduction in colonization after oral vaccination of human subjects have been rather modest<sup>[40,41]</sup>. As new antigens are needed, and there are few studies comparing porin genes among different *H. pylori* strains, we have looked for *H. pylori* porins suitable for vaccine development. The present report provides new information, particularly about Chilean strains, regarding porin expression among clinical isolates.

Folded porins have exposed loops at the bacterial surface which may induce a strong immune response. Porins have been proposed for the design of oral vaccines to eradicate *H. pylori*<sup>[6]</sup>. This pathogen has an extensive collection of OMPs with defined pore characteristics, such as the Hop group. However, some may display a unique on/off mechanism affecting gene expression, based on DNA strand slippage during replication. The instability of expression status makes antigen selection a very important issue. Antigens that will not be permanently expressed in most of the strains should be avoided, since they will provide a limited protection.

PCR assays carried out on 130 Chilean isolates detected *hopE* and *hopV* genes in 46.9% and 63.1%, respectively. However porin expression was infrequently detected in these strains (HopE = 13.1%, HopV = 6.9%), concurring with low seroprevalence of these porins among sera of infected patients, suggesting that these genes may be under DNA strand slippage control.

Infrequent detection of expression cannot be explained by low immunoreactivity of the rabbit antisera resulting from amino acid sequence diversity among strains, since these 2 porin sequences seem to be conserved. HopE and HopV amino acid sequence identities among those from strain CHCTX-1 and the corresponding sequences from fully sequenced *H. pylori* genomes, were in the range of 98%-99% and 94%-96%, respectively.

As a complementary approach, 69 patient sera were tested by Western blotting on *E. coli* clones expressing CagA, VacA, HopE or HopV. It was revealed that HopE and HopV porins were not often recognized within the analyzed sample. Only 6 sera (8.7%) showed IgG-type immune reaction against HopE-containing lysates and 7 (10.1%) against HopV. Similar results were obtained analyzing the IgA response. These results agree with the fact that HopE and HopV porins are sporadically expressed. In contrast, CagA (its gene is present in

about 50% of the strains) and VacA (its gene is present in almost 100% of the strains) reacted with 59.4% and 68.1% of the IgG patient sera, respectively.

Regarding nucleotide sequence features, dinucleotide repeats in *hopE* and *hopV* sequences from the CHCTX-1 strain barely reached 5 nucleotides in length. However, they contained CCCCCC and TTTTTT tracts after codons 58 and 66, respectively. These findings, taken together with the low number of strains expressing these porins, and their low seroprevalence among Chilean patients, suggest that *hopE* and *hopV* may be under strand slippage gene control. Confirmation should be done by sequencing strains carrying silenced genes.

In *H. pylori*, at least 3 porin genes from the Hop family (*hopZ*, *hopP* and *hopO*) may be subjected to this on/off switching<sup>[11,42]</sup>. Another study<sup>[43]</sup> showed a similar case: 3 different *H. pylori* strains re-isolated after *Maccacrus rhesus* infection lost expression of BabA adhesin which binds Lewis b antigen. These observations support the idea that *H. pylori* can modulate expression of some OMP genes. This feature provides an adaptive mechanism to avoid induction of a strong host immune response. This is supported by the large repertoire of OMPs genes in the *H. pylori* genome. Functional redundancy of porins may explain emergence of mutations in these genes without affecting bacteria viability. It has been proposed that the role of such redundancy of outer membrane proteins is to sustain antigenic variation to support pathogen survival by evasion of the host immune response<sup>[44]</sup>. The strand slippage mechanism is not normally operating in *E. coli*, therefore, in most cases, lack of heterologous expression of *H. pylori* genes in *E. coli* should be due to mutations that previously affected the *H. pylori* genome.

In spite of the low content of homopolymeric and dinucleotide repeats found in CHCTX-1 *hopE* and *hopV* genes, some strains may have switched these genes off but, in a few cases, expression could be restored by the same mechanism. This may restrict the use of these *H. pylori* OMPs as a single source of antigens for vaccine design. However, in order to provide a wider and stronger immune response, vaccines based on a mixture of *H. pylori* antigens with the inclusion of HopE and HopV should be considered.

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## COMMENTS

### Background

Few studies have been done on HopE and HopV porins from *Helicobacter pylori* (*H. pylori*). These proteins have been described as part of a large family of outer membrane proteins having similar functions, mainly as gating for influx of nutrients. Compared to other enterobacteria, such as *Escherichia coli* (*E. coli*) with only 3 major porins, *H. pylori* have shown a remarkable redundancy in this kind of outer membrane protein. The explanation for such redundancy has not

arisen. A study looking at how widely these porin genes are distributed and what proportion is actually active among clinical isolates should provide some answers.

### Research frontiers

Switching on/off in outer membrane genes in a few bacteria has been described as a mechanism to distract the immune system during infection by changing the proteins displayed on the surface. The authors found that *H. pylori* HopE and HopV porin genes seemed to be absent in some isolates, and about half of those who carried them did not express them. In addition, sera from infected patients do not frequently recognize these antigens. This feature may contribute to the ability of these bacteria to avoid the host immune response allowing their persistence in humans for an extended period of time.

### Innovations and breakthroughs

Recent reports have shown that some outer membrane protein genes from *H. pylori* could be turned on/off by random nucleotide insertions or deletions either at the promoter or within the coding region, through a mechanism called "strand slippage" during DNA replication. This is the first report proposing that this switching may also occur in the *hopE* and *hopV* genes, explaining why around 70%-90% of these genes are shut down in Chilean clinical isolates.

### Applications

Determining whether a protein is subjected to on/off switching during its expression at the bacterial surface, together with the knowledge of its immunoreactivity will be useful to select potential antigen candidates to be used in the design and development of vaccines.

### Terminology

*H. pylori* HopE and HopV proteins are part of a large family of outer membrane proteins and are located at the bacterial surface. They are defined as porins because they form a pore structure to allow the influx of small size nutrients and other compounds. They may constitute a target for the immune system. The "strand slippage" mechanism to control gene expression is a result of random errors during strand DNA replication consisting of nucleotide insertions or deletions that alter the genetic code of the protein or the functionality of elements (i.e. promoter) that control gene expression.

### Peer review

The manuscript by Lienlaf *et al* assesses the patterns of 2 porin genes in *H. pylori*. *H. pylori* remains a significant problem in developing countries around the world. Although much is now known about the pathogenesis of this bacterium and about host responses to infection, the organism remains a clinical problem. Various investigators have focused upon the establishment of a vaccine for this pathogen. An appropriate selection of a conserved and widely expressed antigen of *H. pylori* clinical isolates will assure a suitable design of a protective vaccine. This work is promising.

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## Aberrant gene methylation in the peritoneal fluid is a risk factor predicting peritoneal recurrence in gastric cancer

Masatsugu Hiraki, Yoshihiko Kitajima, Seiji Sato, Jun Nakamura, Kazuyoshi Hashiguchi, Hirokazu Noshiro, Kohji Miyazaki

Masatsugu Hiraki, Yoshihiko Kitajima, Seiji Sato, Jun Nakamura, Kazuyoshi Hashiguchi, Hirokazu Noshiro, Kohji Miyazaki, Department of Surgery, Saga University Faculty of Medicine, 5-1-1 Nabeshima, Saga 849-8501, Japan

**Author contributions:** Hiraki M, Kitajima Y and Miyazaki K designed this study; Hiraki M performed all of the experiments and analyzed the data; Hiraki M interpreted the analyzed data and wrote the manuscript under the supervision of Kitajima Y; Nakamura J, Hashiguchi K, Sato S and Noshiro H contributed to the sample collection and advice concerning the study; Miyazaki K approved the final version of the manuscript.

**Supported by** Fund from the Department of Surgery, Saga University Faculty of Medicine

**Correspondence to:** Dr. Kohji Miyazaki, MD, PhD, Professor, Department of Surgery, Saga University Faculty of Medicine, 5-1-1 Nabeshima, Saga 849-8501, Japan. [miyazak2@cc.saga-u.ac.jp](mailto:miyazak2@cc.saga-u.ac.jp)

Telephone: +81-952-342349 Fax: +81-952-342019

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### Abstract

**AIM:** To investigate whether gene methylation in the peritoneal fluid (PF) predicts peritoneal recurrence in gastric cancer patients.

**METHODS:** The gene methylation of *CHFR* (checkpoint with forkhead and ring finger domains), *p16*, *RUNX3* (runt-related transcription factor 3), *E-cadherin*, *hMLH1* (mutL homolog 1), *ABCG2* (ATP-binding cassette, sub-family G, member 2) and *BNIP3* (BCL2/adenovirus E1B 19 kDa interacting protein 3) were analyzed in 80 specimens of PF by quantitative methylation-specific polymerase chain reaction (PCR). Eighty patients were divided into 3 groups; Group A ( $n = 35$ ): the depth of cancer invasion was less than the muscularis propria; Group B ( $n = 31$ ): the depth of cancer invasion was beyond the muscularis propria. Both group A and B were diagnosed as no cancer cells in peritoneal cytol-

ogy and histology; Group C ( $n = 14$ ): disseminated nodule was histologically diagnosed or cancer cells were cytologically defined in the peritoneal cavity.

**RESULTS:** The positive rates of methylation in *CHFR*, *E-cadherin* and *BNIP3* were significantly different among the 3 groups and increased in order of group A, B and C (0%, 0% and 21% in *CHFR*,  $P < 0.05$ ; 20%, 45% and 50% in *E-cadherin*,  $P < 0.05$ ; 26%, 35% and 71% in *BNIP3*,  $P < 0.05$ ). In addition, the multigene methylation rate among *CHFR*, *E-cadherin* and *BNIP3* was correlated with group A, B and C (9%, 19% and 57%,  $P < 0.001$ ). Moreover, the prognosis was analyzed in group B, excluding 3 patients who underwent a non-curative resection. Two of the 5 patients with multigene methylation showed peritoneal recurrence after surgery, while those without or with a single gene methylation did not experience recurrence ( $P < 0.05$ ).

**CONCLUSION:** This study suggested that gene methylation in the PF could detect occult neoplastic cells in the peritoneum and might be a risk factor for peritoneal metastasis.

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**Key words:** Ascites; Dissemination; Gastric cancer; Methylation; Peritoneal fluid; Quantitative methylation-specific polymerase chain reaction

**Peer reviewer:** Toru Hiyama, MD, PhD, Health Service Center, Hiroshima University, 1-7-1 Kagamiyama, Higashihiroshima 739-8521, Japan

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## INTRODUCTION

Peritoneal metastasis is the most frequent event in recurrent gastric cancers, and occurs in 34% of patients with recurrences, even after curative resection of the primary tumor<sup>[1,2]</sup>. In addition, peritoneal metastasis shows resistance to various chemotherapeutic drugs and causes massive ascites and intestinal obstruction. In Japan, cytological examination of peritoneal washes using Papanicolaou staining is commonly performed during surgery to detect peritoneal metastasis. However, peritoneal metastasis sometimes occurs even in cases that show a negative cytological examination. The efficacy of immunocytology<sup>[3,4]</sup>, tumor markers<sup>[5,6]</sup> and reverse transcriptase polymerase chain reaction (PCR) analysis of carcinoembryonic antigen (CEA), and cytokeratin (CK) mRNA<sup>[2,7-12]</sup> in the peritoneal washes has been examined.

Epigenetic gene silencing through DNA methylation occurs in various cancers. DNA methylation occurs in the CpG rich promoters of tumor suppressor genes, DNA repair and cell cycle checkpoint genes, resulting in suppressed gene expression<sup>[13,14]</sup>. Numerous studies have investigated gene methylation to assess the correlation with carcinogenesis and tumor progression in various cancers<sup>[15-17]</sup>. Recently, several reports have demonstrated aberrant gene methylation detected in salivary rinses<sup>[18]</sup>, pleural effusion<sup>[19]</sup>, peritoneal fluid (PF)<sup>[20,21]</sup>, lymph node<sup>[22,23]</sup>, breast ductal fluid<sup>[24]</sup>, bile<sup>[25]</sup>, pancreatic juice<sup>[26]</sup>, urine<sup>[27]</sup>, stool<sup>[28]</sup>, serum and plasma<sup>[20,29,30]</sup> from patients with various tumors and suggested the feasibility of methylation analysis in the evaluation of occult neoplastic cells or micrometastasis.

The present study investigated whether DNA methylation using PF is a possible marker for determining gastric cancer micrometastasis to the peritoneum. The DNA methylation levels of 7 genes; *CHFR* (checkpoint with forkhead and ring finger domains), *p16* (cyclin-dependent kinase inhibitor 2A), *RUNX3* (runt-related transcription factor 3), *E-cadherin*, *hMLH1* (mutL homolog 1), *ABCG2* (ATP-binding cassette, sub-family G, member 2), *BNIP3* (BCL2/adenovirus E1B 19 kDa interacting protein 3) in 80 PF specimens were analyzed by quantitative methylation-specific polymerase chain reaction (q-MSP). Furthermore, quantitative reverse transcriptase-PCR (qRT-PCR) of CEA and CK19 mRNA was examined using the same samples and the results were compared with that of q-MSP. The goal of this study was to clarify whether gene methylation in PF is feasible for determining micrometastasis to the peritoneum in gastric cancer.

## MATERIALS AND METHODS

### Ethics

The study protocol was approved by the Ethics Committee of Saga University Faculty of Medicine. Informed consent was obtained from all the patients before collection of the samples.

### Patients and sample collection

Peritoneal lavage fluid was obtained from 80 patients

who underwent surgery at the Department of Surgery, Saga University Hospital from May 2007 to August 2008. A total volume of 200 mL of normal saline was poured into Douglas's pouch and the left subphrenic space. One hundred milliliter of PF was examined by conventional cytological diagnosis with Papanicolaou staining. The remaining PF was centrifuged at 1200 g for 10 min and the pelleted cells were stored at -80°C until the extraction of genomic DNA and RNA. A gastrectomy was subsequently performed in 72 patients. A bypass operation or exploratory laparotomy was carried out in the remaining 8 patients due to either peritoneal dissemination or cytologically positive cancer cells. The histological type, depth of tumor invasion and clinical stage were determined on the basis of the criteria of the Japanese Classification of Gastric Carcinoma guidelines<sup>[31]</sup>. The 80 patients were further divided into 3 groups: Group A ( $n = 35$ ): the depth of cancer invasion was less than the muscularis propria [tumor invasion of mucosa and/or muscularis mucosa (M) or submucosa (SM), tumor involved the muscularis propria (MP)]; Group B ( $n = 31$ ): the depth of cancer invasion was beyond the muscularis propria [tumor involved the subserosa (SS), tumor penetrated the serosa (SE), tumor invasion of adjacent structures (SI)]; Group C ( $n = 14$ ): a peritoneal metastasis was histologically diagnosed [P (+)] or cancer cells were present on peritoneal cytology [CY (+)]. No peritoneal metastasis [P (-)] and benign/ indeterminate cells on peritoneal cytology [CY (-)] were confirmed at surgery in the 66 patients in group A and group B. CY (+) or P (+) was simultaneously diagnosed at surgery in 12 of 14 patients in group C. In the remaining 2 patients, cancerous ascites were collected at the recurrence. The methylation analysis was performed using specimens obtained from all 80 patients. The mRNA analysis was done using 63 samples, because high quality RNA could not be extracted in specimens from the remaining 17 cases.

### DNA extraction, sodium bisulfite modification and q-MSP

The genomic DNA was isolated from cell pellets from the abdominal fluid using an EZ1 DNA tissue kit (Qiagen, Hilden, Germany). Bisulfite modification was carried out using the EpiTet® Bisulfite Kit (Qiagen, Hilden, Germany) with 1500 ng of genomic DNA. Bisulfite-treated DNA was amplified by EpiTect® Whole Bisulfite Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The 80 DNA samples with bisulfite modification were quantitatively analyzed for the methylation levels of 8 genes (*CHFR*, *p16*, *RUNX3*, *E-cadherin*, *hMLH1*, *ABCG2*, *BNIP3* and  $\beta$ -actin; an internal marker). For q-MSP, a 2  $\mu$ L aliquot was amplified by PCR using a primer set along with the Taqman probe specific for methylated sequences. A q-MSP (MethyLight) was carried out with the Light-Cycler™ instrument system (Roche, Mannheim, Germany) using the Light-Cycler® TaqMan® Master (Roche, Mannheim, Germany) according to a previous report<sup>[32]</sup>. The primer sequences are shown in Table 1<sup>[33-37]</sup>. After a denaturing step at 95°C

Table 1 Primer and probe sequence of the LightCycler system for q-MSP

Gene		Primer sequence	Ref.
CHFR	Forward	TTTCGTGATTCGTAGGCGAC	[33]
	Reverse	CGACAATAAAACGAAACCGA	
	Probe	5' FAM-CGCGAAAATAAACGCGTAAAAAACGCTCG-3'BHQ	
p16	Forward	TGGAATTTTCGGTTGATTGGTT	[34]
	Reverse	AACAACGTCCGCACCTCCT	
	Probe	5' FAM- ACCCGACCCCGAACCGCG -3'BHQ	
RUNX3	Forward	CGTTCGATGGTGGACGTGT	[35]
	Reverse	GACGAACAACGTCCTATTACAACGC	
	Probe	5' FAM-CGCACGAACCTCGCTACGTAATCCG-3'BHQ	
E-cadherin	Forward	AATTTTAGGTTAGAGGGTTATCGCGT	[34,36]
	Reverse	TAATAAAAAATTCACCTACCGAC	
	Probe	5' FAM-CGCCCACCCGACCTCGCAT-3'BHQ	
hMLH1	Forward	CGTTATATATCGTTCGTAGTATTCGTGTTT	[34]
	Reverse	CTATCGCCGCTCATCGT	
	Probe	5' FAM-CGCGACGTCAAACGCCACTACG-3'BHQ	
ABCG2	Forward	TTGGGTAATTTGTGCGTTA	[37]
	Reverse	CTACGAAAATCACCAAACGCTC	
	Probe	5' FAM-TTAATCGCCGCTACTAACCAG-3'BHQ	
BNIP3	Forward	TAGGATTCGTTTCGCGTACG	[37]
	Reverse	ACCGCGTCGCCCATTAACCGCG	
	Probe	5' FAM-CGTAAAATACGTATAACACGTACGAC-3'BHQ	
$\beta$ -actin	Forward	TGGTGATGGAGGAGGTTTAGTAAGT	[34]
	Reverse	AACCAATAAAACCTACTCCTCCCTTAA	
	Probe	5' FAM-ACCACCACCCAACACACAATAACAAACACA-3'BHQ	

Table 2 Primer and probe sequence of the LightCycler system for qRT-PCR

Gene		Primer sequence
CEA	Forward	AGTCTATGCAGAGCCACCCAA
	Reverse	GGGAGGCTCTGATTATTTACCCA
	Probe	5' FAM-ACCCCTTCATCACCAGCAACAACCTCCAA-3'BHQ
CK19	Forward	GACATGCGAAGCCAATATGA
	Reverse	TCAGTAACCTCGGACCTGCT
	Probe	5' FAM-CTGGTTCACCAGCCGGACTGAAGAATT-3'BHQ
$\beta$ -actin	Forward	CGAGCGCGGCTACAGCTT
	Reverse	TCCTTAATGTCACGCACGATT
	Probe	5' FAM-ACCACCACGGCCGAGCGG-3'BHQ

for 10 min, PCR amplification was performed with 45 cycles of 15 s denaturing at 95°C, 5 s annealing at 60°C and a 10 s extension at 72°C. These experiments were carried out in triplicate and the mean value was then calculated. CpGenome Universally Methylated DNA (Chemicon, Temecula, CA, USA) was used as a positive control for methylation, and CpGenome Universal Unmethylated DNA (Chemicon) was used as a negative control. The quantified value of DNA methylation of a target gene was normalized by  $\beta$ -actin.

#### RNA extraction, conversion to cDNA and qRT-PCR

Total RNA was extracted from the 80 cell pellets using the ISOGEN kit (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Samples of RNA (100 ng) were converted into cDNA and amplified using the QuantiTect® Whole Transcriptome Kit (Qia-gen, Hilden, Germany) according to the manufacturer's instructions. In order to quantitatively estimate the expression level of CEA and CK19 mRNA, qRT-PCR was performed on a Light-Cycler™ instrument system

(Roche, Mannheim, Germany) using LightCycler® Taq-Man® Master (Roche, Mannheim, Germany) according to the manufacturer's instructions. The primer sequences are shown in Table 2. After denaturing at 95°C for 10 min, qRT-PCR amplification was performed with 45 cycles of 15 s denaturing at 95°C, 5 s annealing at 60°C and a 10 s extension at 72°C. These experiments were all carried out in triplicate and the mean value was then calculated. The quantified value of mRNA expression of a target gene was normalized by  $\beta$ -actin.

#### Comparison of gene methylation between cancer tissue and peritoneal fluid

The conventional qualitative MSP was analyzed using several cancer tissue specimens. Genomic DNA was isolated from the tissue and bisulfite treatment was carried out as described above. The methylation status in the tissue samples was determined by MSP. Amplification was performed using Takara ExTaq Hot Start Version (Takara, Shiga, Japan) according to the manufacturer's instructions. The primer se-

quences are shown in Table 1 and PCR was performed in an iCycler (Bio-Rad, Hercules, CA, USA) under the following conditions. After heating at 96°C for 3 min, 35 cycles at 96°C for 30 s, 60°C for 30 s, 72°C for 30 s and 72°C for 5 min. The PCR products were separated on 1.5% agarose gel, stained with ethidium bromide, and were then observed under ultraviolet light.

### Statistical analysis

A cut-off value for distinguishing methylation status was determined using a receiver-operator characteristic (ROC) curve, which was obtained by comparing methylation values between group A and C. The cut-off value of CEA and CK19 mRNA expression was also determined by a ROC curve as described above. Differences in the frequencies were analyzed by the  $\chi^2$  test, while also applying Fisher's exact test. A statistical analysis was carried out using the SPSS 15.0j statistical software package for Windows (SPSS Japan Inc.). A *P* value of less than 0.05 was considered to be statistically significant.

## RESULTS

The clinicopathological characteristics of the patients in this study are summarized in Table 3. The 80 patients included 28 (35.0%) females and 52 (65.0%) males with a mean age of 65.9 years (range, 39–88 years). The number of patients in group A, B and C was 35 (43.8%), 31 (38.8%) and 14 (17.5%) cases, respectively.

### DNA hypermethylation of peritoneal fluids

Initially, q-MSP of *CHFR*, *p16*, *RUNX3*, *E-cadherin*, *hMLH1*, *ABCG2* and *BNIP3* was examined in 80 PFs. Figure 1 shows the methylation level of each gene in the 3 groups. The 80 cases were then divided into 2 groups, a methylation positive and a methylation negative group as described in the Materials & Methods section. Table 4 shows that the methylation status of *CHFR*, *E-cadherin* and *BNIP3* were significantly different among the 3 groups (*P* < 0.05). In these 3 genes, the positive rate of methylation increased in order of group A, B and C. The correlation of multigene methylation among *CHFR*, *E-cadherin* and *BNIP3* in the 3 groups was further analyzed. In group A and B, 3 of 35 cases (9%) and 6 of 31 cases (19%) showed multigene methylation in 2 or more genes, respectively (Table 5). In contrast, 8 of 14 cases (57%) were methylation positive in 2 or more genes in group C. The results showed a significant relationship between methylation status of more than 2 genes and the 3 groups (*P* < 0.001).

### mRNA expression of CEA and CK19 in peritoneal fluids

The expression of CEA and CK19 mRNAs in the 63 PFs were quantitatively analyzed by qRT-PCR to detect gastric cancer cells in the peritoneal washes. Figure 2 shows the expression level of CEA and CK19 mRNAs in the 3 groups. The mRNA level was further divided into negative and positive groups and compared among the 3 groups. The results showed that CK19, but not CEA, was

Table 3 Clinicopathological factors of the patients (*n* = 80)

Clinicopathological factors	<i>n</i> (%)
Gender	
Female	28 (35.0)
Male	52 (65.0)
Age (yr)	65.9 ± 10.8
Range	39–88
Histological type	
tub	36 (45.0)
por/sig/muc	44 (55.0)
Classification	
Group A: M-MP with CY(-) and P(-)	35 (43.8)
Group B: SS-SI with CY(-) and P(-)	31 (38.8)
Group C: CY(+) or P(+)	14 (17.5)
Clinical stage at the sample collection	
I	37 (46.3)
II	8 (10.0)
III	18 (22.5)
IV	15 (18.8)
Peritoneal recurrence	2 (2.5)

tub: Tubular adenocarcinoma; por: Poorly differentiated adenocarcinoma; sig: Signet-ring cell carcinoma; muc: Mucinous adenocarcinoma. Tumor invasion of mucosa and/or muscularis mucosa (M), muscularis propria (MP) or subserosa (SS), tumor invasion of adjacent structures (SI). Cancer cells on peritoneal cytology (CY), peritoneal metastasis (P).

Table 4 Relationship between the q-MSP results and depth of cancer invasion, CY and P classification *n* (%)

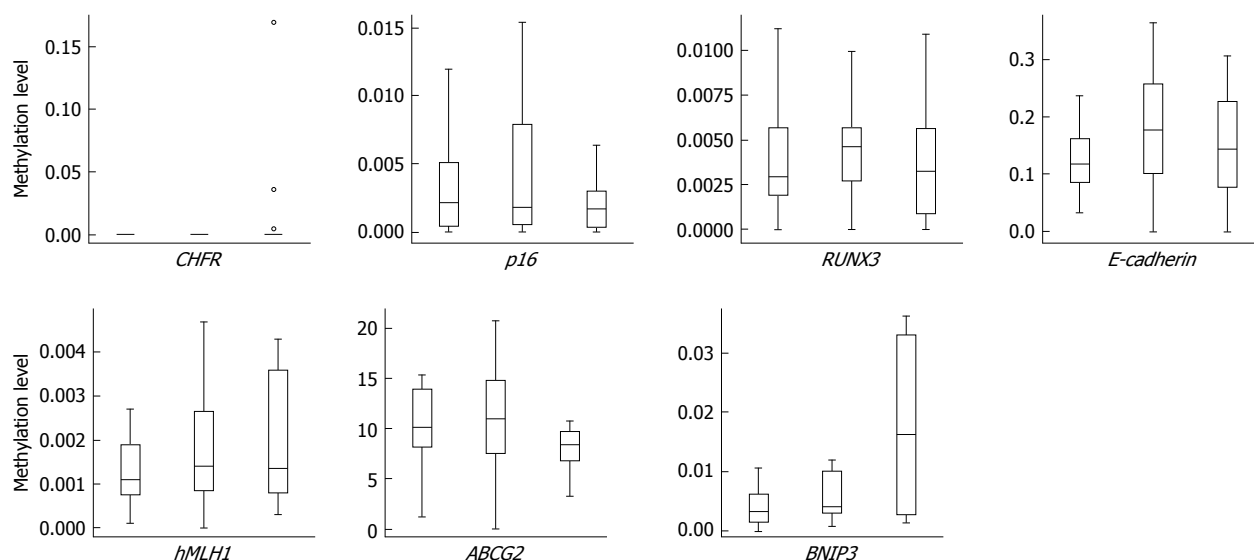
Methylation status	Classification			<i>P</i> -value
	Group A: M-MP with CY(-) and P(-)	Group B: SS-SI with CY(-) and P(-)	Group C: CY(+) or P(+)	
<i>CHFR</i>				< 0.05
(-) 77 (96)	35 (100)	31 (100)	11 (79)	
(+) 3 (4)	0 (0)	0 (0)	3 (21)	
<i>p16</i>				0.821
(-) 33 (41)	14 (40)	14 (45)	5 (36)	
(+) 47 (59)	21 (60)	17 (55)	9 (64)	
<i>RUNX3</i>				0.304
(-) 36 (45)	19 (54)	11 (35)	6 (43)	
(+) 44 (55)	16 (46)	20 (65)	8 (57)	
<i>E-cadherin</i>				< 0.05
(-) 52 (65)	28 (80)	17 (55)	7 (50)	
(+) 28 (35)	7 (20)	14 (45)	7 (50)	
<i>hMLH1</i>				0.861
(-) 43 (54)	20 (57)	16 (52)	7 (50)	
(+) 37 (46)	15 (43)	15 (48)	7 (50)	
<i>ABCG2</i>				0.244
(-) 30 (38)	12 (34)	10 (32)	8 (57)	
(+) 50 (63)	23 (66)	21 (68)	6 (43)	
<i>BNIP3</i>				< 0.05
(-) 50 (63)	26 (74)	20 (65)	4 (29)	
(+) 30 (38)	9 (26)	11 (35)	10 (71)	

significantly correlated with the 3 groups (Table 6, *P* < 0.05, *P* = 0.352). The positive rate of CK19 increased in order of group A (25%), B (46%) and C (73%).

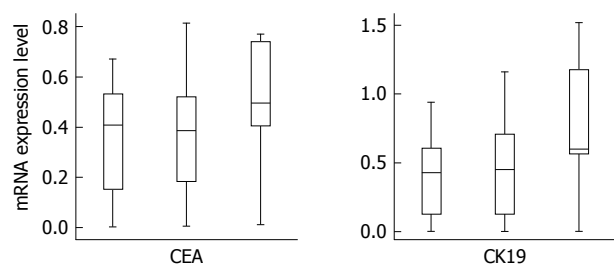
### Comparison of gene methylation between the cancer tissue and ascites fluid

The gene methylation of *CHFR*, *E-cadherin* and *BNIP3* was examined in the primary or metastatic cancer tissue and was compared with those in the corresponding PF





**Figure 1** Methylation level of peritoneal wash specimens from gastric cancer patients measured by q-MSP according to the depth of cancer invasion and positive cancer cell findings. Left: Group A; Middle: Group B; Right: Group C.



**Figure 2** mRNA expression level of peritoneal wash specimens from gastric cancer patients measured by qRT-PCR according to the depth of cancer invasion and positive cancer cell findings. Left: Group A; Middle: Group B; Right: Group C.

in group C. Nine of 14 cases were analyzed because the tissues were not obtained in remaining 5 cases. The same methylation pattern was present in the cancer tissues and ascites in 100% of *CHFR*, 88.9% of *E-cadherin* and 77.8% of *BNIP3* (Table 7).

#### Relationship between multigene methylation or mRNA expression and peritoneal recurrence

The prognosis of patients in group A or B was followed for at least 8 mo after surgery. None of the 35 patients in group A had cancer recurrence (data not shown). The prognosis of only 28 of 31 patients in group B was followed because the remaining 3 patients were excluded from this analysis based on the results of a non-curative resection. In 28 of the patients in group B, 2 of the 5 patients with multigene methylation showed peritoneal recurrence after surgery, while patients without or with a single gene methylation did not experience recurrent cancer (Table 8). There was a significant correlation between peritoneal recurrence and multigene methylation in group B (Table 8,  $P < 0.05$ ). Peritoneal recurrence was observed in only 1 of 10 cases that were CK19 positive, however statistical significance was not observed (Table 8,  $P = 0.943$ ).

**Table 5** Correlation between the multigene methylation of the peritoneal wash specimens from gastric cancer and the depth of cancer invasion, CY and P classification  $n$  (%)

Classification	Multigene methylation ( <i>CHFR</i> , <i>E-cadherin</i> , <i>BNIP3</i> )		<i>P</i> -value
	Less than 2 genes	2 or more genes	
Group A: M-MP with CY(-) and P(-)	32 (91)	3 (9)	< 0.001
Group B: SS-SI with CY(-) and P(-)	25 (81)	6 (19)	
Group C: CY(+) or P(+)	6 (43)	8 (57)	

**Table 6** Relationship between the qRT-PCR results and depth of cancer invasion, CY and P classification  $n$  (%)

Methylation status	Classification			<i>P</i> -value
	Group A: M-MP with CY(-) and P(-)	Group B: SS-SI with CY(-) and P(-)	Group C: CY(+) or P(+)	
CEA				0.352
(-) 39 (62)	17 (61)	17 (71)	5 (45)	
(+) 24 (38)	11 (39)	7 (29)	6 (55)	
CK19				< 0.05
(-) 37 (59)	21 (75)	13 (54)	3 (27)	
(+) 26 (41)	7 (25)	11 (46)	8 (73)	

## DISCUSSION

Postoperative recurrence of gastric cancer usually occurs in the peritoneum, lymph node and liver<sup>[1]</sup>. Peritoneal metastasis occurs most frequently and is highly resistant to various chemotherapies, which leads to a poor prognosis in gastric cancer patients. Peritoneal recurrence has been reported to depend on the depth of invasion, as 97.2% of recurrences occur beyond the MP. In addition, peritoneal recurrence was demonstrated in 34.9% of SS cases, 46.7% of SE cases and 60.0% of SI cases<sup>[2]</sup>.

Table 7 Comparison of the methylation status between cancerous tissue and peritoneal fluid in group C

Sample No.	Pathology	CY	P	Examined tissue	Methylation status					
					Tissue			Peritoneal fluid		
					<i>CHFR</i>	<i>E-cadherin</i>	<i>BNIP3</i>	<i>CHFR</i>	<i>E-cadherin</i>	<i>BNIP3</i>
A-03	por	+	-	Primary cancer	Negative	Positive	Negative	Negative	Positive	Negative
A-09	por	+	-	Primary cancer	Negative	Positive	Positive	Negative	Positive	Positive
A-16	por	+	+	Primary cancer	Positive	Negative	Positive	Positive	Negative	Positive
A-29	tub	+	+	Dissemination nodule	Negative	Positive	Negative	Negative	Positive	Positive
A-51	por	+	-	Metastatic lymph node	Positive	Positive	Positive	Positive	Positive	Positive
A-59	sig	-	+	Primary cancer	Negative	Positive	Positive	Negative	Negative	Negative
A-65	por	-	+	Metastatic ovarian tumor	Negative	Positive	Positive	Negative	Positive	Positive
A-69	sig	+	+	Primary cancer	Negative	Positive	Positive	Negative	Positive	Positive
A-78	tub	+	-	Primary cancer	Negative	Positive	Positive	Negative	Positive	Positive

Abbreviations as in Table 1.

Table 8 Correlation between peritoneal recurrence and multigene methylation and also the CK19 expression results in group B

	Multigene methylation ( <i>CHFR</i> , <i>E-cadherin</i> , <i>BNIP3</i> )		<i>P</i> -value	CK19		<i>P</i> -value
	Less than 2 genes	2 or more genes		Negative	Positive	
Peritoneal recurrence						
Positive	0	2	< 0.05	1	1	0.943
Negative	23	3		10	9	

Another study reported that 50%-60% of gastric cancer patients with serosal invasion after a curative resection eventually developed peritoneal metastasis<sup>[38]</sup>. Furthermore, the average survival after peritoneal recurrence is 4.9 mo<sup>[39]</sup>. Therefore, the detection of micrometastasis in peritoneal lavage is essential, not only to make an accurate diagnosis, but also to start chemotherapy before the metastatic nodule is grossly formed in the peritoneum. The introduction of molecular technology such as RT-PCR of cancer specific genes has addressed the detection of micrometastasis in the LN<sup>[40,41]</sup>, ascites<sup>[2,7-12,38,42,43]</sup>, bone marrow and peripheral blood<sup>[44,45]</sup> in gastric cancer. Various types of mRNA, such as CEA, CK19, and CK20 have been analyzed by RT-PCR and used as molecular markers in detecting micrometastasis in gastric cancer. Several studies have reported that the positive expression of mRNA in PF shows a significant correlation with peritoneal recurrence and survival<sup>[2,8-12,38,43]</sup>.

Recently, an analysis of cancer specific gene methylation has been utilized to detect micrometastasis in salivary rinses for head and neck cancer patients<sup>[18]</sup>, pleural effusion for lung cancer and malignant mesothelioma<sup>[19]</sup>, ductal fluid for breast cancer<sup>[24]</sup>, ascites for ovarian cancer and colorectal cancer<sup>[21,22]</sup>, bile for gallbladder cancer<sup>[25]</sup>, pancreatic juice for pancreatic cancer<sup>[26]</sup>, urine for prostate cancer<sup>[27]</sup>, stool for colorectal cancer<sup>[28]</sup> and serum<sup>[20,29,30]</sup>. However, few studies have addressed gene methylation for the detection of micrometastasis to PF in gastrointestinal cancer<sup>[21]</sup>.

The present study analyzed the promoter methylation of cancer related genes in 80 PFs. *CHFR*, *p16*, *RUNX3*, *E-cadherin*, *bMLH1*, *ABCG2* and *BNIP3* were chosen for the methylation analysis, because the frequent methylation of these 7 genes has been reported in several

malignancies including gastric cancer<sup>[15,16,21,23,25-27,33-37,46-50]</sup>. Peritoneal recurrence has been reported to depend on the depth of invasion<sup>[2]</sup>. Therefore, 80 samples from gastric cancer patients were classified into 3 groups [Group A: cancer invasion was restricted in M, SM, MP, Group B: cancer invasion deeper than MP, Group C: CY(+) or P(+)] and correlated with the gene methylation. As a result, q-MSP analysis using the 80 PFs demonstrated that the methylation status of *CHFR*, *E-cadherin* and *BNIP3* were significantly increased depending on the depth of cancer invasion. In contrast, the methylation status of the other genes was not significantly changed among the 3 groups (Table 4). These results indicate that the increasing value of the methylation of *CHFR*, *E-cadherin* and *BNIP3* from group A to group C was possibly derived from the metastatic cancer cells in the peritoneum. On the other hand, a q-MSP analysis might detect methylation from normal cells in the peritoneum at the basal level in *p16*, *RUNX3*, *bMLH1* and *ABCG2* genes. Based on these findings, *CHFR*, *E-cadherin* and *BNIP3* methylation was thus suggested to be preserved during cancer invasion, finally resulting in the occurrence of peritoneal seeding. Therefore, the methylation in more than 2 genes was compared among the 3 genes in each group. The results showed that there was a significant difference between multigene methylation and the 3 groups (Table 5). Eight of 14 patients (57%) in group C carried the multigene methylation while only 3 of 35 (9%) patients in group A exhibited multigene methylation. On the other hand, a qRT-PCR analysis examined the expression of CEA and CK19 mRNA in 63 samples (Table 6). Unexpectedly, CEA was not correlated with the classification even in group C with the highest positive rate. However, CK19 was significantly increased depending

on the depth of cancer invasion, CY and P classification. It was desirable that gene methylation should be detected in up to 100% of samples in group C with CY1 or P1. However, only 21% of gene methylation was observed in *CHFR*, 46% of *E-cadherin*, 71% of *BNIP3* and 57% of more than 2 genes were methylated in group C, indicating that gene methylation did not universally occur in all cancer cells. Thus, it is important to improve the sensitivity of multigene methylation analysis using PFs by increasing the number of genes that are specifically methylated in cancer cells. To clarify whether the methylation status of the PF originated from the cancer tissue, the methylation status of the primary or metastatic tissue in group C was compared with the methylation in the PF. The methylation status in the primary tumor was highly preserved in the PF (Table 7), thus suggesting that the methylation of the 3 genes assessed in the PF was derived from the primary tumor.

This study finally evaluated whether multigene methylation predicts peritoneal recurrence after surgery. In 21 patients in group B, peritoneal recurrence was found in 2 of 5 patients (40%) carrying multigene methylation. On the other hand, recurrence occurred in only 1 of 10 patients (10%) with positive CK19. There was a significant association between peritoneal recurrence and multigene methylation, but not CK19 (Table 8). These results suggested that multigene methylation may be a risk factor for peritoneal metastasis in the patients in group B even though the metastasis was not detected during surgery. An RT-PCR method using epithelial markers is critical in the diagnosis of micrometastasis. However, these methods only diagnose the presence of cancer cells. A methylation analysis that diagnosed micrometastasis in PFs would provide more information not only concerning the existence of cancer cells but also carcinogenesis, tumor progression and chemosensitivity, based on information on methylation status.

In conclusion, the present study investigated the methylation status in PF by both q-MSP and qRT-PCR analyses. The multigene methylation of *CHFR*, *E-cadherin* and *BNIP* in PF revealed the clinical feasibility of detecting occult neoplastic cells in the peritoneum. A methylation analysis along with a cytological examination might increase the positive detection of cancer cells in PF.

## COMMENTS

### Background

Postoperative recurrence of gastric cancer usually occurs in the peritoneum. Peritoneal recurrence is highly resistant to various chemotherapies, which leads to a poor prognosis in these patients. Therefore, the detection of micrometastasis in peritoneal lavage is essential, not only to make an accurate diagnosis, but also to start chemotherapy before the metastatic nodule is grossly formed in the peritoneum.

### Research frontiers

Epigenetic gene silencing through DNA methylation occurs in various cancers. Recently, several reports have demonstrated aberrant gene methylation detected in various samples from cancer patients and have suggested the feasibility of methylation analysis in the evaluation of occult neoplastic cells or micrometastasis. This study clarified whether gene methylation in peritoneal fluid is feasible for determining micrometastasis to the peritoneum in gastric cancer patients.

### Innovations and breakthroughs

Few studies have addressed gene methylation for the detection of micrometastasis to peritoneal fluid in gastrointestinal cancer. The authors' results indicate that gene methylation in the peritoneal fluid could detect occult neoplastic cells in the peritoneum and might be a risk factor for peritoneal metastasis.

### Applications

The development of this system may improve the accurate diagnosis of peritoneal dissemination and improve the prognosis of gastric cancer patients.

### Terminology

DNA methylation is an epigenetic modification in humans, and changes in methylation patterns play an important role in carcinogenesis, cancer progression and chemosensitivity.

### Peer review

This paper is interesting and written well.

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## Expression of p53, c-erbB-2 and Ki67 in intestinal metaplasia and gastric carcinoma

Yao Zheng, Lin Wang, Jian-Ping Zhang, Jing-Yan Yang, Zhu-Mei Zhao, Xiao-Ying Zhang

Yao Zheng, Lin Wang, Jian-Ping Zhang, Jing-Yan Yang, Zhu-Mei Zhao, Xiao-Ying Zhang, Department of Pathology, Shandong University School of Medicine, Jinan 250012, Shandong Province, China; Department of Pathology, Qilu Hospital of Shandong University, Jinan 250012, Shandong Province, China

Author contributions: Yang JY and Zhao ZM designed and performed the research; Zheng Y, Wang L and Zhang XY analyzed the data; Zheng Y and Zhang JP wrote the paper.

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Correspondence to: Jian-Ping Zhang, MD, Department of Pathology, Qilu Hospital of Shandong University, Jinan 250012, Shandong Province, China. [jzhan3@yahoo.com](mailto:jzhan3@yahoo.com)

Telephone: +86-531-82169221 Fax: +86-531-88383168

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type III IM and gastric carcinomas. The expression of p53, c-erbB-2 and Ki67 proteins in 20 SIM, 27 Atypical IM and 37 gastric carcinomas showed significant differences between SIM and gastric carcinomas while no significant differences were observed between Atypical IM and gastric carcinomas.

**CONCLUSION:** Atypical IM may better reveal the pre-cancerous nature of IM and could be a helpful indicator in the clinical follow up of patients.

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**Key words:** Simple intestinal metaplasia; p53; Atypical intestinal metaplasia; c-erbB-2; Ki67

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### Abstract

**AIM:** To compare two types of classification of intestinal metaplasia (IM) of the stomach and to explore their relationship to gastric carcinoma.

**METHODS:** Forty-seven cases of gastric IM were classified into type I, type II or type III according to mucin histochemical staining and compared with a novel classification in which the specimens were classified into simple IM (SIM) or atypical IM according to polymorphism in terms of atypical changes of the metaplastic epithelium. Forty-seven IM and thirty-seven gastric carcinoma samples were stained for p53, c-erbB-2 and Ki67 proteins by Envision immunohistochemical technique.

**RESULTS:** There were no significant differences in the expression of p53 and c-erbB-2 among type I, type II, type III IM and gastric carcinomas. The positive expression rate of Ki67 was significantly higher in gastric carcinomas than in type I IM while no significant Ki67 expression differences were observed among type II,

### INTRODUCTION

The relationship between intestinal metaplasia (IM) and gastric carcinoma has always been controversial. Correa postulated that gastric cancer develops through a complex sequence of events from normal mucosa to superficial gastritis, chronic atrophic gastritis, IM, dysplasia, and finally to intestinal type gastric carcinoma<sup>[1]</sup>. Many investigators have given credence to the preneoplastic nature of IM<sup>[2]</sup>. Even with common occurrence and presence in gastric biopsies, not all cases of IM may develop into gastric carcinoma. A subtype of IM which

has malignant potential should be classified for clinical follow up. Generally, IM is divided into subtypes on the basis of histochemical characteristics, and IM showing sulphomucin secretion has been considered to be a lesion with precancerous nature<sup>[3-5]</sup>, but some others considered this type of IM had no special link to gastric carcinoma<sup>[2]</sup>, and Rothery<sup>[6]</sup> also found half of the cases with this IM showing sulphomucin secretion exist in benign lesions.

In our study, IM was divided into two subtypes according to the atypical changes of the metaplastic epithelium: simple IM (SIM) and atypical IM (AIM). We detected three tumor-associated proteins, p53, c-erbB-2 and Ki67, in different subtypes of IM in order to find which one is more associated with gastric carcinoma.

## MATERIALS AND METHODS

### Samples

Forty-seven formalin-fixed, paraffin-embedded samples for IM were obtained from endoscopic biopsy and thirty-seven gastric carcinoma specimens from gastrectomy at Qilu Hospital of Shandong University. Mucin histochemical staining for IM subtyping was performed.

Serial sections were cut, stained with hematoxylin-eosin and the following techniques for mucins: Alcian blue pH 2.5-periodic acid-Schiff and high iron-diamine plus Alcian blue pH 2.5 to identify neutral, sialo- and sulphomucins.

Forty-seven cases of IM were classified in accordance with the system used by Jass and Filipe<sup>[7,8]</sup>: Type I (complete): mature absorptive and goblet cells, the latter secreting sialomucins (Figure 1); Type II (incomplete): IM cells with few or absent absorptive cells; presence of columnar “intermediate” cells in various stages of differentiation, secreting neutral and acid sialomucins; goblet cells secreting sialomucins and/or occasionally sulphomucins (Figure 1); Type III (incomplete): the cell dedifferentiation is more marked than in type II; “intermediate” cells secrete predominantly sulphomucins. A variable degree of disorganized glandular architecture is often present (Figure 1).

### IM subtyping according to atypical changes

Forty-seven IM samples were classified into SIM and AIM according to atypical changes of the metaplastic epithelium.

### Immunohistochemical technique

Sections 4  $\mu$ m thick were cut, deparaffinized in xylene, and then dehydrated in descending dilutions of ethanol. For the antigen retrieval regimen, all slides were micro-waved in 10 mmol/L sodium citrate buffer (pH 6.0) at 10 min intervals for a total of 20 min. The endogenous peroxidase activity was blocked by 10 min of incubation with 3% hydrogen peroxidase (reagent A) at room temperature. After washing in PBS, the sections were incubated with monoclonal mouse anti-human antibodies p53 (MAB-0364), c-erbB-2 (CB11) and Ki67 (SP6)

**Table 1** The distribution of 47 IM samples within different subtypes of IM

	Type I	Type II	Type III	Total
SIM	11	7	2	20
AIM	6	11	10	27
Total	17	18	12	47

IM: Intestinal metaplasia; SIM: Simple intestinal metaplasia; AIM: Atypical intestinal metaplasia.

overnight at 4°C. The sections were washed with PBS and incubated with polymerase auxiliaries (reagent B) for 20 min. After washing in PBS, the sections were incubated with biotinylated secondary antibody (reagent C) for 30 min at room temperature and finally DAB was visualized. Tissues were counterstained with hematoxylin. A negative control was designed by using PBS instead of primary antibody.

### Positive criteria of immunohistochemical staining

Sections were scored by light microscopy. The percentage of positively stained cells was calculated after 100 cells were counted in more than 5 high-power ( $\times 40$ ) fields. The following definitions were made: p53 and Ki67: more than 10% positive staining in nuclei was defined as positive staining; c-erbB-2: more than 10% positive staining in cytoplasm was defined as positive staining in IM, and more than 10% positive staining in cell membrane was defined as positive staining in gastric carcinoma<sup>[9]</sup>.

### Statistical analysis

The significance of associations was determined by the  $\chi^2$  test or the Fisher's exact test,  $P$  value  $< 0.05$  was considered statistically significant.

## RESULTS

### Subtype distribution of 47 IM samples

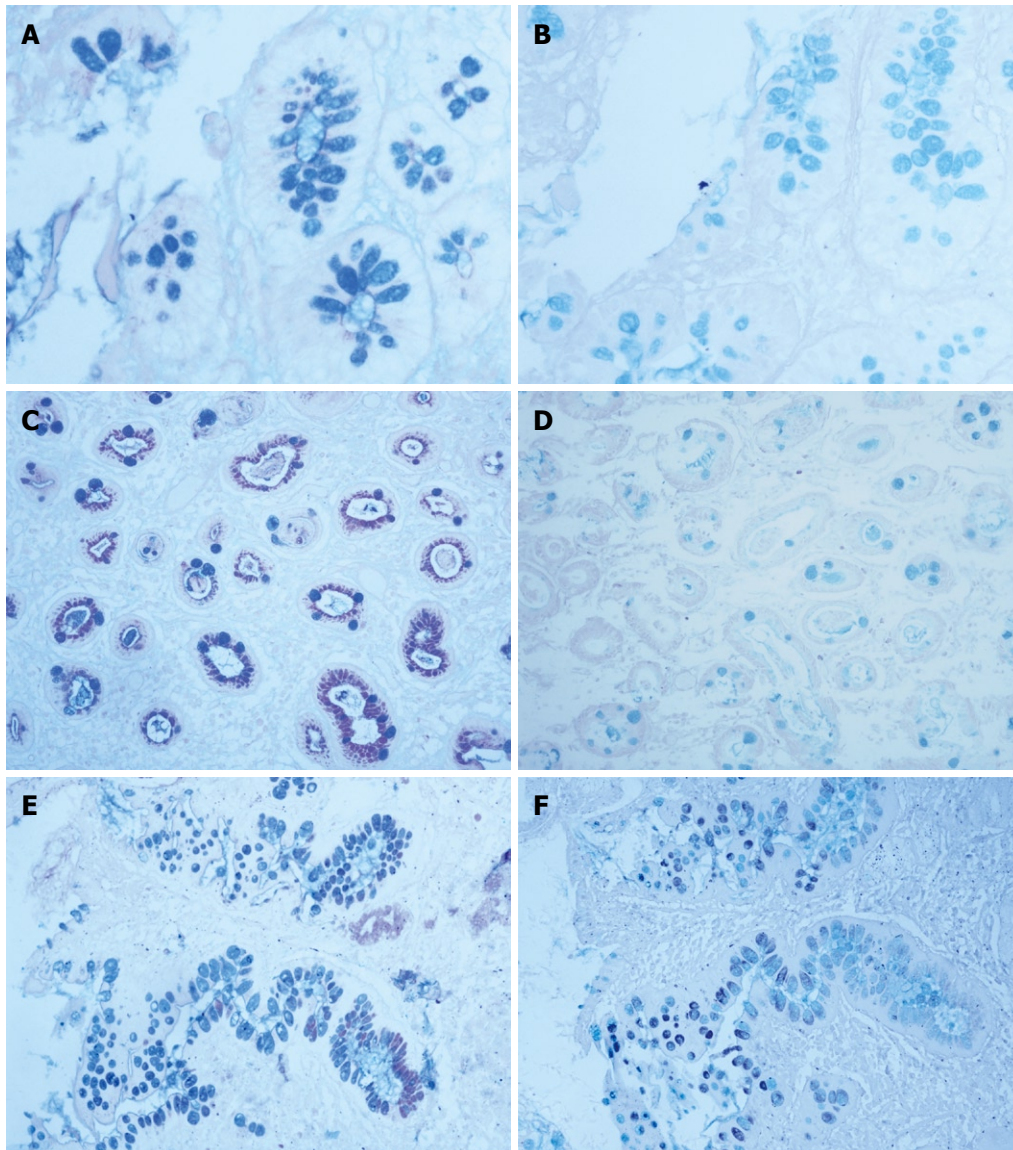
In 47 IM samples, type I, type II and type III IM accounted for 17, 18 and 12 samples, respectively. In 20 SIM, type I, type II and type III IM accounted for 11, 7 and 2 samples, respectively. In 27 AIM, type I, type II and type III IM accounted for 6, 11 and 10 samples, respectively (Table 1).

### Expression of p53 protein

The positive expression rates of p53 in type I, type II, type III IM and gastric carcinomas were 41.18%, 27.78%, 25.00% and 54.05%, respectively. The expression of p53 in gastric carcinomas was not significantly higher than in types I, II and III ( $P > 0.05$ ).

The positive expression rates of p53 in SIM and AIM were 20.00% and 40.74%, respectively. The expression of p53 in gastric carcinomas was significantly higher than in SIM ( $P < 0.05$ ). There was no significant difference in p53 expression between AIM and gastric carcinomas ( $P > 0.05$ ) (Table 2, Figure 2).





**Figure 1** Three types of intestinal metaplasia (IM) according to histochemical stains. A: IM type I : AB-PAS AB positive (blue), PAS negative; B: IM type I : HID-AB AB positive (blue), HID negative; C: IM type II: AB-PAS AB positive (blue), PAS positive (red); D: IM type II: HID-AB AB positive (blue), HID negative; E: IM type III: AB-PAS AB positive (blue), PAS positive (red); F: IM type III: HID-AB AB positive (blue), HID positive (grey and black) ( $\times 200$ ).

**Table 2** The expression of p53, c-erbB-2 and Ki67 proteins in different subtypes of IM and gastric carcinoma (GC)

	Total	p53		c-erbB-2		Ki67	
		+	-	+	-	+	-
SIM	20	4 <sup>a</sup>	16	6 <sup>a</sup>	14	5 <sup>a</sup>	15
AIM	27	11	16	18	9	12	15
Type I	17	7	10	9	8	5 <sup>a</sup>	12
Type II	18	5	13	8	10	8	10
Type III	12	3	9	4	8	4	8
GC	37	20	17	22	15	28	9

<sup>a</sup> $P < 0.05$  vs GC.

#### Expression of c-erbB-2 protein

The positive expression rates of c-erbB-2 in type I, type II, type III IM and gastric carcinomas were 52.94%, 44.44%, 33.33% and 59.46%, respectively. The

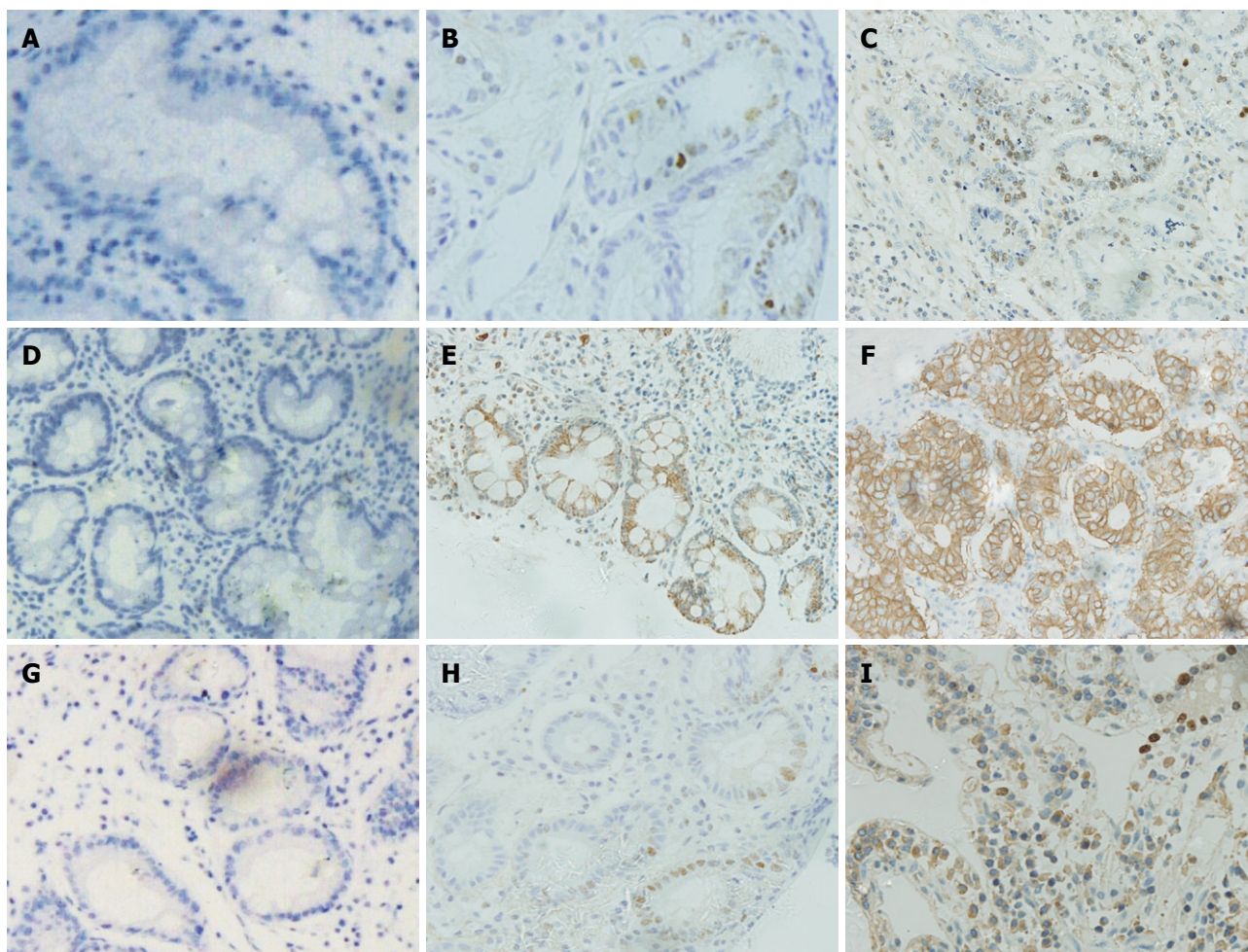
expression of c-erbB-2 in gastric carcinomas was not significantly higher than in types I, II and III ( $P > 0.05$ ).

The positive expression rates of c-erbB-2 in SIM and AIM were 30.00% and 656.67%. The expression of c-erbB-2 in gastric carcinomas was significantly higher than SIM ( $P < 0.05$ ). There was no significant difference between expression in AIM and gastric carcinomas ( $P > 0.05$ ) (Table 2, Figure 2).

#### Expression of Ki67 protein

The positive expression rates of Ki67 in type I, type II, type III IM and gastric carcinomas were 29.41%, 50.00%, 41.67% and 75.68%, respectively. The expression of Ki67 in gastric carcinomas was significantly higher than in type I IM ( $P < 0.05$ ). There was no significant difference in Ki67 expression between gastric carcinomas and type II or type III.





**Figure 2** Expression of p53, c-erbB-2 and Ki67 proteins in simple intestinal metaplasia (SIM), atypical intestinal metaplasia (AIM) and gastric carcinoma (GC). A: p53 negative in SIM; B: p53 positive in AIM (nuclei); C: p53 expression increased in GC (nuclei); D: c-erbB-2 negative in SIM; E: c-erbB-2 positive in AIM (cytoplasm); F: c-erbB-2 expression increased in GC (membrane); G: Ki67 negative in SIM; H: Ki67 positive in AIM (nuclei); I: Ki67 expression increased in GC (Envision method,  $\times 400$ ).

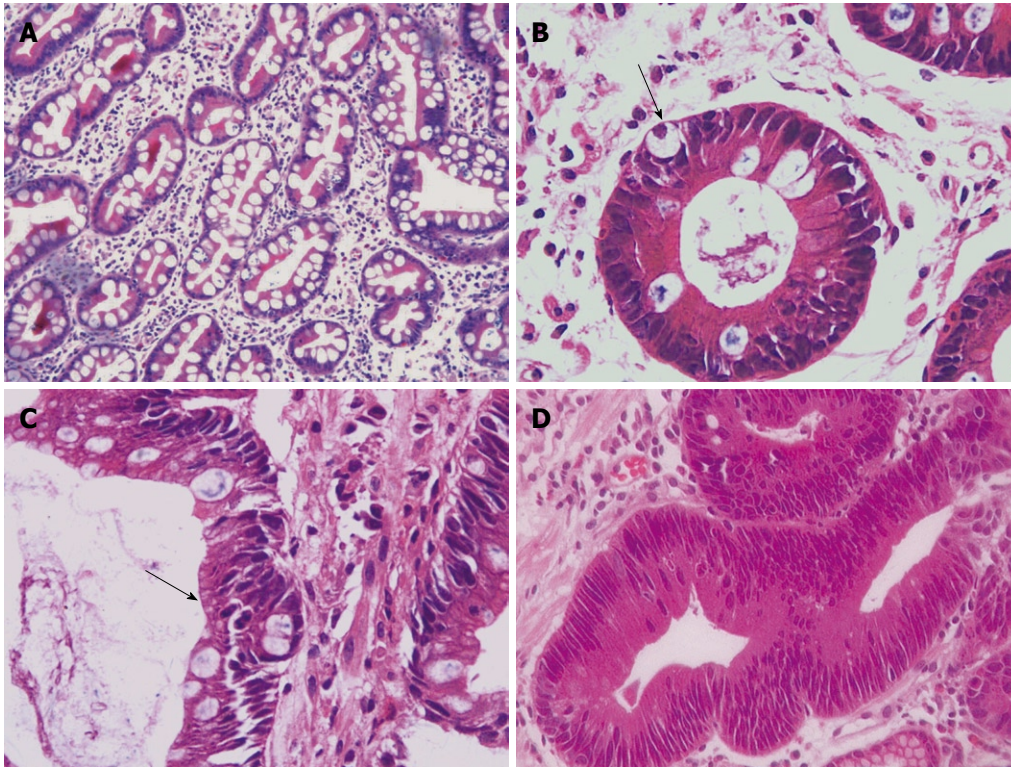
The positive expression rates of Ki67 in SIM and AIM were 25.00% and 51.85%, respectively. The expression of Ki67 in gastric carcinomas was significantly higher than in SIM ( $P < 0.05$ ) while there was no significant difference in Ki67 expression between AIM and gastric carcinomas ( $P > 0.05$ ) (Table 2, Figure 2).

## DISCUSSION

Gastric carcinoma is the fourth most common malignancy worldwide<sup>[10]</sup>, accounting for 10% of newly diagnosed cancers<sup>[11]</sup>. It is one of the most common causes of cancer mortality in China<sup>[12]</sup>. Approximately 84% of patients with gastric cancer will have advanced disease and their median survival time is only 3-4 mo if they are not treated with chemotherapy<sup>[13]</sup>. Therefore, it is necessary to diagnose at an early stage in order to improve the survival rate. Gastric carcinoma is divided into intestinal and diffuse types according to Lauren<sup>[14]</sup> and the intestinal-type of gastric carcinoma is said to have a strong association with IM<sup>[15]</sup>. Since Morson<sup>[16]</sup> pointed out in 1955 that some gastric carcinomas might arise from an area

of IM, IM has been considered to be a possible precancerous state. A large number of IM patients have been found in clinical studies, and a research group reported that the detection rate of IM was 37%<sup>[4]</sup>. Which type of IM is closely related to gastric carcinoma remains an unanswered question. Traditionally, IM is divided according to histochemical characteristics, but research concludes that sulphomucin-positive IM is of no value in surveillance for gastric cancer<sup>[17]</sup>. In view of the inconsistency of this classification, we assigned IM into the groupings of SIM (Figure 3) and IM with atypical changes as AIM (Figure 3). The predominant difference in the two types of IM is the atypical changes of the metaplastic epithelium: SIM glands are arranged neatly and the goblet cells are in normal forms, the mucosa in the foveolae is flat; while AIM glands are crowded and the goblet cells often are associated with immature differentiation, also the mucosa in the foveolae usually become deeper than in SIM. One question should be clarified regarding the difference between AIM and gastric intraepithelial neoplasia (GIN) (Figure 3): the main distinction lies in the goblet cells. There were more goblet cells (over 10%





**Figure 3 Morphological differences in three gastric lesions.** A: SIM; B: AIM: reversed polarity of goblet cell (arrow); C: AIM: mitosis (arrow); D: Gastric intraepithelial neoplasia (GIN) (HE,  $\times 400$ ).

of total cells) in AIM while there were less or no goblet cells in GIN.

In our study, three tumor-associated proteins, p53, c-erbB-2 and Ki67, were selected for immunohistochemical detection. The *p53* gene is localized to chromosome arm 17p13<sup>[18]</sup>. Evidence from *in vitro* models suggests that *p53* acts as a tumor suppressor gene<sup>[19]</sup>. The evidence accumulated so far suggests that mutant *p53* may be the commonest genetic abnormality in human cancer<sup>[20]</sup>. *p53* gene mutation is known to play a considerable role in the carcinogenesis of colonic carcinoma and gastric carcinoma. Shiao *et al*<sup>[21]</sup> reported that 67% of gastric carcinomas, 58% of dysplasias and 38% of metaplastic lesions stained positively for p53. *C-erbB-2* (also called *NEU* and *HER2*) is a proto-oncogene that codes for a protein product which shows considerable homology with EGFR<sup>[22]</sup>. Several studies have reported amplification of the *c-erbB-2* gene in human neoplasms, particularly in adenocarcinomas<sup>[23]</sup>. In recent years, researchers have revealed that c-erbB-2 plays an important role in the occurrence and development of gastric carcinoma. Observations suggested that *c-erbB-2* gene rearrangement non-randomly associated with carcinomas with glandular origin derived from the gastrointestinal tract<sup>[24]</sup>. It is reported that over expression of the *c-erbB-2* gene is at a frequency of 8.2%-45% in gastric carcinoma<sup>[25-27]</sup>. Ki67 is a nuclear proliferation-associated antigen expressed in the growth and synthesis phases of the cell cycle but not in the resting phase<sup>[28]</sup>. This antigen provides information about the proportion of active cells in the cell cycle. Its expression varies

greatly during the cell cycle and is increased in many tumors<sup>[29]</sup>. Studies have revealed that the Ki67 proliferating index increases in the transformation from IM to gastric carcinoma<sup>[30]</sup>. For the reasons above, p53, c-erbB-2 and Ki67 proteins can be regarded as indicators of the pre-cancerous nature of IM in the gastric mucosa. Our results demonstrated that the expressions of p53 and c-erbB-2 in gastric carcinoma were not significantly higher than in types I, II and III IM. The expression of Ki67 in gastric carcinoma was significantly higher than in type I, but not significantly higher than in type II or type III.

It is difficult to determine which subtype of IM has a definite relationship with gastric carcinomas. In SIM and AIM classification, the expressions of all three proteins in gastric carcinomas were significantly higher than in SIM and no significant differences were observed between gastric carcinomas and AIM. Obviously, AIM may have a much more close relationship with gastric carcinoma.

We deduce that SIM may be merely a response to stimuli caused by the changing environment, while AIM may have malignant transformation and could be regarded as preneoplastic lesions. Clinical follow up of AIM patients may be helpful for the diagnosis of early gastric carcinomas. However, since details of the role of AIM in the multiple steps of carcinogenesis of gastric mucosa are still unknown, further study is necessary with regard to AIM, perhaps using more advanced methods. Furthermore, an adequate long term follow up is indispensable to assess the definite value of AIM in the screening for gastric carcinoma.

## COMMENTS

### Background

Gastric carcinoma remains a significant problem globally. The relationship between intestinal metaplasia (IM) and gastric carcinoma has always been controversial. Generally IM is divided into subtypes on the basis of histochemical characteristics; however, this classification is confusing. A new classification of IM is needed in order to follow up patients selectively.

### Research frontiers

By detecting three tumor-associated proteins, p53, c-erbB-2 and Ki67, in IM and gastric carcinoma, this study compared two types of classification in IM of the stomach and explored their relationship to gastric carcinoma.

### Innovations and breakthroughs

In the past, IM was classified according to histochemical characteristics. In this study, IM was first divided into simple IM (SIM) and atypical IM (AIM) was reported to better reveal the precancerous nature of IM and could be a helpful indicator in the surveillance of patients clinically.

### Applications

The new classification of IM could be helpful in the surveillance of patients clinically and useful for the diagnosis of early gastric carcinomas.

### Terminology

IM is defined as the appearance of intestinal epithelium in the stomach. Type I, II and III IM are subtypes of IM classified according to the histochemical characteristics of the mucin-secreting cells. SIM and AIM classification is dependent mainly on the atypical changes of the metaplastic epithelium of the stomach. p53, c-erbB-2 and Ki67 are all tumor-associated proteins that are expressed mainly in metaplastic and tumor tissues.

### Peer review

The study provides important new data about the potential risk of gastric cancer in patients with IM. However, it would be important in the future to investigate the expression of p53 and/or Her2Neu in a prospective study in patients with IM, to confirm that only patients with p53/Her2Neu expression in the IM have actually a higher risk for gastric carcinomas.

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## Prevalence of diverticulosis in recurrent *Clostridium difficile* infection

Michael J Lipp, Odelya E Pagovich, David Rabin, Albert D Min, Brett B Bernstein

Michael J Lipp, Albert D Min, Brett B Bernstein, Division of Digestive Diseases, Beth Israel Medical Center, New York, NY 10003, United States

Odelya E Pagovich, David Rabin, Division of Medicine, Beth Israel Medical Center, New York, NY 10003, United States

**Author contributions:** Lipp MJ designed the study; Lipp MJ and Pagovich OE performed the chart evaluations and data analysis; Lipp MJ and Rabin D wrote the manuscript; Min AD and Bernstein BB were involved with editing the manuscript.

**Correspondence to:** Michael J Lipp, MD, Division of Digestive Diseases, Beth Israel Medical Center, 16th Street at 1st Avenue, 17-Baird Hall, New York, NY 10003, United States. [mlipp@chnpnet.org](mailto:mlipp@chnpnet.org)

Telephone: +1-212-4204183 Fax: +1-646-3493596

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**Peer reviewer:** Dr. Lodewijk Adriaan Anton Brosens, MD, PhD, Department of Pathology, University Medical Center Utrecht, Postbus 85500, 3508 GA Utrecht, The Netherlands

Lipp MJ, Pagovich OE, Rabin D, Min AD, Bernstein BB. Prevalence of diverticulosis in recurrent *Clostridium difficile* infection. *World J Gastroenterol* 2010; 16(3): 345-347 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i3/345.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i3.345>

### Abstract

**AIM:** To re-evaluate the theory that colonic diverticulosis is associated with relapse of *Clostridium difficile* associated disease (CDAD) in light of data suggesting increasing rates of CDAD infection and relapse.

**METHODS:** Charts were reviewed for patients with recurrent CDAD who had also had a prior colonoscopy or flexible sigmoidoscopy. An age and gender matched control group was used to compare the prevalence of diverticulosis.

**RESULTS:** Twenty-two patients met the study criteria, and the prevalence of diverticulosis in patients with CDAD relapse was 23% compared to 32% in age and sex matched controls ( $P = 0.44$ ). A significant proportion of patients with CDAD relapse had comorbidities associated with immune suppression.

**CONCLUSION:** Diverticulosis does not appear to be associated with CDAD relapse.

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### INTRODUCTION

The incidence of *Clostridium difficile* (*C. difficile*) associated disease (CDAD) has reached epidemic proportions, increasing both in terms of frequency and severity. The rates of *C. difficile* associated disease have steadily increased since the 1980s, with a marked increase in infection over the past 10 years. In one region of Canada there was a four-fold increase from 2002 to 2003 alone<sup>[1]</sup>. The cost associated with CDAD in 2008 in the U.S. has been estimated at \$32 million per day with nearly 40 000 extra hospital inpatient days due to *C. difficile* infection<sup>[2]</sup>. The increased incidence and virulence of *C. difficile* infection are felt to be caused by a new hypervirulent strain which produces significantly more toxins A and B. In addition to increasing morbidity and mortality, this hypervirulent strain has also led to an increase in relapse rates, which have been reported to be as high as 47.2%<sup>[3]</sup>, compared to historic relapse rates of approximately 20%<sup>[4]</sup>.

The exact etiology of CDAD relapse is incompletely understood, but it is probably multifactorial. Relapse of CDAD is usually caused by the original strain, although a percentage of patients are infected with a new strain<sup>[5]</sup>. One of the earliest theories for CDAD relapse was the presence of colonic diverticula, which were thought to



serve as reservoirs for *C. difficile* spores. This was largely based on an early study by Tedesco *et al*<sup>[6]</sup> which found that 18 of 22 patients (82%) with recurrent *C. difficile* infection had diverticulosis. It was theorized that *C. difficile* spores in diverticula were impervious to antibiotics and so could germinate after the completion of treatment. Tapered and pulsed antibiotic regimens for recurrent *C. difficile* infection were thus aimed at treating these dormant spores<sup>[7]</sup>. To our knowledge no paper has re-evaluated the association between diverticulosis and *C. difficile* relapse. Our study aimed to re-evaluate the association between diverticulosis and recurrent *C. difficile* infection at our medical center in light of increasing rates of CDAD infection and relapse.

## MATERIALS AND METHODS

We reviewed positive ELISA-based *C. difficile* toxin assays from 2005-2007 at our tertiary level hospital. We reviewed the charts of patients with a minimum of two positive toxin assays more than 14 d apart who also had a prior colonoscopy or flexible sigmoidoscopy at our institution. Patient charts were reviewed for the presence of diverticulosis, prior antibiotic use, comorbidities, recent hospitalizations, *C. difficile* treatment course, and number of relapses.

Relapse was defined as recurrent diarrhea with a positive toxin assay or pseudomembranous colitis after completion of treatment for *C. difficile* infection within the previous 3 mo. To be included in the study, patients must have completed full initial antibiotic therapy for a minimum of 10 d with symptomatic improvement. An age and gender matched control group was used to compare the prevalence of diverticulosis at our institution. Patients in the control group had colonoscopies performed for the purpose of colorectal cancer screening. This study was approved by our medical center's institutional review board.

## RESULTS

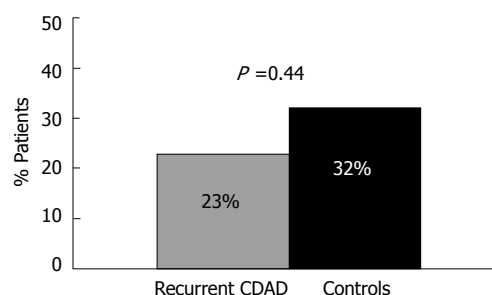
Twenty-two patients met the study criteria, with an average of 2.3 episodes of *C. difficile* infection. All patients were treated with standard metronidazole (*po* or *iv*) and/or oral vancomycin therapy. The prevalence of diverticulosis in patients with *C. difficile* relapse was 23%, with a mean patient age of 62. The prevalence of diverticulosis in the age and sex matched control group was 32% ( $P = 0.44$ , using  $\chi^2$  analysis, Figure 1). Table 1 shows the baseline characteristics of patients with recurrent *C. difficile* relapse. The inpatient mortality in this group was 18%.

## DISCUSSION

Patients with recurrent *C. difficile* infection at our institution did not have a higher prevalence of colonic diverticulosis than age and sex matched controls. The prevalence of diverticulosis in patients with relapsing *C. difficile* and controls was 23% and 32%, respectively ( $P = 0.44$ ), which is consistent with historical controls. To our knowledge this is the first paper to reevaluate

**Table 1** Baseline characteristics of patients with recurrent *Clostridium difficile* associated disease ( $n = 22$ )

Baseline characteristics	
Mean age (yr)	62 (30-90)
Female	13 (59%)
Recent hospitalizations (< 3 mo)	18 (82%)
Lymphoma/malignancy	8 (36%)
HIV/AIDS	3 (14%)
Systemic steroid use	3 (14%)
Liver cirrhosis	2 (9%)



**Figure 1** Prevalence of diverticulosis. CDAD: *Clostridium difficile* associated disease.

the association between diverticulosis and recurrent *C. difficile* infection since first described by Tedesco *et al*<sup>[6]</sup>.

We found that a high percentage of patients with recurrent *C. difficile* infection suffered from co-morbidities often associated with immune suppression. These results are not surprising since growing data suggest that host immunity plays a large role in promoting relapse<sup>[8-14]</sup>. One of the earliest studies to support this theory came from Kyne *et al*<sup>[15]</sup> where 22 patients with resolved *C. difficile* infection were compared to 22 patients with relapsing disease. Patients with relapsing *C. difficile* infection were found to have significantly lower levels of serum antibodies to toxin A. Additional independent risk factors for *C. difficile* recurrence include older age<sup>[16]</sup> and prolonged hospitalization<sup>[17]</sup>.

Patients with impaired host immunity during chemotherapy have been found to have higher rates of CDAD<sup>[8]</sup>, even without prior antibiotic use<sup>[9]</sup>. Higher rates of CDAD infection have been found in patients on nephrology, hematology, and organ transplantation wards<sup>[10]</sup>, as well as in patients with human immunodeficiency virus (HIV) infection<sup>[11]</sup>. These findings have prompted research aimed at improving host immunity in patients with CDAD relapse. Intravenous immunoglobulin (IVIG) has been used in the treatment of severe, refractory, or recurrent *C. difficile* infection<sup>[12,16]</sup>. A human monoclonal antibody against toxins A and B, MDX-1388, was shown to have efficacy in preventing relapse of *C. difficile* infection in hamsters<sup>[13]</sup>. A small study of a toxoid vaccine against *C. difficile* showed benefit in 3 patients with recurrent *C. difficile*, allowing the cessation of antibiotic treatment<sup>[14]</sup>.

Our study had several limitations. Given the small patient size our study may have been underpowered to detect a difference in the prevalence of diverticulosis.

This study was also done retrospectively and all patients originated from a single institution. Our results did not find an association between colonic diverticulosis and recurrent *C. difficile* infection at our institution, however further studies may be needed to verify these findings. The high prevalence of co-morbidities often associated with immune suppression in patients with recurrent *C. difficile*, however, is consistent with current data that suggest host immunity may play a significant role in CDAD relapse.

## COMMENTS

### Background

The incidence of *Clostridium difficile* (*C. difficile*) associated disease (CDAD) has reached epidemic proportions, increasing both in terms of frequency and severity. The exact etiology of CDAD relapse is incompletely understood, and previous research has suggested an association between CDAD relapse and colonic diverticulosis.

### Research frontiers

The increased incidence and virulence of *C. difficile* infection are felt to be caused by a new hypervirulent strain which produces significantly more toxins A and B. In addition to increasing morbidity and mortality, this hypervirulent strain has also led to an increase in relapse rates. In this study the authors did not find an association between colonic diverticulosis and CDAD relapse, but did find that a significant proportion of patients with CDAD relapse had co-morbidities associated with immune suppression.

### Innovations and breakthroughs

Recent reports have suggested that host immunity may play a role in preventing future *C. difficile* relapse. This is the first paper to re-evaluate the association between CDAD relapse and colonic diverticulosis since the emergence of a new hypervirulent strain.

### Applications

By showing a lack of association between colonic diverticulosis and CDAD relapse, this study could help guide future treatments. Previous treatments were originally aimed at treating dormant spores in diverticula with pulsed or tapering antibiotics. This study would suggest that therapy aimed at improving host immunity may be important in CDAD relapse.

### Terminology

*C. difficile* is a gram positive bacterium that may cause a colonic infection, typically after antibiotic use. This infection may become severe and may relapse, requiring multiple antibiotic treatments. Colonic diverticula are outpouchings of the colonic wall.

### Peer review

It is an interesting study that addresses a potentially important relationship between recurrence of *C. difficile* infection and diverticulosis.

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## Body composition changes after transjugular intrahepatic portosystemic shunt in patients with cirrhosis

Jonathan Montomoli, Peter Holland-Fischer, Giampaolo Bianchi, Henning Grønbaek, Hendrik Vilstrup, Giulio Marchesini, Marco Zoli

Jonathan Montomoli, Giampaolo Bianchi, Giulio Marchesini, Marco Zoli, Department of Internal Medicine, Aging and Nephrological Diseases, University of Bologna, I-40138 Bologna, Italy

Peter Holland-Fischer, Henning Grønbaek, Hendrik Vilstrup, Department of Medicine V, Aarhus University Hospital, DK-8000 Aarhus, Denmark

Author contributions: Montomoli J, Holland-Fischer P and Grønbaek H designed the study and performed the majority of experiments; Montomoli J, Bianchi G and Marchesini G produced the statistical analysis of the data and wrote the manuscript; Vilstrup H and Zoli M were involved in study design and final editing of the manuscript.

Correspondence to: Giampaolo Bianchi, MD, Department of Internal Medicine, Aging and Nephrological Diseases, University of Bologna S. Orsola-Malpighi Hospital, Via Albertoni 15, I-40138 Bologna, Italy. [giampaolo.bianchi@unibo.it](mailto:giampaolo.bianchi@unibo.it)

Telephone: +39-51-6364825 Fax: +39-51-6362210

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**RESULTS:** Porto-systemic gradient decreased after TIPS ( $6.0 \pm 2.1$  mmHg vs  $15.8 \pm 4.8$  mmHg,  $P < 0.001$ ). Patients were divided in two groups according to initial body mass index. After TIPS, normal weight patients had an increase in dry lean mass (from  $10.9 \pm 5.9$  kg to  $12.7 \pm 5.6$  kg,  $P = 0.031$ ) and TBW (from  $34.5 \pm 7.6$  L to  $40.2 \pm 10.8$  L,  $P = 0.007$ ), as well as insulin (from  $88.9 \pm 49.2$  pmol/L to  $164.7 \pm 107.0$  pmol/L,  $P = 0.009$ ) and HOMA-IR (from  $3.36\% \pm 2.18\%$  to  $6.18\% \pm 4.82\%$ ,  $P = 0.023$ ). In overweight patients only FFA increased significantly (from  $0.59 \pm 0.24$  mmol/L to  $0.93 \pm 0.34$  mmol/L,  $P = 0.023$ ).

**CONCLUSION:** TIPS procedure is effective in lowering portal pressure in patients with portal hypertension and improves body composition without significant changes in metabolic parameters.

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**Key words:** Insulin resistance; Liver cirrhosis; Malnutrition; Portal hypertension; Transjugular intrahepatic porto-systemic shunt

**Peer reviewers:** Dr. Paolo Del Poggio, Hepatology Unit, Department of Internal Medicine, Treviglio Hospital, Piazza Ospedale 1, Treviglio Bg 24047, Italy; Giuseppe Montalto, Professor, Clinical Medicine and Emerging Diseases, University of Palermo, via del Vespro, 141, Palermo 90100, Italy; Tomoharu Yoshizumi, MD, PhD, Department of Surgery, Saiseikai Fukuoka General Hospital, 1-3-46, Tenjin, Chuou-ku, Fukuoka, 810-0001, Japan

### Abstract

**AIM:** To investigate the effect of transjugular intrahepatic porto-systemic shunt (TIPS) on malnutrition in portal hypertensive cirrhotic patients.

**METHODS:** Twenty-one patients with liver cirrhosis and clinical indications for TIPS insertion were investigated before and 1, 4, 12, 52 wk after TIPS. For each patient we assayed body composition parameters [dry lean mass, fat mass, total body water (TBW)], routine liver and kidney function tests, and free fatty acids (FFA). Glucose and insulin were measured for the calculation of the homeostasis model assessment insulin resistance (HOMA-IR); liver function was measured by the galactose elimination capacity (GEC); the severity of liver disease was graded by model for end-stage liver disease (MELD).

Montomoli J, Holland-Fischer P, Bianchi G, Grønbaek H, Vilstrup H, Marchesini G, Zoli M. Body composition changes after transjugular intrahepatic portosystemic shunt in patients with cirrhosis. *World J Gastroenterol* 2010; 16(3): 348-353 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i3/348.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i3.348>

## INTRODUCTION

Portal hypertension and malnutrition are two important complications of cirrhosis, both affecting prognosis and risk of death<sup>[1-3]</sup>. Portal hypertension is present in 60% of cirrhotic patients<sup>[4]</sup>; the existence of a portal-systemic shunt modifies both fasting and post-prandial metabolism, decreasing the hepatic first-pass effect of nutrients, which become more available for peripheral tissues. Also hormone levels, like insulin, share a similar defect. The lack of a significant first-pass removal by the liver makes insulin flood the systemic circulation and promotes insulin resistance, which is further aggravated by the production of inflammatory molecules [tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), lipopolysaccharide (LPS), *etc.*] which have an anti-insulin effect and contribute to systemic inflammation<sup>[5]</sup>. The final event of the above alterations is a progressive deterioration of liver function, also resulting in poor nutritional status. Malnutrition, in turn, is a risk factor for disease progression, and is associated with the development of complications<sup>[3]</sup>, with a high risk of death<sup>[6]</sup>, as well as increased morbidity and mortality after transplantation<sup>[7]</sup>.

Several studies demonstrated that transjugular intrahepatic porto-systemic shunt (TIPS) using polytetrafluoroethylene (PTFE)-covered stents are the best rescue therapy for failures of medical and endoscopic treatment of portal hypertension<sup>[8-10]</sup>.

The effects of TIPS on metabolism and body composition are not well defined. Allard *et al.*<sup>[11]</sup> and Plauth *et al.*<sup>[12]</sup> found that TIPS insertion in malnourished patients with cirrhosis and hypermetabolism resulted in improved body composition. No studies, however, have clearly defined the relation between TIPS-induced metabolic changes and changes in nutritional status, and/or the underlying mechanism. In particular, no studies addressed the potential impact of pre-TIPS overweight and obesity and associated insulin resistance on the post-TIPS nutritional status and insulin levels.

We aimed to test the effects of TIPS insertion on nutritional status and insulin levels in a group of subjects with cirrhosis and complications of portal hypertension.

## MATERIALS AND METHODS

### Subjects

Twenty-six patients with liver cirrhosis, consecutively undergoing an elective TIPS procedure were included (Table 1). Three patients did not complete the baseline examination protocol due to variceal bleeding requiring intensive care and immediate TIPS insertion. Of the remaining 23 patients (15 males and 8 females), one received an orthotopic liver transplant after TIPS, and one died during follow-up. Three patients missed one or more follow-up evaluations; therefore, only 18 patients had all the evaluations planned by protocol. The diagnosis of cirrhosis was assessed on the basis of biochemical, clinical, and ultrasonographic findings and confirmed by liver biopsy in 8 cases. Among the 21 patients who completed the follow-up, etiology was as follows: alcohol-related, 15;

**Table 1** Modification of values studied in 21 patients (mean  $\pm$  SD)

Variable	Basal Pre-TIPS	After 52 wk	P
HVPG (mmHg)	15.8 $\pm$ 4.8	6.0 $\pm$ 2.1	< 0.001
Bilirubin (mg/dL)	1.26 $\pm$ 1.11	2.85 $\pm$ 2.29	0.017
Creatinine (mg/dL)	1.02 $\pm$ 0.56	1.14 $\pm$ 0.61	0.18
Albumin (g/L)	31.3 $\pm$ 7.7	33.0 $\pm$ 7.7	0.586
GEC (mmol/min)	1.79 $\pm$ 0.48	1.61 $\pm$ 0.41	0.081
MELD (score) (n = 18) <sup>1</sup>	11 (6)	13 (7)	0.804
Blood glucose (mmol/L)	5.8 $\pm$ 1.5	6.3 $\pm$ 1.6	0.345
Plasma insulin (pmol/L)	128.9 $\pm$ 84.0	182.9 $\pm$ 100.6	0.024
HOMA- $\beta$	269 $\pm$ 409	233 $\pm$ 108	0.700
HOMA-IR	5.04 $\pm$ 4.07	7.82 $\pm$ 5.89	0.028
BMI (kg/m <sup>2</sup> )	26.2 $\pm$ 5.8	27.4 $\pm$ 5.6	0.172
FFA (mmol/L)	0.54 $\pm$ 0.23	0.89 $\pm$ 0.44	0.013
Creatinine (mg/dL)	0.98 $\pm$ 0.52	0.83 $\pm$ 0.34	0.053
Fat mass (%)	29.4 $\pm$ 7.7	27.8 $\pm$ 8.7	0.278
Fat mass (kg)	23.4 $\pm$ 10.5	22.9 $\pm$ 10.0	0.812
Dry lean mass (kg)	14.0 $\pm$ 5.8	14.8 $\pm$ 5.0	0.115
TBW (L)	32.3 $\pm$ 9.6	43.3 $\pm$ 10.3	0.017

<sup>1</sup>Median (interquartile range). Values were obtained 2 wk before (Basal) and 52 wk after TIPS insertion. *P* < 0.05 is considered statically significant. TIPS: Transjugular intrahepatic porto-systemic shunt; HVPG: Hepatic venous pressure gradient; GEC: Galactose elimination capacity; MELD: Model for end-stage liver disease; HOMA: Homeostasis model assessment; IR: Insulin resistance; BMI: Body mass index; FFA: Free fatty acids; TBW: Total body water.

autoimmune, 3; primary sclerosing cholangitis, 1; HCV-related, 1; cryptogenic, 1. Abstinence from alcohol was a goal during the study, and to our knowledge all but one adhered to that policy. All patients signed a written informed consent to take part in the study in accordance with the Helsinki II Declaration. The study was approved by the Research Ethics Committee of Aarhus County.

### Experimental design

This was an observational prospective study. TIPS patients were studied approximately 2 wk before TIPS insertion, and were regularly re-evaluated at follow-up visits 1, 4, 12, and 52 wk after the procedure. All examinations were carried out after overnight fasting.

### TIPS procedure

Indications for TIPS insertion were refractory ascites (12 patients), secondary prevention of variceal bleeding (seven patients) or both (two patients). None had active variceal bleeding at the time of TIPS insertion. The TIPS procedure was carried out using covered stents according to the method described by Rössle *et al.*<sup>[13]</sup>. After insertion, a clinical and ultrasonographic control of the shunt was performed after 24 h, 4 wk, and then, at 12-wk intervals during the first year. Ascites was totally removed by paracentesis before TIPS insertion.

### Diet

Each patient had a dietetic investigation and a 7-d diary report before TIPS insertion. During the study, food intake remained unchanged both for quantity and type of nutrients.



### Body composition

Bio-impedance analysis (Quadscan 4000, Bodystat Ltd., Isle of Man, UK) was used to estimate body composition. The predictive equations were taken from Kushner *et al.*<sup>[14]</sup> and Lautz *et al.*<sup>[15]</sup>. Bio-impedance analysis was chosen because, despite some limitations in patients with ascites (not present in our patients both before and after TIPS) it is a bedside tool for the determination of body composition in cirrhotic patients with/without ascites<sup>[16]</sup>. Dry lean body mass was calculated as body weight - fat mass - total body water (TBW). Dry lean mass was preferred to lean mass to reduce the possible interference of changes in TBW due to the fluctuating presence of ascites. According to Tsiaousi *et al.*<sup>[17]</sup>, we considered as malnourished all patients with a body mass index (BMI) lower than 23.

### Liver function

The galactose elimination capacity (GEC) was used to measure quantitatively metabolic liver function, from blood concentration decay curves corrected for urinary excretion, as described by Tygstrup<sup>[18]</sup>. The clinical status was assessed according to the model for end-stage liver disease score (MELD)<sup>[19]</sup>.

### Biochemical analyses

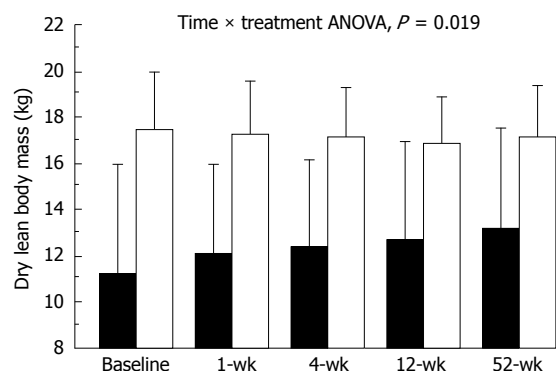
Glucose, creatinine, bilirubin and prothrombin time were routinely assayed by automatic analyzer on fresh serum/plasma samples. Free fatty acids (FFA) were determined with a colorimetric method using a commercial kit (Wako Chemicals, Neuss, Germany). Blood aliquots for insulin concentrations were stored at -80°C and measured by a two-site immunospecific insulin enzyme-linked immunosorbent assay<sup>[20]</sup>.

### Insulin secretion and sensitivity

Basal insulin secretion and sensitivity were assessed by means of the homeostasis model assessment (HOMA)<sup>[21,22]</sup>. Secretion was estimated using the Beta index (HOMA- $\beta$ ), while peripheral sensitivity was measured by HOMA-IR, with the following equations: HOMA- $\beta$  =  $20 \times$  fasting plasma insulin (mU/L)/[fasting plasma glucose (mmol/L) - 3.5]; HOMA-IR = fasting plasma insulin (mU/L)  $\times$  fasting plasma glucose (mmol/L)/22.5.

### Statistical analysis

Data analysis was performed using STATA 10 statistical software (StataCorp LP, Texas, USA). Results are given as mean  $\pm$  SD. Changes from baseline were explored by analysis of variance (ANOVA) for repeated measurements. Changes from baseline to end-of-observation and between groups were also tested by parametric and non-parametric paired and unpaired methods, whenever appropriate. Due to non-systematic factors, blood samples were missing from 8 examinations in 6 patients. For the statistical analyses these missing values were replaced by the mean of adjacent values. A *P*-value < 0.05 was considered significant in a two-tailed test.



**Figure 1** Time course of dry lean body mass in normal weight (black bars) and overweight subjects (white bars) following the TIPS procedure. The data are presented as means and standard error. Trend shows significant difference (*P* = 0.019).

## RESULTS

TIPS was well tolerated by all patients, and there were no procedure-related complications. TIPS insertion produced a reduction in hepatic venous pressure gradient (*P* < 0.001) (Table 1). Three patients needed a stent revision during follow-up. Transient hepatic encephalopathy was observed in three patients and was reversed by diet and oral disaccharides without the need for shunt reduction.

At the end of the study period, a non-significant increase in BMI was observed in the whole population (Table 1). Patients were divided in two groups according to their BMI at enrolment [BMI  $\leq$  25 kg/m<sup>2</sup>, normal/underweight (NW), *n* = 12; BMI > 25, overweight/obesity (OW), *n* = 9]. A few NW cases (*n* = 7; 58%) showed clinical evidence of malnutrition, but only 3 had a BMI below the lower cut-off of normality (18.5 kg/m<sup>2</sup>). Analysis of variance (ANOVA) for repeated measurements did not show differences in the time trend of BMI after TIPS between the two groups. Also the non-parametric analysis of 52-wk changes failed to detect differences in BMI changes over time.

### Body composition

Dry lean mass increased in NW patients by 14.5% (from  $10.9 \pm 5.9$  kg to  $12.7 \pm 5.6$  kg, *P* = 0.031), but did not change in OW patients; the trend was significant on ANOVA test for repeated measures (*P* = 0.002) (Figure 1). The increase in dry lean body mass in normal weight patients paralleled a significant increase in TBW of 14.3%, (from  $34.5 \pm 7.6$  L to  $40.2 \pm 10.8$  L, *P* = 0.007). Fat mass, both as absolute value or as percentage of body weight, did not vary significantly in the 2 groups (ANOVA, *P* = 0.815).

Regarding liver function, GEC did not vary over time in TIPS-treated patients; similarly MELD increased non-significantly by over 2 points during the observation period (Table 1). These changes were largely due to increased MELD values in NW patients, while no changes were observed in the OW group. Similarly, GEC remained stable in NW patients while in the OW group there was a trend toward a significant deterioration (Table 2).

**Table 2** Anthropometric, pressure and laboratory values recorded in 12 normal weight (NW: BMI  $\leq 25$  kg/m<sup>2</sup>) and in 9 overweight (OW: BMI  $> 25$ ) patients with liver cirrhosis (mean  $\pm$  SD)

Variable		Basal Pre-TIPS	After 52 wk	P
HVPG (mmHg)	NW	16.4 $\pm$ 5.0	6.3 $\pm$ 2.3	< 0.001
	OW	15.0 $\pm$ 4.7	5.7 $\pm$ 2.1	< 0.001
Albumin (g/L)	NW	30.8 $\pm$ 8.4	32.6 $\pm$ 8.1	0.871
	OW	31.9 $\pm$ 7.1	33.4 $\pm$ 7.6	0.581
Bilirubin (mg/dL)	NW	1.20 $\pm$ 0.47	3.21 $\pm$ 2.82	0.061
	OW	1.71 $\pm$ 0.81	2.44 $\pm$ 1.61	0.164
GEC (mmol/min)	NW	1.62 $\pm$ 0.36	1.57 $\pm$ 0.47	0.623
	OW	1.96 $\pm$ 0.55	1.65 $\pm$ 0.36	0.088
MELD (score) (n = 9)	NW	11 (5)	13 (4)	0.727
	OW	11 (6)	12 (8)	0.289
Blood glucose (mmol/L)	NW	5.7 $\pm$ 1.5	5.7 $\pm$ 0.8	0.941
	OW	5.9 $\pm$ 1.5	6.8 $\pm$ 1.9	0.271
Insulin (pmol/L)	NW	88.9 $\pm$ 49.2	164.7 $\pm$ 106.9	0.009
	OW	171.4 $\pm$ 94.4	203.0 $\pm$ 94.9	0.466
HOMA- $\beta$	NW	152 $\pm$ 88	253 $\pm$ 118	0.042
	OW	413 $\pm$ 588	209 $\pm$ 95	0.316
HOMA-IR	NW	3.36 $\pm$ 2.18	6.18 $\pm$ 4.82	0.023
	OW	6.91 $\pm$ 4.94	9.64 $\pm$ 6.70	0.262
BMI (kg/m <sup>2</sup> )	NW	21.4 $\pm$ 2.6	24.1 $\pm$ 4.0	0.220
	OW	30.9 $\pm$ 3.7	30.8 $\pm$ 5.1	0.944
FFA (mmol/L)	NW	0.49 $\pm$ 0.22	0.86 $\pm$ 0.53	0.124
	OW	0.59 $\pm$ 0.24	0.93 $\pm$ 0.34	0.023
Creatinine (mg/dL)	NW	0.84 $\pm$ 0.43	0.76 $\pm$ 0.39	0.149
	OW	1.17 $\pm$ 0.59	0.92 $\pm$ 0.26	0.157
Fat mass (%)	NW	27.6 $\pm$ 7.2	25.3 $\pm$ 8.3	0.334
	OW	31.5 $\pm$ 7.9	30.5 $\pm$ 8.7	0.638
Fat mass (kg)	NW	16.9 $\pm$ 3.7	17.5 $\pm$ 6.7	0.782
	OW	30.5 $\pm$ 11.0	28.8 $\pm$ 9.9	0.649
Dry lean mass (kg)	NW	10.9 $\pm$ 5.9	12.7 $\pm$ 5.6	0.031
	OW	17.4 $\pm$ 3.3	17.1 $\pm$ 2.9	0.557
TBW (L)	NW	34.5 $\pm$ 7.6	40.2 $\pm$ 10.8	0.007
	OW	44.6 $\pm$ 8.9	46.6 $\pm$ 9.2	0.459

P < 0.05 is considered statistically significant.

### Metabolic parameters

Plasma insulin and HOMA-IR significantly increased in TIPS-treated cirrhotic patients without any change in fasting glucose (Table 1). The changes were mainly limited to normal weight patients who also had a significant increase in HOMA- $\beta$  (Table 2). However, no significant differences were demonstrated by repeated-measures ANOVA.

After TIPS insertion, changes in FFA plasma levels (Table 1) were exclusively observed in OW patients (Table 2).

No systematic changes in serum creatinine were observed (Tables 1 and 2), but patients with mild renal failure (creatinine levels  $\geq 1.2$  mg/dL, n = 7) slightly improved their values (from  $1.75 \pm 0.37$  mg/dL to  $1.45 \pm 0.43$  mg/dL, P = 0.23).

## DISCUSSION

The main finding of this study is that dry lean body mass significantly increases in NW patients with cirrhosis after TIPS insertion. Advanced cirrhosis is associated with reduced lean mass<sup>[23]</sup>; a significant protein-calorie malnutrition is present in at least 30% of patients with cirrhosis<sup>[24]</sup>. Both parameters appear to be related to the severity of the liver disease<sup>[25]</sup>. Considering that a large

fraction of our NW patients showed signs of malnutrition at baseline, TIPS appears to modify the natural history of malnutrition. This finding agrees with the results obtained by Plauth *et al*<sup>[12]</sup> and Allard *et al*<sup>[11]</sup>. Both reported an increase in dry lean mass but many questions on the metabolic mechanisms involved remain unresolved<sup>[26]</sup>. The significant increase in dry lean mass of about 1.84 kg in the year after TIPS insertion is paralleled by an increase in TBW that, in the absence of ascites and/or regional edemas, is indicative of muscle tissue formation<sup>[27]</sup>. Our results are in keeping with a very recent preliminary report by Camci *et al*<sup>[28]</sup>, on six TIPS-treated malnourished cirrhotic patients.

This beneficial effect of TIPS on lean body mass was not observed in overweight or obese subjects at baseline. The reasons for this different metabolic response are not easy to determine. NW cases were characterized by lower insulin resistance at baseline, but also lower insulin and lower insulin secretion. In these normal- or under-weight individuals, the additional metabolic and hormonal abnormalities already in the group with excess body weight do not increase the cirrhosis-related insulin resistance<sup>[29]</sup>. Muscle tissue takes up 80%-85% of glucose infused during hyperinsulinemic euglycemic clamp<sup>[30,31]</sup>. After TIPS placement, these cases were characterized by a significant increase in serum insulin, without changes in blood glucose levels, and consequently by increased HOMA-IR and HOMA- $\beta$ , and stable liver function. Under these conditions, the improved hemodynamic state, in the presence of a normal intake of nutrients, could promote insulin production and insulin action, favoring the improved nutritional state. This mechanism might not be operative in the OW group, with a much higher degree of insulin-resistance than NW cases, and where insulin did not increase further after TIPS placement. In these patients, the TIPS procedure was followed by a remarkable reduction of HOMA- $\beta$ , and the decreased insulin production could not sustain lean body mass formation.

After TIPS the plasma levels of FFA increased significantly only in the OW group. In agreement with the hypothesis of Yki-Järvinen *et al*<sup>[32,34]</sup>, the increase in FFA levels is likely to stimulate hepatic gluconeogenesis and competition with glucose in muscle metabolism. These metabolic changes could further promote insulin resistance.

Reduced portal hypertension might be an additional factor playing a role in the improved metabolic and nutritional status. Portal hypertension increases the permeability of enteric mucosa that promotes intestinal bacterial translocation and the systemic diffusion of LPS and other pro-inflammatory molecules, ultimately producing interleukin-1 $\beta$ , interleukin-6 and TNF- $\alpha$ . These molecules promote insulin-resistance and have an anti-insulin, catabolic effect, leading to protein mass wasting. Any mechanism mediated by anti-insulin molecules might play a differing effect in relation to BMI and to the levels of insulin resistance, much higher in obesity. Obesity *per se* is a chronic inflammatory state that sustains insulin-resistance<sup>[35]</sup>, in the presence of obesity the multiple factors sustaining insulin resistance could not be totally

removed by TIPS; in contrast, in subjects with poor nutritional status the less severe insulin resistance might be removed by TIPS, thus explaining improved nutritional status. Larger studies are needed to explore the potential benefits of TIPS on long-term survival of underweight patients with cirrhosis, at higher risk of morbidity and mortality according to several previous studies<sup>[36]</sup>.

Finally, the presence of insulin-resistance might be related to advanced liver disease and this condition could justify the absence of any improvement in OW patients with liver cirrhosis. We used GEC, an estimate of the functional liver mass with a prognostic value in the medium term interval<sup>[37]</sup>, and there was no overall change in GEC over time, however, in OW patients a trend towards a decrease in GEC was observed. During the study, food intake remained unchanged both for quantity and type of nutrients. Further, it is our policy that alcohol has to be withdrawn in patients before TIPS insertion, as alcohol itself increases portal hypertension and may thus be involved in both the risk of variceal bleeding and ascites formation. During follow up, alcohol abstinence is important and this policy is reflected in the high number of alcohol abstainers in the present study. We therefore suggest that changes in body composition do not reflect a change in food and/or alcohol intake but are an effect of TIPS and of the reduction in portal pressure *per se*.

Finally, the study confirmed the effectiveness of TIPS on portal hypertension with a reduction in Hepatic venous pressure gradient (HVPG) to a value of 6 mmHg, lower than the 12 mmHg threshold value of increased risk for variceal bleeding, re-bleeding and mortality<sup>[1,4]</sup>. The absence of re-bleeding episodes and the disappearance of ascites confirm this hypothesis. Furthermore, patients with functional renal failure at baseline improved their creatinine levels at follow-up. Considering that lean mass increased, this value does not reflect a worsening of malnutrition<sup>[38]</sup> but is due to a positive effect of TIPS on the hemodynamic state<sup>[39]</sup>.

In conclusion, the TIPS procedure is effective in lowering portal pressure in patients with portal hypertension and improves body composition without significant changes in metabolic parameters.

## COMMENTS

### Background

Malnutrition in portal hypertensive cirrhotic patients increases the risk and the severity of clinical complications. Transjugular intrahepatic porto-systemic shunt (TIPS) is a well established therapy for complications of portal hypertension in cirrhotic patients. However, the effect of TIPS on malnutrition is unclear.

### Research frontiers

The article provides insight into the beneficial effects of TIPS in regard to improvement in nutritional status. Malnutrition in liver diseases is an important area of research as it is an independent factor resulting in increased morbidity and mortality. Furthermore, the article addresses the effects of shunting of hormones/peptides *etc.* especially insulin, that relate to the possible mechanisms behind insulin resistance in patients with liver cirrhosis.

### Innovations and breakthroughs

Related papers have focussed on improvement in body composition without addressing the pre-TIPS nutritional status. With a simple yet comprehensive separation into normal weight and overweight subjects, the results suggest

additional nutritional benefits in the group of malnourished patients from TIPS insertion (see next section).

### Applications

TIPS treatment of the complications of portal hypertension seems to improve nutritional status in liver cirrhosis, especially in patients suffering from malnutrition. Ultimately, malnutrition may provide an additional reason/indication for TIPS insertion in patients with liver cirrhosis.

### Terminology

The TIPS procedure is a minimally invasive procedure used in patients with liver cirrhosis to reduce portal hypertension and thereby ameliorating complications of portal hypertension. Using a catheter technique *via* the right jugular vein, a stent is placed within the liver connecting the portal vein and the hepatic vein and thus reducing portal hypertension.

### Peer review

This is an interesting and novel study showing an amelioration of nutritional parameters after TIPS especially in lean cirrhotic patients. An improvement in the body composition (nutritional status) of liver cirrhosis patients after TIPS implementation has been studied previously. And this study is well conducted and long term follow up data are provided.

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## Immunogenetic characteristics of patients with autoimmune gastritis

Aino Mirjam Oksanen, Katri Eerika Haimila, Hilpi Iris Kaarina Rautelin, Jukka Antero Partanen

Aino Mirjam Oksanen, Herttoniemi Hospital, Health Centre, City of Helsinki, PO Box 6300, 00099 Helsinki, Finland  
Katri Eerika Haimila, Finnish Red Cross Blood Service, Clinical Laboratory, 00310 Helsinki, Finland  
Hilpi Iris Kaarina Rautelin, Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki, 00014 Helsinki, Finland; HUSLAB, Helsinki University Central Hospital, 00029 Helsinki, Finland; Department of Medical Sciences, University and University Hospital of Uppsala, 75185 Uppsala, Sweden

Jukka Antero Partanen, Finnish Red Cross Blood Service, Research and Development, 00310 Helsinki, Finland

Author contributions: Oksanen AM designed the study, enrolled patients, and wrote the manuscript; Haimila KE performed the immunogenetic studies; Rautelin HIK analyzed the data and revised the manuscript; Partanen JA designed and analyzed the immunogenetic studies.

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Correspondence to: Aino Mirjam Oksanen, MD, Herttoniemi Hospital, Health Centre, City of Helsinki, PO Box 6300, 00099 Helsinki, Finland. [aino.oksanen@hel.fi](mailto:aino.oksanen@hel.fi)

Telephone: +358-9-3105511 Fax: +358-9-31055894

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interleukin (IL)-1 gene cluster, IL-2, IL-4, IL-6, IL-10, IL-12, interferon  $\gamma$ , transforming growth factor  $\beta$ , and tumor necrosis factor  $\alpha$ . Variation in *KIR* genes was also explored. The results were compared with prevalence of the polymorphisms in Finnish or European populations.

**RESULTS:** All patients had pepsinogen I levels below normal (mean: 11  $\mu\text{g/L}$ , range: < 5 to 25  $\mu\text{g/L}$ ). Three patients had elevated *H. pylori* IgG antibodies, while *H. pylori* serology was negative in the rest of the patients. AIG patients carried significantly more often HLA-DRB1\*04 (58%) and DQB1\*03 (83%) than the general Finnish population did (28% and 51%, respectively;  $P = 0.045$  and  $0.034$  by the Fisher's exact test). No patient was positive for HLA-B8-DRB1\*03, a well-established autoimmune marker. Neither cytokine polymorphisms nor *KIR* gene variation showed association with AIG.

**CONCLUSION:** As explored with modern DNA-based methods, HLA-DRB1\*04 and DQB1\*03 alleles, but not HLA-B8-DRB1\*03, may predispose to AIG.

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**Key words:** Atrophic gastritis; Autoimmune diseases; Cytokines; Genetic polymorphisms; Human leukocyte antigens; Killer cell immunoglobulin-like receptor

**Peer reviewers:** David J McGee, PhD, Associate Professor, Department of Microbiology & Immunology, Louisiana State University Health Sciences Center-Shreveport, 1501 Kings Highway, Shreveport, LA 71130, United States; Tamara Vorobjova, Senior Researcher in Immunology, Department of Immunology, Institute of General and Molecular Pathology, University of Tartu, Ravila, 19, Tartu, 51014, Estonia, Tamara

Oksanen AM, Haimila KE, Rautelin HIK, Partanen JA. Immunogenetic characteristics of patients with autoimmune gastritis. *World J Gastroenterol* 2010; 16(3): 354-358 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i3/354.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i3.354>

### Abstract

**AIM:** To explore whether predisposition to autoimmune gastritis (AIG) is found in human leukocyte antigen (HLA), cytokine or killer cell immunoglobulin-like receptor (*KIR*) gene variations.

**METHODS:** Twelve Finnish patients with autoimmune-type severe atrophy of the gastric corpus were included. The patients' serum was analyzed for pepsinogen I and *Helicobacter pylori* (*H. pylori*) antibodies. DNA was separated and the patients were genotyped for HLA-A, B, Cw, DRB1 and DQB1 antigens, and studied for single nucleotide polymorphisms for the following cytokines:

## INTRODUCTION

Autoimmune gastritis (AIG) is an organ-specific autoimmune disease, in which inflammation of the mucosa of the gastric corpus results in total loss of corpus-type glands, and achlorhydria. AIG patients typically have a low serum pepsinogen I (PG I) concentration, and most of them also have parietal cell antibodies (PCAs). In many, but not all patients, vitamin B12 absorption is deficient, which leads to pernicious anemia (PA)<sup>[1]</sup>.

The occurrence of AIG and PA has long been recognized to be determined strongly by genetic factors, which, however, are largely unexplored. The most important genetic association found in human AIG so far is a link with the human leukocyte antigen (HLA) region. The observed association between AIG and certain HLA antigens has, however, not been strong enough to explain the familial clustering of AIG<sup>[2]</sup>.

Polymorphisms in the genes that encode immune regulator molecules may affect the secretion or function of the corresponding proteins, and thus influence immune responses, inflammation and tissue injury. Cytokine genes have been studied widely in autoimmune diseases and associations have been found between, for instance, tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and interleukin (IL)-10 polymorphisms and autoimmune hepatitis and pemphigus, respectively<sup>[3,4]</sup>. Also *Helicobacter pylori* (*H. pylori*)-associated atrophic gastritis has been shown to be more frequent in patients with proinflammatory polymorphisms of genes for *IL-1* gene cluster, and TNF $\alpha$ <sup>[5]</sup>.

Killer cell immunoglobulin-like receptors (KIRs) are members of a diverse family of regulatory molecules expressed on subsets of T cells. KIRs play a role in the control of the natural killer (NK) cell immune response. The KIR receptors recognize certain HLA class I determinants and regulate NK cell activity. The number and type of *KIR* genes vary between individuals who can carry anything from seven to 12 *KIR* genes, of which, some encode activating and others inhibiting receptors<sup>[6,7]</sup>. *KIR* genes can be divided into two main haplotype groups. Group A contains only one activating and six inhibiting *KIR* genes, whereas group B haplotypes are more variable and contain several activating *KIR* genes<sup>[8]</sup>. In addition to the copy-number variation, individual *KIR* genes exhibit allelic variation. *KIR* genes have been shown to be associated with various diseases, including some autoimmune diseases<sup>[9]</sup>.

Recently, we sequenced the coding regions of genes for  $\alpha$ - and  $\beta$ -subunits of H<sup>+</sup>/K<sup>+</sup>-ATPase, which is the main autoantigen in AIG, in AIG patients, but no disease-associated polymorphisms could be found<sup>[10]</sup>. In the present study, a number of genes involved in immune activation were explored in patients with AIG, by modern molecular genetic methods. The aim of this study was to determine whether variations in the immune regulator genes, such as HLA, cytokine or KIR, are associated with the presence of AIG.

## MATERIALS AND METHODS

### Clinical information

A total of 18 patients, who had earlier undergone gastroscopy at Herttoniemi Hospital and were known to have severe atrophic corpus gastritis without any history of *H. pylori* infection, and who were under 65 years of age, were invited by letter to participate in the study. Twelve patients gave written informed consent, donated a blood sample, and completed a questionnaire about their possible vitamin B12 replacement therapy and thyroid diseases, as well as the occurrence of AIG in the family. Signs of other autoimmune diseases were looked for in the patient records. The study was approved by the Ethical Committee for Internal Medicine at Helsinki University Central Hospital.

### Blood tests

EDTA blood and serum samples were kept at -20°C until analyzed. DNA was extracted from the EDTA blood sample using a DNA purification kit (PureGene®; Centralsystems, Minneapolis, MN, USA), according to the manufacturer's instructions. Serum samples were analysed for PG I, PCAs and *H. pylori* antibodies.

For serum PG I concentrations, an immunoenzymometric assay (Gastroset PG1; Orion Diagnostica, Espoo, Finland) was used. The lower normal limit of the assay was 28 µg/L. PCAs were determined by an enzyme immunoassay (Varelixa Parietal Cell Antibodies; Pharmacia Diagnostics, Freiburg, Germany), which used H<sup>+</sup>/K<sup>+</sup>-ATPase as the antigen. Concentrations < 10 U/mL were normal, according to the manufacturer. For *H. pylori* antibodies, an in-house immunoassay that measured IgG antibodies was used, and titers  $\geq 700$  were considered elevated<sup>[11]</sup>.

### Immunogenetics

*HLA* genes were explored using the INNO-LiPA kit (Innogenetics, Ghent, Belgium) according to the manufacturer's instructions. The *HLA-A*, *B*, *Cw*, *DRB1* and *DQB1* genes were amplified by polymerase chain reaction (PCR), and the biotinylated PCR products were hybridized with sequence-specific oligonucleotides on membrane-based strips. Results were analyzed by the LiRAS (Innogenetics) interpretation software.

Cytokine polymorphisms in the genes of *IL-1* gene cluster, *IL-2*, *IL-4*, *IL-6*, *IL-10*, *IL-12*, interferon (IFN)- $\gamma$ , transforming growth factor (TGF)  $\beta$ , and TNF $\alpha$  were genotyped using the Cytokine Genotyping Kit (Pel-Freez Clinical Systems, Brown Deer, WI, USA). Cytokine profiles (high/intermediate/low producer) based on the polymorphisms were formed according to the published phenotypes also mentioned in the product insert of the kit. *KIR* genes (*KIR2DL1*, *KIR2DL2*, *KIR2DL3*, *KIR2DL4*, *KIR2DL5*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS4*, *KIR2DS5*, *KIR3DL1*, *KIR3DL2*, *KIR3DL3*, *KIR3DS1*, *KIR2DP1* and *KIR3DP1*) were determined using the KIR Genotyping Kit (Pel-Freez Clinical Systems), following the manufacturer's instructions. Both genotyping kits were

based on PCR amplification with sequence-specific primers that were designed to detect polymorphisms of cytokine/*KIR* genes. The PCR products were separated by gel electrophoresis, and the genotype results were interpreted on the basis of specific amplification patterns.

Prevalence of the HLA genotypes was compared with HLA frequency in the Finnish population, based on the data collected in Clinical Laboratory of Finnish Red Cross blood service. The cytokine polymorphisms and *KIR* genes were compared with the frequency of polymorphisms and *KIR* genes in populations of Finnish of European ancestry published previously<sup>[12-15]</sup>.

### Statistical analysis

Fisher's exact test was used to compare the prevalence of genotypes between patients and the populations used as controls.

## RESULTS

Demographic and clinical characteristics of the patients are summarized in Table 1. All patients had total atrophy in the gastric corpus. The mucosa of the gastric antrum was normal in eight patients, and mild chronic inflammation or sparse intestinal metaplasia was detected in four. All patients had PG I levels below normal (mean: 11 µg/L, range: < 5 to 22 µg/L), and elevated PCAs (median: 185 U/mL, range: 20-509 U/mL). Three patients (numbers 3, 10 and 11 in Tables 1 and 2) had elevated *H. pylori* IgG antibodies (titers: 730-2200), whereas *H. pylori* serology was entirely negative in the rest of the patients (titers: 50-100). All patients but one (number 2 in Tables 1 and 2) had vitamin B12 replacement therapy.

### Immunogenetics of the patients

The HLA-A, B, Cw, DRB1 and DQB1 alleles in the AIG patients are shown in Table 2. DRB1\*04 was present in seven out of 12 (58%) patients, whereas 28% of the Finnish general population carry the allele ( $P = 0.045$  by Fisher's exact test). Ten patients (83%) had DQB1\*03; its allele frequency in the Finnish population is 51% ( $P = 0.034$  by Fisher's exact test).

Only one of the 12 patients carried the DRB1\*0301-DQB1\*0201 haplotype, which is an established susceptibility factor for various autoimmune diseases<sup>[16]</sup>. It is of particular note that the only DRB1\*0301-positive patient did not have the classical A\*01-B\*08 haplotype.

The frequencies of polymorphisms in the genes of the *IL-1* gene cluster, *IL-2*, *IL-4*, *IL-6*, *IL-10*, *IL-12*, *IFNγ*, *TGFβ* and *TNFα* did not differ significantly from those found in Finnish (where data were available) or other European populations. The results for genotyping the *IL-1* gene cluster, *TNFα* and *IL-10* are shown in Table 3.

All 14 characterized *KIR* genes, *KIR2DL1*, *KIR2DL2*, *KIR2DL3*, *KIR2DL4*, *KIR2DL5*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS4*, *KIR2DS5*, *KIR3DL1*, *KIR3DL2*, *KIR3DL3* and *KIR3DS1*, were determined, as well as two *KIR* pseudogenes *KIR2DP1* and *KIR3DP1*. Ten patients

Table 1 Clinical characteristics of the 12 AIG patients

Patient	Sex	Age (yr)	Years from diagnosis	Other autoimmune diseases	AIG in family
1	F	50	7		-
2	F	56	3	RA	+
3	F	49	2		+
4	M	62	19		+
5	F	63	2	HT	+
6	F	54	3		-
7	F	38	4		+
8	F	56	5	PBC	-
9	F	52	0		-
10	F	52	0		-
11	F	47	15	T1D	-
12	F	52	0		-

AIG: Autoimmune gastritis; RA: Rheumatoid arthritis; HT: Hyperthyreosis; PBC: Primary biliary cirrhosis; T1D: Type 1 diabetes.

Table 2 HLA-A, B, Cw, DRB1 and DQB1 genotypes of the 12 AIG patients

Patient	HLA-A		HLA-B		HLA-Cw		HLA-DRB1		HLA-DQB1	
1	03	11	07	44	01	07	12	15	0301	0602
2	02	-	15	51	04	14	04	08	0302	0402
3	02	24	15	39	04	07	04	15	0302	0602
4	02	03	07	18	07	-	04	15	0302	0602
5	02	26	15	40	03	04	01	08	0402	0501
6	02	03	15	-	03	-	04	08	0302	0402
7	02	-	13	51	06	15	07	09	0202	0303
8	02	32	40	51	02	15	09	15	0303	0602
9	03	-	07	51	03	15	04	13	0301	0303
10	02	03	35	40	04	07	04	12	0301	0302
11	02	-	27	35	01	03	03	08	0201	0402
12	03	24	13	35	04	06	04	-	0302	-

carried both A and B *KIR* haplotypes; two patients were homozygotes for A haplotype. *KIR* genotype and haplotype frequencies of the patients did not differ from those reported earlier in the Finnish population<sup>[15]</sup>.

## DISCUSSION

In Finnish AIG patients, the HLA-DRB1\*04 and DQB1\*03 alleles were more frequent than in the general population, which implies an association between certain HLA-DRB1 and DQB1 haplotypes and AIG. The well-known autoimmune markers HLA-B8, DRB1\*03 and DQB1\*02 were practically missing in the AIG patients. This suggests that the immunogenetics of AIG are different to that of many classical autoimmune diseases.

The co-localization of susceptibility foci in experimental AIG and type 1 diabetes (T1D) is the strongest known between two autoimmune diseases<sup>[2]</sup>, and the most prominent susceptibility locus for both diseases is located in the HLA region. Individuals with T1D also have PCAs more often than population controls do<sup>[17]</sup>. Over 90% of Caucasians with T1D carry the DR3 or DR4 haplotype, and the DQB1\*0302 allele is associated strongly with T1D<sup>[18]</sup>. In the present study, the DRB1\*04 allele was more frequent in AIG patients

**Table 3** IL-1, IL-10, and TNF $\alpha$  polymorphisms in 12 AIG patients, and in Finnish, Italian and Czech populations

	Genotype	AIG patients	Finnish <sup>[11]</sup> %	Italian <sup>[12]</sup> %	Czech <sup>[13]</sup> %
TNF $\alpha$ -308	AA <sup>1</sup>	-	3	2	2
	GA	-	21	14	38
	GG	12	76	84	60
IL-1 $\beta$ -511	TT <sup>1</sup>	-		9	10
	CT	8		41	45
	CC	4		50	45
IL1RA	CC <sup>1</sup>	1		3	8
mspa111100	TC	8		41	45
	TT	3		56	47
IL-10	ATA/ATA <sup>1</sup>	1		5	
	ATA/ACC	1		21	
	ACC/ACC	1		7	
	GCC/ATA	5		24	
	GCC/ACC	4		31	
	GCC/GCC	-		12	

<sup>1</sup>The genotypes are the most proinflammatory ones.

than in the general population, but the DRB1\*03 allele was only carried by the patient with T1D. Six of our 12 patients had DQB1\*0302, the prevalence of which in the Finnish population is 13% ( $P = 0.005$  by Fischer's exact test). The AIG patient with T1D was also the only one to carry the DQB1\*02 haplotype, which is present in 91% of Finnish celiac disease patients<sup>[19]</sup>, and in 17% of the general Finnish population. Thus, Finnish AIG patients seem to share some of the haplotypes that are common in patients with T1D, but not those seen in patients with celiac disease.

In the 1970s, several studies were carried out to find a possible association between AIG or PA and HLA antigens. Increased frequency of HLA antigens A3, B7 or both has been found in AIG and PA patients<sup>[20-22]</sup>; however, these findings were not confirmed by others<sup>[23]</sup>. Subgroups of AIG patients have shown associations with different HLA antigens. Patients with a concomitant endocrine disease showed an increased frequency of the B8, B18 and BW15 antigens, and those without endocrine disease that of the B7 and B12 antigens<sup>[24]</sup>.

Of the class II HLA antigens, PA patients showed increased frequency of the DR2 and DR4 antigens and a decreased presence of the DR3 antigen, as compared to controls. PA patients with a concomitant endocrine disease showed DR3/DR4 antigens more often, and those without autoimmune endocrine disease showed DR2/DR4 and DR4/DR5 antigens, as compared to controls<sup>[25]</sup>. Possibly because of the small number of patients in the present study, no significant difference could be found between those with and without concomitant autoimmune disease.

The role of *H. pylori* in AIG and PA is still poorly understood<sup>[26]</sup>. On one hand, patients with *H. pylori* infection often develop atrophic gastritis and even autoimmune characteristics, such as PCAs<sup>[27]</sup>. On the other hand, AIG patients without any signs of *H. pylori* infection, such as the majority of patients in the present study, may be found. In studies before the *Helicobacter* era, the role of *H. pylori* in atrophic gastritis was not recognized, and patients with

*H. pylori*-associated autoimmunity may have been included; this may have made it more difficult to detect associations between AIG and, for example, HLA antigens. Our patients were relatively young with a median age of 52 years and the majority were women, which is typical for the classic AIG<sup>[1]</sup>. The three patients with positive *H. pylori* serology showed no clinical difference from the others.

In *H. pylori*-positive individuals, proinflammatory polymorphisms of the IL-1 $\beta$  gene cluster have been found to be associated with atrophic gastritis, achlorhydria<sup>[28,29]</sup>, and even gastric cancer<sup>[30]</sup>, which often is a late sequel of atrophic gastritis. Patients carrying proinflammatory IL-1 $\beta$ -511T and TNF $\alpha$ -308A, and who are homozygous for IL-1RN\*2\*2, had an OR of 5.8 for developing atrophic gastritis<sup>[31]</sup>. In addition, patients that carried three or more of the proinflammatory polymorphisms (carriage of IL-1 $\beta$ -511T+ or TNF $\alpha$ -308A; homozygosity for IL-1RN\*2\*2 or IL-10 ATA/ATA) had an OR of 26.3 for non-cardia gastric cancer<sup>[32]</sup>. However, the association between gastric cancer and IL-1 $\beta$  polymorphisms has not been seen in all studies<sup>[33]</sup>. Despite the fact that all our patients had profound atrophy in the gastric corpus at a relatively young age, the frequencies of these particular genotypes did not differ from those found in populations with European ancestry. Even though the small number of patients and the lack of controls in the present study make it impossible to detect small or modest associations, our results suggest that these polymorphisms are not crucial for the development of AIG.

In conclusion, HLA DRB1\*04 and DQB1\*03 were more frequent in AIG patients than in the general Finnish population, which suggests an association between certain HLA-DRB1 and DQB1 haplotypes and AIG. Also, the well-known autoimmune markers HLA-B8, DRB1\*03 and DQB1\*02 were practically missing in the AIG patients. However the number of patients in the present study was small, and larger studies are needed to confirm these findings.

## COMMENTS

### Background

Autoimmune gastritis (AIG) is chronic inflammation in the mucosa of the gastric body, which may lead to vitamin B12 deficiency of and pernicious anemia. The cause of this inflammation is not known, but its occurrence is known to be strongly determined by genetic factors.

### Research frontiers

In earlier studies using antigen determination for the detection of human leukocyte antigen (HLA) tissue determinants, an association was found between HLA tissue antigens and AIG. Before the present study, this association had not been studied by modern DNA-based methods.

### Innovations and breakthroughs

The study is believed to be the first to show an association between AIG and certain HLA genotypes, as explored with modern DNA-based methods.

### Applications

The study included a small number of patients. These results may in the future contribute to exploring the mechanisms of AIG and possibly other autoimmune diseases. AIG is also a risk factor for gastric cancer; thus, understanding the evolution of AIG may contribute to exploring the development of cancer.

### Peer review

This is a good pilot study that indicates the need for a much bigger, longer-term study.



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## ***NOD2*, *IL23R* and *ATG16L1* polymorphisms in Lithuanian patients with inflammatory bowel disease**

Jurgita Sventoraityte, Aida Zvirbliene, Andre Franke, Ruta Kwiatkowski, Gediminas Kiudelis, Limas Kupcinskas, Stefan Schreiber

Jurgita Sventoraityte, Aida Zvirbliene, Gediminas Kiudelis, Limas Kupcinskas, Department of Gastroenterology, Kaunas University of Medicine, LT-44307 Kaunas, Lithuania  
Andre Franke, Ruta Kwiatkowski, Stefan Schreiber, Institute for Clinical Molecular Biology, Christian-Albrechts-University Kiel, D-24105 Kiel, Germany

**Author contributions:** Sventoraityte J and Zvirbliene A contributed equally to this work; Zvirbliene A, Kiudelis G and Kupcinskas L contributed human subject blood samples; Kupcinskas L, Zvirbliene A and Kwiatkowski R designed the research; Zvirbliene A and Sventoraityte J performed the research and analyzed the data; Sventoraityte J wrote the paper; Franke A and Kupcinskas L revised the manuscript; Schreiber S and Franke A co-ordinated and provided the financial support for this work.

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**Correspondence to:** Limas Kupcinskas, Professor, Department of Gastroenterology, Kaunas University of Medicine, A. Mickeviciaus Street 9, LT-44307 Kaunas, Lithuania. [likup@takas.lt](mailto:likup@takas.lt)

Telephone: +370-37-326092 Fax: +370-37-326508

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### **Abstract**

**AIM:** To investigate the frequency of *NOD2*, *IL23R* and *ATG16L1* genetic variants in a case-control panel for inflammatory bowel disease (IBD) from Lithuania.

**METHODS:** One hundred and eighty unrelated IBD patients [57 Crohn's disease (CD) and 123 ulcerative colitis (UC)] and 186 healthy controls were genotyped for the following known genetic susceptibility variants: *NOD2* - Arg702Trp (rs2066844), Gly908Arg (rs2066845) and Leu1007insC (rs2066847), as well as *IL23R* - Arg381Gln (rs11209026) and *ATG16L1* - Thr300Ala (rs2241880).

**RESULTS:** The effect that carriership of at least one *NOD2* risk allele predisposes to CD was replicated in the

Lithuanian population (41.1% CD vs 16.9% controls,  $P = 2 \times 10^{-4}$ , OR = 3.48, 95% CI: 1.81-6.72). In the allelic single marker analysis, Leu1007insC was strongly associated with CD (21.4% CD vs 4.7% controls,  $P = 3.687 \times 10^{-8}$ , OR = 5.54, 95% CI: 2.85-10.75). Neither the other two *NOD2* variants, nor the known variants in *IL23R* and *ATG16L1* were found to be risk factors for CD, UC or IBD. However, our relatively small study population was underpowered to demonstrate such weak to moderate disease associations.

**CONCLUSION:** The results support a strong association between CD susceptibility and the Leu1007insC variant in *NOD2* in the Lithuanian study population.

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**Key words:** *NOD2*; *IL23R*; *ATG16L1*; Single nucleotide polymorphisms; Crohn's disease; Ulcerative colitis; Lithuania

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### **INTRODUCTION**

The inflammatory bowel diseases (IBD) refer to two

clinically defined conditions, ulcerative colitis (UC) and Crohn's disease (CD) that represent major burdens of morbidity in Western countries, with prevalence rates in North America and Europe ranging from 21 to 246 per 100 000 inhabitants for UC and 8 to 214 per 100 000 inhabitants for CD<sup>[1]</sup>. Although the exact aetiology of IBD remains unclear, accumulating data suggests that IBD occurs from the combined effects of genetic predisposition and environmental factors<sup>[2]</sup>.

Linkage, candidate gene, targeted association mapping and genome-wide association studies have identified many common variants associated with IBD and have rapidly expanded our fundamental knowledge of complex disease biology. The first and most consistently replicated genetic susceptibility variants, were found in the *NOD2* gene<sup>[3-5]</sup>, attributed to the recognition of bacterial products, along with several other genetic loci coding for cytokines involved in acquired immune responses (*IL23R*<sup>[6]</sup>) and genes related to the autophagy pathway (*ATG16L1*<sup>[7]</sup>).

Given the heterogeneity in allele frequencies reported for the genetic factors involved in the pathogenesis of IBD in different European populations<sup>[8]</sup>, we aimed to perform the first genetic study of IBD in a low-incidence population<sup>[9,10]</sup> of North-Eastern Europe - Lithuania. We examined the frequencies of the previously described variants in the *NOD2*, *IL23R* and *ATG16L1* genes in a Lithuanian IBD study population.

## MATERIALS AND METHODS

### Patients

The study included 57 unrelated patients with CD, 123 with UC and 186 healthy, age- and gender-matched controls. All study participants were of Caucasian ethnicity. The recruitment of the study individuals was performed at the Department of Gastroenterology, Kaunas University of Medicine Hospital during the period from 2003 to 2006. Written informed consent from all participants and approval of the Kaunas Regional Biomedical Research Ethics Committee (Protocol No. 84/2003) was obtained. The diagnosis of either CD or UC was based on standard clinical, endoscopic, radiological and histological criteria<sup>[11]</sup>. Patients' demographic and phenotypic details are summarized in Table 1. The clinical characteristics provided in the table are given according to the Montreal classification<sup>[12]</sup>.

### Genotyping

Genomic DNA was isolated from EDTA peripheral blood using the Invisorb Blood Giga Kit from Invitex (Berlin, Germany). The three *NOD2* variants - Arg702Trp (rs2066844), Gly908Arg (rs2066845) and Leu1007insC (rs2066847), and the *IL23R* variant Arg381Gln (rs11209026) were genotyped using Applied Biosystem's (Foster City, CA, USA) allele-specific TaqMan™ or TaqMan-MGB assays (Table 2); *ATG16L1* variant Thr300Ala (rs2241880) detection was performed using a pre-designed TaqMan® single nucleotide polymorphism

**Table 1** Summary of clinical and demographic characteristics of the IBD patients *n* (%)

Characteristics	CD	UC
Gender (male/female)	27/30	68/55
Age (years ± SD)	40.5 ± 14.9	45.4 ± 16.4
Age at diagnosis (years ± SD)	31.7 ± 16.6	34.3 ± 14.7
Familial IBD	0	0
Surgery treatment	15 (26.3)	3 (2.4)
Disease extension in UC		
Proctitis	-	26 (21.1)
Left-sided colitis	-	61 (49.5)
Extended colitis	-	36 (29.3)
Disease localization in CD		
Terminal ileum, L1	17 (29.8)	-
Colon, L2	16 (28.1)	-
Ileocolon, L3	23 (40.3)	-
Upper GI, L4	1 (1.8)	-
Disease Behavior in CD		
Non-stricturing, non-penetrating, B1	41 (71.9)	-
Stricturing, B2	5 (8.8)	-
Penetrating, B3	11 (19.3)	-
Perianal disease, B4	-	-
Extraintestinal manifestations		
Joints	6 (10.5)	13 (10.6)
Cutaneous	3 (5.3)	4 (3.3)
Ocular	1 (1.8)	0
Hepatobiliary	0	2 (1.6)

IBD: Inflammatory bowel diseases; CD: Crohn's disease; UC: Ulcerative colitis.

(SNP) genotyping assay (ID C\_9095577\_20). Genotyping was performed on an automated platform using the TaqMan® (Applied Biosystems, Foster City, CA, USA) technique as previously described<sup>[13]</sup>. All genotyped markers had a call rate greater than 95% in case and healthy control samples.

### Statistical analysis

Each SNP was checked for conformance with Hardy-Weinberg equilibrium in the control group using Fisher's exact test ( $P_{HWE} > 0.01$ ). Single-marker association analyses between cases and controls were performed using  $\chi^2$  statistics or Fisher's exact genotypic test. The significance level of the tests for considering *P*-values as significant was set to  $< 0.05$ . Data were evaluated using the web interface SISA<sup>[14]</sup>. Carriership of mutated alleles in case and control populations was estimated by direct counting.

The population attributable risk percentage (PAR%) was calculated as the attributable risk percentage (AR%) multiplied by the proportion of exposed cases, where AR% was estimated from the odds ratio (OR), assuming that the exposure of the control population to the disease-associated variant reflects the true prevalence of the variant in the general population<sup>[15]</sup>.

## RESULTS

All five SNPs were successfully genotyped in our North-Eastern European IBD case-control panel comprising 57 CD and 123 UC patients from Lithuania. The distribution of genotypes within the control group was

Table 2 TaqMan® primer and probe sequences of *NOD2* and *IL23R* assays

Marker	Primers	Probes
<i>NOD2</i>		
rs2066844	5'-TTCCTGGCAGGGCTGTGTC 5'-AGTGGAAGTGCTTGCAGGAGG	TET-CCTGCTC <b>T</b> GGCGCCAGGCC FAM-CCTGCTC <b>C</b> GGCGCCAGGC
rs2066845	5'-ACTCACTGACACTGTCTGTGACTCT 5'-AGCCACCTCAAGCTCTGGTG	TET-TTCAGATTCTGG <b>C</b> GCAACAGAGTGGGT FAM-TTTTCAGATTCTGG <b>G</b> GCAACAGAGTGGGT
rs2066847	5'-CCAGGTTGTCCAATAACTGCATC 5'-CCTTACCAGACTTCCAGGATGGT	VIC-TGCAGG <b>C</b> CCCTTG FAM-CTGCAG <b>G</b> CCCTTG
<i>IL23R</i>		
rs11209026	5'-CGTCTTTGCTGTATGTGTCAATTCTT 5'-AGAAAACAGAAATCTGCAAAAACCTACC	VIC-CAGATCATTC <b>A</b> AACTG FAM-ACAGATCATTC <b>G</b> AACTG

The examined alleles are highlighted by bold underlined typing.

Table 3 Association statistics for the *NOD2*, *ATG16L1* and *IL23R* variants in the Lithuanian IBD population

Gene marker	Minor allele	Controls ( <i>n</i> = 186)			CD ( <i>n</i> = 56)				UC ( <i>n</i> = 123)			
		GT (11/12/22)	MAF	<i>P</i> <sub>HWE</sub>	GT (11/12/22)	MAF	<i>P</i> <sub>CCA</sub>	OR (95% CI)	GT (11/12/22)	MAF	<i>P</i> <sub>CCA</sub>	OR (95% CI)
<i>NOD2</i>												
rs2066844	T	0/9/171	0.025	> 0.99	0/2/54	0.018	> 0.99	0.71 (0.15-3.33)	0/10/113	0.041	0.278	1.65 (0.66-4.13)
rs2066845	C	1/7/169	0.025	0.099	0/3/53	0.027	> 0.99	1.06 (0.28-3.97)	0/1/121	0.004	0.055	0.16 (0.02-1.25)
rs2066847	insC	2/13/166	0.047	0.048	4/16/36	0.214	3.687 × 10 <sup>-8a</sup>	5.54 (2.85-10.75)	1/8/114	0.041	0.711	0.86 (0.39-1.91)
<i>ATG16L1</i>												
rs2241880	G	44/89/53	0.476	0.560	16/28/11	0.546	0.199	1.32 (0.86-2.03)	33/61/25	0.534	0.164	1.26 (0.91-1.75)
<i>IL23R</i>												
rs11209026	A	3/16/167	0.059	0.017	0/4/52	0.036	0.335	0.59 (0.20-1.75)	0/11/109	0.045	0.477	0.76 (0.36-1.61)

Minor allele frequencies (MAF), genotype counts (GT; 11 = homozygous for minor allele; 12 = heterozygous for common allele; 22 = homozygote for common allele), allelic test *P* values (<sup>a</sup>*P*<sub>CCA</sub> < 0.05), and odds ratios (OR, shown for the minor allele) with 95% confidence intervals (CI) are depicted for both the CD and UC case-control population.

consistent with Hardy-Weinberg equilibrium (Table 3). For each of the variants studied, the risk of carrying the variant was compared between the CD, UC and healthy controls groups. The genotype and minor allele frequencies are presented in Table 3.

As expected, none of the studied individuals were carriers of all three *NOD2* risk alleles. However, two CD patients were determined as compound heterozygotes. The combined allele carriership in the group of patients with CD was much higher than in controls (41.1% *vs* 16.9%) and resulted in significant association (*P* = 2 × 10<sup>-4</sup>, OR = 3.48, 95% CI: 1.81-6.72) whereas no significant difference was observed between UC patients and controls. The PAR% in CD patients was 29.5% for possession of one or more *NOD2* variant alleles at any of the three sites.

In the allelic single marker analysis of the *NOD2* variants, a significant association was detected only between CD and Leu1007insC. For this variant, both the allelic and genotypic tests revealed *P*-values < 10<sup>-4</sup> (OR<sub>allele</sub> = 5.54, 95% CI: 2.85-10.75; OR<sub>carriership</sub> = 6.12, 95% CI: 2.88-13.15), resulting from the increased minor allele frequency (MAF) in cases (21.4%) *vs* controls (4.7%). In the UC group, the risk allele frequency of 4.1% was almost identical with the frequency detected in the controls. The frequencies of the other two *NOD2* variants: Arg702Trp and Gly908Arg were low in both controls and IBD pa-

tients groups and were not statistically significant.

The allele frequencies distribution for the *IL23R* and *ATG16L1* disease associated variants were almost identical between cases and controls and did not demonstrate significant differences.

## DISCUSSION

This is the first report on the prevalence of the previously defined *NOD2*, *ATG16L1* and *IL23R* disease associated variants in an IBD case-control sample from Lithuania. Baltic countries still observe low IBD incidence rates, especially for CD in their populations. In Estonia (1993-1998) the incidence rate of CD was reported to be 1.4 per 100 000 inhabitants<sup>[16]</sup>; and in Lithuania (2006) - 2.0 per 100 000 inhabitants<sup>[9,10]</sup>. Therefore, analysis of the genetic contribution to disease susceptibility in this region was of great interest.

Since 2001, following the identification of *NOD2* as the first gene conferring susceptibility to CD<sup>[3-5]</sup> a significant number of studies have replicated the association of the Arg702Trp, Gly908Arg and Leu1007insC variants with the development of CD in populations of Caucasian origin from Europe and North America<sup>[17]</sup>. However, significant heterogeneity in the frequencies of these variants has been observed not only between ethnically divergent populations<sup>[18,19]</sup>, but also within Europe<sup>[17]</sup>.



Our study results add to this pattern. The carriage of at least one *NOD2* variant was highest in the CD patients group (41.1%) compared to the control group (16.9%) and resulted in the OR = 3.48 (95% CI: 1.81-6.72). These data are in concordance with previously reported rates of 30%-50% in CD and 7%-20% in controls from other European regions<sup>[17]</sup>. The Leu1007insC variant was responsible for the major contribution of *NOD2* to disease susceptibility in the Lithuanian CD population (MAF = 21.4%, OR = 5.54, 95% CI: 2.89-10.75). These data are consistent with previous reports from Central Europe and North America (MAF = 6.6%-16%)<sup>[17]</sup> and contrast markedly with studies performed in Northern Europe, where carriage rates of Leu1007insC and other *NOD2* variants are relatively low, i.e. the carriage of at least one *NOD2* variant varies from 2.8% to 22%<sup>[20,21]</sup>. However, we were not able to confirm the association between Arg702Trp, Gly908Arg and IBD susceptibility in our study group. These findings are in contrast with previous reports from Southern and Central European populations, where a positive association between Arg702Trp, Gly908Arg and CD was detected. The reported alleles frequency rates in these European countries vary from 6.7% to 12.5% for Arg702Trp, from 3.3% to 6.1% for Gly908Arg, respectively, in CD patients and from 3.5% to 6.9% and from 0.6% to 3.0%, respectively, in controls<sup>[17]</sup>.

Moreover, the PAR%, an indication of the contribution of a mutation to the disease in a specific area, was 29.5% in the present study and contrasts with the other Northern European populations reporting lowest PAR% (range: 1.88%-11%)<sup>[20,21]</sup>. The PAR% measured in the Central European populations and North America was around 30%<sup>[3,5,17]</sup>. Therefore, the results of our study indicate that CD in Lithuania has a strong genetic background that is related partially to *NOD2* susceptibility variants. Interestingly, the relatively high carriership frequency of any of the three *NOD2* alleles in the healthy controls (16.9%) in our study is in contrast with data of low CD incidence in Lithuania<sup>[9]</sup>. This indicates the importance of environmental factors (e.g. diet, lifestyle) in disease development.

The first two genome wide association studies identified genetic alterations within *IL23R*<sup>[6]</sup> a component of the adaptive immune system - and *ATG16L1*<sup>[7]</sup> - a protein involved in autophagic processes - to be associated with IBD and CD. These findings broadened the understanding of the different pathways that are involved in IBD susceptibility and/or pathogenesis. In addition, the *IL23R* and *ATG16L1* findings were confirmed in large independent Caucasian samples<sup>[22-34]</sup>. A study in Japan<sup>[35]</sup> failed to replicate these results, supporting the previously reported distinct ethnic difference of the genetic background of CD. Upon analysis of the Lithuanian IBD population we were not able to confirm any of these findings. We were just able to observe trends for possible associations with *ATG16L1* risk allele. The frequencies and contributable risk of the *ATG16L1* G allele reported in our study (55% CD and 48% controls, OR: 1.32) were similar to the published studies performed in Germany (59% CD and 52% controls, OR: 1.35)<sup>[22]</sup>, UK

(57% CD and 51% controls, OR: 1.30)<sup>[23]</sup>, Hungary (58% CD and 50% controls, OR: 1.39)<sup>[26]</sup> and pooled study of German, Dutch and Hungarian cohorts (57% CD and 51% controls, OR: 1.32)<sup>[24]</sup>. The allele frequency distribution of the protective *IL23R* variant in our control samples (5.9%) was similar to previous reports in Caucasian populations (approx. 6%), whereas the allele frequencies in our both IBD cases groups were higher (3.6% CD and 4.5% UC) compared to the results of other studies (1.87%-2.85% CD and 1.9%-2.68% UC)<sup>[26-34]</sup>.

It must be noted that our relatively small study population was underpowered to demonstrate such weak to moderate disease associations. The panel had a power of 80% to detect an odds ratio of 1.8 or higher at the 5% significance level, assuming a frequency of the disease-associated allele of at least 30% in the controls. Therefore, larger-sized case-control panels will be needed in order to further evaluate the importance of the herein tested loci.

In summary, our study provides clear evidence that the *NOD2* Leu1007insC variant increases susceptibility to CD in the Lithuanian study population, whereas associations of *IL23R* and *ATG16L1* variants with any of the distinct IBD subtypes need to be further evaluated in larger North-Eastern European IBD sample collections.

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## COMMENTS

### Background

Numerous genome-wide and linkage studies have identified and replicated significant association between inflammatory bowel disease (IBD) development and polymorphisms of genes attributed to recognition of bacterial products (*CARD15*), adaptive immune responses (*IL23R*), and autophagosome pathways (*ATG16L1*). However, there has been reported a heterogeneity in allele frequencies of genetic factors involved in the pathogenesis of IBD in different European populations. The genetic association with IBD susceptibility has never been investigated in Lithuania previously.

### Research frontiers

The research was performed to obtain data about the frequency of *NOD2*, *IL23R* and *ATG16L1* genetic variants in a case-control study group for IBD from Lithuania.

### Innovations and breakthroughs

The results of the authors' study indicate that Crohn's disease (CD) in Lithuania has a strong genetic background that relates partially to *NOD2* susceptibility variants, especially Leu1007insC. The relatively high carriership frequency of any of the three *NOD2* alleles in the healthy controls (16.9%) in this study is in contrast with the data of low CD incidence in Lithuania. This indicates the importance of environmental factors (e.g. diet, lifestyle) in disease development.

### Applications

This is one of the first studies investigating the genetic association with IBD in a North-Eastern European country. The results of this study confirm that the heterogeneity of variants might be observed within Europe and will further help to understand the role of interplay between genetic and environmental factors in the development of complex diseases. Future studies in larger study groups

and further analysis of the biological functions of the identified variants are required to understand their role in determining the risk of CD and ulcerative colitis in ethnically divergent populations.

### Terminology

*NOD2* is a member of the NACHT-LRR receptor (NLR) protein family, which is known to be involved in recognition of microbial structures. *ATG16L1* encodes a protein which is part of a larger family of proteins that are required for the intracellular degradation system - autophagy process. *IL23R* encodes a protein which is a subunit of the receptor for IL23A/IL23 and participates in JAK-STAT3 signaling pathway.

### Peer review

The authors concluded that the *NOD2* Leu1007insC variant increases susceptibility to CD in the Lithuanian study population, whereas associations of *IL23R* and *ATG16L1* variants with any of the distinct IBD subtypes need to be further evaluated in larger Eastern European IBD sample collections. The study was conducted with good design and convincing analysis, and the manuscript has been well written and solid conclusions have been drawn.

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## Leukocyte-technetium-99m uptake in Crohn's disease: Does it show subclinical disease?

Luciene G Mota, Luiz GV Coelho, Carlos JR Simal, Maria LA Ferrari, Clodomiro Toledo, Josep Martin-Comin, Simone OF Diniz, Valbert N Cardoso

Luciene G Mota, Nuclear Engineering Department, Federal University of Minas Gerais, 31270-901, Belo Horizonte, Brazil  
 Luiz GV Coelho, Maria LA Ferrari, Clodomiro Toledo, Gastroenterology ALFA Institute, Clinical Hospital, Federal University of Minas Gerais, 31270-901, Belo Horizonte, Brazil

Carlos JR Simal, Nuclear Medicine, Felício Rocho Hospital, Medicine Faculty, Federal University of Minas Gerais, 31270-901, Belo Horizonte, Brazil

Josep Martin-Comin, Medicine Nuclear Center, Bellvitge Hospital, 08907, Barcelona, Spain

Simone OF Diniz, Valbert N Cardoso, Radioisotope Laboratory, Pharmacy Faculty, Federal University of Minas Gerais, 31270-901, Belo Horizonte, Brazil

**Author contributions:** Mota LG, Cardoso VN, Diniz SOF and Simal CJR were responsible for radiolabeling and scintigraphic images; Coelho LGV, Ferrari MLA and Toledo C contributed to the selection of patients; Martin-Comin J provided the initial idea for this study.

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**Correspondence to:** Valbert N Cardoso, PhD, Radioisotope Laboratory, Pharmacy Faculty, Federal University of Minas Gerais, Avenue Antônio Carlos 6627, 31270-901, Belo Horizonte, Minas Gerais, Brazil. [cardosov@farmacia.ufmg.br](mailto:cardosov@farmacia.ufmg.br)

Telephone: +55-31-34096892 Fax: +55-31-34096985

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C-reactive protein (CRP) of each patient were performed 7 d before the scintigraphic images. The leukocytes were labeled according to the International Society of Radiolabeled Blood Elements (ISORBE) consensus protocol and the scintigraphic images, including single photon emission computed tomography, were obtained 30 min and 2 h after injection of the radiolabeled leukocytes.

**RESULTS:** The labeling yield of the leukocytes with the lipophilic complex 99mTc-HMPAO was 55.0% ± 10%. Six of the 20 patients (30%) presented congruent results for the three parameters investigated (CDAI, Scintigraphic Index and CRP). On the other hand, 14 patients (70%) did not show congruent results. There was no significant correlation between the indices analyzed according to the Spearman test ( $P > 0.05$ ,  $n = 20$ ).

**CONCLUSION:** The results suggest that 99mTc-HMPAO-labeled leukocyte scintigraphy could be important for determining inflammatory activity in CD even in the absence of clinical symptoms.

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**Key words:** Technetium-99m; Hexamethylpropyleneamine oxime; Leukocytes; Inflammatory bowel disease; Scintigraphy

**Peer reviewer:** Amado S Peña, Professor, Department of Pathology, Immunogenetics, VU University Medical Centre, De Boelelaan 1117, PO Box 7057, Amsterdam 1007 MB, The Netherlands

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### Abstract

**AIM:** To evaluate inflammatory activity in patients with Crohn's disease (CD) using technetium-99m-hexamethylpropyleneamine oxime (99mTc-HMPAO) granulocyte scintigraphy.

**METHODS:** Twenty patients (7 male and 13 female) with CD and five healthy volunteers were selected for 99mTc-HMPAO granulocyte scintigraphy. The Crohn's Disease Activity Index (CDAI), blood tests and



## INTRODUCTION

Crohn's disease (CD) is characterized by chronic intestinal inflammation of unknown etiology that can affect any segment of the digestive tract from the mouth to the rectum<sup>[1]</sup>. The diagnosis of this disease is based on clinical manifestations, radiological, endoscopic, surgical and anatomical pathological observations. However, none of these findings is considered pathognomonic of the disease<sup>[2-4]</sup>.

The initial clinical symptoms of CD may be subtle, variable, nonspecific and easily overlooked. Recurrent episodes of inflammation in the gastrointestinal tract are typical. This inflammation underlies many of the symptoms and signs of the disease, thus its detection and monitoring are of the utmost importance in clinical management<sup>[5]</sup>. The follow-up of patients in clinical remission is based currently on the calculate of clinical activity indexes, including the Crohn's Disease Activity Index (CDAI) that is based on clinical and laboratory parameters, and measures CD activity as a sum of points<sup>[6]</sup>. CDAI < 150 is characteristic of remission of the disease. Values between 150 and 250 are associated with mild inflammatory activity. Inflammatory activity is considered moderate when the values lie between 250 and 350, and CDAI > 350 characterizes intense activity<sup>[7]</sup>. Another parameter used in the CD is the Vienna Classification that considers data constant such as age (A), location (L) and behavior (B) of the disease. It has as its aim phenotype standardization to evolutionary studies and works involving genetic, biological and environment factors<sup>[3]</sup>.

Gastrointestinal inflammation is not directly observable by patients or physicians, therefore, many methods have been developed to quantify the severity and extent of this inflammation. Therefore, a simple, rapid, sensitive, specific, inexpensive, noninvasive method to detect and monitor intestinal inflammation in CD is needed. According to Annovazzi *et al*<sup>[6]</sup>, if relapse or subclinical inflammation can be predicted in CD, it is likely to change the approach to treatment<sup>[6]</sup>. In this case, the use of a functional imaging method such as technetium-99m-hexamethylpropyleneamine oxime (99mTc-HMPAO) granulocyte scintigraphy could be more important to elucidate the location of the inflammatory site in the bowel. Scintigraphic images are based on functional alterations of the tissue, which permit an early diagnosis of the inflammation and infection when the anatomical alterations are not visible<sup>[4,8]</sup>. Among the scintigraphic methods used in the identification of the inflammatory and infectious foci, the use of radiolabeled leukocytes has been employed as a specialized technique that explores the natural migratory behavior of the white blood cells<sup>[9]</sup>.

Arndt *et al*<sup>[10]</sup> have demonstrated that 99mTc-HMPAO-labeled leukocyte scintigraphy is better than the Van Hees activity index and laboratory parameters for the evaluation of the inflammatory activity of intestinal diseases. Other authors have reported that autologous radiolabeled leukocyte scintigraphy can be utilized in

the monitoring of patients to evaluate the efficiency of therapy, differentiation between fibrotic and inflammatory stenosis, and the recurrence of the disease after surgery<sup>[11,12]</sup>. Among the treatment options for CD, the following stand out: aminosalicylates, corticosteroids, immunomodulators and biological therapy for control of inflammatory activity<sup>[2]</sup>.

The aim of the present study was to evaluate the presence of the inflammatory activity in patients with CD, who were subjected to usual treatment using 99mTc-HMPAO-labeled leukocyte scintigraphy.

## MATERIALS AND METHODS

### Materials

HMPAO (Ceretek) was supplied by Amersham Health (UK). Technetium-99m was obtained from a molybdenum generator (IPEN/Brazil). All other chemicals and reagents used were of analytical grade.

### Subjects

Twenty patients (mean age 38.7 years, 7 male and 13 female) with previous diagnosis of CD were selected at the Gastroenterology Alfa Institute of the Clinical Hospital at Federal University of Minas Gerais in the period between September 2007 and June 2008. The diagnoses were based on the patient's clinical history and physical examination, as well as the results of radiological and endoscopic examinations. The patients were being treated with corticosteroids, aminosalicylates, antibiotics, immunomodulators and biological therapy (Table 1). Informed consent was obtained from all patients admitted to the study. This study was approved by the Ethical Committee at Federal University of Minas Gerais. Seven days before 99mTc-HMPAO-labeled leukocyte scintigraphy, patients were subjected to determination of complete hemography, erythrocyte sedimentation rate and C-reactive protein (CRP) level. The CRP reference value was considered < 8 mg/L. In this same period, all patients filled in the card to calculate CDAI<sup>[6]</sup>. Five healthy volunteers were invited to participate in this study as controls.

### Cell labeling with 99mTc-HMPAO

The labeling of leukocytes with 99mTc-HMPAO was performed in accordance with the method described by Martin-Comin *et al*<sup>[13]</sup>. Briefly, blood samples (45 mL) were withdrawn from patients and healthy volunteers with a syringe that contained 6.0 mL anticoagulant [citric acid-citrate-dextrose (ACD)]. The leukocyte-rich pellet was obtained according to the established protocol of the International Society of Radiolabeled Blood Elements (ISORBE)<sup>[14]</sup>. The leukocyte-rich pellet was gently resuspended in 0.5 mL cell-free plasma using a polypropylene Pasteur pipette. The HMPAO was labeled with a solution of sodium pertechnetate ( $\text{Na}^{99\text{m}}\text{TcO}_4$ ) with 1480 MBq of activity. After labeling, the radiochemical purity of the 99mTc-HMPAO was determined by partition between 0.9% saline and chloroform<sup>[15]</sup>. Freshly prepared 99mTc-

Table 1 Summary of data from patients with CD

Patients (sex, yr)	CDAI	Vienna classification	SI	CRP (mg/L)	Treatment
I.M.F. (M, 41)	330.0	A1L1B2	11	30.0	Methotrexate
A.A.A. (M, 40)	53.4	A1L1B2	4	2.1	Prednisone
J.A.N. (M, 44)	2.0	A1L1B3	0	4.0	Prednisone, mesalazine, azathioprine
L.M.T.(F, 54)	59.3	A2L1B1	5	7.3	Prednisone, azathioprine
L.J.O. (F, 37)	126.6	A1L4B1	0	12.0	Mesalazine, azathioprine
T.F.C. (M, 24)	56.3	A1L3B2	0	9.2	Without medication
F.R.F. (F, 44)	52.6	A1L1B1	2	2.5	Prednisone, mesalazine, azathioprine
A.A.S. (F, 24)	1.7	A1L1B1	3	6.0	Mesalazine
L.D. (F, 24)	47.1	A1L1B1	8	34.1	Azathioprine
A.F.S. (F, 35)	62.9	A1L3B3	5	3.0	Prednisone, mesalazine, ciprofloxacin, azathioprine, infliximab
L.P.M. (M, 38)	146.6	A1L1B1	3	17.5	Prednisone, mesalazine
M.O.R. (F, 30)	197.4	A1L3B3	4	6.0	Hydrocortisone, mesalazine, ceftriaxone, azathioprine
G.E.S.F.(M, 37)	162.1	A1L1B3	5	16.0	Sulfasalazine, ciprofloxacin/metronidazole, thalidomide
E.C.S. (F, 31)	122.1	A1L1B3	3	8.0	Prednisone, ciprofloxacin, azathioprine
J.G.R. (M, 41)	97.5	A1L1B3	3	6.0	Prednisone, mesalazine, azathioprine
J.D.G. (F, 52)	126.2	A1L2B2	3	14.0	Prednisone, mesalazine
R.M.M.(F, 2)	73.4	A1L1B1	0	6.1	Azathioprine
A.B.O. (F, 58)	81.3	A2L3B1	2	2.5	Mesalazine
M.F.L. (F, 46)	84.3	A1L1B2	3	48.0	Prednisone
M.J.A. (F, 51)	179.9	A2L1B1	2	3.2	Mesalazine, azathioprine

CD: Crohn's disease; CDAI: Crohn's Disease Activity Index; SI: Scintigraphic index; CRP: C-reactive protein.

HMPAO (0.7 mL, approximate 600 MBq) was added to the leukocyte-rich pellet. This preparation was incubated at 37°C for 15 min. Aliquots of 4 mL of cell-free plasma were added to the test tube. The tube was centrifuged (150 g) for 5 min. The plasma supernatant that contained unbound 99mTc-HMPAO was removed, and the 99mTc-HMPAO-labeled leukocyte pellet was suspended in 4.0 mL cell-free plasma. The labeling yield was calculated from: Labeling yield = {[cpm (precipitate)]/[cpm (precipitate) + cpm (supernatant)]} × 100; cpm = counts per minute or disintegrations per minute.

### Scintigraphic imaging

Images were obtained at 30 min and 2 h after injecting patients and healthy volunteers with the labeled leukocytes (mean activity approximate 273 MBq). Abdominal scans were obtained in the anterior and caudal views (patients sat on the camera bed with the detector head positioned below the bed) using a wide field gamma camera (Orbiter, Siemens, Germany and Millennium MG, General Electric Company, Milwaukee, WI, USA)<sup>[16]</sup>. The time of each image was approximately 10 min or one million counts<sup>[17]</sup>.

The single photon emission computed tomography (SPECT) study was performed just after completing the 30-min and 2-h planar images<sup>[16]</sup>. SPECT was acquired using the following parameters: a matrix size of 64 × 64, 360° circular rotation, and a 5° step angle with a 20-s time frame.

### Scintigraphic index (SI)

SI was calculated according to the method of Ybern *et al.*<sup>[18]</sup>. Briefly, regions of interest (ROIs) were outlined over the liver, spleen, iliac crest and abnormal accumulations when present (Figure 1). The processing program of the gamma camera furnished the number of counts/area proportional

to the radioactivity and the average value of the activity per pixel present in each region. The abdomen was divided into five zones: right, top, left, bottom and center, which corresponded approximately to the ascending colon, transverse colon, descending colon, sigmoid colon/rectum and small bowel. The SI was calculated in all scans in the anterior view:  $SI = (\sum A_i) + B$ . "A<sub>i</sub>" represents the degree of activity of the accumulations in each zone (1 = activity less than bone activity; 2 = activity greater than bone activity; 3 = activity greater than liver activity; 4 = activity greater than spleen activity). "B" indicates the number of zone with abnormal accumulations of labeled leukocytes (1 = one or more accumulations in one zone; 2 = accumulations in two or three zones; 3 = abnormal accumulations in four or five zones). SI > 2 was considered as active disease<sup>[18]</sup>.

### Statistical analysis

CDAI, SI and CRP were compared using the Spearman's rank correlation.

## RESULTS

The radiochemical purity of the lipophilic complex 99mTc-HMPAO presented a mean labeling percentage of the order of 85.0% ± 9.0% for the 25 samples. The mean yield for labeling of the autologous leukocytes with the lipophilic complex 99mTc-HMPAO was 55.0% ± 10.0%.

Six of the 20 patients (30%) presented congruent results for the three parameters investigated (CDAI, SI and CRP), which were two patients (I.M.F.; G.E.S.F.) with inflammatory activity and four (J.A.N.; F.R.F.; R.M.M.; A.B.O.) with disease in remission. On the other hand, 14 patients (70%) did not show congruent results for CDAI, SI and CRP (Table 1). Twelve patients showed results that were congruent with the Vienna Classification

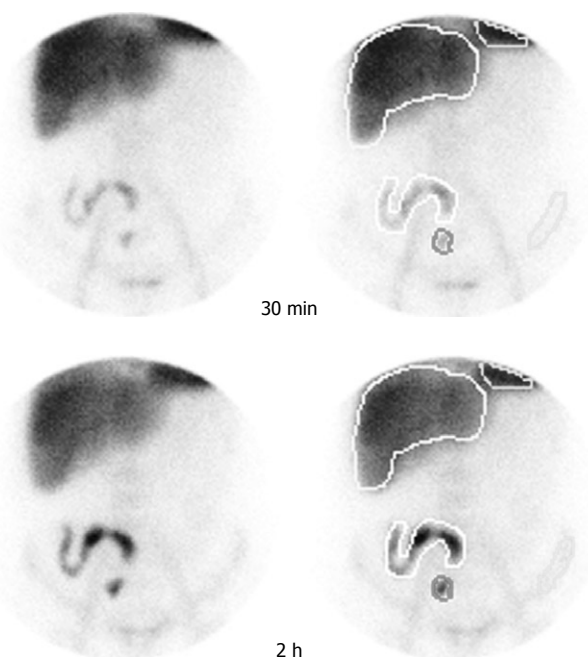


Figure 1 Regions of interest (ROIs).

(Table 1) and technetium-99m-HMPAO-labeled leukocyte scintigraphy with regard to disease location. Besides, there was no correlation for both parameters that were described above in five patients; since scintigraphy images showed positive areas while these regions were not included in the Vienna Classification. In three other patients, the scintigraphy results did not show radioactivity uptake in the intestinal segments.

The images from a healthy volunteer in the anterior and caudal projections at 30 min and 2 h after injection of <sup>99m</sup>Tc-HMPAO-labeled leukocytes are presented in Figure 2. Intense accumulation in the liver and the spleen could be seen at 30 min post-injection, as well as accumulation in the bone marrow. Because it was a control case, there was no abnormal accumulation in the digestive tract.

Accumulation of <sup>99m</sup>Tc-HMPAO-labeled leukocytes in the region of the terminal ileum, which suggested the presence of an inflammatory process at this location (anterior view), is shown in Figure 3A. At 2 h, uptake of labeled leukocytes increased, which showed the concentration of radiotracer in the indicated region. No pathological accumulation of <sup>99m</sup>Tc-HMPAO-labeled leukocytes could be seen in the caudal view, which indicated that there was no inflammation in the sigmoid and/or rectum.

The presence of inflammatory foci in the terminal ileum, descending colon, sigmoid and rectum, revealed by the intense accumulation of radiolabeled leukocytes, is shown in Figure 3B. Regions of the sigmoid and rectum affected by inflammation can be seen in the caudal projection. An increase in the radioactivity with time can also be seen.

According to the Spearman test, there was no significant correlation between the CDAI, SI and CRP in any of the investigated cases (Table 2).

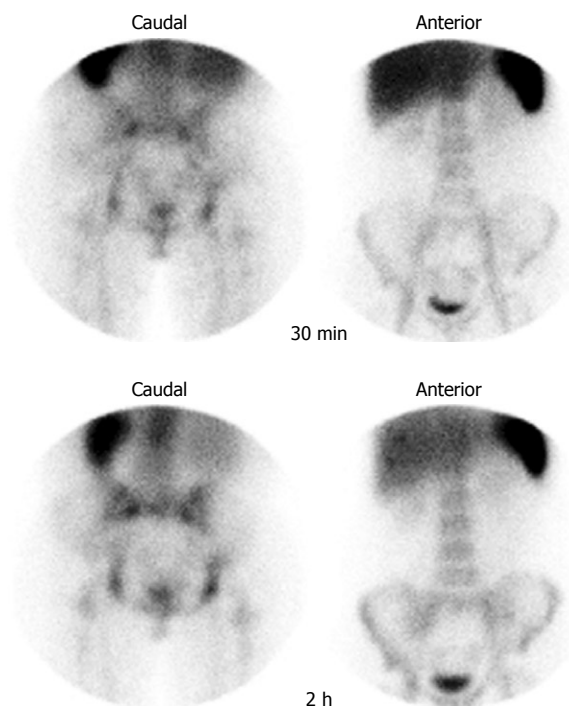


Figure 2 Images of a healthy volunteer (control) in the anterior and caudal projections at 30 min and at 2 h after injection of radiolabeled leukocytes.

Table 2 Spearman's rank correlation ( $n = 20$ )

	Spearman's rank	Correlation coefficient ( $\rho$ )	
		$t$	$P$
CDAI and SI	0.1835	0.7918	0.4388
CDAI and CRP	0.3373	1.5204	0.1457
SI and CRP	0.2783	1.2293	0.2347

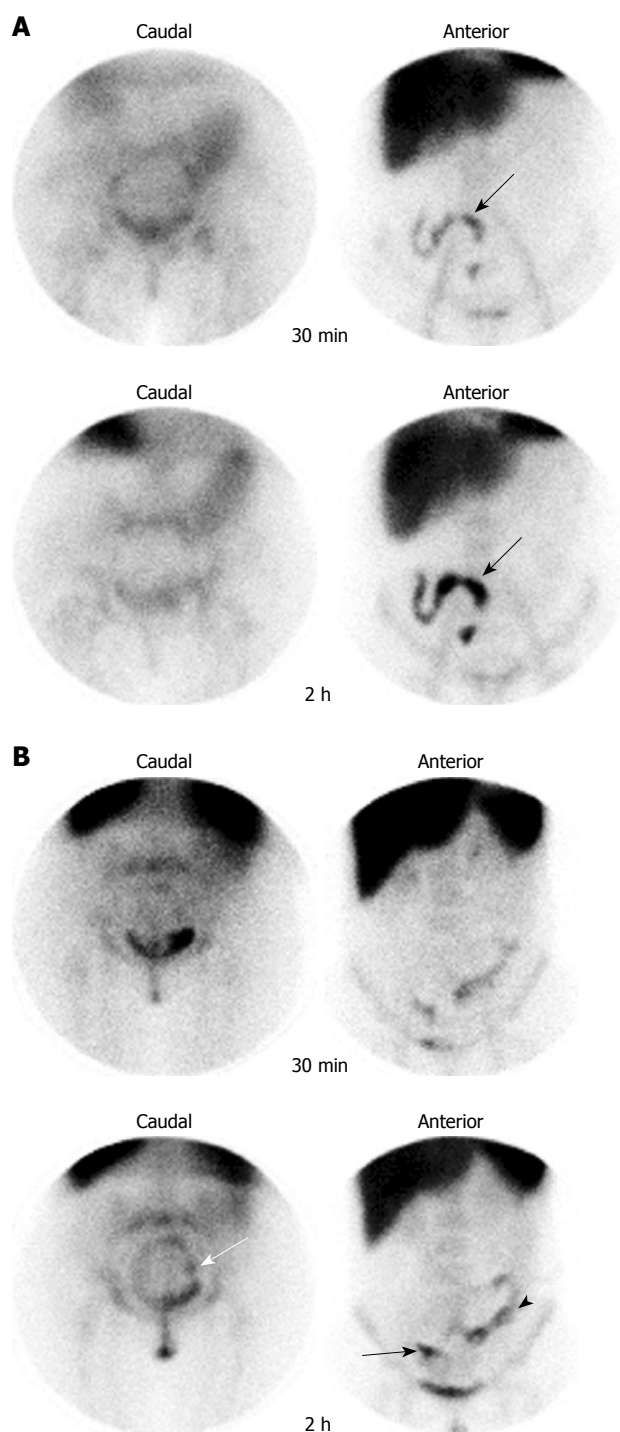
## DISCUSSION

The radiochemical purity of the lipophilic complex (HMPAO) labeled with technetium-99m was  $85.0\% \pm 9.0\%$ . The data suggest that 85% of the technetium-99m atoms were bound to HMPAO molecules. This result is in agreement with other data described in the literature<sup>[15]</sup>. On the other hand, the labeling yield for leukocytes was  $55.0\% \pm 10\%$ . Labeling yields of 46% and 65.5% have been reported previously<sup>[19,20]</sup>. Thus, the value obtained in the present work is supported by published data, which suggests that the manipulation process utilized in the preparation of the radiolabeled cells was adequate.

<sup>99m</sup>Tc-HMPAO granulocyte scintigraphy of a healthy volunteer (Figure 2) showed uptake of radiolabeled leukocytes by the liver, spleen and bone marrow, which reflected physiological retention of labeled white blood cells<sup>[11]</sup>.

The scintigraphic images were based on physiological alterations such as an increase in blood flow, vasodilatation, increased permeability and cellular leakage, which permit the precocious detection of inflammatory foci. These findings support the use of <sup>99m</sup>Tc-HMPAO granulocyte scintigraphy as an important examination for

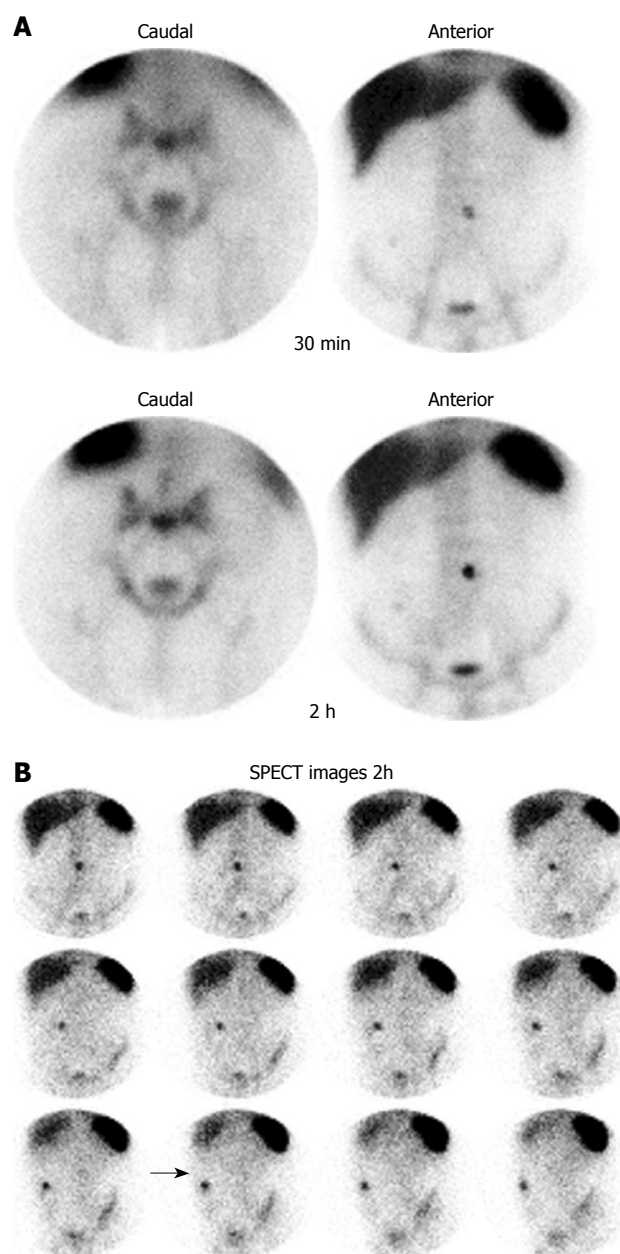




**Figure 3** Images obtained 30 min and 2 h post-injection of  $^{99m}\text{Tc}$ -HMPAO-labeled leukocytes in the caudal and anterior views. A: The arrows indicate the region of the intestine affected by the inflammation (L.M.T., female, 54 years); B: The arrows indicate  $^{99m}\text{Tc}$ -HMPAO-labeled leukocyte uptake in the terminal ileum, descending colon and rectum-sigmoid (L.D., female, 24 years). The black arrows indicate terminal ileum; the arrowhead indicates descending colon; the white arrow indicates rectum-sigmoid.

diagnostic screening of inflammatory diseases<sup>[4]</sup>. Cardoso *et al*<sup>[19]</sup> have observed sensitivity, specificity and accuracy values of  $> 87.5\%$  in studies performed in patients highly suspected of having inflammatory bowel disease.

The data obtained in the present study showed that, in 70% of investigated cases, the results did not show correspondence with CDAI, CRP and SI (Table 1). It



**Figure 4**  $^{99m}\text{Tc}$ -HMPAO granulocyte scintigraphy of patient J.A.N., male, 44 years. A: Images obtained at 30 min and at 2 h after injection of the radiolabeled leukocytes, in the caudal and anterior projections; B: SPECT images 2 h after injection of the radiolabeled leukocytes. The arrow indicates the fistula region of the patient's abdomen.

is known that CDAI is quite subjective and is based on a group of signs and clinical symptoms associated with erythrocyte sedimentation rate. This method was able to indicate inflammatory activity in four patients, while the SI and CRP indicated inflammatory activity in 13 and nine patients, respectively. Therefore, it is reasonable to suppose that the intestines of patients may have inflammatory foci that attract radiolabeled leukocytes in the specific case of CD, which results in a positive SI, although the signs and symptoms may still be absent.

As example of this observation, the patient L.M.T. (female, 54 years, Figure 3A) showed retention of radiolabeled leukocytes in the terminal ileum. On the other hand, the CDAI index (59.3) indicated disease remission



and the CRP value (7.3 mg/L) suggested the absence of inflammatory activity. This patient was receiving treatment with prednisone and azathioprine, but inflammatory activity was present, which justified the uptake of <sup>99m</sup>Tc-HMPAO-labeled leukocytes in the region, however, the patient was asymptomatic.

A case that deserves special attention is the pathological accumulation of radiolabeled leukocytes in the terminal ileum, descending colon, sigmoid and rectum, which resulted in an SI of 8 (disease activity) as illustrated in Figure 3B (patient L.D., female, 24 years). Before performing <sup>99m</sup>Tc-HMPAO-labeled leukocyte scintigraphy, it was known that the CD in this patient was located only in the region of the terminal ileum, with absence of clinical signs, and the patient was receiving immunomodulator treatment. Seven days before <sup>99m</sup>Tc-HMPAO granulocyte scintigraphy, CRP level was elevated (34.1 mg/L). Despite all the segments that were identified by scintigraphy, the disease was considered to be in remission according to the CDAI (47.1). Thus, this result showed the importance of <sup>99m</sup>Tc-HMPAO-labeled leukocyte scintigraphy as a early diagnosis method for identifying affected regions not previously known. In addition, the therapeutic response should be also evaluated using this method, since the dose of immunomodulator administered to the patient probably was not sufficient to control the disease<sup>[11,12]</sup>.

<sup>99m</sup>Tc-HMPAO-labeled leukocyte scintigraphy performed in the patient J.A.N. (male, 44 years; Figure 4A) showed uptake of radiolabeled leukocytes in the central region of the abdomen, which indicated the presence of enterocutaneous fistula. On the other hand, the results did not show uptake of <sup>99m</sup>Tc-HMPAO-labeled leukocytes in segments of the intestine. SPECT showed that this radioactive uptake in the planar images corresponded to the external surface of the patient's enterocutaneous fistula (Figure 4B). According to Arndt *et al.*<sup>[10]</sup>, the capture of leukocytes in areas outside the intestinal segments, such as abscesses and fistulas, should be analyzed separately, but not considered for the calculation of SI<sup>[10]</sup>. In our opinion, this uptake should be considered for SI calculation, because a fistula is a clinical feature that is suggestive of inflammatory activity and possible complications of CD.

The data obtained in the present work show that <sup>99m</sup>Tc-HMPAO-labeled leukocyte scintigraphy of the intestine could be useful for the evaluation of CD inflammatory activity, even in the absence of clinical signs and symptoms (disease remission). A functional method (scintigraphy) was compared with subjective (CDAI) and nonspecific (CRP) tests. Therefore, further studies will be necessary to prove the real utility of radiolabeled leukocytes to diagnosis and monitor patients with CD. Thus, it will be interesting to compare the scintigraphic method with another examination such as CT enterography.

scintigraphy was able to identify inflammatory activity in patients with Crohn's disease (CD) even in the absence of signs and clinical symptoms.

### Research frontier

Scintigraphic images are based on functional alterations of the tissues, and permit the early diagnosis of inflammatory bowel disease, when the anatomical alterations are not visible.

### Innovations and breakthroughs

In the authors' clinical routine, the Crohn's disease activity index is used normally to evaluate the presence of inflammatory activity in patients. However, this index is subjective and sometimes is not able to detect the presence of inflammatory activity in subclinical disease. Thus, <sup>99m</sup>Tc-HMPAO granulocyte scintigraphy could contribute to follow-up of these patients.

### Applications

The results obtained suggest that <sup>99m</sup>Tc-HMPAO-labeled leukocyte scintigraphy could be used to monitor inflammatory bowel disease and evaluate the efficiency of therapy.

### Terminology

Scintigraphic images are obtained using compounds or cells labeled with radioactive isotopes such as technetium-99m, iodine-131 and gallium-67. CD is a chronic intestinal inflammation of unknown etiology that can affect any segments of the digestive tract.

### Peer review

The number of patients is small and therefore their positive experience with the technique may decrease with time and number of patients. The figures are of high quality and indicate the expertise of the authors.

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## COMMENTS

### Background

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## Genetic variants involved in gallstone formation and capsaicin metabolism, and the risk of gallbladder cancer in Chilean women

Sergio Báez, Yasuo Tsuchiya, Alfonso Calvo, Martha Pruyas, Kazutoshi Nakamura, Chikako Kiyohara, Mari Oyama, Masaharu Yamamoto

Sergio Báez, Alfonso Calvo, Department of Surgery, Sótero del Río Hospital, Puente Alto, Santiago, Chile  
 Yasuo Tsuchiya, Kazutoshi Nakamura, Mari Oyama, Department of Community Preventive Medicine, Niigata University Graduate School of Medical and Dental Sciences, Niigata 951-8510, Japan

Martha Pruyas, Department of Anatomic Pathology, Sótero del Río Hospital, Puente Alto, Santiago, Chile

Chikako Kiyohara, Department of Preventive Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan

Masaharu Yamamoto, Faculty of Health Sciences, Niigata University of Health and Welfare, Niigata 950-3198, Japan

**Author contributions:** Báez S and Tsuchiya Y contributed equally to this work; Báez S, Tsuchiya Y and Yamamoto M designed the research; Báez S, Tsuchiya Y, Calvo A and Pruyas M performed the research; Nakamura K and Oyama M analyzed the data; Báez S, Tsuchiya Y and Kiyohara C wrote the paper.  
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**Correspondence to:** Yasuo Tsuchiya, PhD, Division of Social and Environmental Medicine, Department of Community Preventive Medicine, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Chuo-ku, Niigata 951-8510, Japan. [troof@med.niigata-u.ac.jp](mailto:troof@med.niigata-u.ac.jp)

Telephone: +81-25-2272121 Fax: +81-25-2270764

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patients with gallstones, and 70 controls were enrolled in this study. DNA was extracted from their blood or paraffin block sample using standard commercial kits. The statuses of the genetic variants were assayed using Taqman® SNP Genotyping Assays or Custom Taqman® SNP Genotyping Assays.

**RESULTS:** The non-ancestral T/T genotype of apolipoprotein B rs693 polymorphism was associated with a decreased risk of GBC (OR: 0.14, 95% CI: 0.03-0.63). The T/T genotype of cholesteryl ester transfer protein (CETP) rs708272 polymorphism was associated with an increased risk of GBC (OR: 5.04, 95% CI: 1.43-17.8).

**CONCLUSION:** Genetic variants involved in gallstone formation such as the apolipoprotein B rs693 and CETP rs708272 polymorphisms may be related to the risk of developing GBC in Chilean women.

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**Key words:** Genetic risk factor; Gallbladder cancer; Gallstone; Genetic polymorphism; Apolipoprotein B; Cholesteryl ester transfer protein

**Peer reviewer:** Guang-Wen Cao, MD, PhD, Professor and Chairman, Department of Epidemiology, Second Military Medical University, 800 Xiangyin Road, Shanghai 200433, China

### Abstract

**AIM:** To determine the effects of genetic variants associated with gallstone formation and capsaicin (a pungent component of chili pepper) metabolism on the risk of gallbladder cancer (GBC).

**METHODS:** A total of 57 patients with GBC, 119

Báez S, Tsuchiya Y, Calvo A, Pruyas M, Nakamura K, Kiyohara C, Oyama M, Yamamoto M. Genetic variants involved in gallstone formation and capsaicin metabolism, and the risk of gallbladder cancer in Chilean women. *World J Gastroenterol* 2010; 16(3): 372-378 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v16/i3/372.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i3.372>

## INTRODUCTION

There is a prominent worldwide geographical and racial variability in the incidence of gallbladder cancer (GBC), which correlates with the prevalence of cholelithiasis<sup>[1]</sup>. High incidences of GBC are observed in specific countries and in confined areas. For example, the incidence of GBC is very high in northern Indian cities (5-7 per 100 000 women) and low (0-0.7 per 100 000 women) in southern India, possibly reflecting the different ethnic origins of these populations<sup>[2]</sup>. The evidence suggests that the incidence of GBC is associated with the presence of both geographically-specific environmental factors and environmental factor-related genetic factors.

Recent studies have shown that the incidence rate for GBC is higher in Chile than in other countries<sup>[3-5]</sup>. According to a previous epidemiological study, the consumption of high levels of red chili pepper has been identified as an important risk factor for GBC in Chilean women who carry gallstones (GS)<sup>[6]</sup>. However, the pathogenic mechanism by which GBC occurs *via* chili pepper consumption in the presence of GS remains uncertain.

Although GS is the main cause of GBC, not all patients with GS develop GBC. While the standard mortality rates for GBC between 1985 and 2002 remained unchanged at 11.3 per 100 000 (0.0113%) in Chile<sup>[7]</sup>, 38.8% of adult women and 14.9% of adult men, which are staggeringly high rates, were GS carriers between 1972 and 1995<sup>[8]</sup>. Red chili pepper is a widely consumed spice among the Chilean population. Therefore, the development of GBC in Chilean women cannot be completely explained by the presence of GS and chili pepper consumption alone.

In some but not all studies, lipid metabolism-related gene variants have been associated with GS formation. Apolipoprotein (apo) B, apo E, and cholesteryl ester transfer protein (CETP) polymorphisms have been associated with increased risk for cholelithiasis<sup>[9-11]</sup>. On the other hand, capsaicin (8-methyl-N-vanillyl-6-nonenamide) is the active ingredient that makes chili peppers pungent. Although previous findings regarding the potential genotoxicity of capsaicin are inconsistent<sup>[12-15]</sup>, it is possible that GBC can be caused by high consumption of red chili pepper that contains capsaicin. Therefore, genes involved in the metabolism of capsaicin, such as cytochrome P450 (CYP) 2E1, CYP2C9, and CYP3A4, may be related to increased risk for GBC<sup>[16-18]</sup>. However, no study has examined the association between the risk of GBC in Chile and the genetic variants involved in GS formation and capsaicin metabolism.

We hypothesized that individuals with a genotype promoting greater lipid metabolism/capsaicin metabolism would be more prevalent among the GS and GBC patients than among healthy subjects. We conducted a hospital-based case-control study in a Chilean population with special reference to polymorphism-polymorphism combination.

## MATERIALS AND METHODS

### Study subjects

A total of 57 female patients with GBC who had been diagnosed by histological examination of tissue at Sótero del Río Hospital in Santiago, Chile, between January 2007 and February 2008 were enrolled in this case-control study. A total of 119 female patients with GS who were diagnosed by an ultrasonic diagnostic method were also enrolled in the study. Seventy controls, who were patients with hernia or varicose veins of the legs who had no history of GS or any cancer and who were diagnosed by an ultrasonic diagnostic method, were selected randomly at the same hospital over the same period.

All patients gave their written informed consent, and our study protocol was approved by the Ethics Committee at Sótero del Río Hospital.

### DNA extraction and storage

Samples collected in the hospital were sent to Niigata University, Japan, for DNA extraction and genotyping assay. Genomic DNA was extracted from the blood or paraffin block samples using standard commercial kits for blood samples (DNA Extractor WB-rapid, WAKO Pure Chemicals Industries, Ltd., Osaka, Japan) and for paraffin block samples (Dexpat, Takara Bio Co. Ltd., Tokyo, Japan). The extracted DNA samples were stored in a freezer at -80°C until genetic polymorphism analyses were performed.

### Genotyping assay

The statuses of the genetic variants of apo B rs693, apo E rs7412, rs429358, CETP rs708272, CYP2C9 rs1057910, CYP2C9 rs1799853, and CYP3A4 rs12721627 were assayed using TaqMan® SNP Genotyping Assays purchased from Applied Biosystems (Foster City, CA, USA). The assay IDs were C\_7615420\_20 for rs693, C\_904973\_10 for rs7412, C\_3084793\_20 for rs429358, C\_9615318\_10 for rs708272, C\_27104892\_10 for rs1057910, C\_25625805\_10 for rs1799853, and C\_30634207\_10 for rs12721627. Genotyping for the presence of CYP2E1 polymorphisms (rs2031920, rs6413432) was performed using the Custom Taqman® SNP Genotyping Assays purchased from Applied Biosystems. The reaction components for a single 10 µL reaction (using a 96-well plate) included sample genomic DNA, TaqMan® Genotyping Master Mix (Applied Biosystems), SNP Genotyping Assay-Mix (Applied Biosystems), DNase free water was used. The thermal cycle (PE 9700, Applied Biosystems) conditions were as follows: 95°C for 10 min, followed by 40 cycles of 92°C for 15 s and 60°C for 1 min. After the PCR reaction, the plate read-out and allele discrimination were analyzed using a multiplex real-time QPCR system (Mx3000P, Stratagene Japan, Tokyo).

For quality control, all genotyping assays were reconfirmed according to the PCR-restriction fragment length polymorphism method and the replicates were 100% concordant.



Table 1 Demographic characteristics and food intake frequency of study subjects (mean  $\pm$  SD)

	Controls <i>n</i> = 70	GS patients <i>n</i> = 119	GBC patients <i>n</i> = 57	<i>P</i> value		
				Control <i>vs</i> GS	GS <i>vs</i> GBC	Control <i>vs</i> GBC
Age (yr)	45.8 $\pm$ 14.1	42.7 $\pm$ 9.2	56.5 $\pm$ 11.2	NS	<i>P</i> < 0.001	<i>P</i> < 0.001
Height (m)	1.56 $\pm$ 0.07	1.57 $\pm$ 0.07	1.54 $\pm$ 0.06	NS	NS	NS
Weight (kg)	72.1 $\pm$ 15.5	69.3 $\pm$ 12.8	64.1 $\pm$ 12.8	NS	NS	NS
BMI (kg/m <sup>2</sup> )	29.5 $\pm$ 6.1	28.2 $\pm$ 4.8	27.3 $\pm$ 5.6	NS	NS	NS
Chili pepper	0.8 $\pm$ 1.0	0.9 $\pm$ 1.1	1.7 $\pm$ 1.4	NS	<i>P</i> < 0.05	<i>P</i> < 0.01
Beef	1.2 $\pm$ 0.6	1.5 $\pm$ 0.7	1.8 $\pm$ 0.5	NS	NS	<i>P</i> < 0.01
Pork	0.8 $\pm$ 0.6	1.0 $\pm$ 0.8	1.4 $\pm$ 0.8	NS	<i>P</i> < 0.05	<i>P</i> < 0.01
Chicken	1.6 $\pm$ 0.6	2.0 $\pm$ 0.8	2.0 $\pm$ 0.5	<i>P</i> < 0.01	NS	NS
Fish	1.0 $\pm$ 0.6	1.1 $\pm$ 0.6	1.5 $\pm$ 0.7	NS	<i>P</i> < 0.05	<i>P</i> < 0.01
Fried food	1.0 $\pm$ 0.9	1.3 $\pm$ 1.1	2.2 $\pm$ 1.1	NS	<i>P</i> < 0.001	<i>P</i> < 0.001
Butter	2.3 $\pm$ 1.5	2.6 $\pm$ 1.6	3.2 $\pm$ 1.1	NS	NS	<i>P</i> < 0.05
Cheese	1.1 $\pm$ 1.0	1.1 $\pm$ 0.9	2.0 $\pm$ 1.0	NS	<i>P</i> < 0.001	<i>P</i> < 0.001

Food intake was grouped into the following 5 categories, and a sequence number was assigned to each category: (0) never; (1) 1-3 times/mo or less; (2) 1-3 times/wk; (3) 4-6 times/wk; and (4) everyday. Data on height, weight, and food intake was collected from 70 controls, 119 GS patients, and 26 GBC patients. Data were evaluated by one-way analysis of variance (ANOVA) followed by Scheffe's multiple comparison test. GS: Gallstone; GBC: Gallbladder cancer; BMI: Body mass index; NS: No significant difference.

### Measurement of dietary intake

An interviewer-administrated food-frequency questionnaire was used in the present study. Our subjects were asked to report the frequencies of their long-term intake of red chili pepper, vegetables, fruits, beef, pork, chicken, fish, milk, butter, cheese, and fried foods. Food intake was grouped into the following 5 categories, and a sequence number was assigned to each category: (0) never; (1) 1-3 times/mo or less; (2) 1-3 times/wk; (3) 4-6 times/wk; and (4) every day. Each dietary intake assessment was made according to the score obtained from each subject.

### Statistical evaluation

Statistical analyses were performed using SAS software (Release 6.12, SAS Institute Inc., Cary, NC, USA) and STATA software (SE 8.0, STATA Corporation, TX, USA). Fisher's exact probability test was used to assess the association between the genotypes or alleles and GBC risk. The age-adjusted odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated from logistic regression coefficients. Data on demographic characteristics and food intake frequencies were evaluated by one-way analysis of variance (ANOVA) followed by Scheffe's multiple comparison test. *P* values of less than 0.05 were considered to indicate statistical significance. The genotypic distribution of the polymorphisms in the controls was compared with that expected based on Hardy-Weinberg equilibrium (HWE) by the  $\chi^2$  (Pearson) test. When the *P* values exceeded 0.05, we estimated that the sample was under the HWE.

## RESULTS

Table 1 shows the demographic characteristics and food intake frequencies of our subjects. The GBC patients had the highest mean age among the three groups, showing significant differences from the other two groups. No significant differences were found in the height, weight, or body mass index (BMI) among the three groups.

Significantly higher consumption of red chili pepper was observed in the GBC patients compared with the controls (*P* < 0.01) and the GS patients (*P* < 0.05). This difference in the consumption of red chili pepper between the GBC and GS patients was in agreement with the result of a previous study<sup>[6]</sup>. In the GBC patients, the consumption of pork, fish, fried foods, and cheese were significantly higher than in the controls and the GS patients. Overall, higher consumption of the foods we asked about was observed in the GBC patients.

Table 2 shows the GBC risk associated with the apo B rs693 polymorphism. The genotype distributions were consistent with HWE in the controls (*P* = 0.44). The frequency of the T/T genotype was significantly lower in the GBC patients than in the controls; the age-adjusted OR for the GBC risk was 0.14 (95% CI: 0.03-0.63, *P* = 0.0099). The T allele was associated with a decreased risk of GBC (OR: 0.49, 95% CI: 0.29-0.84, *P* = 0.010). Based on these results, we designated the C allele that is presumed to increase the risk of GBC as the "at-risk" allele. No significant differences in the genotypic and allelic distribution were detected between the controls and the GS patients.

Table 3 shows the risk of GBC associated with the CETP rs708272 polymorphism. In the GBC patients, the frequencies of the C/C, C/T and T/T genotypes were 28.1%, 40.3% and 31.6%, respectively. The frequencies of the C/C, C/T, and T/T genotypes in the controls were 25.7%, 57.2% and 17.1%, respectively, and in the patients with GS they were 35.3%, 47.9% and 16.8%, respectively. The distribution of the genotypes of the rs708272 polymorphism agreed with HWE in the controls (*P* = 0.20). The frequency of the T/T genotype was significantly higher in the GBC patients than in the GS patients (*P* = 0.012), though no significant differences were found between the controls and the GS patients, or between the controls and the GBC patients. Based on these results, we designated the T allele that is presumed to increase the risk of GBC as the "at-risk" allele.

**Table 2** Association of the apo B rs693 polymorphism with gallbladder cancer risk

Genotypes and alleles	Frequency (%)		Age-adjusted OR	95% CI	P value
	Controls	GS patients			
C/C	25 (35.7)	41 (34.5)	1.0		
C/T	31 (44.3)	65 (54.6)	1.27	0.66-2.47	NS
T/T	14 (20.0)	13 (10.9)	0.59	0.24-1.45	NS
C	81 (57.9)	147 (61.8)	1.0		
T	59 (42.1)	91 (38.2)	0.85	0.56-1.30	NS
	$P_{HWE} = 0.442$ $P_{HWE} = 0.088$				
	Controls	GBC patients			
C/C	25 (35.7)	30 (52.6)	1.0		
C/T	31 (44.3)	24 (42.1)	0.67	0.30-1.51	NS
T/T	14 (20.0)	3 (5.3)	0.14	0.03-0.63	0.010
C	81 (57.9)	84 (73.7)	1.0		
T	59 (42.1)	30 (26.3)	0.49	0.29-0.84	0.010
	$P_{HWE} = 0.516$				
	GS patients	GBC patients			
C/C	41 (34.5)	30 (52.6)	1.0		
C/T	65 (54.6)	24 (42.1)	0.70	0.31-1.59	NS
T/T	13 (10.9)	3 (5.3)	0.14	0.02-1.05	NS
C	147 (61.8)	84 (73.7)	1.0		
T	91 (38.2)	30 (26.3)	0.58	0.35-0.94	0.028

NS: No significant difference;  $P_{HWE}$ : P for Hardy-Weinberg equilibrium test.

The apo E and capsaicin metabolism-related gene variants were not associated with either GBC or cholelithiasis risk.

Table 4 shows the risk of GBC associated with the combined “at-risk” genotypes of the apo B rs693 and CETP rs708272 polymorphisms. The frequencies of the combined “at-risk” genotypes of the C/C genotype of the apo B rs693 polymorphism and the T/T genotype of the CETP rs708272 polymorphism were 4.3% in the controls, 6.7% in the GS patients, and 19.3% in the GBC patients. Compared with all remaining combinations combined, the frequency of the C/C plus T/T genotypes was significantly higher in the GBC patients than in the controls; the age-adjusted OR for the GBC risk was 4.75 (95% CI: 1.16 -19.4,  $P = 0.030$ ).

## DISCUSSION

In this hospital-based case-control study, the T allele carriers of the apo B rs693 polymorphism were associated with a decreased risk of GBC. In contrast, the T/T genotype of the CETP rs708272 polymorphism was associated with an increased risk of GBC. However, the capsaicin metabolism-related gene variants were not associated with GBC risk.

Singh *et al.*<sup>[19]</sup> found that the frequency of the C allele of the apoB rs693 polymorphism was significantly higher in GBC patients than in GS patients or healthy subjects. Their data also showed that the C/T and T/T genotypes are associated with a lower risk for GBC compared with the C/C genotype (ORs: 0.28 and 0.34, 95% CI: 0.17-0.46, and 0.13-0.89, respectively). They suggested that the apo B rs693 polymorphism confers susceptibility to GBC under specific environmental conditions. Our results were

**Table 3** Association of the CETP rs708272 polymorphism with gallbladder cancer risk

Genotypes and alleles	Frequency (%)		Age-adjusted OR	95% CI	P value
	Controls	GS patients			
C/C	18 (25.7)	42 (35.3)	1.0		
C/T	40 (57.2)	57 (47.9)	0.60	0.30-1.19	NS
T/T	12 (17.1)	20 (16.8)	0.68	0.27-1.70	NS
C	76 (54.3)	141 (59.2)	1.0		
T	64 (45.7)	97 (40.8)	0.82	0.54-1.24	NS
	$P_{HWE} = 0.204$ $P_{HWE} = 0.930$				
	Controls	GBC patients			
C/C	18 (25.7)	16 (28.1)	1.0		
C/T	40 (57.2)	23 (40.3)	0.75	0.30-1.93	NS
T/T	12 (17.1)	18 (31.6)	1.80	0.62-5.26	NS
C	76 (54.3)	55 (48.2)	1.0		
T	64 (45.7)	59 (51.8)	1.27	0.78-2.09	NS
	$P_{HWE} = 0.147$				
	GS patients	GBC patients			
C/C	42 (35.3)	16 (28.1)	1.0		
C/T	57 (47.9)	23 (40.3)	1.31	0.52-3.28	NS
T/T	20 (16.8)	18 (31.6)	5.04	1.43-17.8	0.012
C	141 (59.2)	55 (48.2)	1.0		
T	97 (40.8)	59 (51.8)	1.56	0.99-2.44	NS

CETP: Cholesteryl ester transfer protein.

in agreement with their findings, showing an association between the T allele and the lower risk of GBC. Since apo B is a key protein in lipid metabolism<sup>[20]</sup>, the apo B variant may be related to a higher incidence of GS and subsequently GBC. Generally, the T/T genotype has been reported to have significantly higher serum total cholesterol, low density lipoprotein cholesterol (LDL), and apo B levels compared with the C/C genotype<sup>[21,22]</sup>. Therefore the T/T genotype or the T allele appears to relate to a higher risk of GS or GBC. However, our findings showed that the T allele may work as a preventive factor for GBC. The inverse association between the T/T genotype of the apo B polymorphism and the GBC risk may be explained by structural changes of apo B as proposed by Boekholdt *et al.*<sup>[23]</sup>. Their hypothesis about the inverse association between the T/T genotype and ischemic heart disease (IHD) is as follows: the apo B variant causes hypercholesterolemia, modifies LDL to become a less atherogenic particle, and causes IHD. We could not clarify by what mechanism the T allele decreases the risk of GBC, and the mechanism research will be the topic of our next trial.

Some genetic variants may exert population-specific effects that are independent of the other genetic profiles of the individual and of environmental exposures, while other population-specific effects may be generated under differential gene-gene interactions in different populations, differential gene-environment interactions, or both<sup>[24]</sup>.

As reported by some researchers, GS which is a main risk factor for GBC, has been associated with both decreased levels of high-density lipoprotein (HDL) cholesterol and increased levels of LDL cholesterol and triglyceride<sup>[25-27]</sup>. CETP has a central role in the metabolism of HDL and therefore might relate to the susceptibility to

**Table 4** Effects of the combined genotypes of the apo B rs693 and CETP rs708272 polymorphisms on the risk of gallbladder cancer

Genotypes apo B-CETP	Frequency (%)		OR	95% CI	P value
	Controls	GS patients			
Others	70 (95.7)	111 (93.3)	1.0		NS
C/C-T/T	3 (4.3)	8 (6.7)	1.64	0.42-6.46	
	Controls	GBC patients			
Others	70 (95.7)	46 (80.7)	1.0		
C/C-T/T	3 (4.3)	11 (19.3)	4.75	1.16-19.4	0.030
	GS patients	GBC patients			
Others	111 (93.3)	46 (80.7)	1.0		
C/C-T/T	8 (6.7)	11 (19.3)	2.77	0.85-9.04	NS

Others: All remaining combinations combined.

cholelithiasis<sup>[28]</sup>. The CETP variant has been reported to be associated with higher plasma CETP levels and lower HDL cholesterol levels<sup>[29]</sup>. For this reason, we examined the association between the genotypic frequencies of the CETP variant and GBC risk. No significant difference in the CETP variant was found between the controls and the patients with GS or between the patients with GS and those with GBC. However, the frequency of the T/T genotype of CETP rs708272 polymorphism was significantly higher in the GBC patients than in the patients with GS (OR = 5.04,  $P = 0.012$ ). Hassanzadeh *et al*<sup>[30]</sup> reported that the C allele is associated with higher HDL cholesterol levels and lower CETP activity. Since the C allele is the major allele in GS patients<sup>[31]</sup>, the frequency of the C allele or that of the C/C genotype may have been higher in the GS patients than in the GBC patients. Obesity is one of the risk factors for GBC, and an association between obesity and low HDL cholesterol level has been found<sup>[32,33]</sup>. Since the CETP variant has been associated with lower HDL cholesterol levels<sup>[29]</sup>, the risk of progression from cholelithiasis to GBC may be increased by obesity through an abnormality in the lipid metabolism of HDL cholesterol. However, the difference in the frequency of the T/T genotype may be caused by the small sample size of the controls and the GBC patients in our study, because the 95% CI was quite broad, ranging from 1.43 through 17.8. Further study in which the numbers of cases and controls are increased is needed to demonstrate our finding more clearly.

We also examined the combined effects of the apo B rs693 and CETP rs708272 polymorphisms on the risk of GBC. As shown in Table 4, the frequency of the combined C/C genotype of the apo B rs 693 polymorphism and the T/T genotype of the CETP rs708272 polymorphism was significantly higher in the GBC patients (19.3%) than in the controls (4.3%,  $P = 0.030$ ). The OR for the “at-risk” T/T genotype of the rs708272 polymorphism alone was 5.04 (95% CI: 1.43-17.8), and that for the combined “at-risk” genotype of the C/C and T/T was 4.75 (95% CI: 1.16-19.4); their 95% CIs were widely overlapping. Thus, the T/T genotype of the CETP polymorphism appeared to be a

good candidate gene for the genetic factor independently.

The other genetic variants involved in GS formation that we evaluated in this study did not reach conventional levels of statistical significance. As patients with hernia or varicose veins of the legs who had no history of GS or cancer were used as controls in this study, the association may be attenuated. Since both disease incidences might affect the genotype distribution, healthy subjects having no GS or cancer may be needed to detect significant differences between the controls and cases.

The genotypic and allelic frequencies in the capsaicin metabolism-related gene polymorphisms were not significantly different among the three groups. Previous studies indicated that pure capsaicin was a non-mutagenic substance when tested using the Ames test<sup>[12,13]</sup>, but other studies showed that capsaicin and chili extract both acted as tumor promoters, carcinogens, and potential mutagens<sup>[14,15]</sup>. Capsaicin is catalyzed by CYP 2E1, CYP2C9, and CYP3A4 to reactive species<sup>[16-18]</sup>. On the basis of this evidence, we examined the effects of the CYP2E1, CYP2C9, and CYP3A4 variants on the GBC risk. No significant differences in the genotypic and allelic frequencies were found between the three groups. Some other constituents of the chili pepper, e.g., aflatoxin contamination, may be associated with the GBC risk rather than capsaicin itself.

Identification of the high-risk group characterized in terms of genetic measures is important for GBC screening studies. The high-risk group also should be a target of chemoprevention and treatment trials. In addition to genetic association studies of apo B, CETP, CYP2C9 and CYP3A4, further genetic association studies of inflammatory (cyclooxygenase and ATP-binding cassette half-transporters, interleukin-1 beta) genes are needed to help illuminate the complex landscape of GBC risk and genetic variations. We also anticipate that in future genetic association studies of GBC, new approaches will facilitate the evaluation of haplotype effects, either for selected polymorphisms that are physically close to each other or for multiple genes in the overall gallbladder carcinogenesis pathway.

This study had the following limitation. Our sample size was small, and the GBC patients had a bias in age distribution with respect to the controls and the GS patients. Thus, our results may have reduced statistical power to detect a possible association between genetic variants and GBC risk, or they may have failed to reflect precisely the genetic risk factors for the development of GBC. Nonetheless, our finding of the apo B rs693 polymorphism was in agreement with the result of the Indian study<sup>[19]</sup>. To the best of our knowledge, the present study is the first to demonstrate that the T/T genotype of the CETP rs708272 polymorphism was associated with an increased risk of GBC. An additional study including a greater number of controls and cases is required to clarify the association between these genetic factors and the GBC risk.

In conclusion, the C/C genotype of the apo B rs693 polymorphism and the T/T genotype of the CETP

rs708272 polymorphism were associated with increased risk of GBC in Chilean women. While our findings require further confirmation, they provide evidence that the apo B and CETP genes are associated with a higher risk of GBC in Chilean women.

## COMMENTS

### Background

Gallbladder cancer (GBC) is the most common type of biliary tract cancer which results from a complex interplay of genetic and environmental risk factors, like other common multifactorial diseases such as cardiovascular disease, diabetes mellitus and autoimmune disease. Whether genetic variants involved in gallstone formation and capsaicin metabolism affect the risk of GBC in Chilean women is unknown.

### Research frontiers

In this study, the frequency of the cholesteryl ester transfer protein (CETP) rs708272 T/T genotype was significantly higher in the GBC patients than in the gallstone patients. This is the first analysis of the association between genetic predisposition and GBC risk in Chilean women.

### Innovations and breakthroughs

Variants of gallstone formation-related genes such as apolipoprotein B and CETP were associated with an increased risk of GBC in Chilean women. However, capsaicin metabolism-related gene variants were not associated with GBC risk. To clarify the role of genetic predisposition in the development of GBC, we may have to pay more attention to other genes such as inflammatory genes.

### Applications

The apolipoprotein B rs693 T/T and CETP rs708272 T/T genotypes can be used as biomarkers for selecting patients from the group of individuals at high risk for GBC in Chile. Identifying such susceptibility polymorphisms may lead to the development of tests that allow more focused follow-ups of high-risk groups.

### Terminology

CETP is a protein that facilitates the exchange of cholesteryl esters for triglycerides between high-density lipoproteins (HDL) and triglyceride-rich lipoproteins. Therefore, CETP has a central role in the metabolism of both of these types of lipoproteins. Although the C/C genotype was associated with higher plasma CETP and lower HDL cholesterol levels, there is no consistent result regarding the role of the C/C genotype in the development of gallstones.

### Peer review

This study provided some useful data on genetic predisposition and risk of GBC. Methods used in this study are generally reliable, the results are reasonable and convincing. The manuscript is also well written. Authors also pointed out the shortcoming of this study-small sample size.

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## HBV genotype C is independently associated with cirrhosis in community-based population

Jian-Hua Yin, Jun Zhao, Hong-Wei Zhang, Jia-Xin Xie, Wei-Ping Li, Guo-Zhang Xu, Jie Shen, Hong-Jun Dong, Jun Zhang, Lin Wang, Jian-Kang Han, Hong-Yang Wang, Guang-Wen Cao

Jian-Hua Yin, Hong-Wei Zhang, Jia-Xin Xie, Guang-Wen Cao, Department of Epidemiology, Second Military Medical University, Shanghai 200433, China

Jun Zhao, Department of Hepatobiliary Surgery, the 3rd Affiliated Hospital, Second Military Medical University, Shanghai 200438, China

Wei-Ping Li, Department of Ultrasonography, the 1st Affiliated Hospital, Second Military Medical University, Shanghai 200433, China

Guo-Zhang Xu, Hong-Jun Dong, Municipal Center for Disease Control and Prevention of Ningbo, Ningbo 315010, Zhejiang Province, China

Jie Shen, Jun Zhang, Municipal Center for Disease Control and Prevention of Soochow, Soochow 215007, Jiangsu Province, China

Lin Wang, District Center for Disease Control and Prevention of Yangpu, Shanghai 200090, China

Jian-Kang Han, Municipal Center for Disease Control and Prevention of Huzhou, Huzhou 313000, Zhejiang Province, China

Hong-Yang Wang, Laboratory for Signal Transduction, the 3rd Affiliated Hospital, Second Military Medical University, Shanghai 200438, China

**Author contributions:** Yin JH, Zhao J and Zhang HW contributed equally to this work; Yin JH, Zhao J and Zhang HW were involved in experimental work and data analysis; Xie JX was involved in HBV genotyping; Li WP was responsible for ultrasonographic examination; Xu GZ, Shen J, Dong HJ, Zhang J, Wang L, and Han JK were responsible for field study; Wang HY provided partial financial support; Cao GW designed the study and wrote the manuscript.

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**Correspondence to:** Dr. Guang-Wen Cao, Professor of Medicine, Chairman, Department of Epidemiology, Second Military Medical University, 800 Xiangyin Rd., Shanghai 200433, China. [gcao@smmu.edu.cn](mailto:gcao@smmu.edu.cn)

Telephone: +86-21-81871060 Fax: +86-21-81871060

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### Abstract

**AIM:** To determine the association of hepatitis B virus (HBV) genotypes with probable cirrhosis and fatty liver in community-based populations.

**METHODS:** A multi-stage cluster probability sampling method was applied to recruit 10 167 subjects aged between 6 and 72 years from our epidemiological bases in Eastern China. After excluding the subjects co-infected with hepatitis C or hepatitis D viruses, the hepatitis B surface antigen (HBsAg)-positive subjects were examined for HBV genotype, serum viral load, alanine aminotransferase (ALT), hepatitis B e antigen (HBeAg) status, and ultrasonographic changes. Logistic regression models were used to determine the factors associated with probable cirrhosis and fatty liver.

**RESULTS:** Of 634 HBsAg-positive subjects with HBV genotype determined, 82 had probable cirrhosis (ultrasonographic score  $\geq 5$ ), 42 had ultrasonographic fatty liver. Probable cirrhosis was only found in the HBeAg-negative subjects, and more frequently found in the subjects with genotype C than in those with genotype B (14.8% vs 8.0%,  $P = 0.018$ ). In HBeAg-negative subjects, high viral load was frequently associated with abnormal ALT level, while ALT abnormality was more frequent in those with probable cirrhosis than those without (19.5% vs 7.8%,  $P = 0.001$ ). Univariate analysis showed that age, sex, HBV genotypes, and viral load were not significantly associated with ultrasonographic fatty liver, whereas ALT abnormality was significantly related to ultrasonographic fatty liver (OR = 4.54, 95% CI: 2.11-9.75,  $P < 0.001$ ). Multivariate analysis demonstrated that HBV genotype C, age ( $\geq 45$  years), male sex, and ALT abnormality were independently associated with probable cirrhosis (AOR = 2.30, 95% CI: 1.26-4.19; AOR = 1.81, 95% CI: 1.10-2.99; AOR = 1.74, 95% CI: 1.03-2.95; AOR = 2.98, 95% CI: 1.48-5.99, respectively).

**CONCLUSION:** A crude prevalence of probable cirrhosis is 12.9% in the community-based HBV-infected subjects. HBV genotype C is independently associated with probable cirrhosis in the HBeAg-negative subjects.

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**Key words:** Hepatitis B virus; Genotype; Viral load; Alanine aminotransferase; Probable cirrhosis; Ultrasonography

**Peer reviewer:** Raffaele Pezzilli, MD, Department of Internal Medicine and Gastroenterology, Sant'Orsola-Malpighi Hospital, Via Massarenti, 9, Bologna 40138, Italy

Yin JH, Zhao J, Zhang HW, Xie JX, Li WP, Xu GZ, Shen J, Dong HJ, Zhang J, Wang L, Han JK, Wang HY, Cao GW. HBV genotype C is independently associated with cirrhosis in community-based population. *World J Gastroenterol* 2010; 16(3): 379-383 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i3/379.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i3.379>

## INTRODUCTION

Hepatitis B virus (HBV) infection is a serious public health problem. Approximately 2 billion people have been exposed to HBV, and more than 300 million are chronically infected with HBV. Chronic HBV infection is the most important risk factor of liver cirrhosis and hepatocellular carcinoma (HCC) in HBV endemic areas<sup>[1]</sup>. Liver fibrosis, which is the natural wound healing process to necroinflammation frequently caused by chronic HBV infection, is the essential pathogenic process that leads to cirrhosis. Metabolic syndrome is also an independent risk factor of liver cirrhosis in the patients with chronic hepatitis B<sup>[2]</sup>. Subclinical liver cirrhosis diagnosed by ultrasonography is significantly associated with the risk for HCC<sup>[3]</sup>.

HBV genotypes have distinct geographical distributions, and have been shown to differ with regard to clinical outcome and prognosis<sup>[4]</sup>. Genotypes B and C are endemic in most parts of Asia<sup>[5]</sup>. Genotype C is associated with HCC in the aged<sup>[6,7]</sup>. Genotype B is associated with HCC in the young, relapse of HCC, and acute hepatitis B in adults<sup>[8-10]</sup>. However, the relationship between HBV genotypes and liver cirrhosis remains controversial. Some studies suggested that genotype C had a higher risk of cirrhosis, whereas other studies indicated that the progression to cirrhosis did not differ among genotypes B- and C-related chronic liver diseases<sup>[11-13]</sup>. In addition, the association between HBV genotypes and subclinical cirrhosis has not been evaluated in community-based studies in the HBV endemic areas.

Our objective was to determine the prevalence of probable liver cirrhosis in community-based subjects who were seropositive for hepatitis B surface antigen (HBsAg), and to evaluate the viral and demographic factors contributing to subclinical cirrhosis.

## MATERIALS AND METHODS

### Study population and epidemiological survey

The study was carried out at our epidemiological bases in Eastern China, from February to July 2009. A multistage cluster probability sampling method was applied to select the study population. A total of 10167 residents aged between 6 and 72 years were involved in this study. The participants were interviewed by the trained research assistants using a standard questionnaire requesting information about sociodemographic characteristics. Fasting blood samples (4 mL) were collected with vacuum blood collection tube (BD Diagnostics, Plymouth, UK) without anticoagulant. The serum was separated by centrifugation at 4°C at the Centers for Disease Control and Prevention, transported on dry ice and stored at -40°C in the Department of Epidemiology, Second Military Medical University. Informed consent in writing was obtained from each participant or guardian. Each resident who agreed to participate in the study completed a questionnaire and provided blood samples. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the Institutional Review Board of this university.

### Examination of HBV serological markers, serum viral load, and serum alanine aminotransferase level

All participants received HBsAg examination. Those positive for HBsAg were examined for hepatitis B e antigen (HBeAg), serum viral load, and alanine aminotransferase (ALT). HBsAg was examined using enzyme linked immunosorbent assay (Kehua, Shanghai, China) according to the manufacturer's instructions. Serological testing for HBeAg, antibody to hepatitis C virus (HCV), and antibody to hepatitis D virus (HDV), liver function tests, and  $\alpha$ -fetoprotein examination were performed as previously described<sup>[9]</sup>. Upper limit of normal ALT was 45 U/L. Viral load was measured in the LightCycler™ 480 (Roche, Basel, Switzerland) using quantitative HBV PCR fluorescence diagnostic kits (Fosun Diagnostics, Shanghai, China). The kit has a certified lower limit of detection of 500 copies/mL, which was standardized using the Abbott reagents (Abbott Laboratories, North Chicago, IL).

### HBV genotyping

HBV DNA was extracted from 200  $\mu$ L HBsAg-positive serum using High Pure Viral Nucleic Acid Kits (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instruction. HBV genotype was determined using a multiplex PCR assay<sup>[9,14]</sup>. HBV genotypes of samples with low level of HBV DNA were identified by nested multiplex PCR. Outer primers were 5'-TTTGCGGGTCACCATAATTCTTGG-3' and 5'-CGA ACCACTGAACAAATGGCACTAG-3'. An Autorisierter Thermocycler (Eppendorf AG, Hamburg, Germany) was programmed to initially denature the samples for 3 min at 95°C, followed by 35 cycles consisting of 94°C for 60 s, 58°C for 60 s, 72°C for 60 s, followed by a final elongation step at 72°C for 10 min. The products (2  $\mu$ L) were used as templates for multiplex PCR<sup>[14]</sup>.

### Ultrasonographic examination of liver cirrhosis and fatty liver

With the use of a Philips iU22 scanner (Philips Medical Systems, Best, the Netherlands) equipped with a 2-4 MHz variable convex probe or Toshiba systems (SSA-340; Toshiba, Tokyo, Japan) with a 3.75 MHz convex probe, probable liver cirrhosis and fatty liver were determined. Each subject was examined by two independent operators who were blinded to the clinical details. Discrepancies were resolved by consensus. The ultrasonographic scoring system consisting of liver surface, parenchyma, vascular structure, and splenic size was used to describe the existence and the severity of cirrhosis. The scores ranged from 4 for a normal liver to 11 for advanced cirrhosis<sup>[15]</sup>. A score of 8 or more was used as the cutoff point for ultrasonographic cirrhosis. The subjects with the score from 5 to 7 were diagnosed as having cirrhosis-like ultrasonographic abnormality. A score of 5 or more was defined as probable cirrhosis. The subject with an ultrasonographic steatosis score of 2 or more was diagnosed as having fatty liver<sup>[16]</sup>.

### Statistical analysis

$\chi^2$  test was used to determine the differences in categorical variables, such as HBeAg positivity and the percentage of HBV genotypes. Continuous variables, like serum viral load and ALT level with skewed distribution, were adjusted to normal distribution by transformation into logarithmic function, and then tested by Student's *t* test. Univariate and multivariate regression analyses were performed to obtain the odds ratio (OR) and adjusted odds ratio (AOR) of factors for the risk of probable liver cirrhosis or ultrasonographic fatty liver and their 95% confidence intervals (CI). All statistical tests were two-sided, and performed using the Statistical Program for Social Sciences (SPSS15.0 for Windows, SPSS, Chicago, IL). A *P* value of < 0.05 was considered as statistically significant.

## RESULTS

Of the 10167 participants, 793 were HBsAg positive; 745 of the 793 subjects were free of antibodies to HCV or HDV; and 634 of 745 subjects had HBV genotyped. Ten of the 634 subjects (8 with genotype C, one with genotype D, and one with genotype B) were diagnosed as having ultrasonographic cirrhosis (score 8 or higher), while 72 had cirrhosis-like ultrasonographic abnormalities (scores 5-7). Of the 634 subjects, one was diagnosed as having HCC. A crude prevalence of probable cirrhosis (ultrasonographic cirrhosis and cirrhosis-like ultrasonographic abnormalities) was 12.9%. Table 1 shows the demographic and viral characteristics and liver abnormalities of the 634 subjects. There were no significant differences in proportions of age, sex, ALT level, HBeAg positivity, and fatty liver between the subjects infected with genotype B and those with genotype C. Compared with genotype C, HBV genotype B was more frequently seen in those with a high viral load ( $\log_{10}$  copies/mL  $\geq 4$ ). Of the 634 subjects, 39 were positive for HBeAg. The HBeAg-positive subjects were significantly younger than the HBeAg-negative

Table 1 Demographic and viral characteristics and liver abnormalities of 634 subjects seropositive for hepatitis B surface antigen *n* (%)

Variables	Genotype B ( <i>n</i> = 199)	Genotype C ( <i>n</i> = 411)	Others ( <i>n</i> = 24) <sup>1</sup>	<i>P</i> value <sup>2</sup>
Age (yr) <sup>3</sup>	42.1 $\pm$ 13.0	41.3 $\pm$ 12.8	43.8 $\pm$ 12.8	0.493
Gender				
Male	116 (58.3)	228 (55.5)	16 (66.7)	0.511
Female	83 (41.7)	183 (44.5)	8 (33.3)	
ALT ( $\log_{10}$ U/L) <sup>3</sup>	1.4 $\pm$ 0.3	1.4 $\pm$ 0.3	1.4 $\pm$ 0.2	0.238
$\leq 45$ U/L	174 (87.4)	373 (90.8)	23 (95.8)	0.207
> 45 U/L	25 (12.6)	38 (9.2)	1 (4.2)	
HBeAg				
Negative	186 (93.5)	385 (93.7)	24 (100.0)	0.922
Positive	13 (6.5)	26 (6.3)	0	
HBV DNA levels ( $\log_{10}$ copies/mL) <sup>3</sup>	3.7 $\pm$ 1.7	3.5 $\pm$ 1.7	3.1 $\pm$ 1.1	0.194
< 4	151 (75.9)	347 (84.4)	23 (95.8)	0.011
$\geq 4$	48 (24.1)	64 (15.6)	1 (4.2)	
Fatty liver				
Yes	16 (8.0)	24 (5.8)	2 (8.3)	0.303
No	183 (92.0)	387 (94.2)	22 (91.7)	
Cirrhosis status				
Normal	183 (92.0)	350 (85.2)	19 (79.2)	0.018
Probable cirrhosis <sup>4</sup>	16 (8.0)	61 (14.8)	5 (20.8)	

<sup>1</sup>23 cases for genotype mixture, 1 case for genotype D; <sup>2</sup>Genotype C *vs* genotype B; <sup>3</sup>mean  $\pm$  SD; <sup>4</sup>Ultrasonographic cirrhosis and cirrhosis-like ultrasonographic abnormalities (ultrasonographic score  $\geq 5$ ). ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus.

subjects (25.4  $\pm$  11.2 years *vs* 42.7  $\pm$  12.4 years, *P* < 0.001). The subjects with probable cirrhosis were significantly older than those without probable cirrhosis (45.3  $\pm$  10.5 years *vs* 41.1  $\pm$  13.3 years, *P* = 0.001). Probable cirrhosis was only found in the HBeAg-negative subjects, and more frequently in the subjects with genotype C than in those with genotype B (14.8% *vs* 8.0%, *P* = 0.018). Serum viral load was significantly higher in the HBeAg-negative subjects with abnormal ALT levels than in the HBeAg-negative subjects with normal ALT levels (4.54  $\pm$  2.16  $\log_{10}$  copies/mL *vs* 3.31  $\pm$  1.36  $\log_{10}$  copies/mL; *P* < 0.001). However, this association was not found in the HBeAg-positive subjects. Serum ALT level was significantly higher in the subjects with high viral load ( $\geq 1 \times 10^4$  copies/mL) than in those with low viral load ( $< 1 \times 10^4$  copies/mL) (33.9  $\pm$  2.4 U/L *vs* 22.4  $\pm$  1.9 U/L, *P* < 0.001). Serum ALT level was significantly higher in the subjects with ultrasonographic cirrhosis (score  $\geq 8$ ) than in the HBeAg-negative subjects with ultrasonographic score less than 7 (41.1  $\pm$  1.6 U/L *vs* 24.1  $\pm$  2.0 U/L, *P* = 0.026). ALT abnormality was more frequent in HBeAg-negative subjects with probable cirrhosis than those without probable cirrhosis (19.5% *vs* 7.8%, *P* = 0.001).

Table 2 shows the factors associated with probable liver cirrhosis in the HBeAg-negative subjects by univariate and multivariate regression analyses. Age ( $\geq 45$  years *vs* < 45 years), sex (male *vs* female), HBV DNA ( $\geq 4 \log_{10}$  copies/mL *vs* < 4  $\log_{10}$  copies/mL), ALT (> 45 U/L *vs*  $\leq 45$  U/L), and HBV genotypes (genotype C *vs* genotype B) were included in the models. It was found that age ( $\geq 45$  years), male sex, genotype C, and ALT abnormality were independently associated with



**Table 2** Univariate and multivariate regression analyses for the risk factors of probable liver cirrhosis in the 595 HBeAg negative subjects infected with HBV

Variables	Controls ( <i>n</i> = 513)	Cases ( <i>n</i> = 82)	OR (95% CI)	AOR (95% CI)
Age (yr)				
< 45	284 (55.4)	31 (37.8)		
≥ 45	229 (44.6)	51 (62.2)	2.04 (1.26-3.29)	1.81 (1.10-2.99)
Sex				
Female	235 (45.8)	25 (30.5)		
Male	278 (54.2)	57 (69.5)	1.93 (1.17-3.18)	1.74 (1.03-2.95)
ALT (U/L)				
≤ 45	473 (92.2)	66 (80.5)		
> 45	40 (7.8)	16 (12.9)	2.87 (1.52-5.41)	2.98 (1.48-5.99)
Viral load (Log <sub>10</sub> copies/mL)				
< 4	432 (84.2)	67 (81.7)		
≥ 4	81 (15.8)	15 (18.3)	1.19 (0.65-2.19)	1.06 (0.54-2.08)
Genotype				
B	170 (34.4)	16 (20.8)		
C	324 (65.6)	61 (79.2)	2.00 (1.12-3.58)	2.30 (1.26-4.19)

AOR: Adjusted odds ratio; OR: Odds ratio.

probable cirrhosis (AOR = 1.81, 95% CI: 1.10-2.99; AOR = 1.74, 95% CI: 1.03-2.95; AOR = 2.30, 95% CI: 1.26-4.19; AOR = 2.98, 95% CI: 1.48-5.99, respectively).

Forty-two (6.6%) of the 634 subjects had ultrasonographic fatty liver, including 11 with abnormal ALT levels. Ultrasonographic fatty liver was not found in the subjects with probable cirrhosis. In the subjects with high viral load (log<sub>10</sub> copies/mL ≥ 4), ultrasonographic fatty liver was more frequently found in those with genotype B than in those with genotype C (12.5% *vs* 0.0%, *P* = 0.005). Univariate analysis showed that age, sex, HBV genotypes, and viral load were not significantly associated with ultrasonographic fatty liver, whereas ALT abnormality was significantly associated with ultrasonographic fatty liver (OR = 4.54, 95% CI: 2.11-9.75, *P* < 0.001).

## DISCUSSION

This large epidemiological study for the first time described the prevalence of probable liver cirrhosis in community-based, HBV-infected subjects who were free of HCV or HDV infection. About 13% of HBV-infected subjects had probable cirrhosis. The subjects with probable cirrhosis were significantly older than the subjects without cirrhosis. Probable cirrhosis was only found in the HBeAg-negative subjects. The HBeAg-positive subjects were significantly younger than the HBeAg-negative subjects. These results indicate that age is an important determinant for the development of probable liver cirrhosis. High viral load and ALT abnormality are associated with liver fibrosis in the HBeAg-negative patients<sup>[17]</sup>. We further demonstrated that high viral load was associated with increased serum ALT levels in the HBeAg-negative subjects and high ALT levels were frequently found in the subjects with probable cirrhosis, indicating that continuing HBV replication and hepatocyte damage contribute to the development of liver cirrhosis.

Importantly, the occurrence of probable liver cirrhosis

was significantly higher in the subjects with genotype C than in those with genotype B. Multivariate analysis indicated that genotype C was significantly associated with an increased risk of probable liver cirrhosis. This was probably related to the prolonged immune clearance and delayed HBeAg seroconversion<sup>[18,19]</sup>. Although genotype B is associated with acute hepatitis<sup>[10]</sup>, it tends to be self-limiting and short-living. However, genotype C was associated with the longer duration of liver damage in the HBeAg-negative subjects<sup>[12,20]</sup>, which may be the main reason for the development of liver cirrhosis. In addition, genotype C-specific viral mutations are associated with probable cirrhosis<sup>[11,21,22]</sup>. Our recent meta-analysis has shown that PreS deletion, C1653T, T1753V, and A1762T/G1764A are increasingly more prevalent as chronic HBV infection progressed from the asymptomatic HBsAg carrier to cirrhosis or HCC<sup>[23]</sup>. Further studies are needed to probe into the different mutation patterns between genotypes B and C and their roles in the development of liver cirrhosis.

Since metabolic syndrome increased the risk of liver cirrhosis in the patients infected with HBV<sup>[2]</sup>, we evaluated the prevalence and possible risk factors of ultrasonographic fatty liver in the 634 HBV-infected subjects. Interestingly, ultrasonographic fatty liver was not found in those with probable cirrhosis, while ultrasonographic fatty liver was more frequently found in those with genotype B than in those with genotype C at high viral load levels. This suggests that ultrasonographic fatty liver is unlikely to be a late event during the development of probable cirrhosis.

In conclusion, this study found that HBV genotype C, age (≥ 45 years), ALT abnormality, and male sex are independently associated with an increased risk of probable cirrhosis. Ultrasonographic fatty liver is not found in the subjects with probable cirrhosis. Although cirrhosis-like ultrasonographic abnormalities are not clinical liver cirrhosis, it is an early event during the development of clinical cirrhosis. Genotype C HBV-infected male residents at the age of 45 years or older should be routinely examined for active hepatitis and early cirrhosis. Early intervention to the HBV-infected subjects with high risks of cirrhosis might be effective for decreasing the overall mortality from liver cirrhosis and subsequent HCC.

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## COMMENTS

### Background

Chronic hepatitis B virus (HBV) infection is the most important risk factor of liver cirrhosis and hepatocellular carcinoma (HCC) in HBV endemic areas. Metabolic syndrome has been found to be an independent risk factor of liver cirrhosis in the

patients with chronic hepatitis B. The relationship between HBV genotypes and liver cirrhosis remains controversial. Furthermore, the association between HBV genotypes and subclinical cirrhosis has not been evaluated in community-based population.

### Research frontiers

HBV genotypes have distinct geographical distributions and differ with regard to clinical outcome, prognosis, and response to interferon treatment. The role of genotype B and C, the two major HBV genotypes endemic in East Asia, in the development of liver cirrhosis has not been unequivocally addressed. In this study, the authors demonstrate that infection with HBV genotype C is closely associated with subclinical cirrhosis in the community-based subjects with increasing age.

### Innovations and breakthroughs

Recent reports have highlighted the importance of HBV genotypes, alanine aminotransferase (ALT), age, and sex in hepatocarcinogenesis and the development of clinical liver cirrhosis. Metabolic syndrome has been found to be independently associated with liver cirrhosis in the patients with chronic hepatitis B. This is the first study to report that HBV genotype C, age ( $\geq 45$  years), male sex, and ALT abnormality are independently associated with probable cirrhosis in community-based HBV-infected subjects, especially with the subclinical liver cirrhosis. Furthermore, this study suggested that fatty liver may not be associated with probable liver cirrhosis.

### Applications

This study suggests that genotype C HBV-infected male residents at the age of 45 years or older should be routinely examined for active hepatitis and early cirrhosis. Early intervention to the HBV-infected subjects with high risks of cirrhosis might be effective for decreasing the overall mortality from liver cirrhosis and subsequent HCC.

### Terminology

Probable cirrhosis is referred to ultrasonographic cirrhosis (ultrasonographic score  $\geq 8$ ) and cirrhosis-like ultrasonographic abnormalities (ultrasonographic score from 5 to 7). Probable cirrhosis is not histologically confirmed liver cirrhosis. Ultrasonography is an imaging examination which is widely accepted by the community-based HBV-infected subjects without apparent clinical manifestations.

### Peer review

The results of this study provide sufficient experimental evidences or data from which scientific conclusions can be drawn. The discussion is well organized and an overall theoretical analysis is given.

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## ***Gentiana manshurica* Kitagawa prevents acetaminophen-induced acute hepatic injury in mice *via* inhibiting JNK/ERK MAPK pathway**

Ai-Yan Wang, Li-Hua Lian, Ying-Zi Jiang, Yan-Ling Wu, Ji-Xing Nan

Ai-Yan Wang, Li-Hua Lian, Ying-Zi Jiang, Yan-Ling Wu, Ji-Xing Nan, Key Laboratory for Natural Resource of Changbai Mountain & Functional Molecules, Ministry of Education, College of Pharmacy, Yanbian University, Yanji 133002, Jilin Province, China

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Correspondence to: Ji-Xing Nan, Professor, Key Laboratory for Natural Resource of Changbai Mountain & Functional Molecules, Ministry of Education, College of Pharmacy, Yanbian University, Yanji 133002, Jilin Province, China. [jxnanybu@gmail.com](mailto:jxnanybu@gmail.com)

Telephone: +86-433-2660603 Fax: +86-433-2660631

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### Abstract

**AIM:** To investigate the *in vivo* hepatoprotective effects and mechanisms of *Gentiana manshurica* Kitagawa (GM) in acetaminophen (APAP)-induced liver injury in mice.

**METHODS:** GM (200, 150 or 50 mg/kg body weight) or N-acetyl-L-cysteine (NAC; 300 mg/kg body weight) was administrated orally with a single dose 2 h prior to APAP (300 mg/kg body weight) injection in mice.

**RESULTS:** APAP treatment significantly depleted hepatic glutathione (GSH), increased serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and malonyldialdehyde (MDA) and 4-hydroxynonenal levels, and decreased hepatic activity of glutathione peroxidase (GSH-px) and superoxide dismutase (SOD). However, the pretreatment of GM significantly alleviated APAP-induced oxidative stress by increasing

GSH content, decreasing serum ALT, AST and MDA, and retaining the activity of GSH-px and SOD in the liver. Furthermore, GM pretreatment can inhibit caspase-3 activation and phosphorylation of c-Jun-NH<sub>2</sub>-terminal protein kinase 2 (JNK1/2) and extracellular signal-regulated kinase (ERK). GM also remarkably attenuated hepatocyte apoptosis confirmed by the terminal deoxynucleotidyl transferase mediated dUTP nick end-labeling method.

**CONCLUSION:** Hepatoprotective effects of GM against APAP-induced acute toxicity are mediated either by preventing the decline of hepatic antioxidant status or its direct anti-apoptosis capacity. These results support that GM is a potent hepatoprotective agent.

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**Key words:** *Gentiana manshurica* Kitagawa; Acetaminophen; Oxidative stress; Caspase-3; JNK/ERK MAPK

**Peer reviewers:** Isabel Fabregat, PhD, Associate Professor, Laboratory of Molecular Oncology, Institute of Biomedical Research of Bellvitge, Gran Via Km 2, 7, L'Hospitalet, 08907 Barcelona, Spain; Maurizio Parola, Professor, Department of Experimental Medicine and Oncology, University of Turin, Corso Raffaello 30, 10125 Torino, Italy

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### INTRODUCTION

Acetaminophen (APAP) is a widely used analgesic and antipyretic drug. It is safe at therapeutic doses, however,



when taken at high doses, APAP can precipitate severe liver injury that may develop into a liver failure<sup>[1,2]</sup>. Overdoses of APAP lead to the generation of high amounts of the toxic metabolite N-acetyl-quinoneimine (NAPQI) by the cytochrome P-450 isoenzyme mixed-function oxidase system, which is immediately conjugated with glutathione (GSH), forming the nontoxic metabolites cysteine and mercapturic acid conjugates. Although a number of P450 enzymes can metabolize APAP, the most relevant isoenzyme is CYP2E1<sup>[3]</sup>. There is an alternative view that oxidative stress plays a role in hepatotoxicity. Oxidative stress in APAP hepatotoxicity is characterized by several features, including lipid peroxidation, mitochondrial damage and ATP depletion in proteins<sup>[4]</sup>. NAPQI reacts with GSH spontaneously or catalyzed by glutathione-S-transferases to form a GSH-adduct. Thus, GSH depletion and formation of protein adducts are key mechanisms of APAP-induced cell death<sup>[5-7]</sup>.

Many antioxidant agents have been studied in experimental and clinical studies to reduce or prevent APAP-induced hepatotoxicity. The most popular antioxidant for APAP hepatotoxicity is N-acetyl-L-cysteine (NAC)<sup>[8]</sup>. Protection by NAC is believed to be attributable to its ability to regenerate GSH stores because of its capacity to provide cysteine residues<sup>[9]</sup>.

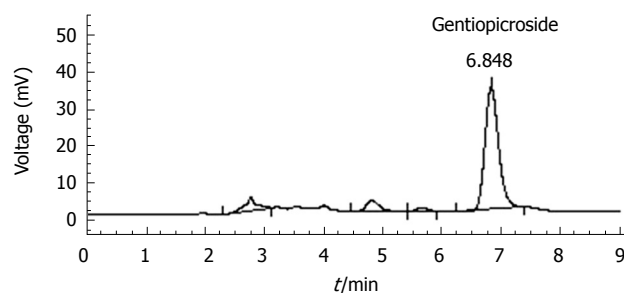
In recent years, plant-derived natural products have received considerable attention due to their diverse pharmacological properties. A growing interest has been observed in the analysis of these natural entities for their potential benefits to human health on hepatoprotective effects. *Gentiana manshurica* Kitagawa (GM) is distributed in northeastern China and reputed “Dongbei longdan”, which is one of the most common herbal medicines used by Chinese people suffering from chronic liver diseases. As an iridoid containing plant, GM has various pharmacological activities. Previous phytochemical studies reported that GM includes loganic acid, 6-O- $\beta$ -d-glucopyranosylgentiopicroside, swertiamarin, gentiopicroside, sweroside and 2-(o,m-dihydroxybenzyl)-sweroside<sup>[10]</sup>. It was reported that gentiopicroside involves down-regulation of NR2B receptors in the anterior cingulate cortex to persistent inflammatory pain<sup>[11]</sup>. Animal experiments have revealed adaptogenic<sup>[12]</sup> and anti-inflammatory<sup>[13]</sup> activities. GM has also been used traditionally as a folk remedy for healing wound<sup>[14,15]</sup>. However, no research has been conducted about its hepatoprotective patterns.

Since a high dose APAP-induced hepatotoxicity resulted from the generation of free radicals during its metabolism at liver, the possible protection by GM was evaluated and the results are presented in this paper.

## MATERIALS AND METHODS

### Chemicals

APAP and NAC were purchased from Sigma Chemicals (St. Louis, MO, USA). Detection kit for GSH and malondialdehyde (MDA), glutathione peroxidase (GSH-px) and superoxide dismutase (SOD) were purchased



**Figure 1** HPLC chromatograms of *Gentiana manshurica* Kitagawa (GM). Column: Diamonsil C18 (250 mm  $\times$  4.6 mm); Flow rate: 1 mL/min; Mobile phase: Methanol and H<sub>2</sub>O (35:65).

from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals used were of analytical grade.

### Preparation of GM

The rhizomes and roots GM were purchased from Yanbian Puhe, China in March 2006 and authenticated by Prof. Hui-Zi Lv of College of Pharmacy, Yanbian University. The rhizomes and roots of GM (1 kg) were extracted three times with methanol (10 L) and boiled under reflux for 4 h at 40°C, and then the percolated was concentrated in a rotary vacuum evaporator followed by lyophilization. The yield (w/w) of extract was about 22.35%. The freeze-dried extract was used in both chemical analysis and pharmacological studies. GM extract was analyzed on HPLC to confirm the presence of gentiopicroside (Figure 1). The content of gentiopicroside was 2.48% in GM extract. The extract was pre-solubilized in distilled saline for the *in vivo* studies.

### Animals and treatment

Male Kunming mice (20-22 g) were provided by Yanbian University Laboratory Animal Center, fed with a standard chow diet and given tap water *ad libitum*. Animals were housed in plastic cages and maintained at 22  $\pm$  2°C and 50%-60% relative humidity, with a 12-h light-dark cycle throughout the experiment. Animal experiments were performed under the latest edition of “Guiding Principles in the Use of Animals in Toxicology” adopted by the Society of Toxicology (USA).

The mice were fasted overnight (16 h) prior to administration of a single intraperitoneal dose (300 mg/kg) of APAP dissolved in sterile phosphate buffered saline (PBS, pH 7.4) warmed to 40°C<sup>[16]</sup>. Animals were divided into 7 groups of ten animals each. Animals of group 1 received vehicle only and served as normal and group 2 treated intraperitoneally with a single dose of APAP (300 mg/kg) was kept as control. Groups 3, 4 and 5 were administered orally with GM extract (50, 100 or 200 mg/kg) 2 h before APAP injection, and served as GM *per se*. Group 6 was treated with NAC (300 mg/kg) 2 h before APAP injection, and served as positive control. Group 7 received only GM extract (200 mg/kg) 2 h after saline injection, instead of APAP injection. Animals were sacrificed and blood was collected from the carotid artery 12 h after administration of APAP. Serum was then separated by



centrifugation at 4°C, 3000 r/min for 30 min. The liver was removed immediately from each mouse, and kept at -80°C until analyzed.

### Blood biochemistry

Blood was collected at 12 h after APAP administration. Serum levels of AST and ALT were detected using an Autodry Chemistry Analyzer (SPOTCHEM™ SP4410, Arkray, Japan).

### GSH, SOD, GSH-px activities and hepatic lipid peroxidation assay

The removed liver tissue was homogenized in 9 volumes of cold buffer (0.01 mol/L Tris-HCl, 0.0001 mol/L EDTA-2Na, 0.01 mol/L sucrose, and 0.8% saline, pH 7.4) on ice. The homogenate was centrifuged at 4°C (3000 r/min, 15 min) and the supernatant was used for the determination of GSH and MDA, GSH-px and SOD following the manufacturer's instructions. Briefly, the MDA was detected using the thiobarbituric acid reactive substances (TBARS) methods; 4-hydroxynonenal (4-HNE) was detected as a fluorimetric derivative<sup>[17]</sup>; the GSH activity was detected through yellow tetramethyl-benzidine and oxidised GSH produced by the combination of GSH and dithio-nitrobenzene; SOD activity was examined through nitroblue tetrazolium coloration; and GSH-px activity was determined through detecting selenium cysteine, the active centre of GSH-px. Protein content was determined with a Bio-Rad Protein Assay Kit (Bio-Rad, USA).

### Histopathological analysis

Liver samples obtained at different time points after the APAP injection were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin for histopathological analysis. The sections were examined under light microscopy and graded for the presence and intensity of lesions using a scale from 0 to 5 (0 = no lesions; 1 = minimal lesions involving a single or few necrotic cells; 2 = mild lesions, 10%-25% necrotic cells or mild diffuse degenerative changes; 3 = moderate lesions, 25%-40% necrotic or degenerative cells; 4 = marked lesions, 40%-50% necrotic or degenerative cells; and 5 = severe lesions, more than 50% necrotic or degenerative cells)<sup>[18,19]</sup>.

### Transferase-mediated dUTP nick end-labeling (TUNEL) assay

Apoptotic cells were detected by the terminal deoxynucleotidyl TUNEL method using an *in situ* cell detection kit (Roche, Mannheim, Germany) for the detection and quantification of apoptosis at a single-cell level. Staining of tissue sections was performed according to the manufacturer's protocol, as follows. Paraffin-embedded sections were dewaxed in xylene and rehydrated by passing through a graded series of ethanol solutions, ending with phosphate-buffered saline. Sections were permeabilized with proteinase K (20 µg/mL in 10 mmol/L Tris-HCl,

pH 7.4-8.0) at 37°C for 15 min. After washing, sections were stained with fluorescent anti-TdT. Sections were viewed and photographed using standard fluorescent microscopic techniques.

### Western blotting analysis

The total protein extracts were made by pulverization in a grinder with liquid nitrogen, then using a ratio of 1 mL lysis buffer (150 mmol/L NaCl, 1.0% NP-40, 0.5% NaVO<sub>4</sub>, 0.1% SDS, 50 mmol/L Tris, pH 7.5) containing 1 mmol/L PMSF for each 100 mg powdered liver sample. Liver lysates (40 µg) were electroblotted onto a PVDF membrane following separation on 8%-12% SDS-polyacrylamide gel electrophoresis. Blotted membranes were blocked with 5% skim milk in incubation buffer at room temperature, followed by incubation overnight at 4°C with 1:1000 dilution of caspase-3, JNK, ERK (Santa Cruz biotechnology, CA, USA) and phospho-JNK, phospho-ERK (Cell Signaling Technology, MA, USA) primary antibody. Bound antibody was detected with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA) and immunodetected proteins were visualized using WEST-ZOL™ (plus) Western blotting detection system (iNtRON Biotechnology, Gyeonggi, Korea). Loading accuracy was evaluated by membrane rehybridization with monoclonal antibodies against  $\alpha$ -tubulin (Sigma, St. Louis, MO, USA). Densities of the immunoreactive bands were analyzed with the Image Master 1D Elite software (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

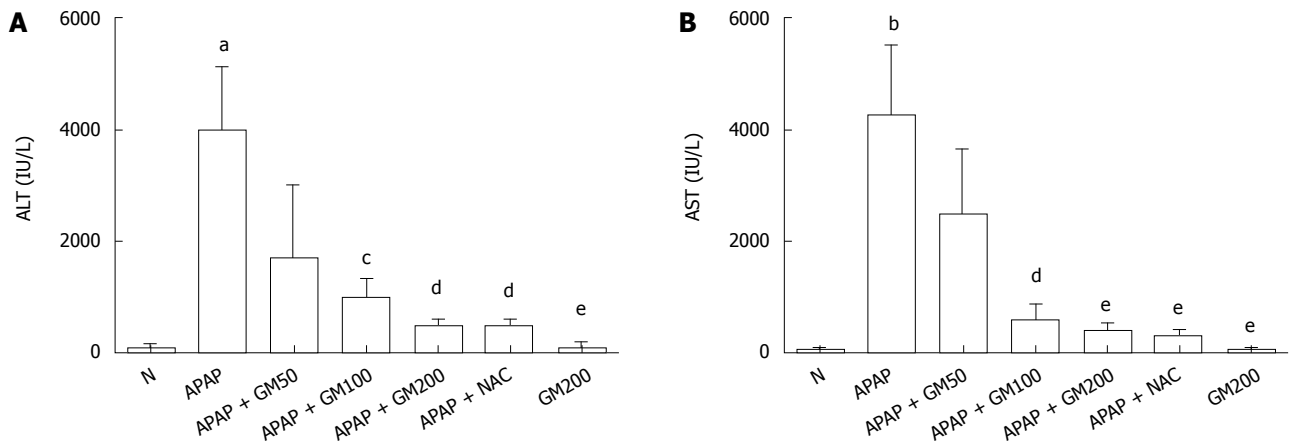
### Statistical analysis

All values were expressed as mean  $\pm$  SD. All other results, except pathological findings, were evaluated by one-way ANOVA and Tukey's multiple comparison tests. Liver histopathological examination data were analyzed by the Kruskal-Wallis nonparametric test, followed by a Mann-Whitney test. Statistically significant differences between groups were defined as *P* values less than 0.05. Calculations were performed with the GraphPad Prism program (Graphpad Software, Inc, San Diego, USA).

## RESULTS

### Effects of GM on serum AST and ALT levels

Serum activities of ALT and AST are shown in Figure 2. A single dose of APAP significantly elevated the serum ALT (*P* < 0.05) and AST (*P* < 0.01) activities when compared with the normal animals. Pretreatment with GM 2 h prior to APAP administration lowered markedly both serum ALT and AST levels. Serum ALT levels were significantly decreased to 43%, 26%, 13% or 13% in GM groups (200, 100 and 50 mg/kg) or NAC group (300 mg/kg) compared with APAP alone group, respectively. Serum AST levels were significantly decreased to 58%, 14%, 10% or 8% in GM groups (200, 100 and 50 mg/kg) or NAC group (300 mg/kg) compared with APAP alone group, respectively.



**Figure 2** Protective effects of GM on serum biochemical parameters after acetaminophen (APAP) administration. Mice were intraperitoneally injected with APAP (300 mg/kg body weight). GM (200, 100 or 50 mg/kg body weight) was orally administered at 2 h before APAP injection. All data are presented as mean  $\pm$  SD,  $n = 10$ /group. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , significantly different when compared with normal controls. <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$ , <sup>e</sup> $P < 0.001$  significantly different when compared with APAP alone group.

### Effects of GM on GSH, SOD, GSH-px activities, MDA and 4-HNE contents

Twelve hours after APAP administration, GSH, GSH-px and SOD concentrations were significantly decreased to 44% ( $P < 0.05$ ), 35% ( $P < 0.05$ ) and 55% ( $P < 0.05$ ) respectively in APAP group compared with the normal group. However, pretreatment with a high dose of GM (200 mg/kg) significantly alleviated subsequent APAP-induced GSH depletion to  $1561 \pm 186$  ( $P < 0.05$ ). SOD ( $P < 0.01$ ) and GSH-px activities ( $P < 0.05$ ) were significantly enhanced in GM 200 mg/kg plus APAP treated group. MDA levels increased by 252% in mice treated with APAP, indicating that APAP administration significantly increased lipid peroxidation in liver ( $P < 0.01$ ). Briefly, in mice receiving GM (200 or 100 mg/kg) plus APAP, the MDA levels were significantly reduced to 42% ( $P < 0.01$ ) or 43% ( $P < 0.01$ ) as compared with the APAP treated mice. In mice receiving GM (200 mg/kg) plus APAP, the 4-HNE levels were significantly reduced to 59% ( $P < 0.05$ ) compared with the APAP treated mice (Figure 3).

### Effects of GM on histopathology

Histopathological analysis of the APAP alone treated animal showed severe centrilobular necrosis, fatty infiltration and lymphocytes infiltration (data not shown), which were significantly less in the GM plus APAP treated groups with mild sinusoidal congestion, less inflammatory cell infiltration, and well preserved hepatocytes with less area of necrosis (Table 1 and Figure 4).

### GM protects against APAP-induced hepatocyte apoptosis in mice via inhibiting caspase-3 and JNK/ERK MAPK pathway

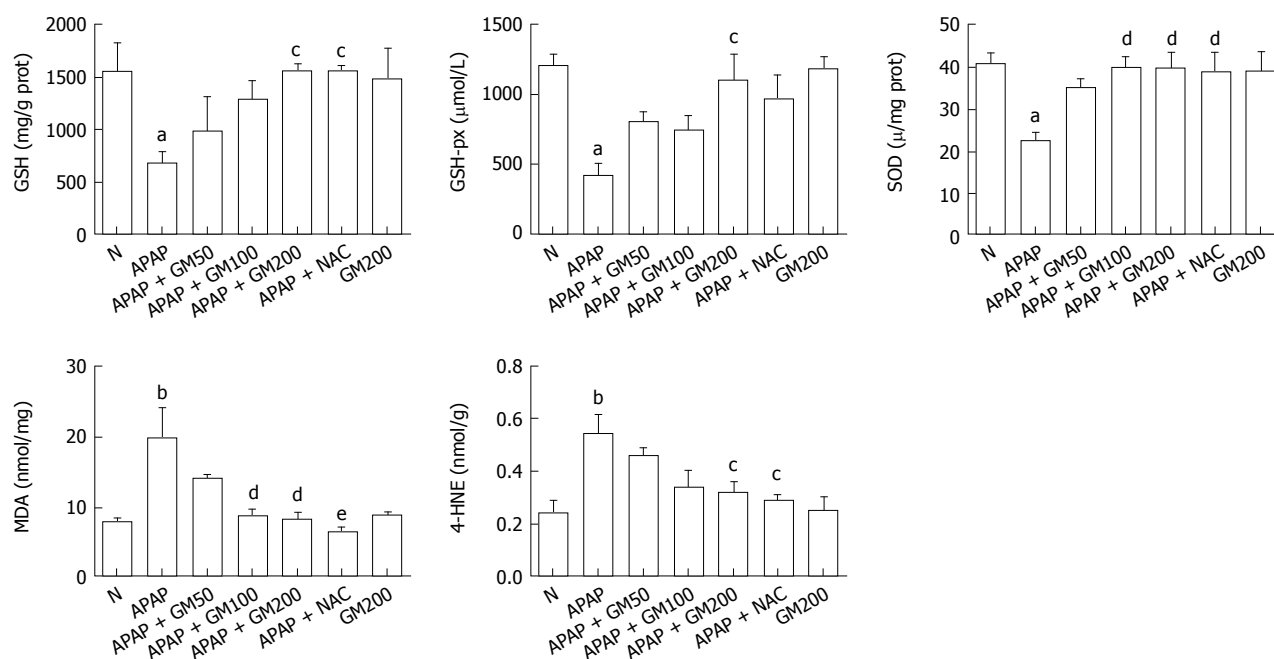
Apoptotic hepatocytes were detected by TUNEL staining. A large number of TUNEL-positive hepatocytes were seen in the livers of APAP-treated mice 12 h after injection, however, a few TUNEL-positive hepatocytes were found in livers from animals pretreated with GM (Figure 4). The protease activity of caspase-3 was measured in APAP-induced liver injury mice treated with

and without GM. Caspase-3 was proteolytically processed to the active p17 fragment 12 h after APAP treatment in mice (Figure 5). However, GM significantly inhibited caspase-3 activity. It has recently been reported that c-Jun-NH2-terminal protein kinase 2 (JNK1/2) plays a critical role in mediating APAP hepatotoxicity in mice<sup>[20]</sup>. JNK1/2 activation is an early key signal in mediating mitochondria-mediated lethal cell injury triggered by toxicants in hepatocytes<sup>[21]</sup>. Therefore, we investigated whether APAP-induced JNK activation was attenuated by GM. Our data revealed that phosphorylated JNK and phosphorylated ERK, significantly increased after treatment with APAP when oxidative stress in the liver had been significantly enhanced as described above, while the JNK and ERK total protein level were almost normal 12 h after APAP treatment (Figure 5). After administrated with various doses of GM 2 h *via* APAP injection, phosphorylated JNK and phosphorylated ERK levels were declined (Figure 5). These data are consistent with our hypothesis that GM inhibits JNK/ERK MAPK signaling pathway.

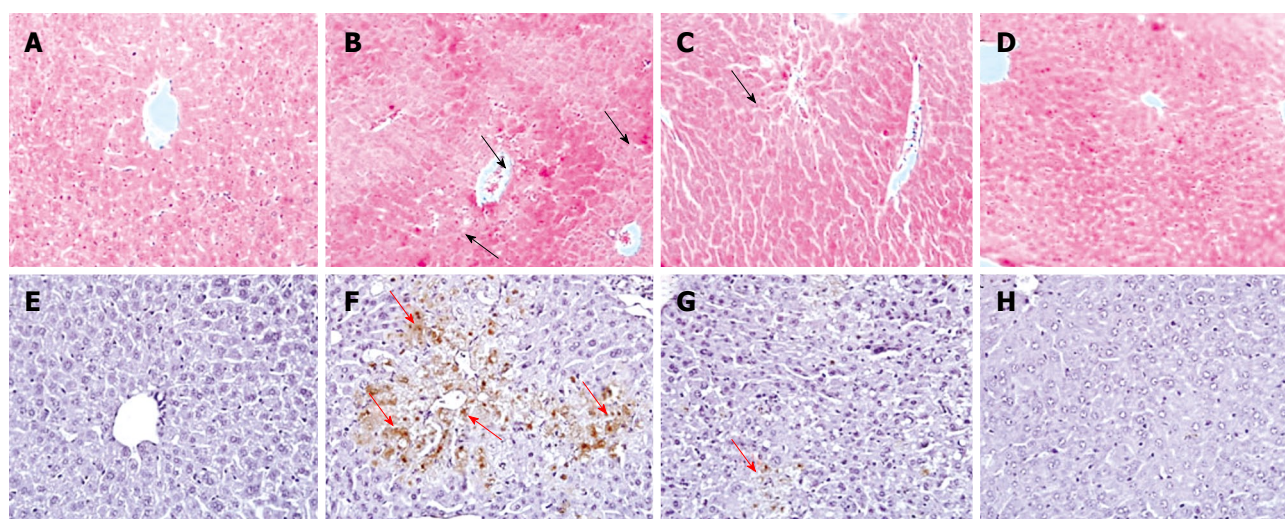
## DISCUSSION

Progress has been achieved in research of the chemical constituents and pharmacological activities of genus *Gentiana*. It is reported that gentianine from *Gentiana Macrophylla* Radix, one of *Gentiana* species, might express anti-inflammatory activities at least partly through preventing the immune cells, including macrophages, from producing TNF- $\alpha$  and IL-6, pro-inflammatory cytokines in male *Sprague Dawley* rats treated with LPS<sup>[22]</sup>. Hepatoprotective effects of *Gentiana olivieri* Griseb, flowering herbs on subacute administration were studied using *in vivo* models in rats, and the remarkable hepatoprotective activity of *Gentiana olivieri* might be due to the potent antioxidant activity of iso-orientin<sup>[23]</sup>. However, this is the first study to report the hepatoprotective effects of GM against APAP-related liver toxicity.

APAP is a safe and effective analgesics when used at therapeutic doses, an overdose of APAP, however, can



**Figure 3** Protective effects of pretreatment with GM against APAP-induced glutathione (GSH) depletion, malonyldialdehyde (MDA) levels, 4-hydroxynonenal (4-HNE) levels and superoxide dismutase (SOD), glutathione peroxidase (GSH-px) activity in liver of mice. APAP was given intraperitoneally with a single dose of 300 mg/kg. GM was given orally with a single dose of 200, 100 or 50 mg/kg. All data are presented as mean  $\pm$  SD,  $n = 10$ /group. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , significantly different when compared with normal controls. <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$ , <sup>e</sup> $P < 0.001$  significantly different when compared with APAP alone group.



**Figure 4** Liver histopathology and transferase-mediated dUTP nick end-labeling (TUNEL) assay. APAP was given intraperitoneally with a single dose of 300 mg/kg. GM was given orally with a single dose of 200 mg/kg. A and E: Normal group; B and F: APAP group; C and G: APAP plus GM 200 mg/kg; D and H: Only GM 200 mg/kg. Upper panels stand for HE staining and the lower panels for TUNEL assay. The regions by black arrows in the upper figures indicate the hepatocyte necrosis or hepatocyte degeneration; the regions by red arrows in the lower figures indicate the TUNEL-positive apoptotic cells ( $\times 100$  magnification).

induce severe hepatotoxicity in experimental animals and in human<sup>[24,25]</sup>. Liver injury induced by APAP is commonly used for the screening of hepatoprotective drugs<sup>[16]</sup>. NAC is used currently in clinical treatment for APAP overdose. Hereby, we used NAC as positive control, to compare with GM on hepatoprotective effects.

Administration of a single high dose of APAP significantly ( $P < 0.01$ ) elevated the serum transaminase activities compared with the normal controls (Figure 2). The significantly decreased serum transaminases activities in the GM administered groups prior to APAP demonstrated

its hepatoprotective effects. Thus, a single dose of methanol extract of GM could render a complete protection.

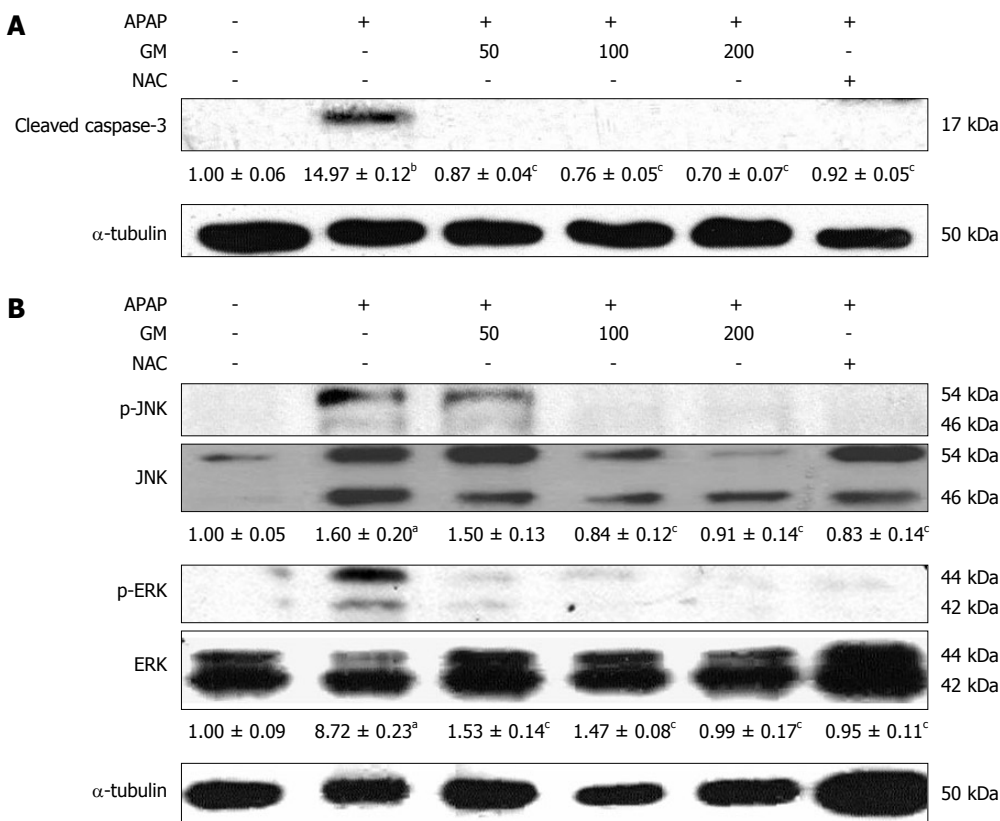
Cytochrome P-450 enzymes are the major catalysts involved in the metabolism of drugs. APAP is mainly metabolized by cytochrome P-450 to form an electrophilic metabolite, N-acetyl-p-benzoquinonimine, which is primarily inactivated by conjugation with GSH<sup>[26,27]</sup>. A large number of the metabolites produced by APAP are found to generate superoxide anion and other free radicals in the biological systems<sup>[28]</sup>. However, at a higher dose of APAP, intermediate metabolites accumulate and cause



Table 1 Effects of GM on hepatic damage induced by APAP in mice

Treatment	Dose (mg/kg)	Histopathological scores						Average
		0	1	2	3	4	5	
Saline + Saline	-	10	0	0	0	0	0	0.0
APAP + Saline	-	0	0	1	3	4	2	3.7
APAP + GM	200	0	4	3	2	1	0	2.0 <sup>b</sup>
	100	0	3	3	2	2	0	2.3 <sup>a</sup>
	50	0	2	3	2	2	1	2.7
APAP + NAC	300	0	3	4	2	1	0	2.1 <sup>b</sup>

APAP was given intraperitoneally with a single dose of 300 mg/kg. GM was given orally with a single dose of 200, 100 or 50 mg/kg. Livers were graded from 0 to 5 as described in Materials and Methods. Each value is the number of animals with grading changes. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 significantly different when compared with APAP alone group. GM: *Gentiana manshurica* Kitagawa; APAP: Acetaminophen.



**Figure 5 Western blotting analysis of caspase-3 and JNK/ERK MAPK.** GM (200, 100 or 50 mg/kg), NAC (300 mg/kg) or saline was orally administered 2 h before APAP injection. Active form of caspase-3, phospho-JNK and phospho-ERK were detected by Western blot. The active form of caspase-3 levels corresponding to each immunoreactive band were digitized and expressed as a percentage of the α-tubulin levels. Densitometric scanning data of phospho-JNK and phospho-ERK levels were expressed as the ratio of JNK or ERK, respectively. The ratio of the normal group band was set to 1.00. Data of three independent experiments are expressed as mean ± SD. <sup>a</sup>*P* < 0.01, <sup>b</sup>*P* < 0.001, significantly different when compared with the normal group. <sup>c</sup>*P* < 0.001 significantly different when compared with APAP alone group.

liver damage. Depletion of GSH beyond certain critical level can lead to oxidative stress and development of overt hepatotoxicity<sup>[29]</sup>.

The decreased hepatic antioxidant status is related to oxidative stress and elevation of lipid peroxidation that lead to the leakage of hepatic enzymes to serum in APAP alone treated animals. To determine whether GM could inhibit APAP-induced GSH depletion, we measured the hepatic GSH levels. Our results showed that co-treatment with GM and APAP inhibited APAP-induced GSH depletion (Figure 3). NAC reduced APAP hepatic toxicity

by increasing hepatic GSH levels. The increasing GSH levels had no significant differences between the group pretreated with GM (200 mg/kg) and NAC. The elevated hepatic reduced GSH level could partially explain the hepatoprotective mechanism of GM. Reduced GSH can function as a reductant in the metabolism of hydrogen peroxide and various organic peroxides. The GSH-px present in the cells can catalyze this reaction<sup>[30]</sup>. It is reported that depletion of GSH below a threshold value was associated with a significant conversion of xanthine dehydrogenase to reversible xanthine oxidase, a superoxide



radical generation reaction catalyzing enzyme. Therefore, the enhanced hepatic GSH-px and SOD activities in the GM plus APAP treated group further support its hepatoprotective effects (Figure 3). MDA and HNE are major end-products of oxidation of polyunsaturated fatty acids, and are frequently measured as indicators of lipid peroxidation and oxidative stress *in vivo*. Thereby, the elevated antioxidant status in the liver of GM plus APAP treated group is related to the decreased MDA level and 4-HNE level, could maintain the membrane integrity and prevented lipid peroxidation and was comparable to NAC (Figure 3). The histopathological analysis of liver section indicates a moderate centrilobular necrosis, fatty infiltration and lymphocytic infiltration in the GM plus APAP treated animals with respect to the APAP alone treated animals (Table 1 and Figure 4).

APAP was believed to induce apoptosis based on the observation that APAP treatment results in the activation of caspase-3. In this study, pretreatment with GM prior to APAP inhibited caspase-3 cleavage (Figure 5). Thus, the active form of caspase-3 was not observed in GM or NAC pretreated groups. Microscopic observation on TUNEL-stained sections demonstrated that GM significantly decreased the TUNEL-positive apoptotic hepatocytes. Furthermore, it was reported that p38 MAPK, JNK, and ERK were activated by APAP<sup>[31]</sup>. JNK2 plays a protective role against APAP-induced liver injury in mice, in part, by modulating hepatocellular regeneration and repair, which further suggests the use of JNK inhibitors as a potential treatment for APAP-induced liver injury<sup>[32]</sup>. In both cultured hepatocytes and *in vivo* livers, treatment with APAP induced a sustained activation of JNK as reflected in increased phospho-c-jun levels<sup>[33]</sup>. A number of studies have suggested that oxidative stress leads to JNK activation, either through redox alteration of the sequestration of JNK or through inhibition of JNK phosphatase<sup>[34,35]</sup>. Our data showed that phospho-JNK1/2 expression was greatly increased 12 h after APAP administration (Figure 5). Coinciding with the expression of phospho-JNK1/2, APAP also increased the level of phospho-ERK1/2. However, GM pretreatment can effectively inhibit phosphorylation of ERK1/2 (Figure 5). These data suggest that GM inhibits the JNK/ERK signaling pathway at a more proximal regulatory step, resulting in inhibition of its downstream effector mechanisms.

In conclusion, in this study, GM can significantly prevent the APAP-induced acute hepatotoxicity by enhancing the hepatic antioxidant activity, and inhibiting the caspase-3 cleavage and JNK/ERK MAPK activation. GM exerts some effects which resemble those of an antidote of acetaminophen such as NAC. However, further detailed studies are required to confirm its clinical application.

## COMMENTS

### Background

Acetaminophen (APAP) is a widely used analgesic and antipyretic drug that is safe at therapeutic doses, however, when taken at high doses, APAP can precipitate severe liver injury that can develop into a liver failure. Overdoses of APAP lead to the generation of high amounts of the toxic metabolite N-acetyl-

quinoneimine (NAPQI). NAPQI reacts with glutathione (GSH) spontaneously or catalyzed by glutathione-S-transferases to form a GSH-adduct. Thus, GSH depletion and formation of protein adducts are key mechanisms of APAP-induced cell death.

### Research frontiers

In recent years, plant-derived natural products have received considerable attention due to their diverse pharmacological properties. Further studies on hepatoprotective effect of these natural entities and its possible mechanism are important for understanding the mechanism of APAP-induced liver injury.

### Innovations and breakthroughs

The authors investigated the effects of *Gentiana manshurica* Kitagawa (GM) on APAP-induced liver injury in mice and whether GM prevents APAP-induced hepatocyte apoptosis *in vivo*. The present study concluded that GM can significantly prevent the APAP-induced acute hepatotoxicity by enhancing the hepatic antioxidant activity, and inhibiting the caspase-3 cleavage and JNK/ERK MAPK activation, and GM exerts some effects which resemble those of an antidote of acetaminophen such as NAC.

### Applications

The results provide significant evidence illustrating the key feature of recovery from APAP-induced acute liver injury.

### Peer review

This is an interesting paper that, although descriptive, points out to the potential hepatoprotective effect of a herbal compound (*Gentiana manshurica* Kitagawa: GM) on the acetaminophen-induced acute hepatotoxicity. Results are clear and manuscript is well written.

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## A laterally-spreading tumor in a colonic interposition treated by endoscopic submucosal dissection

Hideaki Bando, Hiroaki Ikematsu, Kuang-I Fu, Yasuhiro Oono, Takashi Kojima, Keiko Minashi, Tomonori Yano, Takahisa Matsuda, Yutaka Saito, Kazuhiro Kaneko, Atsushi Ohtsu

Hideaki Bando, Hiroaki Ikematsu, Yasuhiro Oono, Takashi Kojima, Keiko Minashi, Tomonori Yano, Kazuhiro Kaneko, Atsushi Ohtsu, Department of Gastrointestinal Oncology & Endoscopy, National Cancer Center Hospital East, Kashiwanoha 6-5-1, Kashiwa, Chiba 277-8577, Japan

Kuang-I Fu, Department of Gastroenterology, Juntendou University Nerima Hospital, Tokyo 177-0033, Japan

Takahisa Matsuda, Yutaka Saito, Endoscopy Division, National Cancer Center Hospital, Tokyo 104-0045, Japan

**Author contributions:** Bando H designed the research, collected, analyzed and interpreted the data; Ikematsu H, Fu KI, Oono Y, Kojima T, Minashi K, Yano T, Matsuda T, Saito Y, Kaneko K and Ohtsu A wrote and revised the paper.

**Correspondence to:** Hiroaki Ikematsu, MD, Department of Gastrointestinal Oncology & Endoscopy, National Cancer Center Hospital East, Kashiwanoha 6-5-1, Kashiwa, Chiba 277-8577, Japan. [hikemats@east.ncc.go.jp](mailto:hikemats@east.ncc.go.jp)

Telephone: +81-4-71331111 Fax: +81-4-71346928

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**Peer reviewers:** Christopher Mantyh, MD, Associate Professor, Department of Surgery, Duke University Medical Center, Box 3117, Durham, NC 2771, United States; Ming-Te Huang, Professor, Department of Surgery, Taipei Medical University-Shuang Ho Hospital, Taipei 23561, Taiwan, China

Bando H, Ikematsu H, Fu KI, Oono Y, Kojima T, Minashi K, Yano T, Matsuda T, Saito Y, Kaneko K, Ohtsu A. A laterally-spreading tumor in a colonic interposition treated by endoscopic submucosal dissection. *World J Gastroenterol* 2010; 16(3): 392-394 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v16/i3/392.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i3.392>

### Abstract

Herein we describe an early colonic carcinoma which developed in a colonic interposition 14 years after surgery for esophageal cancer, which was successfully treated by endoscopic submucosal dissection (ESD). An 80-year-old man underwent colonic interposition between the upper esophagus and stomach after surgery for an early esophageal squamous cell carcinoma in 1994. He received a surveillance endoscopy, and a laterally-spreading tumor of granular type, approximately 20 mm in size, was identified in the colonic interposition. An endoscopic biopsy revealed moderately differentiated adenocarcinoma histologically, however, we diagnosed the lesion as an intramucosal carcinoma based on the endoscopic findings. The lesion was safely and completely removed *en bloc* by ESD using a bipolar knife. Histologically, the lesion was an intramucosal moderately differentiated adenocarcinoma in a tubular adenoma.

### INTRODUCTION

Although rarely reported, adenoma and adenocarcinoma can occur as a late complication in colon segments used to replace the esophagus. Herein, we describe an early colonic carcinoma which developed in a colonic interposition 14 years after surgery for esophageal cancer, which was successfully treated by endoscopic submucosal dissection (ESD).

### CASE REPORT

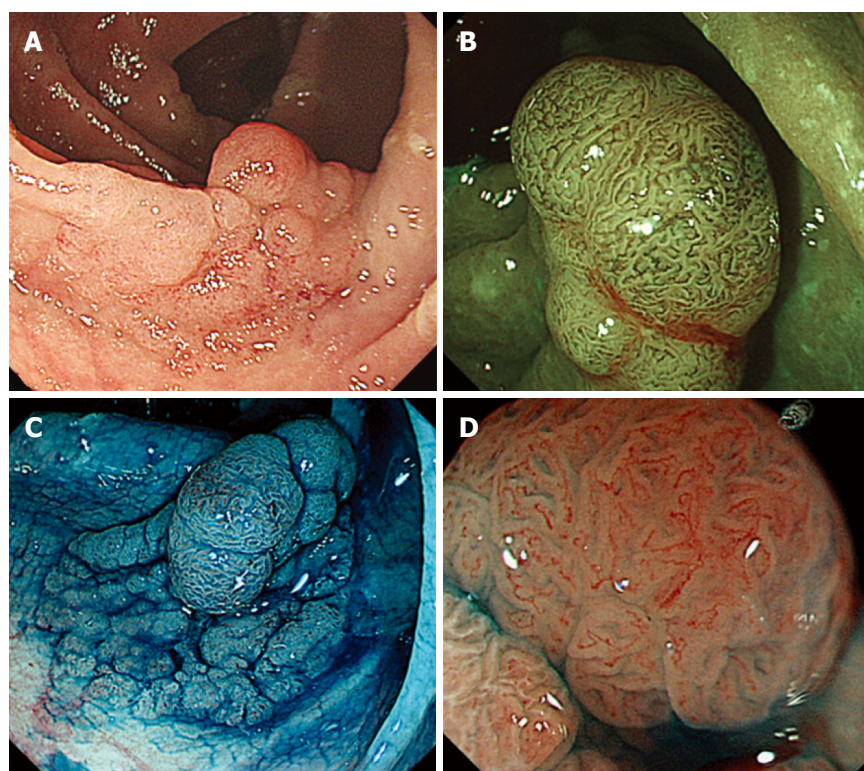
An 80-year-old man underwent colonic interposition between the upper esophagus and stomach after surgery for an early esophageal squamous cell carcinoma (T1, N0, M0, stage I according to the TNM classification) in 1994. He received an esophagogastroduodenoscopy for surveillance and a laterally-spreading tumor of granular type (LST-G), approximately 20 mm in size, was identified in the colonic interposition. On conventional view, a



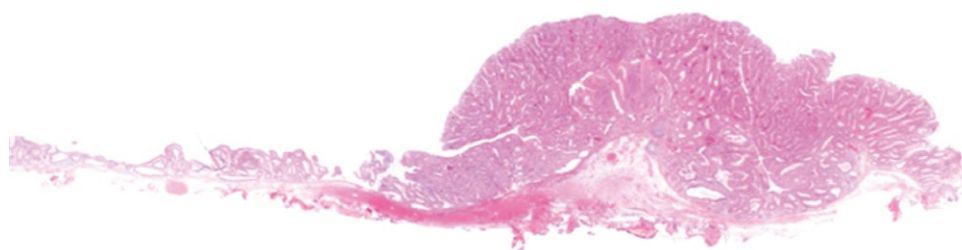
Table 1 Summary of reported cases of neoplasia arising in a colonic interposition

Case	Authors	Age	Gender	Size (mm)	Histology	Period after surgery (yr)	Follow up	Therapy	Course
1	Goldsmith <i>et al</i> <sup>[5]</sup> , 1968	48	F	50	Adenocarcinoma	2	+	Surgery	Follow up
2	Szántó <i>et al</i> <sup>[6]</sup> , 1981	65	M	5	Adenomatous polyp	1	-	Polypectomy	Follow up
3	Haerr <i>et al</i> <sup>[7]</sup> , 1987	72	M	NI	Adenocarcinoma	9	+	Radiation chemotherapy	Death
4	Houghton <i>et al</i> <sup>[8]</sup> , 1989	64	M	NI	Adenocarcinoma	20	-	Surgery	Follow up
5	Theile <i>et al</i> <sup>[9]</sup> , 1992	68	M	29	Adenocarcinoma	12	NI	Surgery	Follow up
6	Lee <i>et al</i> <sup>[10]</sup> , 1994	75	F	NI	Adenocarcinoma	20	+	Surgery	Follow up
7	Altörjay <i>et al</i> <sup>[11]</sup> , 1995	NI	M	60	Adenomatoid polyp Carcinoma	6	+	Surgery	Death
8	Kovacs <i>et al</i> <sup>[12]</sup> , 1997	8	M	9 11	Tubular adenoma Tubular adenoma	13	+	Polypectomy	Follow up
9	Altomare <i>et al</i> <sup>[13]</sup> , 2006	64	M	6	Tubular adenoma	7	+	Polypectomy	Follow up
10	Present case, 2008	80	M	25	Adenocarcinoma in tubular adenoma	14	-	ESD	Follow up

ESD: Endoscopic submucosal dissection; NI: No information.



**Figure 1** A laterally-spreading tumor of granular type (LST-G) in the colonic interposition was shown at colonoscopy. Narrow-band imaging with magnification revealed a capillary pattern type II. Magnifying chromoendoscopy using 0.4% indigo carmine revealed a type IV pit pattern. A: Conventional view; B: Narrow-band imaging with magnification; C: Chromoendoscopy with 0.4% indigo carmine; D: Magnifying chromoendoscopy using 0.4% indigo carmine dye spraying.



**Figure 2** Histologically, the resected specimen showed an intramucosal adenocarcinoma in a tubular adenoma. Cross sectional view (HE, magnification  $\times 5$ ).

large, reddish nodule was detected in the lesion. With magnifying narrow-band imaging (NBI) observation, the lesion revealed a capillary pattern type II according to Sano's classification<sup>[1]</sup>, and a type IV pit pattern according to Kudo's classification was detected under magnifying chromoendoscopy using 0.4% Indigo carmine dye

spraying<sup>[2]</sup>. An endoscopic biopsy was taken from the large nodule and a histological diagnosis of moderately differentiated adenocarcinoma was established, however, we diagnosed the lesion as an intramucosal carcinoma based on the above endoscopic findings (Figure 1). Thus, the lesion was considered a good candidate for endoscopic



resection. The lesion was safely and completely removed *en bloc* by ESD using a bipolar knife (B-knife® XEMEX Co. Ltd. Tokyo, Japan)<sup>[3,4]</sup>. Histologically, the lesion was an intramucosal moderately differentiated adenocarcinoma in a tubular adenoma. Lateral and vertical margins of the specimen were negative. There was no lymphatic and venous invasion (Figure 2). The patient was hospitalized for 6 d after ESD to confirm the absence of complications such as delayed perforation, and was then discharged.

## DISCUSSION

Despite the fact that many interposition grafts are performed for malignant esophageal disease, to the best of our knowledge, there have only been 10 reported cases, including four adenomatous polyps and six adenocarcinomas, arising in a colonic interposition (Table 1)<sup>[5-13]</sup>. Because the sizes of the adenomatous polyps in the reported cases were small, they were treated with polypectomy. Reoperation or chemoradiotherapy was performed in patients with cancers. Therefore, this is the first case of an early adenocarcinoma in a colonic interposition resected by ESD.

We performed ESD instead of endoscopic mucosal resection (EMR) in this case, as the lesion was not well-elevated even after submucosal injection of glycerol. This phenomenon is the so-called “non-lifting sign positive” as determined by Uno *et al.*<sup>[14]</sup>. As our endoscopic diagnosis of an intramucosal carcinoma was established with magnifying NBI and chromoendoscopy, submucosal benign fibrosis rather than desmoplastic reaction created by invasive cancer was considered to cause the non-lifting sign positive. EMR for the lesion with the non-lifting sign positive may result in incomplete resection or unfavorable complications such as colonic perforation. During ESD, hyaluronic acid was additionally injected into the submucosal layer and a transparent hood was attached to the tip of the scope for better submucosal dissection<sup>[15]</sup>. To reduce deep burn to the muscle layer, we used a bipolar knife instead of a monopolar knife. To reduce operating time, we used a bipolar snare to remove the lesion after adequate dissection. These efforts enabled us to completely and safely remove the lesion *en bloc* without complication. Furthermore, the patient's colonic interposition was reconstructed using the subcutaneous route, and thus the risk of mediastinitis even if perforation occurred was lower than that if reconstructed substantially.

Despite the fact that many interposition grafts are performed for malignant esophageal disease, few reports of adenocarcinoma arising in a colonic interposition have been reported. It is commonly thought that patients who have esophageal malignancy carry a dismal prognosis, and few of these patients will survive long enough to develop colonic adenocarcinoma. However, with recent progress in chemotherapy, many patients have long-term survival. Almost all case reports presenting with adenoma or adenocarcinoma arise five or more years after colonic interposition surgery, and there are only two case reports where adenoma or adenocarcinoma in the

colonic interposition has arisen 1 or 2 years after surgery (Table 1). In our case, adenocarcinoma in a tubular adenoma was detected 14 years postoperatively. Colonoscopic screening is usually performed before colonic interposition. However, Heresbach *et al.*<sup>[16]</sup> reported an overall miss rate of 23.4% in the colonoscopic detection of neoplasia including both adenomas and colorectal cancers. Therefore, we recommend upper endoscopic screening within 1 year of colonic interposition and periodic surveillance, as lesions may be detected early and removed safely by endoscopy.

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## Actinomycosis of the appendix mimicking appendiceal tumor: A case report

Sang-Yun Lee, Hee-Jin Kwon, Jin-Han Cho, Jong-Young Oh, Kyung-Jin Nam, Jin-Hwa Lee, Seong-Kuk Yoon, Myong-Jin Kang, Jin-Sook Jeong

Sang-Yun Lee, Hee-Jin Kwon, Jin-Han Cho, Jong-Young Oh, Kyung-Jin Nam, Jin-Hwa Lee, Seong-Kuk Yoon, Myong-Jin Kang, Department of Radiology, Dong-A University Hospital, Dongdaesindong 3ga, Seo-gu, Pusan 602-715, South Korea  
 Jin-Sook Jeong, Department of Pathology, Dong-A University Hospital, Dongdaesindong 3ga, Seo-gu, Pusan 602-715, South Korea

**Author contributions:** Lee SY and Kwon HJ contributed equally to the work; Lee SY, Kwon HJ, Cho JH, Yoon SK and Kang MJ designed the research; Lee SY, Kwon HJ and Cho JH performed the research; Kwon HJ, Cho JH, Oh JY, Nam KJ and Lee JH contributed new reagents/analytic tools; Kwon HJ and Jeong JS analyzed the data; Lee SY and Kwon HJ wrote the paper.

**Correspondence to:** Hee-Jin Kwon, MD, Department of Radiology, Dong-A University Hospital, Dongdaesindong 3ga, Seo-gu, Pusan 602-715, South Korea. [risual@dau.ac.kr](mailto:risual@dau.ac.kr)  
 Telephone: +82-51-2405367 Fax: +82-51-2534931

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**Peer reviewer:** Clifford S Cho, MD, Assistant Professor, Department of Surgery, Section of Surgical Oncology, University of Wisconsin School of Medicine and Public Health, H4/724 Clinical Sciences Center, 600 Highland Avenue, Madison, WI 53792, United States

Lee SY, Kwon HJ, Cho JH, Oh JY, Nam KJ, Lee JH, Yoon SK, Kang MJ, Jeong JS. Actinomycosis of the appendix mimicking appendiceal tumor: A case report. *World J Gastroenterol* 2010; 16(3): 395-397 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i3/395.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i3.395>

### INTRODUCTION

Actinomycosis is a chronic progressive suppurative disease caused by *Actinomyces israelii*, and is characterized by the formation of multiple abscesses, draining sinus, abundant granulation, and dense fibrous tissue<sup>[1]</sup>. Several reports have described the radiological findings of abdominopelvic actinomycosis. The infiltrative mass with unusual aggressiveness is the one of important radiological findings<sup>[1-3]</sup>. Also, some reports of appendiceal actinomycosis have described wall thickening and peri-appendiceal inflammation, with contrast enhancement<sup>[4,5]</sup>. We experienced pathologically confirmed appendiceal actinomycosis that presented as a small intraluminal mass without peri-appendiceal infiltration. Here, we report the computed tomography (CT) and ultrasound (US) findings.

### CASE REPORT

A 50-year-old woman was found incidentally to have a small appendiceal mass during routine screening. Past clinical history, physical examination, and laboratory examination were all unremarkable. Contrast-enhanced abdominal CT showed a well-defined small mass at the origin of the appendix. The length of the mass on CT

### Abstract

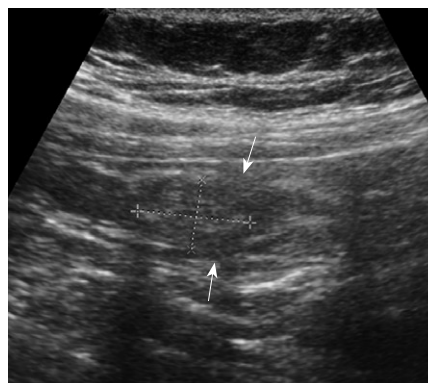
Actinomycosis is an uncommon chronic infectious disease. Common sites of involvement include the cervicofacial, thoracic and abdominopelvic regions. In abdominopelvic actinomycosis, the ileocecal region, including the appendix, is the most commonly involved site. In some reports, limited appendiceal actinomycosis has revealed a thickened appendiceal wall with peri-appendiceal inflammation as acute appendicitis or perforated appendicitis. We experienced pathologically confirmed intraluminal limited appendiceal actinomycosis without peri-appendiceal infiltration. Here, we report the computed tomography and ultrasound findings.

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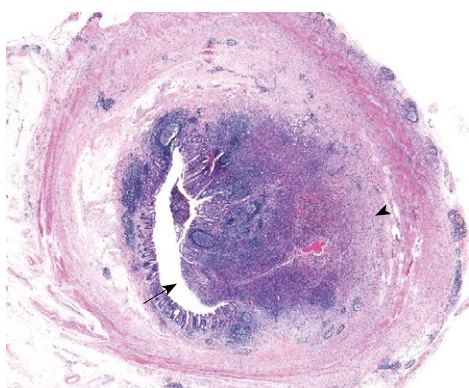
**Key words:** Actinomyces; Actinomycosis; Appendiceal neoplasms; Appendicitis



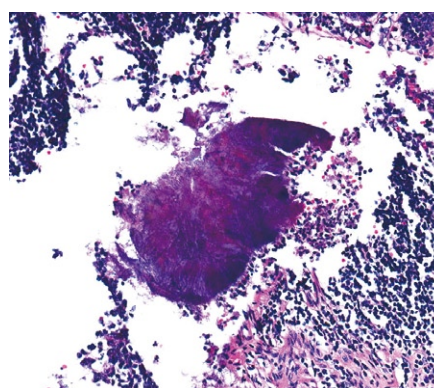
**Figure 1** Contrast-enhanced CT revealed a well-defined solid mass with strong enhancement in the base of the appendix (arrow). Peri-appendiceal infiltration was not seen.



**Figure 2** US showed a heterogeneous, hyperechoic, intraluminal mass at the base of the appendix, without peri-appendiceal infiltration. We also noted focal defects at the echogenic inner mucosal layer (arrows).



**Figure 3** Microscopy of appendiceal actinomycosis. An abscess composed of chronic and acute inflammatory cells was observed in a mass-like lesion (arrow), from the mucosal surface to the superficial submucosa (arrowhead) (HE,  $\times 10$ ).



**Figure 4** Higher magnification showed a typical sulfur granule surrounded by neutrophils in the clefted abscess center (HE,  $\times 200$ ).

images was 2 cm. This mass showed dense enhancement of 100 HU at the arterial phase and 125 HU at the portal phase. Infiltration around the meso-appendix and peri-appendiceal area was not demonstrated by CT, nor was regional lymph node enlargement detected (Figure 1). Positron emission tomography (PET) with [F18]-2-fluoro-2-deoxy-D-glucose (FDG) showed a high metabolic rate, with a maximum standardized uptake value (SUV) of 3.64.

US images obtained with a 9.4-MHz linear probe showed a well-defined intraluminal mass with heterogeneous echogenicity in the base of the appendix. The diameter of the mass was 1.6 cm. Peri-appendiceal infiltration was not demonstrated by US. US revealed focal defects at the echogenic inner mucosal layer. However, relatively well preserved mural echogenicity was noted (Figure 2).

From these findings, appendiceal neoplasms of mucosal origin, such as mucinous adenocarcinoma and carcinoid, were diagnosed, and a laparoscopic partial cecectomy was performed. Surgical findings revealed an intraluminally growing small mass with no sign of macroscopic serosal invasion. Also, there was no sign of infiltration or invasion of the meso-appendix and other surrounding organs.

Gross pathology revealed an ill-defined, yellowish

white, protruding mass lesion, which originated from the basal portion of the appendix. Adjacent appendiceal and cecal wall showed edema, and the serosal surface was relatively clear. Upon microscopy, the appendix showed a localized, mass-like abscess formation of the appendiceal wall (Figure 3). With higher magnification, a typical sulfur granule surrounded by neutrophils was found (Figure 4), which was confirmed as appendiceal actinomycosis. At the submucosa, the outer portion of the abscess showed evidence of organization, with vascularized granulation tissue and fibrosis in addition to chronic inflammatory cell infiltration.

## DISCUSSION

Actinomycosis has a worldwide distribution and is found with equal frequency in urban and rural dwellers. In humans, *Actinomyces israelii* is the most common cause of the disease. These organisms are indigenous in the oral cavity, gastrointestinal tract, and genital tract, with opportunistic infection occurring when the mucosal barrier is broken, which leads to multiple abscess formation, fistula, or mass lesions<sup>[6,7]</sup>. Actinomycosis commonly occurs in three distinct forms that may occasionally overlap; most clinical disease is cervicofacial (55%), with only 20% occurring in the abdominopelvic



form and 15% in the thoracic form<sup>[8]</sup>. Although the clinical features depend on which organs are involved, common symptoms and signs include fever and leukocytosis<sup>[3,6]</sup>.

Abdominopelvic actinomycosis is associated with abdominal surgery (such as appendectomy), bowel perforation, or trauma<sup>[9]</sup>. In addition, the presence of a long-standing intrauterine device (IUD) is a reported risk factor in young women<sup>[10]</sup>. Although the pathogenesis of abdominopelvic actinomycosis is not well understood, the appendix is the most commonly involved intra-abdominal organ, the colon, stomach, liver, gallbladder, pancreas, small intestine, pelvis, and abdominal wall may also be involved<sup>[6]</sup>. However, development of abdominopelvic actinomycosis after acute appendicitis has decreased because of early diagnosis, a lower incidence of perforated appendicitis, and improved antibiotic therapy<sup>[8]</sup>.

As a result of its resemblance to other diseases such as appendicitis, diverticulitis, colon carcinoma, Crohn's disease, ulcerative colitis, and tubo-ovarian abscess, the diagnosis of abdominopelvic actinomycosis is difficult<sup>[11]</sup>. A definite diagnosis is generally based on histological identification of actinomycotic granules or culture of the *Actinomyces* species, or both. High-dose intravenous penicillin injection is the treatment of choice and has a favorable response<sup>[11]</sup>. Therefore, early diagnosis is important to minimize morbidity of the disease and prevent unnecessary surgery.

Direct spread into adjacent tissue is the most common primary route of propagation after penetration of the organism through the mucosal barrier. Therefore, infiltration has been well described as an important radiological characteristic<sup>[1,3]</sup>. CT is an important imaging modality for suggesting the diagnosis and determining the anatomical location and extent of the disease, as well as monitoring the effectiveness of treatment<sup>[2,3,6]</sup>. Important CT features are an infiltrative mass (predominantly cystic or solid) adjacent to the other involved organs or anatomical structures, and the main CT feature when the gastrointestinal tract is involved is bowel wall thickening<sup>[1-3]</sup>. After infusion of contrast material, dense contrast enhancement in the mass or involved bowel has been reported, which may be caused by abundant granulation and dense fibrous tissue<sup>[1,3,10]</sup>. Although these findings are nonspecific, actinomycosis should be included in the differential diagnosis, especially in patients with abdominal pain, fever, leukocytosis, or

long-term IUD use<sup>[6,9]</sup>. CT features of abdominopelvic actinomycosis closely resemble complicated gastrointestinal malignancy or other chronic inflammatory disease (such as intestinal tuberculosis or Crohn's disease). However, because of the size of the bacterium, it usually does not spread via the lymphatic system; therefore, regional lymphadenopathy is uncommon or develops late<sup>[8,9]</sup>.

In our case, appendiceal actinomycosis showed dense contrast enhancement, especially at the portal phase, and vascularized granulation tissue with fibrosis in the submucosa. There was no sign of regional lymphadenopathy. However, appendiceal actinomycosis presented as an intraluminal limited mass without peri-appendiceal infiltration, which is an unusual finding. A review of the literature about abdominopelvic actinomycosis did not reveal any similar cases.

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## Cerebral lipiodol embolism following transcatheter arterial chemoembolization for hepatocellular carcinoma

Lu Wu, Ye-Fa Yang, Jun Liang, Shu-Qun Shen, Nai-Jian Ge, Meng-Chao Wu

Lu Wu, Ye-Fa Yang, Jun Liang, Shu-Qun Shen, Nai-Jian Ge, Meng-Chao Wu, The First Department of Interventional Radiology, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200438, China

Author contributions: Wu L, Yang YF, Liang J, Shen SQ and Ge NJ collected the data and performed the research; Wu L wrote the manuscript; Yang YF and Wu MC revised the article.

Correspondence to: Dr. Ye-Fa Yang, The First Department of Interventional Radiology, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200438, China. [electchina@hotmail.com](mailto:electchina@hotmail.com)

Telephone: +86-21-81875323 Fax: +86-21-65562400

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### INTRODUCTION

Transcatheter arterial chemoembolization (TACE) is utilized worldwide in the treatment of patients with unresectable hepatocellular carcinoma (HCC). The documented complications of TACE include post-embolization syndrome, septicemia, acute hepatic failure, liver infarction or abscess, intrahepatic biloma, embolization of extrahepatic organs, pseudoaneurysm formation, cholecystitis, tumor rupture, splenic infarction, gastritis, duodenitis, gastroduodenal ulceration, variceal bleeding, and iatrogenic dissection<sup>[1-5]</sup>. Cerebral lipiodol embolism (CLE) is a rare complication of TACE. The authors report a case of CLE that occurred after the second TACE, and present its clinical and imaging findings, as well as a review of the literature.

### Abstract

Cerebral lipiodol embolism (CLE) is an extremely rare complication of transcatheter arterial chemoembolization for hepatocellular carcinoma (HCC). The authors present a case of CLE that occurred after the second hepatic arterial chemoembolization for HCC, and attempt to introduce several plausible mechanisms of CLE, after reporting the clinical and radiological findings and reviewing the medical literature.

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**Key words:** Intracranial embolism; Lipiodol; Chemo-therapeutic embolization; Hepatocellular carcinoma

**Peer reviewer:** De-Liang Fu, MD, PhD, Professor, Department of Surgery, Pancreatic Disease Institute, Fudan University, 12 Wulumqi Road (M), Shanghai 200040, China

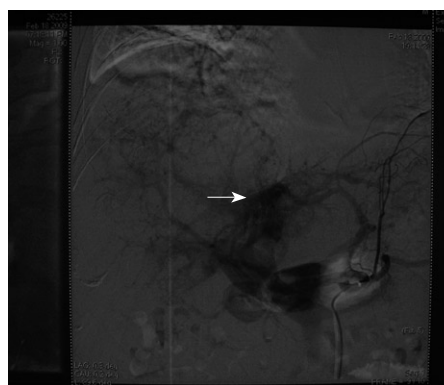
Wu L, Yang YF, Liang J, Shen SQ, Ge NJ, Wu MC. Cerebral lipiodol embolism following transcatheter arterial chemoembolization for hepatocellular carcinoma. *World J Gastroenterol* 2010; 16(3): 398-402 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i3/398.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i3.398>

### CASE REPORT

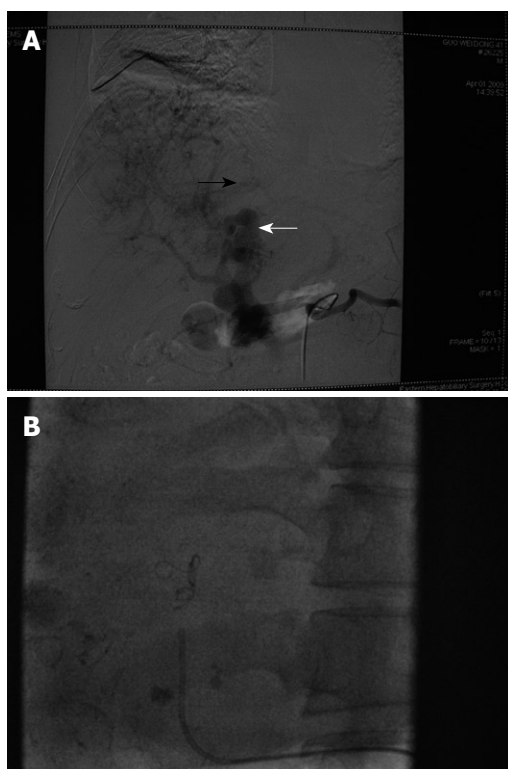
A 41-year-old man with multiple HCC accompanied by portal vein invasion (Figures 1 and 2) underwent a second course of TACE via the proper hepatic artery by using a mixture of 30 mL lipiodol, 10 mg hydroxyl camptothecin and 40 mg pirarubicin, as well as gelatin sponge particles (1400-2000  $\mu$ m) and coils (5 mm  $\times$  8 mm) (Figure 3). The tumor marker test showed 136.9  $\mu$ g/L alpha fetoprotein (AFP), 0.7  $\mu$ g/L carcinoembryonic antigen (CEA), and of 28.2  $\mu$ g/mL carbohydrate antigen 19-9 (CA19-9). The liver function was ranked A in Child-Pugh classification. During the procedure, the patient experienced cough, visual loss, headache and motor weakness of the left upper limb and left lower limb. Upon physical examination, blood pressure was 158/93 mmHg, and pulse rate, respiratory rate and body temperature were normal. The muscle strength of the left upper limb was III+, while the left lower limb was IV+. No neurological pathological reflex was observed. The peripheral oxygen saturation was 94%, and with a nasal catheter, the arterial oxygen saturation was elevated and maintained higher than 97%. The patient was given neurotrophins, cerebrovascular dilators and supportive therapy immediately. Functional exercise was added in the following days.



**Figure 1** Computed tomography (CT) scan obtained before the first Transcatheter arterial chemoembolization (TACE) procedure. Portal vein was shown during the arterial period, which suggested that the tumor embolus invaded the portal vein (arrow).

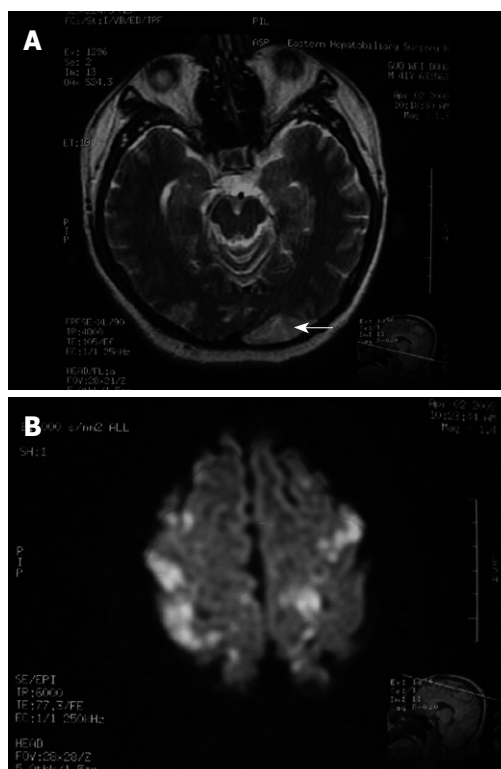


**Figure 2** First TACE procedure. Arterial-portal fistula (arrow).



**Figure 3** Second TACE procedure. A: Hepatic arteriovenous fistula (black arrow), arterial-portal fistula (white arrow); B: Coils (5-8 mm).

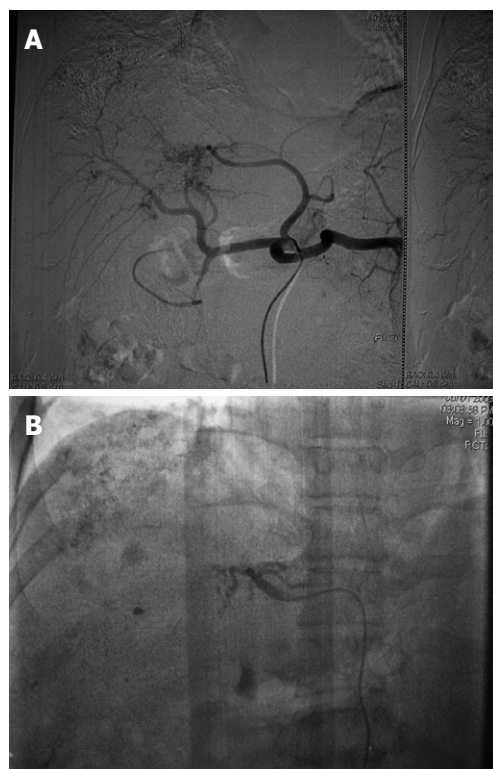
The laboratory data after TACE revealed a white blood cell count of  $13.51 \times 10^9/L$  (normal:  $4 \times 10^9$  to  $10 \times 10^9/L$ ), platelet count of  $83 \times 10^9/L$  (normal:  $100 \times 10^9$  to  $300 \times 10^9/L$ ), total bilirubin of  $28.6 \mu\text{mol/L}$ , direct bilirubin of  $11.0 \mu\text{mol/L}$ , alanine aminotransferase of  $187.4 \text{ U/L}$ , aspartate aminotransferase of  $429.7 \text{ U/L}$ , and normal renal function. Consultation with a neurologist suggested a diagnosis of cerebral embolism induced by the introduction of iodized oil. Magnetic resonance imaging (MRI) was performed 19 h after the TACE procedure, which revealed multiple abnormal signals that indicated ischemic foci of the centrum semiovale, and both parietal and occipital lobes (Figure 4). On the 10th day, with the



**Figure 4** Cranial magnetic resonance imaging (MRI) obtained after the second TACE procedure. A: cerebral lipiodol embolism (arrow); B: cerebral lipiodol embolism (multiple high signals).

appearance of melena, erythrocyte suspension and plasma were transfused along with hemostatic agents. Melena disappeared 2 d later. Vision and muscle strength of the affected extremities also improved gradually. Six weeks after CLE, the patient completely recovered without any neurological sequelae.

Two months after the second TACE procedure, the patient came back for follow-up. The tumor markers were tested: AFP  $11.0 \mu\text{g/L}$ , CEA  $0.8 \mu\text{g/L}$ , CA-19-9  $12.5 \text{ U/mL}$ , and digital subtraction angiography (DSA) revealed that an arterial-portal fistula was still present but improved, meanwhile, hepatic arteriovenous fistula was not seen. Therefore, we performed a third course of TACE. A mixture of 8 mL lipiodol, 10 mg hydroxyl



**Figure 5 Third TACE procedure.** A: Hepatic arteriovenous fistula was not seen, and the arterial-portal fistula was still present but improved; B: Lipiodol and gelatin sponge were injected through the left gastric artery and the arterial-portal fistula was embolized.

camptothecin and 40 mg pirarubicin, as well as gelatin sponge particles (760-1000  $\mu\text{m}$ ) was injected through the right hepatic artery, left hepatic artery and left gastric artery (Figure 5). The patient did not have any respiratory or neurological complaints and was discharged 2 d later, and was confirmed to be in good condition until the present follow-up.

## DISCUSSION

CLE is an extremely rare complication of the invasive procedure TACE<sup>[1-3,5]</sup>. Only 11 papers have reported 16 cases of lipiodol-associated embolic brain damage following TACE. In China, the first case report of CLE was by Li *et al*<sup>[6]</sup> in 2001, while the first overseas was by Yoo *et al*<sup>[11]</sup> in 2004.

The symptoms of CLE are nonspecific, including visual loss, headache, motor weakness, and change of mental status. These symptoms vary in severity according to the site of iodized oil deposition. In addition, if the lipiodol enters the lungs as well, the patient complains of chest pain and dyspnea<sup>[7]</sup>. Actually, among the twelve prior cases presented, 10 were confirmed with pulmonary embolism after initial diagnosis of respiratory manifestations. Most CLE occurs during or immediately after TACE and is identified initially by one or more symptoms mentioned above, however, a delayed type of CLE also has been reported<sup>[8]</sup>. Although some of the patients who suffered from CLE after TACE die, despite

**Table 1 Analysis of 13 cases of CLE following TACE ( $n = 12$ )** [1-3,5-8,11,13,19]

	<i>n</i>
Sex	
Male	8
Female	4
Age (yr)	
≥ 50	10
< 50	2
Course of TACE	
First	3
Second	6
Third	2
More than third	1
Doses of lipiodol (mL)	
≥ 20	6
< 20	3
ND	3
Gelatin sponge particles	
Yes	5
No	7
Embolization through extra-hepatic collateral artery	
Yes	6
No	4
ND	2
Tumor site including right lobe	
Yes	8
No	0
ND	4
Size of tumor/invasion of diaphragm	
Large/multiple	9
Minor	1
ND	2
Vascular invasion	
Yes	3
No	2
ND	7
Arteriovenous fistula	
Yes	2
No	5
ND	5
Right-to-left shunt	
Yes	0
No	6
ND	6
Pulmonary embolism	
Yes	10
No	1
ND	1
Time of neurologic symptoms	
During or shortly after TACE	11
69 h after TACE	1
Recovery time (All ≤ 6 wk)	
≥ 4 wk	3
< 4 wk	5
ND	1
Death	3

CLE: Cerebral lipiodol embolism; TACE: Transcatheter arterial chemo-embolization; ND: Not described.

positive interventions, most of them recover completely without any neurological sequelae<sup>[5]</sup> (Table 1). In our case, the patient developed CLE during the TACE procedure, and after 6 wk of supportive therapy, he recovered, leaving no neurological symptoms.

The radiological findings of CLE on computed tomography (CT) and MRI in the previously reported

cases are similar. The site of lipiodol deposition includes the basal ganglia, thalamus, gray-white matter junction, and both parietal and frontal cortices. Cranial CT/MRI taken after the neurological symptoms disappeared is usually clear, which infers that the lipiodol in the brain could have been cleared entirely<sup>[5,8]</sup>. In our case, the sites of iodized oil deposition were mainly on the centrum semiovale, and both parietal and occipital lobes.

Ten papers covering 12 cases (the present case included) have described in detail the clinical and radiological data of CLE following TACE, and have been selected to make an analysis (Table 1) (in fact, 12 papers in total were searched, and because Zhao *et al*<sup>[9]</sup> and Li *et al*<sup>[10]</sup> provided no detailed information, their two cases were excluded). In six TACE procedures that were complicated by CLE, lipiodol was infused through extra-hepatic collateral arteries, and mostly the inferior phrenic arteries. In all 12 cases, only eight of them described the tumor sites, all of which involved the right liver lobes. This implies that invasion of the diaphragm is probably common in patients with CLE after TACE, although it was confirmed only in three cases<sup>[2,5,11]</sup>.

Six authors have analyzed the mechanisms of CLE following TACE treatment of HCC. Wu *et al*<sup>[3]</sup> have shown that the most probable cause of lipiodol-induced brain embolism after TACE is a combination of a right-to-left shunt and the dose-dependent effect of the drug<sup>[3,12]</sup>. The communication between the systemic and pulmonary vessels might develop via adhesive pleurae or tumor invasion of the diaphragm, thus a right-left shunt is formed. When injecting iodized oil via the inferior phrenic artery, some oil droplets might enter the brain, thus bypassing the right-left shunt. Combined with an increased dose of lipiodol during the second course of TACE, the patient has a greater risk of CLE<sup>[3]</sup>. Wu *et al*<sup>[11]</sup> have added that an intra-pulmonary arteriovenous shunt might appear during the pulmonary lipiodol embolism because of increasing pulmonary artery pressure or hypoxia<sup>[1,11]</sup>. Cui *et al*<sup>[13]</sup> have observed that the contrast medium injected into the hepatic artery enters the pulmonary veins directly during angiography in patients with CLE after TACE. This phenomenon supports the mechanism of intra-pulmonary arteriovenous shunting suggested by Wu *et al*<sup>[3]</sup>. Choi *et al*<sup>[7]</sup> have performed echocardiography and DSA to exclude intra-cardiac and intra-tumoral shunts, to support the mechanism that the communication between the inferior phrenic artery and pulmonary vessels occurs via adherent pleura, and tumor recurrence causes the lipiodol-induced brain embolism after TACE. Li *et al*<sup>[6]</sup> and Matsumoto *et al*<sup>[5]</sup> have made similar speculation.

Yoo *et al*<sup>[1]</sup> have presented three cases of CLE following TACE; all of which had evidence of pulmonary involvement but without a demonstrable intra-cardiac shunt. Yoo *et al*<sup>[1]</sup> have speculated that since it has been verified that fat globules < 7  $\mu\text{m}$  in diameter can pass directly through the pulmonary arteriolar network (i.e. trans-pulmonary shunt) and cause cerebral injury<sup>[14]</sup>, the presence of an intra-cardiac right-to-left shunt might not be necessary<sup>[1]</sup>.

In another case reported by Wu *et al*<sup>[8]</sup>, pulmonary and cerebral embolism occurred 34 and 69 h, respectively, after TACE treatment of HCC. Wu *et al*<sup>[8]</sup> have concluded that the rapid-flow, tumor-feeding artery washes out the iodized oil, which leads to embolic damage of the lungs. Then, the lipiodol that is deposited in the lungs is washed out again and enters the systemic circulation, thus causing embolism of the brain.

In the present case, the most probable mechanism of pulmonary and cerebral embolism is attributed to a hepatic venous-arterial shunt and an intra-pulmonary or intra-cardiac shunt. Lipiodol entered the pulmonary circulation through a hepatic arteriovenous fistula (Figure 3), which caused pulmonary embolism that increased pulmonary artery pressure, and produced a temporary pulmonary arteriovenous fistula. Lipiodol entered the brain through this temporary fistula. In addition, the use of a large dose of lipiodol also promotes CLE.

It is well known that CLE after TACE might be associated with intra-cardiac shunt, intra-pulmonary shunt and infusion of large doses of lipiodol<sup>[7]</sup>. Intra-cardiac right-to-left shunt can occur in some congenital heart diseases, such as patent foramen ovale. Research has revealed that among 1100 non-selected autopsies, 386 cases showed patent foramen ovale and 83% of them were < 0.2 cm in diameter<sup>[15]</sup>. The small foramen ovale, being often undetectable on routine echocardiography, might allow transient right-to-left shunting when the right heart pressure increases following oil trapping in the lungs<sup>[3]</sup>. Pulmonary arteriovenous shunts usually are associated with congenital pulmonary vascular malformations, acute or chronic pulmonary diseases, pulmonary tumors and advanced liver diseases<sup>[3,16]</sup>. The communication that can develop between the inferior phrenic artery and the pulmonary vessels also suggests a possible route for right-to-left shunting<sup>[4]</sup> (Table 1). Large dose of lipiodol infusion might increase the risk of extra-hepatic embolism. Some authors have suggested that a lipiodol dose less than 15 or 20 mL can prevent ectopic embolism<sup>[17,18]</sup>. However, in most prior cases of CLE, including the one described in this study, the HCCs were single large or multiple tumors (Table 1). As a result, limiting the dose of lipiodol to < 20 or 15 mL routinely might not be reasonable, especially when there is insufficient evidence to confirm that a large dose of lipiodol is a determinant factor for CLE following TACE. Three previous cases have revealed that CLE also occurs when the lipiodol dose is < 15 mL<sup>[1,3,5]</sup>. Therefore, all procedures must be individualized.

It is not necessary to search routinely for shunts prior to TACE, especially when right-to-left shunting is often undetectable in routine examination, as in chest or abdominal CT, DSA, and echocardiography<sup>[7]</sup>. In the present case, the patient was subjected to echocardiography and no positive evidence of right-to-left shunting was revealed. The patient had a history of bleeding gastroesophageal varices, therefore, transesophageal echocardiography was not performed. Direct evidence of a shunt has not yet been reported in previous studies (Table 1). Kim *et al*<sup>[19]</sup> have presented one case of CLE



following TACE during which the presence of a right-to-left shunt (was demonstrated by the presence of microbubbles in the left middle cerebral artery and left atrium, while trans-cranial Doppler and transesophageal echocardiography were performed during the intravenous injection of agitated saline.

The mechanism of CLE following TACE has not yet been elucidated. Intracardiac or intrapulmonary right-to-left shunts and infusion of large doses of lipiodol might contribute to the increased risk of CLE following TACE. An individualized plan of therapy, including lipiodol dose determination, shunting detection, as well as selecting vessels for the lipiodol infusion prior to TACE is of great importance to achieve an efficient overall result<sup>[11]</sup>.

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**Luigi E Adinolfi, Professor**, Division of Internal Medicine & Hepatology, Seconda Università di Napoli, Facoltà di Medicina e Chirurgia, Via Cotugno, 1 (c/o Ospedale Gesù e Maria), 80135 Naples, Italy

**Meenakshisundaram Ananthanarayanan, Associated Professor**, Department of Pediatrics, Annenberg Bldg, Rm.14-24A, Box 1664, The Mount Sinai Medical Center, One Gustave L. Levy Place, New York, NY 10029, United States

**Andrew Seng Boon Chua, MD**, Department of Gastroenterology, Gastro Centre Ipoh, 1, lorong Rani, 31, lebuhraya Tmn Ipoh, Ipoh Garden South, IPOH 30350, Malaysia

**Massimo Colombo, Professor**, 1st Division of Gastroenterology, Fondazione IRCCS Maggiore Hospital, Policlinico, Mangiagalli e Regina Elena, University of Milan, Via F. Sforza 35, 20122 Milan, Italy

**Paolo Del Poggio, Dr.**, Hepatology Unit, Department of Internal Medicine, Treviglio Hospital, Piazza Ospedale 1, Treviglio Bg 24047, Italy

**Kevin Cheng-Wen Hsiao, MD, Assistant Professor**, Colon and rectal surgery, Tri-Service General Hospital, No. 325, Sec. 2, Cheng-Kung Rd, Nei-Hu district, 114 Taipei, Taiwan, China

**Robert J Korst, MD**, Department of Cardiothoracic Surgery, Weill Medical College of Cornell University, Room M404, 525 East 68th Street, New York, NY 10032, United States

**Shiu-Ming Kuo, MD**, University at Buffalo, 15 Farber Hall, 3435 Main Street, Buffalo, NY 14214, United States

**Sara Lindén, Dr., PhD, Assistant Professor**, Mucosal immunobiology and Vaccine Center, Gothenburg University, Box 435, Göteborg, 40530, Sweden

**Giulio Marchesini, Professor**, Department of Internal Medicine and Gastroenterology, "Alma Mater Studiorum" University of Bologna, Policlinico S. Orsola, Via Massarenti 9, Bologna 40138, Italy

**Kenji Miki, MD**, Department of Surgery, Showa General Hospital, 2-450 Tenjin-cho, Kodaira, Tokyo 187-8510, Japan

**Bernardino Rampone, Dr.**, Department of General Surgery and Surgical Oncology, University of Siena, viale Bracci, Siena 53100, Italy

**Vasiliy I Reshetnyak, MD, PhD, Professor, Scientist Secretary** of the Scientific Research Institute of General Reanimatology, 25-2, Petrovka str., 107031, Moscow, Russia

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

**Norbert Senninger, Professor**, Department of General Surgery, University Clinics, Westphalian-Wilhelm's-University, Waldeyerstrasse 1, D-48149 Muenster, Germany

**Jon Arne Søreide, Professor, MD, PhD, FACS**, Department of Surgery, Stavanger University Hospital, N-4068 Stavanger, Norway

**Sumita Verma, Dr., Senior Lecturer** Medicine (Hepatology), Brighton and Sussex Medical School, Medical School Building, University of Sussex, Falmer, Brighton, BN1 9PX, United Kingdom

**Satoshi Yamagiwa, MD, PhD**, Division of Gastroenterology and Hepatology, Niigata University Graduate School of Medical and Dental Sciences, 757 Asahimachi-dori, Chuo-ku, Niigata 951-8510, Japan



## Meetings

### Events Calendar 2010

January 25-26  
Tamilnadu, India  
International Conference on Medical  
Negligence and Litigation in Medical  
Practice

January 25-29  
Waikoloa, HI, United States  
Selected Topics in Internal Medicine

January 26-27  
Dubai, United Arab Emirates  
2nd Middle East Gastroenterology  
Conference

January 28-30  
Hong Kong, China  
The 1st International Congress on  
Abdominal Obesity

February 11-13  
Fort Lauderdale, FL, United States  
21th Annual International Colorectal  
Disease Symposium

February 26-28  
Carolina, United States  
First Symposium of GI Oncology at  
The Caribbean

March 04-06  
Bethesda, MD, United States  
8th International Symposium on  
Targeted Anticancer Therapies

March 05-07  
Peshawar, Pakistan  
26th Pakistan Society of  
Gastroenterology & Endoscopy  
Meeting

March 09-12  
Brussels, Belgium  
30th International Symposium on  
Intensive Care and Emergency  
Medicine

March 12-14  
Bhubaneswar, India  
18th Annual Meeting of Indian  
National Association for Study of  
the Liver

March 23-26  
Cairo, Egypt  
14th Pan Arab Conference on  
Diabetes PACD14

March 25-28  
Beijing, China  
The 20th Conference of the Asian

Pacific Association for the Study of  
the Liver

March 27-28  
San Diego, California, United States  
25th Annual New Treatments in  
Chronic Liver Disease

April 07-09  
Dubai, United Arab Emirates  
The 6th Emirates Gastroenterology  
and Hepatology Conference, EGHG  
2010

April 14-17  
Landover, Maryland, United States  
12th World Congress of Endoscopic  
Surgery

April 14-18  
Vienna, Austria  
The International Liver Congress™  
2010

April 28-May 01  
Dubrovnik, Croatia  
3rd Central European Congress  
of surgery and the 5th Croatian  
Congress of Surgery

May 01-05  
New Orleans, LA, United States  
Digestive Disease Week Annual  
Meeting

May 06-08  
Munich, Germany  
The Power of Programming:  
International Conference on  
Developmental Origins of Health  
and Disease

May 15-19  
Minneapolis, MN, United States  
American Society of Colon and  
Rectal Surgeons Annual Meeting

June 04-06  
Chicago, IL, United States  
American Society of Clinical  
Oncologists Annual Meeting

June 09-12  
Singapore, Singapore  
13th International Conference on  
Emergency Medicine

June 14  
Kosice, Slovakia  
Gastro-intestinal Models in  
the Research of Probiotics and  
Prebiotics-Scientific Symposium

June 16-19  
Hong Kong, China  
ILTS: International Liver  
Transplantation Society ILTS Annual  
International Congress

June 20-23  
Mannheim, Germany  
16th World Congress for  
Bronchoesophagology-WCBE

June 25-29  
Orlando, FL, United States  
70th ADA Diabetes Scientific  
Sessions

August 28-31  
Boston, Massachusetts, United States  
10th OESO World Congress on  
Diseases of the Oesophagus 2010

September 10-12  
Montreal, Canada  
International Liver Association's  
Fourth Annual Conference

September 11-12  
La Jolla, CA, United States  
New Advances in Inflammatory  
Bowel Disease

September 12-15  
Boston, MA, United States  
ICAAC: Interscience Conference  
on Antimicrobial Agents and  
Chemotherapy Annual Meeting

September 16-18  
Prague, Czech Republic  
Prague Hepatology Meeting 2010

September 23-26  
Prague, Czech Republic  
The 1st World Congress on  
Controversies in Gastroenterology &  
Liver Diseases

October 07-09  
Belgrade, Serbia  
The 7th Biannual International  
Symposium of Society of  
Coloproctology

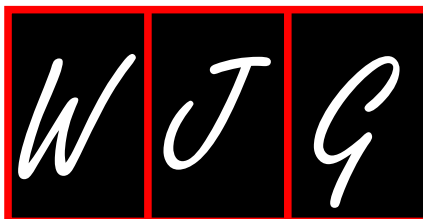
October 15-20  
San Antonio, TX, United States  
ACG 2010: American College of  
Gastroenterology Annual Scientific  
Meeting

October 23-27  
Barcelona, Spain  
18th United European  
Gastroenterology Week

October 29-November 02  
Boston, Massachusetts, United States  
The Liver Meeting® 2010--AASLD's  
61st Annual Meeting

November 13-14  
San Francisco, CA, United States  
Case-Based Approach to the  
Management of Inflammatory Bowel  
Disease

December 02-04  
San Francisco, CA, United States  
The Medical Management of HIV/  
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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

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- 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]

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 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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- 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. *HRSA 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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- 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. Wiczorek 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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- 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>

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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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Editorial Board of *World Journal of Gastroenterology*, Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China  
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## Probiotics and gut health: A special focus on liver diseases

Silvia Wilson Gratz, Hannu Mykkanen, Hani S El-Nezami

Silvia Wilson Gratz, Rowett Institute of Nutrition and Health, University of Aberdeen, Greenburn Road, Aberdeen AB21 9SN, United Kingdom

Hannu Mykkanen, School of Public Health and Clinical Nutrition, University of Kuopio, PO Box 1627, 70211 Kuopio, Finland

Hani S El-Nezami, Food and Health Research Centre, University of Kuopio, PO Box 1627, 70211 Kuopio, Finland; School of Biological Sciences, University of Hong Kong, S5-13 Kadoorie Biological Sciences Building, Pokfulam, Hong Kong, China

Author contributions: Gratz SW, Mykkanen H and El-Nezami HS wrote the article.

Correspondence to: Hani S El-Nezami, Professor, School of Biological Sciences, University of Hong Kong, S5-13 Kadoorie Biological Sciences Building, Pokfulam, Hong Kong, China. [elnezami@hku.hk](mailto:elnezami@hku.hk)

Telephone: +852-22990835 Fax: +852-22990364

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### Abstract

Probiotic bacteria have well-established beneficial effects in the management of diarrhoeal diseases. Newer evidence suggests that probiotics have the potential to reduce the risk of developing inflammatory bowel diseases and intestinal bacterial overgrowth after gut surgery. In liver health, the main benefits of probiotics might occur through preventing the production and/or uptake of lipopolysaccharides in the gut, and therefore reducing levels of low-grade inflammation. Specific immune stimulation by probiotics through processes involving dendritic cells might also be beneficial to the host immunological status and help prevent pathogen translocation. Hepatic fat metabolism also seems to be influenced by the presence of commensal bacteria, and potentially by probiotics; although the mechanisms by which probiotic might act on the liver are still unclear. However, this might be of major importance in the future because low-grade inflammation, hepatic fat infiltration, and hepatitis might become more prevalent as a result of high fat intake and the increased prevalence of obesity.

### INTRODUCTION

Probiotics have been well defined and long used in human and animal health and nutrition. Many of the probiotic strains used today have been isolated from the human gut flora, and it is therefore more a reintroduction of organisms rather than a novel concept. The beneficial effects of probiotics and, especially, the clinical use of probiotics in the management of specific diarrhoeal diseases, including Rotavirus diarrhoea, Traveller's diarrhoea and others are well accepted<sup>[1]</sup>. These effects are mainly based on colonisation resistance or the influence of probiotics on microflora balance. When discussing probiotics, it must be remembered that the intestinal microflora, resident in the large intestine, will always outnumber the probiotics that can be administered. Furthermore, probiotic processes will always be confounded by the diversity of the human microbiota and its variability in the face of varied human diets and genetic backgrounds<sup>[2]</sup>.

Within the human gut, we have to separate processes occurring in the distal small intestine from those happening in the colon. The small intestine harbours relatively low numbers of resident intestinal bacteria,

but at the same time contains the major part of the gut associated lymphoid tissue (GALT), which samples intestinal microbes<sup>[3]</sup>. Hence modulation of systemic immune and allergic phenomena might be primarily mediated by the GALT of the small bowel. Supplementing qualitatively and quantitatively optimised microbes to this part of the gut might stimulate Treg cell development and consequent immunomodulation<sup>[1]</sup>. Within the large intestine, bacterial intervention might cause modulation of the microbial fermentation activity and direct action on the colonic epithelium to alter (suppress) innate immunity. These processes might explain the impact of probiotics on inflammatory and functional bowel disorders.

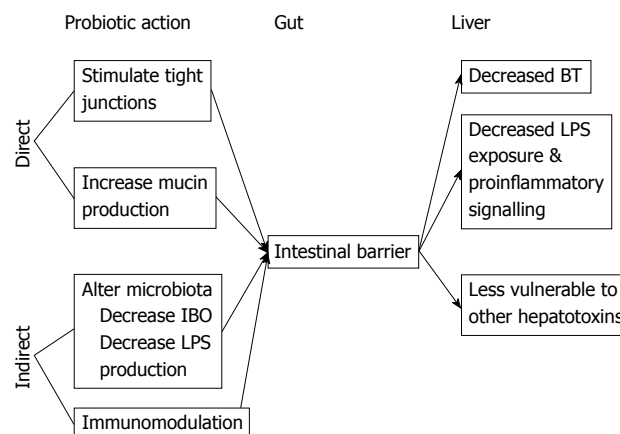
The research field of probiotics is very heavily reviewed, using different clinical and microbiological angles to elucidate the topic. The aim of this review is to summarise the most recent trends in probiotic research, focusing on the clinical use of probiotics and their effects on the healthy and diseased gut and liver.

The direct and indirect actions of probiotics on intestinal cells and their consequences on liver (summarised in Figure 1) will be discussed in this review.

## GUT HEALTH

### Probiotics and intestinal barrier function

The intestinal barrier is seen as the first line of defence against pathogens and food allergens entering the intestinal tract, and probiotics have been intensely studied for their involvement in maintaining this barrier. In colonic epithelia, probiotics are suggested to stimulate mucin production, and therefore enhance the self protecting properties of the intestinal epithelium<sup>[4]</sup>. Furthermore, tight junctional proteins, important for the physical tightness of the epithelial cell layer, are found to be enhanced by probiotics<sup>[5,6]</sup> and the disruption of tight junctions by pathogens can be counteracted<sup>[7]</sup>. Besides these effects on the physical barrier function, evidence is mounting that both commensal microflora and specific probiotic supplementations specifically enhance the immunological barrier function of the small intestinal mucosa<sup>[8]</sup>. For this cross talk between bacteria and the immune system, bacteria derived products including metabolites, cell wall components and DNA, can be sensed by enterocytes and immune competent cells<sup>[9]</sup>. Commensals, unlike pathogens, are efficiently killed by intestinal macrophages, therefore avoiding an inflammatory response in the mucosa<sup>[10]</sup>. At the same time, dendritic cells can sample commensals, incorporate them, and transport them to mesenteric lymph nodes. Here, commensal-loaded dendritic cells induce a local immune response with the activation of specific B-cells to produce secretory IgA against these commensals<sup>[8,10]</sup>. This appears to be a paradox, but this specific and local immune stimulation by commensals and probiotics can actually be considered non-inflammatory in the mucosal environment and the host systemics<sup>[8]</sup>. Therefore GALT,



**Figure 1** Potential mechanisms of action by which probiotics can promote GI health and the consequences for the liver. Probiotics and surface-layer proteins competitively exclude microbial pathogens from mucosal surfaces. Tight junction proteins, such as zona occludins-1 and claudin1, remain intact and thereby prevent both uptake of intact macromolecules and translocation of viable organisms (BT) to mesenteric lymph nodes, and ultimately to the liver. Through a cascade of signalling events, probiotics enhance production and secretion of anti-inflammatory cytokines, including interleukin-10 and transforming growth factor- $\beta$ , by a subset of immune cells, referred to as T regulatory cells. Innate immune responses to probiotics include increased mucin and trefoil factor production by goblet cells and enhanced production of antibacterial defensins by Paneth cells and intestinal epithelia. Probiotics might alter the intestinal microbiota and hence limit intestinal bacteria overgrowth (IBO) and the production of lipopolysaccharides (LPS).

and specifically the mesenteric lymph nodes, can be considered another layer of intestinal barrier function<sup>[3,10]</sup>.

### Probiotics and inflammatory bowel diseases (IBD)

The pathogenesis of IBD [ulcerative colitis (UC) and Crohn's disease] remains unknown, but the intestinal microflora appears to play an important role. Changes in microflora composition have been observed in UC patients, with increased pro-inflammatory bacteria, including Enterobacteriaceae, increased *Bacteroides fragilis* within the mucosal microflora<sup>[11]</sup>, and decreased protective bacteria, including lactobacilli and bifidobacteria<sup>[12]</sup>. Probiotic treatment has the potential to decrease the severity of symptoms in IBD *via* interaction with gut epithelium<sup>[13]</sup>. Proposed mechanisms include changes in short chain fatty acids (SCFA) production patterns, reduction in pro-inflammatory cytokine secretion, improving Th1/Th2 ratios, and eliminating pathogens. For example, the production of reuterin has been shown *in vitro* to reduce growth of pathogens, including *Escherichia coli* (*E. coli*), *Salmonella enterica*, *Shigella sonnei*, and *Vibrio cholera*. Adhesion of probiotics to enterocytes and enhancement of barrier function (secretion of B-defensins and mucus, TJ proteins) have also been shown for specific strains *in vitro*. However, it is unclear whether this strengthening of the intestinal barrier function would also occur in the large intestine, the area most affected by intestinal disease. Some evidence from a mouse model of colitis [interleukin (IL)-10 deficient mice] suggests that probiotic lactobacilli and the VSL#3 mix reduced



bacterial translocation (BT) and intestinal permeability in the colon<sup>[14,15]</sup>. Mechanistic effects can also be observed with non-viable probiotics and culture supernatants *in vitro* and in animals, which might be a safer therapeutic for patients with impaired intestinal barrier and increased risk of sepsis. Bacteria-free culture supernatant from *Lactobacillus plantarum* was shown to inhibit inflammatory pathways important for intestinal inflammation, such as nuclear factor (NF)- $\kappa$ B binding activity and proteasome activity, in a young adult mouse colon cell line and in macrophages<sup>[16]</sup>. This might provide a novel and safe strategy for treatment of IBD, if results can be repeated *in vivo*.

### Probiotics and irritable bowel syndrome (IBS)

IBS includes a range of symptoms, such as abdominal pain, altered bowel habits, bloating and flatulence in the absence of structural abnormalities in the intestine. As no curative treatment is available for IBS, therapy is palliative and supportive, targeting special symptoms, and is notoriously unsatisfactory. Studies have observed alterations in intestinal microflora in patients and increased symptoms following enteric infections, therefore probiotics might be a useful tool to improve symptoms. A meta analysis of probiotic treatment and IBS<sup>[17]</sup> included 20 trials with 23 probiotic treatments. This study showed that probiotics were associated with improved global IBS symptoms [risk ratio (RR) = 0.77] and with decreased abdominal pain (RR = 0.78). Due to the large variety in probiotic strains used, no analysis on strain type was possible. Probiotics used included *B. infantis*, lactobacilli (*L. acidophilus*, *L. plantarum*, *L. reuteri*, *L. rhamnosus*), *Saccharomyces boulardii*, *Streptococcus faecium*, VSL#3 (mix of eight), and other mixes. In a recent systematic review, Brenner *et al.*<sup>[18]</sup> claimed that 16 randomized controlled human trials met their inclusion criteria, of which 11 had suboptimal study design. They concluded that only one study using a specific strain of *B. infantis* 35624 efficiently improved IBS symptoms. Probiotics might offer a treatment possibility for IBS symptoms, but more controlled studies are needed to identify the ideal strain, dose, and duration of treatment.

### Probiotics after gut surgery

Morbidly obese patients can undergo Roux-en Y gastric bypass surgery (one type of bariatric surgery) for effective and enduring weight loss. This procedure uses restriction in stomach size and intestinal malabsorption as measures for achieving substantial weight loss. Problems that occur postoperatively are alteration of microflora with bacterial overgrowth (BO) in the blind sac of the intestine, intestinal pain, and possibly impaired vitamin B<sub>12</sub> status (due to lack of intrinsic factor production from the stomach). This study<sup>[19]</sup> used probiotic treatment (6 mo, commercial product of unspecified lactobacilli) in 44 post operational patients. BO (measured with hydrogen breath test) improved only after 6 mo, but not earlier. Vitamin B<sub>12</sub> status improved, gastrointestinal

symptoms remained unchanged, but post operational weight loss was significantly increased in the probiotic group. The authors speculated that increased weight loss might be due to changes in microflora towards extracting fewer calories from the diet, although this was not studied.

Probiotics/symbiotics have also been used to prevent postoperative infections in patients undergoing abdominal surgery (biliary cancer, liver transplantation, and pancreaticoduodenectomy). Major infections were pneumonia, urinary tract infection, wound infection, intra-abdominal abscess, and cholangitis. A recent meta analysis<sup>[20]</sup> found that probiotics reduced overall infections [odds ratio (OR) = 0.26], length of antibiotic treatment need (OR = -4.01), reduced length of postoperative hospital stays (OR = -2.7), but did not change overall mortality (OR = 0.98). Overall the use of probiotics is very promising, although data are very variable (type of surgery, type of infection, and type of probiotic treatment). However, bacteremia might be a potential hazard in these vulnerable patients. The appropriate therapeutic route, length of therapy, time of administration, dosage, and kind of probiotic remain controversial and no uniform preventative strategy can be suggested on based of the current literature.

## LIVER HEALTH

There is a longstanding practice of using lactulose in the treatment of hepatic encephalopathy, which suggests involvement of gut microflora in the management of chronic liver disease. Loguercio *et al.*<sup>[21]</sup> use the phrase “gut liver axis” and suggest that the microflora might affect the liver and be cofactor in aetiology of chronic liver damage. This could happen *via* modulating chronic damage by ethanol or by contributing to complications such as encephalopathy (production of ammonia, ethanol, acetaldehyde, phenols, endotoxin, and benzodiazepines)<sup>[21]</sup>. Probiotic actions most relevant to liver disease are modification of intestinal barrier function and prevention of BT. Gram-negative BO, increased permeability, and impaired immunity all contribute to increased BT, and there is a strong correlation between the rate of BT and the severity of cirrhosis. Probiotics might alter gut flora towards protective organisms and increase barrier function<sup>[22]</sup>.

### Probiotics and non alcoholic fatty liver disease (NAFLD)

NAFLD is the most common form of liver disease in the US; its incidence is rising together with rising problems of obesity and Type II diabetes. NAFLD includes a spectrum of pathologies. Steatosis (fatty liver), is clinically asymptomatic, but might predispose the liver to other insults, such as lipopolysaccharides (LPS) or hepatotoxins, that might lead to cirrhosis. Non-alcoholic steatohepatitis (NASH) is an intermediate state where lobular inflammation occurs. Cirrhosis is the most severe form, responsible for most liver specific morbidity and mortality<sup>[23]</sup>.

Histopathological changes are very similar to alcoholic liver disease, and there might be a common pathway of development. Data suggest a “multi hit” hypothesis, where initial hits such as obesity and sub-clinical insulin resistance might promote the development of steatosis<sup>[23]</sup>. This enhances the fatty liver’s vulnerability to subsequent insult (e.g. ethanol and LPS) that increase the production of pro-inflammatory cytokines [e.g. tumor necrosis factor (TNF)- $\alpha$ ]. This aggravates insulin resistance and leads to oxidative stress (increased production of reactive oxygen by hepatocytes and liver macrophages) and organelle dysfunctions, which kill hepatocytes and promote accumulation of inflammatory cells in the liver and the development of NASH<sup>[24]</sup>. Following years of chronic inflammation this might develop into fibrogenic response and cirrhosis.

The contribution of microflora in the development of NAFLD is mainly based on increased hepatic oxidative stress by increased production of ethanol and LPS in the intestinal lumen, and subsequent release of inflammatory cytokines in intestinal epithelia and liver macrophages. Both processes then lead to injury of the intestinal epithelium and disrupted intestinal barrier function, which in turn increases hepatic exposure to intestine-derived toxins. This hypothesis is further supported by evidence that intestinal BO exacerbates fatty liver disease in rodents and humans, and that obese patients with NASH have increased prevalence of BO. Furthermore, obese subjects are known to have decreased intestinal motility and are therefore more prone to BO. A recent clinical trial confirms that patients with NASH have increased intestinal permeability and small intestinal BO<sup>[25]</sup>.

From this data, an obvious way to control the development of NAFLD seems to be a manipulation of the gut microflora, mainly by the reduction of BO. This can be achieved by antibiotic treatment, which has been used successfully, but is controversial due to its unspecific impairment of all microflora and its severe side effects<sup>[23]</sup>. Probiotic therapy, on the other hand, has been suggested to counteract the development of NAFLD on various different levels. Competitive inhibition of pathogens by probiotics might alter their inflammatory effects in intestinal BO, which is associated with NAFLD. Furthermore, improved intestinal epithelium function and decreased BT and endotoxemia following probiotic treatment have been observed in experimental animals and humans<sup>[26]</sup>. In a series of feeding studies in mice, Cani *et al.*<sup>[27]</sup> claimed that high-fat feeding changes the intestinal microflora composition (less bifidobacteria), which led to increased LPS levels in plasma, pro-inflammatory cytokines and increased intestinal permeability. All these effects could be counteracted by prebiotic treatment and increasing bifidobacteria species (summarized in<sup>[27]</sup>). This would indicate a direct effect of intestinal bacteria on low-grade inflammation, insulin insensitivity, and fat deposition in the liver.

Other research suggests a direct decrease in pro-inflammatory cytokines e.g. TNF- $\alpha$  *via* downregulation of

NF- $\kappa$ B activity by probiotic treatments<sup>[14,28-30]</sup>. In a study in ob/ob mice, probiotics (VSL#3) and TNF- $\alpha$  antibodies were used to treat NAFLD. Both treatments improved liver function, reduced hepatic fatty acid content, and both interfered with NF- $\kappa$ B signalling and reduced hepatic fatty acid  $\beta$ -oxidation close to levels in lean mice. The authors suggest that this effect might result from improved hepatic insulin resistance<sup>[28]</sup>. Another study, using the same probiotic in normal mice, measured hepatic natural killer T (NKT)-cell depletion in high-fat fed animals<sup>[31]</sup>. NKT are unconventional T cells that express both T cell and Killer cell receptors. They regulate hepatic inflammatory process by balancing production of pro- and anti-inflammatory cytokines. Alterations of NKT function might lead to overproduction of TNF- $\alpha$ , causing inflammation in insulin resistance. High-fat diet induced the depletion of NKT from the liver, leading to insulin resistance and steatosis. Probiotics significantly improved all these symptoms and the effect resulted from TNF- $\alpha$  signalling and led to improved insulin signalling<sup>[31]</sup>. There is some suggestive evidence that probiotics might have efficacy in NAFLD in humans, but more controlled trials are needed<sup>[30]</sup>.

### Probiotics and alcoholic liver disease

In alcoholic liver disease, bowel liver interactions are well described, and relationships include increased gut permeability, endotoxemia, and TNF- $\alpha$  production<sup>[32]</sup>. In rats, *Lactobacillus* GG has been shown to reduce alcohol induced gut leakiness and steatohepatitis<sup>[33]</sup>. The same group also found that the mucosa associated microflora were altered in rats on a high alcohol feed, and this dysbiosis could be counteracted by *Lactobacillus* GG or oat supplementation<sup>[34]</sup>. Furthermore, alcoholics have altered microflora with decreased numbers of bifidobacteria and lactobacilli. A recent pilot study<sup>[32]</sup> compared control subjects with alcoholics in a clinic, either on standard treatment for alcoholic disease (Vitamin B<sub>1</sub> & B<sub>6</sub> supplements and diazepam,  $n = 34$ ) or on standard treatment + probiotics ( $0.9 \times 10^8$  CFU *B. bifidum* and  $0.9 \times 10^9$  CFU *L. plantarum*,  $n = 32$ ) for five consecutive days. No placebo was included in the control group. They found that lactobacilli, bifidobacteria, and enterococci were reduced in alcoholics and the numbers were restored in the probiotic treatment group. *E. coli* levels were not altered. Liver function parameters [alanine aminotransferase (ALT) and aspartate aminotransferase] were significantly improved by probiotics compared to standard treatment, but remained below levels found in healthy controls. Other liver enzymes (GGT and LDH) were not significantly altered. In the subgroup of alcoholics with well-defined hepatitis, standard treatment only altered total bilirubin, but probiotics improved all the liver parameters mentioned above. The authors concluded that bifidobacteria seemed to play an important role in steatohepatitis of alcoholic and non alcoholic causes, including obesity<sup>[35]</sup>. In a double blind placebo

controlled (sucrose capsule) intervention Lata *et al*<sup>[36]</sup> studied the effect of probiotic *E. coli* Nissle in cirrhotic patients ( $n = 39$ , 34 with alcoholic cirrhosis). They found an improvement of intestinal colonization in faeces, and a restoration of “physiological microflora” in faeces (more patients with normal microflora containing lactobacilli and bifidobacteria, less patients with potentially pathogenic bacteria). They also showed trends towards a reduced endotoxin level in blood ( $P = 0.07$ ) and a reduced Child-Pugh score ( $P = 0.07$ ), which is a measure for severity of overall liver disease. Improved liver function by probiotics has already been published<sup>[5]</sup>, but the mechanism remains unclear. They assume that microflora reduce the toxic load of liver, e.g. reduced endotoxin, which stimulates pro-inflammatory cytokines (TNF- $\alpha$ , IL-1, 6)<sup>[22]</sup>.

### Probiotics to cure/alleviate cirrhosis?

In cirrhosis there are many conditions that alter microflora and the function of the intestinal epithelium, as recently reviewed in detail<sup>[37]</sup>. BT is caused by BO, increased permeability, and altered host defence. BT and microflora imbalance are strongly correlated with the severity of cirrhosis<sup>[38]</sup>. In a rat model of acute liver injury (using either D-galactosamine or endotoxin), probiotic lactobacilli and bifidobacteria attenuated liver injury (ALT and bilirubin), reduced BT, and normalized hepatic TNF- $\alpha$  and glutathione levels compared to liver injury controls<sup>[39,40]</sup>.

Lactobacilli might counteract BT by (1) promoting growth of anaerobes and Gram-positive bacteria while inhibiting gram-negative bacteria, or (2) increasing SCFA while decreasing pH, inducing growth factors and proliferation of microflora, and inhibiting adherence and invasion of pathogens. Patients with liver cirrhosis have imbalanced intestinal microflora with increased aerobes (enterobacter and enterococci) and anaerobes (clostridia), and decreased bifidobacteria counts in stool. In a probiotic intervention trial in cirrhotic patients<sup>[38]</sup> (mainly caused by hepatitis virus B and C; HBV and HCV) patients received two different probiotic capsules [bifidobacteria + *L. acidophilus* + *Enterococcus* or *Bacillus subtilis* (*B. subtilis*) + *Enterococcus faecium* (*E. faecium*)], two capsules per day for 14 d. In both probiotic groups, bifidobacteria counts increased with treatment, while faecal pH and ammonia levels in faeces and blood decreased. Additionally, *B. subtilis* + *E. faecium* decreased clostridia counts and endotoxin levels in the blood of cirrhotic patients.

Flora imbalance in cirrhotics might be caused by decreased gut motility, diminished excretion of secretory IgA, lysozyme, mucus, acids, increased pH, shortage of bile acids, and excessive alcohol intake. Elevated blood ammonia is a crucial factor in hepatic encephalopathy aetiology.

Loguerio *et al*<sup>[21]</sup> conducted a pilot study in patients suffering from hepatitis of various causes (HCV, alcoholism, and NASH). The patients received a probiotic

mix (*Lactobacillus acidophilus*, *L. bifidus*, *L. rhamnosus*, *L. plantarum*, *L. salivarius*, *L. bulgaricus*, *L. lactis*, *L. casei*, *L. brevis* + fructo-oligosaccharides + vitamins). Interestingly, the authors reported no effect of probiotics in HCV patients. In NASH patients some liver function parameters (ALT and  $\gamma$ -glutamyltransferase) improved, TNF- $\alpha$  decreased and plasma malondialdehyde decreased in some patients with probiotic treatment. The strongest effect of probiotics was seen in patients with alcoholic liver cirrhosis, where all parameters of liver function improved, as did TNF- $\alpha$  and malondialdehyde.

From these studies in humans, it appears that the microflora is an important cofactor in the aetiology of chronic liver disease, and that probiotics might have a therapeutic role.

### Probiotics to bind toxins and carcinogens

Some experimental evidence suggests that probiotics could be used to bind and immobilise toxic compounds within the gut lumen. Through this process, the negative effects of dietary toxins could be reduced and gut and liver health improved. *In vitro*, *L. rhamnosus* GG is able to bind mycotoxins known to interfere with intestinal mucosal barrier. In Caco-2 cells, the negative effects of mycotoxins on cell differentiation and intestinal integrity can be attenuated with *L. rhamnosus* GG<sup>[41,42]</sup>. In rats, genotoxic effects of food carcinogens, such as heterocyclic amines, on the colonocytes and hepatocytes were counteracted by the use of different probiotics<sup>[43,44]</sup>. Probiotics have also been shown to attenuate hepatotoxic effects of aflatoxin, a well known liver carcinogen, in rats<sup>[45]</sup> and to reduce biomarkers of liver cancer risk in a human intervention trial<sup>[46]</sup>.

### Safety considerations of probiotics

Generally recognized as safe (GRAS) status is defined by the Food and Drug Administration for food adjuncts that might not meet the usual requirements for safety assessment but have been used extensively without demonstrable harm. Probiotics are claimed to be GRAS as they comprise organisms identical to those in human gut and vaginal flora, although strain dependence needs to be considered and GRAS should only be granted to one specific probiotic preparation used in one specific food product<sup>[47]</sup>. Previous probiotic studies stress the safety of probiotic preparations and their ability to reduce BT of pathogenic bacteria to host organs and tissues. Probiotic BT from the intestine is difficult to induce in healthy animals, and therefore hard to study. From animal studies, NOAEL (no observed adverse effect levels) can be determined and ADI (acceptable daily intake) extrapolated for humans. These calculations suggest that up to  $10^{14}$  cfu/d of lactobacilli and bifidobacteria, a dose way beyond the usual intake of  $10^9$ - $10^{11}$  cfu/d, are acceptable for human consumption. In healthy humans, probiotic BT occurs occasionally, but detrimental effects are rare. Salminen *et al*<sup>[48]</sup> assessed the frequency of lactobacillus bacteraemia in the Finnish population



following the increased consumption of probiotic products in the years 1995-2000 and found no trends towards increased lactobacillus bacteraemia over this period. A recent meta analysis summarised the safety of probiotics in pregnancy and concluded no effect of lactobacillus and bifidobacterium species on incidence of caesarean section, birth weight, or gestational age<sup>[49]</sup>. In immunocompromised individuals however, this might be different. Cannon *et al.*<sup>[50]</sup> summarised over 200 clinical cases of lactobacillus infections and found association with endocarditis and bacteraemia. *L. casei* and *rhamnosus* were most common, and the overall mortality rate was nearly 30%. They also reported that the main underlying conditions were cancer, diabetes, antibiotic therapy, organ transplantation, and abscesses. Salminen *et al.*<sup>[51]</sup> investigated the severity and outcome of lactobacillus bacteraemia in 89 patients, and report mortality of 26% 1 mo after illness onset, but only in patients with severe underlying comorbidities.

### Future of probiotic research

Most recent developments in probiotic and prebiotic research use a new systems biology approach to assess the complex relationship between the microflora, probiotic modulations, and the impact on host metabolism in multiple compartments. A major finding is the impact of microflora modulation on host energy metabolism, especially lipid metabolism in the liver where marked decreases in plasma lipoproteins and hepatic glutamine and glycogen levels are observed<sup>[52]</sup>. This group uses a germ free mouse model colonized with human baby flora, to study the effects of a probiotic intervention (*L. paracasei* or *L. rhamnosus*) on gut flora composition, SCFA in caecal content, plasma, urine, faecal and liver metabolomics, and bile acids in ileal flushes.

The integration of multicompartment metabolic data using hierarchical principal component analysis showed that probiotic lactobacilli induce changes in hepatic influx and efflux of fatty acids, increased enterohepatic recycling of bile acids and dietary fats, lowered plasma LP, and stimulated glycolysis. Probiotic intervention also changed the proteolytic activity and bacterial metabolism of AA and SCFA in the gut<sup>[53]</sup>. In a mouse model of high-fat feeding and NAFLD, an association was shown between the metabolism of choline by microbiota and the host. The author suggested a contribution of microflora to the development of the NAFLD phenotype<sup>[54]</sup>. This complex analytical and statistical methodology allows investigation of the impact of microflora and probiotics on various body compartments simultaneously and might, in the future, lead to a better understanding of the influence of probiotics on the host. Similarly, the complex metabolic relationships between the microflora and the host have also been studied in human cohorts and this approach might be used to gain further understanding of the relation between changes in the microflora (dysbiosis) and disease<sup>[55]</sup>.

## CONCLUSION

It appears that specific clinical applications of probiotics are safe, effective, and can clearly be recommended. However, the importance of probiotic food items in the “maintenance of health” in healthy individuals as marketed by food industries remains questionable. To date, no generalisation can be made from health effects of one probiotic strain to another one and this remains a serious problem within the probiotic research field and its applications. Multi-dimensional research approaches, studying the microflora composition, its metabolic profile, and the impact on host metabolism appear a promising way forward to further describe and explore these complex relationships within the microflora-host “superorganism”.

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## Endoscope-guided pneumatic dilation for treatment of esophageal achalasia

Seng-Kee Chuah, Keng-Liang Wu, Tsung-Hui Hu, Wei-Chen Tai, Chi-Sin Changchien

Seng-Kee Chuah, Keng-Liang Wu, Tsung-Hui Hu, Wei-Chen Tai, Chi-Sin Changchien, Gastrointestinal Motility Unit, Division of Hepatogastroenterology, Chang Gang Memorial Hospital, Kaohsiung Medical Center, No. 123, Ta-Pei Road, Niasung Hsiang, Kaohsiung 833, Taiwan, China; Chang Gung University College of Medicine, No. 123, Ta-Pei Road, Niasung Hsiang, Kaohsiung 833, Taiwan, China

Author contributions: Chuah SK and Changchien CS drafted the editorial; all other authors wrote the final version.

Correspondence to: Chi-Sin Changchien, MD, Gastrointestinal Motility Unit, Division of Hepatogastroenterology, Chang Gang Memorial Hospital, Kaohsiung Medical Center, No. 123, Ta-Pei Road, Niasung Hsiang, Kaohsiung 833, Taiwan, China. chuahsk@seed.net.tw

Telephone: +886-7-7317123-8301 Fax: +886-7-7322402

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**Peer reviewers:** Kyoichi Adachi, MD, Department of Gastroenterology and Hepatology, Shimane University, School of Medicine Shimane, 89-1 Enya-cho, Izumo-shi Shimane 693-8501, Japan; Fabio Pace, Professor, Division of Gastroenterology, "L. Sacco" University Hospital, University of Milan, Via G. B. Grassi, 74, Milano 20157, Italy

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### Abstract

Pneumatic dilation (PD) is considered to be the first line nonsurgical therapy for achalasia. The principle of the procedure is to weaken the lower esophageal sphincter by tearing its muscle fibers by generating radial force. The endoscope-guided procedure is done without fluoroscopic control. Clinicians usually use a low-compliance balloon such as Rigiflex dilator to perform endoscope-guided PD for the treatment of esophageal achalasia. It has the advantage of determining mucosal injury during the dilation process, so that a repeat endoscopy is not needed to assess the mucosal tearing. Previous studies have shown that endoscope-guided PD is an efficient and safe nonsurgical therapy with results that compare well with other treatment modalities. Although the results may be promising, long-term follow-up is required in the near future.

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**Key words:** Esophagoscopy; Dilatation; Esophageal achalasia

### INTRODUCTION

Achalasia occurs at all ages; the mean occurrence is in middle age and affects both sexes and all races equally. The diagnosis is made by examinations such as barium esophagography, and esophageal manometry and endoscopy, after it has been distinguished from secondary achalasia caused by malignancy<sup>[1-5]</sup>. The truth we are facing in dealing with esophageal achalasia is that there is so far no cure for the disease. There are currently three main treatment modalities: pneumatic dilation (PD), surgery and botulinum toxin (BT) injection therapy. All therapeutic approaches are to loosen the lower esophageal sphincter (LES), because LES dysfunction leads to obstruction of the esophagus<sup>[6-20]</sup>. The goals are to relieve symptoms, improve esophageal emptying and avoid megaesophagus<sup>[21]</sup>. Traditional smooth muscle relaxants in the form of nitrates and calcium channel antagonists play little role. Besides, there are many intolerable side effects such as headaches, hypotension, and eventual tachyphylaxis<sup>[22,23]</sup>.

No doubt, PD is considered to be the first line nonsurgical therapy for achalasia. The principle of the procedure is to weaken the LES by tearing its muscle fibers by generating radial force. For many decades, there have been many reports about long-term efficacy of

PD in the treatment of achalasia under the guidance of fluoroscopy<sup>[16-20]</sup>. However, some factors may hinder the practice of PD by some gastroenterologists. Some may have fear of the misplaced risk of perforation, and the overall decreased immediate morbidity from laparoscopic myotomy. It is the responsibility of gastroenterologists to continue the tradition of PD as a generally available technique, or otherwise, myotomy may become the routine available therapy for achalasia.

Another reason may be concern about exposure to the X-rays during the procedure under fluoroscopic guidance<sup>[24-26]</sup>. Some of the highest doses to both patients and medical workers only arise from some other interventional radiology procedures<sup>[12,26]</sup>. Potential high doses during interventional procedures have been reached despite the procedure being carried out on equipment that normally has effective local shielding, so that the dose outside the lead coat is relatively low during fluoroscopy<sup>[12,27]</sup>. However, fluoroscopic-guided PD requires positioning of the balloon, which may need longer time, thus increasing the radiation exposure. Besides, the entire endoscope-guided PD procedure is done under direct visual control. It is easy to determine the mucosal injury during the dilation. Unlike fluoroscopic-guided PD, a repeat endoscopy to assess the mucosal tearing is not needed. The issue of the endoscope-guided PD for the treatment of esophageal achalasia is reviewed and discussed in this paper.

## ENDOSCOPE-GUIDED PD

Levine *et al.*<sup>[25]</sup> first proposed a safe and convenient PD technique under endoscopic guidance without the use of fluoroscopy. Since then, this technique has been used by many physicians<sup>[11,12,23,28-35]</sup>. There are many types of pneumatic dilators commercially available. The high-compliance balloons are the Rider-Moeller device and the Brown-McHardy dilator (Narco Scientifics, Piling Division, Fort Washington, PA, USA), Witzel dilator (ABS, par d' Activite Saint Michel, France) while the low-compliance balloons such as Gruntzig-type dilator (Rigiflex dilator; Microvasive, Watertown, MA, USA). Clinicians usually prefer using the low-compliance balloon (Gruntzig-type, Rigiflex dilator), because it has various theoretical advantages over a high-compliance balloon<sup>[34]</sup>. Rigiflex dilator is designed so that it can be inflated to a desired maximum diameter. Further inflation can only result in the increase of the pressure but not the diameter<sup>[35]</sup>. Therefore, the wall tension is increased maximally at the stenotic zone. In contrast, an inflated high-compliance balloon may lead to an increase in the esophageal wall tension more proximal to the stenotic zone more than the stenotic zone itself, which may lead to perforation, by the Laplace law<sup>[36-38]</sup>. Such speculation is supported theoretically by the fact that the most common site of esophageal perforation is proximal to the cardia on the left lateral side of the esophagus in clinical practice<sup>[34,35]</sup>.

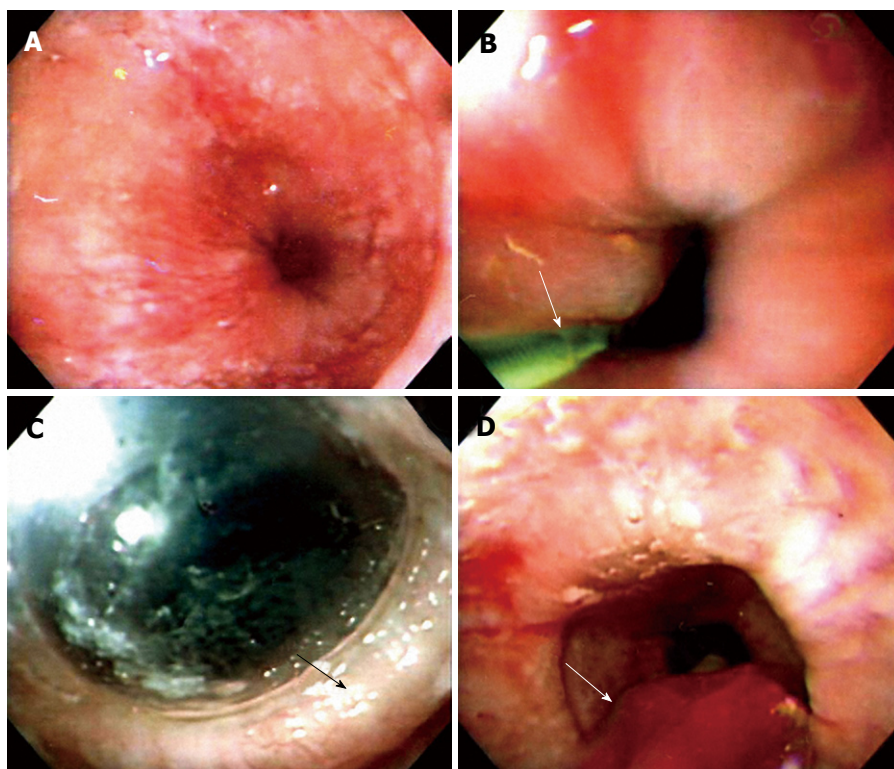
## TECHNIQUE OF ENDOSCOPE-GUIDED PD

There is so far no clear consensus on the optimal method for performing PD with regard to balloon diameter, and the amount and rate of inflation pressure. It has been shown that the risk of perforation increases with the size of the balloon<sup>[39]</sup>. Mikaeli's<sup>[39]</sup> and Karamanolis' groups<sup>[14]</sup> have reported that graded pneumatic balloon dilatation with a 30-mm diameter and slower rate of balloon inflation is an effective and safe initial method of therapy for achalasia. According to our experience, we recommend that a 3.0-cm dilator with a smaller average inflation pressure of 10-12 psi is sufficient to attain satisfactory clinical remission, at least for oriental populations<sup>[14]</sup>.

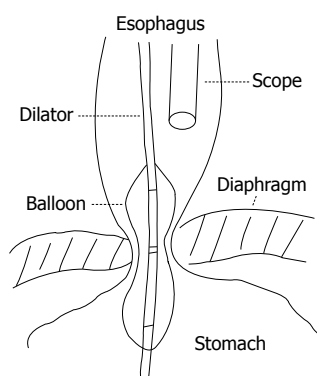
Endoscope-guided PD is carried out by choosing a desirable diameter of balloon dilator, under conscious sedation, with informed consent after an overnight fast. Nowadays, the low-compliance Rigiflex dilator is preferred by most doctors. The endoscope is inserted down to the duodenum (Figure 1A). A guide-wire is placed into the duodenum under endoscopic guidance and then the endoscope is removed (Figure 1B). A Rigiflex balloon dilator of a desirable diameter, which is marked with a thick colored marker at the mid-section of the balloon, is passed over the guide wire to the stomach. The endoscope is reinserted to serve as a guide to control the position of the balloon in the esophagus. The balloon is withdrawn to the esophagus, until the mark reaches the gastroesophageal junction. The balloon is inflated up to 12 psi and maintained for 60 s, until an ischemic ring at the LES can be seen by the endoscope through the transparent balloon (Figure 1C). The same inflation procedure is repeated once more and held for another 30 s. The balloon is flattened completely and removed together with the endoscope (Figure 1D). Gastrografin ingestion is performed immediately after the dilation to determine possible esophageal perforation. Chest pains and vital signs are monitored closely. Chest X-rays or computer tomography are carried out should severity of the chest pains imply any possibility of rupture.

Studies on the techniques of Rigiflex balloon dilation of achalasia by positioning the endoscope above the balloon without fluoroscopy have shown results comparable with studies when using fluoroscopy (Figure 2). The advantages are performing this procedure without any extra equipment in a time and cost-effective manner. However, Rai's group<sup>[28]</sup> has introduced a novel technique by the presence of the endoscope across the gastroesophageal junction during the dilation procedure, with good efficacy reported after dilation. However, despite the safety report from Rai's group, the potential danger of increased perforation is a concern of many other clinicians. Some have argued that such a technique is likely to interfere with the application of uniform radial force on the spastic sphincter. The effect of dilation toward the side of the endoscope can be compromising, and lead to a decrease in overall efficacy of the procedure and the possibility of generating an unequal radial force on the sphincter<sup>[40,41]</sup>.





**Figure 1** Technique of endoscope-guided pneumatic dilation (PD). A: A dilated low esophageal lumen with tight gastroesophageal junction under endoscope-guided PD; B: The endoscope is inserted down to the duodenum. A guide-wire (arrow) is placed into the duodenum under endoscopic guidance and the endoscope is removed. A Regiflex balloon dilator, which is marked with a thick colored marker at the mid-section of the balloon, is passed over the guide wire to the stomach; C: The endoscope is reinserted to serve as a guide to control the position of the balloon in the esophagus. The balloon is withdrawn to the esophagus, until the mark reaches the gastroesophageal junction. The balloon is then inflated up to 12 psi and maintained for 60 s, until an ischemic ring (arrow) at the LES is seen by the endoscope through the transparent balloon; D: The balloon is flattened completely and removed together with the endoscope (arrow).



**Figure 2** Almost all endoscope-guided PD are performed by using a low compliance Regiflex balloon dilator by positioning the endoscope above the balloon without fluoroscopy. The advantages are that the procedure can be performed without any extra equipment in a time and cost-effective manner.

## POST-DILATION INVESTIGATIONS

### Assessment by symptom scores vs esophagography

Usually, structured interviews are performed using validated symptom score methods<sup>[10,28,42]</sup> at the initial investigation, 6 wk later, and every year thereafter. Depending on whether dysphagia, regurgitation, and chest pain occur occasionally, daily, or several times during the day, a symptom score can be determined. In the system validated by Eckardt<sup>[42]</sup>, a symptom score of 0-3 was assigned to the degree of weight loss. Thus a completely asymptomatic patient would have a symptom score of 0, whereas a severely affected patient could have a high symptom score. In most of these symptom scoring systems, patients were considered to have reached clinical remission if symptoms had totally disappeared or if they had improved by attaining a certain drop in score. Patients who requested further therapy despite having a certain drop in score were considered treatment failures.

A discrepancy between objective parameters and the subjective symptomatic improvement after PD exists in clinical practice. Radiographic findings do not reliably correlate the symptoms to improved esophageal emptying after PD in some large studies<sup>[43-45]</sup>. However, Vaezi *et al.*<sup>[46-49]</sup> have reported that there was a significant association between improvement in patient symptoms and barium height. They believe that radiographic findings can reliably predict clinical remission and have suggested strongly the need for further treatment in those patients with poor esophageal clearance after each dilation, to avoid possible future complications such as sigmoid-type achalasia.

Large-scale, long-term follow-up investigations<sup>[15,50,51]</sup> have reported unfavorable recurrence in patients who have undergone fluoroscopic-guided PD. During the prolonged observation period (median, 13.8 years) in a prospective follow-up investigation study conducted by Eckardt *et al.*<sup>[15]</sup>, only 40% of patients treated with a single round of PD remained in remission at 5 years. We used endoscope-guided PD to treat achalasia and attained cumulative remissions of 86.7% in first 2 years, which had dropped to 72.9% after 5 years, but it remained at 61.7% in years 6 and 7, but patients were assessed by clinical symptom scores<sup>[11]</sup>. Bias probably existed when using subjective symptomatic scoring assessment to determine clinical remission. We agree with Vaezi's group who claimed that underscores may occur by only using an objective assessment. However, we believe that esophagography can only offer an additional objective assessment to the response to achalasia treatment, especially in patients who report symptomatic improvement, but the evidence is not strong enough to overthrow the

**Table 1** Cumulative effectiveness of endoscope-guided pneumatic dilators for the treatment of achalasia by using low compliance Regiflex dilators

Author (yr)	n	Study design	Dilator size (cm)	Improvement (%) (excellent/good)	Follow-up (yr) mean (range)	Perforation (%)
Levine <i>et al</i> <sup>[25]</sup> (1987)	62	Retrospective	3.0-3.5	85/88	-	0
Lambroza <i>et al</i> <sup>[24]</sup> (1995)	27	Retrospective	3.0	67	1.8 (0.1-4.8)	0
Dobrucali <i>et al</i> <sup>[28]</sup> (2004)	43	Prospective	3.0-3.5	54/79	2.4 (0.5-5)	0
Rai <i>et al</i> <sup>[29]</sup> (2005)	56	Prospective	3.5	92.9/89.3	2	0
Chuah <i>et al</i> <sup>[11]</sup> (2009)	32	Prospective	3.0	69/91	4.5 (2.5-7)	3.3

traditional assessment of clinical remission by using subjective symptom score assessment<sup>[52]</sup>. Although an additional objective parameter such as esophagography to the subjective symptom scores should be more optimal in assessing clinical remission, further investigations that include larger sample sizes and longer follow-up periods are required for clarification of this issue.

### Manometric studies

Manometry is an important predictor of treatment failure with balloon dilation, other than younger age (< 40 years), male sex, pulmonary symptoms, and failed response to one or two initial dilations<sup>[15,41,53,54]</sup>. It has been demonstrated previously that post-dilated LES pressure is relevant to better remission. In general, decreases in LES pressure of > 50% after PD, or an absolute end-expiratory LES pressure of < 10 mmHg, are more indicative of clinical success<sup>[15,52,53]</sup>. Therefore, it is suggested strongly that manometry be performed routinely before and after PD. One recent advance in the diagnosis of esophageal achalasia is the use of updated high resolution manometry (HRM) with pressure topography plotting<sup>[55]</sup>. We are optimistic that more promising evidence may emerge on the use of HRM in the near future.

## EFFECTIVENESS AND POST-DILATION SAFETY OF ENDOSCOPE-GUIDED PNEUMATIC DILATORS FOR ACHALASIA

Table 1 summarizes studies on the effectiveness of graded endoscope-guided pneumatic dilators for achalasia. Most studies were retrospective except for three prospective, longitudinal cohort studies with a mean follow-up period of 2-4.5 years<sup>[11,24,25,28,29]</sup>. All studies attained an acceptable clinical remission rate of 54%-91%, which was comparable to those reported by using fluoroscopy-guided PD<sup>[32,50,56-59]</sup>. Although the existing mid-term follow-up results are encouraging, further long-term follow-up is required in the near future.

The major adverse event caused by PD is esophageal perforation, with a 2% overall cumulative rate, and may occur in up to as many as 5% of all the reported cases of fluoroscopy-guided PD<sup>[32,49,56-59]</sup>. As shown in Table 1, the reported perforation rates was 0%-3.3% for endoscope-guided PD<sup>[11,24,25,28,29]</sup>. This implies the

relative safety of endoscope-guided PD compared to fluoroscopy-guided PD. However, esophageal perforation is a potential hazard after PD<sup>[60,61]</sup>. Usually, gastrograffin is ingested immediately after each PD to detect extravasation, which implies the presence of perforation. However, on rare occasions, immediate gastrograffin ingestion may not always detect perforation, which can become clinically evident several hours later after delayed presentation (> 24 h)<sup>[62,63]</sup>. Therefore, we must observe the clinical symptoms and signs closely, such as severe chest pain and fever, which imply the potential presence of perforation after PD.

Reflux symptoms after PD are usually mild and transient and should be easily controlled with proton-pump inhibitors<sup>[19]</sup>. However, objective assessment of gastroesophageal reflux after PD has rarely been studied. Other complications are usually minor, and include intramural hematoma, diverticula at the gastric cardia, mucosal tears, prolonged post-procedure chest pain, hematemesis without change in hematocrit, fever and angina.

## LAPAROSCOPIC MYOTOMY VS ENDOSCOPE-GUIDED PD

Like every other treatment of achalasia, the goal of surgery is to assuage the esophageal obstruction by myotomy of the LES. Minimally invasive laparoscopic myotomy with a variety of fundoplication procedures has evolved to be a primary approach for many surgeons and gastroenterologists in a majority of patients with achalasia<sup>[64-67]</sup>. However, there are only limited systematic reviews and meta-analyses that have compared existing treatment methods for achalasia and all favor surgery to PD<sup>[64,68,69]</sup>. With overall success rates of 47%-82% at 10 years, laparoscopic Heller myotomy with partial fundoplication appears to have evolved into the surgical procedure of choice<sup>[64,65]</sup>. Despite this, the major concern for myotomy is still that it can be complicated by severe acid reflux disease, and the role of fundoplication with myotomy continues to be controversial<sup>[21,69-72]</sup>. Hence, it is generally accepted that myotomy is usually suggested for younger male patients (< 40 years), those with pulmonary symptoms, and those who have failed to respond to one or two initial dilations; older age appears to be associated with favorable outcomes of PD<sup>[70,71]</sup>.

## BT INJECTION THERAPY VS ENDOSCOPE-GUIDED PD

As a result of its wider safety margin and fewer complications, BT injections have been practiced widely in past decades, with excellent immediate responses (success rates of > 90%). Unfortunately, the duration of response for BT injections is relatively discouraging (6-9 mo on average) in most patients, and only half of all patients benefit for > 1 year<sup>[6,10,73]</sup>. The effect of BT injections vanishes with time in elderly patients, which necessitates repeated injections to keep the patients symptom-free. As a result of the number of repeated injections required, this procedure is more expensive than PD by  $\geq 50\%$ . However, it has been reported that the long-term success is highest among elderly patients and in those with an LES pressure that did not exceed the upper normal level before treatment<sup>[6,10,74,75]</sup>. Also, younger patients (< 55 years) with a severe increase in LES pressure do not seem to benefit from BT injections, and PD or minimally invasive myotomy are more advantageous<sup>[10]</sup>. Generally, minimally invasive myotomy is recommended in younger patients.

In short, PD is more efficacious than BT injections for sustained symptomatic relief in patients with achalasia. BT is as good as PD in achieving a short-term improvement in achalasia. It is also effective in patients with tortuous megaesophagus and previous failed PD. However, as mentioned earlier, recurrence is high during 1-year follow-up<sup>[76]</sup>. Furthermore, some surgeons may be concerned that previous BT injections make subsequent minimally invasive myotomy riskier and more difficult<sup>[77]</sup>. Therefore, BT injections are recommended as a suitable alternative only for a minority of older or high-risk patients.

## CONCLUSION

Endoscope-guided PD is an efficient and safe nonsurgical therapy with results comparable to other treatment modalities. Besides, it has the advantage that the entire procedure is done without fluoroscopic control, and the mucosal injury during the dilation can be determined by direct visual observation. Long-term follow-up studies are required in the near future.

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## Present and future possibilities for early diagnosis of hepatocellular carcinoma

Piotr Stefaniuk, Janusz Cianiara, Alicja Wiercinska-Drapalo

Piotr Stefaniuk, Janusz Cianiara, Alicja Wiercinska-Drapalo, Department of Hepatology and Acquired Immunodeficiencies, Warsaw Medical University, 37, Wolska St, Warsaw 01-201, Poland

**Author contributions:** Stefaniuk P, Cianiara J and Wiercinska-Drapalo A were responsible for the review of the literature and initial preparation of the paper; Stefaniuk P prepared the final version of the manuscript.

**Correspondence to:** Piotr Stefaniuk, MD, Department of Hepatology and Acquired Immunodeficiencies, Warsaw Medical University, 37, Wolska St, Warsaw 01-201, Poland. [pstefaniuk@gmail.com](mailto:pstefaniuk@gmail.com)

Telephone: +48-22-3355222 Fax: +48-22-6310535

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$\gamma$ -glutamyl transferase, glypican-3, squamous cell carcinoma antigen, a new generation of immunoglobulin M-immunocomplexes, and very promising gene-expression profiling.

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### Abstract

Hepatocellular carcinoma (HCC) represents the fifth most common cancer in the world, and the third most frequent oncological cause of death. The incidence of HCC is on the increase. HCC typically develops in patients with chronic liver diseases, and cirrhosis, usually with viral etiology, is the strongest predisposing factor. Nowadays HCC diagnosis is a multistage process including clinical, laboratory, imaging and pathological examinations. The prognosis of HCC is mostly poor, because of detection at an advanced, non-resectable stage. Potentially curative treatment (surgery) is limited and really possible only for cases with small HCC malignancies. For this reason, more effective surveillance strategies should be used to screen for early occurrence of HCC targeted to the population at risk. So far, the generally accepted serological marker is  $\alpha$ -fetoprotein (AFP). Its diagnostic accuracy is unsatisfactory and questionable because of low sensitivity, therefore there is a strong demand by clinicians for new HCC-specific biomarkers. In this review, we will focus on other biomarkers that seem to improve HCC diagnosis, such as AFP-L3, des- $\gamma$ -carboxyprothrombin,  $\alpha$ -L-fucosidase,

### INTRODUCTION

Hepatocellular carcinoma (HCC) is a major challenge in contemporary medicine. The incidence of HCC is on the increase and it is becoming more and more significant both clinically and epidemiologically. Now HCC represents the fifth most common cancer in the world and the third most frequent cause of mortality amongst oncological patients<sup>[1]</sup>. It is responsible for more than 500 000 deaths with over 600 000 new cases yearly worldwide<sup>[2]</sup>. Incidence rates are different in various countries: highest in South-East Asia and Sub-Saharan Africa (around 120/100 000) and lowest in the USA (1.8/100 000) and Western Europe (3-5/100 000)<sup>[1,2]</sup>. Although Poland belongs to the group of countries with a relatively small incidence rate: lower than 6/100 000 in men and 3/100 000 in women, HCC causes the death of more than 2500 Poles every year and, according to statistically observed trends, the mortality rate will gradually increase<sup>[3]</sup>.

More than 95% of HCC patients present underlying hepatopathy - in particular of viral etiology (Table 1)<sup>[4]</sup>. The majority of the cases (> 85%) have liver cirrhosis, which masks symptoms of cancer progression. The clinical course of HCC is mostly asymptomatic. Suspected focal liver changes are often detected incidentally while monitoring the patient's condition during abdominal ultrasound (US) examination, and often are too large and too advanced for the tumor to be subjected to potentially effective and radical therapy.

In 1999 in "Hepatology", Llovet *et al.*<sup>[5]</sup> published the results of an analysis of clinical data of 102 patients with unresectable HCC. They found that 80% of patients with asymptomatic unresectable HCC survived for 1 year, 65% for 2 years, and 50% for 3 years. Only 29% of patients with clinical symptoms who did not have radical therapy survived for 1 year, 16% for 2 years, and 8% for 3 years<sup>[5]</sup>. Despite great medical progress since the times of Llovet's report and huge developments in medicine, patients suffering from HCC presently cannot be offered much more. Because of serious limitations of the surgical and oncological treatment available, it seems necessary to concentrate on the earliest possible diagnosis, particularly sensitive detection of resectable focal liver changes - preferably when tumors are less than 2 cm in diameter<sup>[6]</sup>. For this reason, surveillance with US techniques and serum  $\alpha$ -fetoprotein (AFP) analyses is recommended for all cirrhotic patients and other specific risk groups (Table 2)<sup>[7]</sup> every 6 mo.

## RADIOLOGICAL TECHNIQUES FOR HCC DIAGNOSIS

US is the most popular method for HCC screening. Diagnostic success of US for HCC surveillance depends on many factors, but mostly on the size and character of the focal liver changes, as well as the experience of the sonographer and the technical quality of the US equipment. According to the literature<sup>[8,9]</sup> US sensitivity rises from 70% for lesions of about 1 cm in diameter, towards 90% when the tumor diameter is more than 5 cm. The specificity is variable between 48% and 94%<sup>[8,9]</sup>. HCC does not have a specific morphology on US, whereas smaller lesions, less than 3 cm in diameter, are homogenic and hypoechoic. As they increase and form focal necrosis and microbleeding, they become more and more heterogenic and hyperechoic. This feature together with arterial vascularity are typical of increased malignancy and poor prognosis. Doppler or contrast-enhanced US leading to a better visualization of the relation between organic neoplasms and vascular structures may be used for clear differentiation of those lesions. Because US examination is subjective and non-repetitive, all focal liver lesions suspected on US should be verified using: computer tomography (CT) and/or magnetic resonance imaging (MRI). The use of these methods leads to a much more accurate diagnosis of HCC: sensitivity up to 89% and specificity reaching 99%<sup>[9]</sup>. Unfortunately, the diagnosis seems not to be so precise when

Table 1 HCC risk development factors (%)<sup>[4]</sup>

	Europe	North America	Asia & Africa	Japan
HCV	60-70	50-60	20	70
HBV	10-15	20	70	10-20
ALC	20	20	10	10
Other	10	10	0	0

HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HBV: Hepatitis B virus; ALC: Alcohol abuse.

Table 2 HCC specific risk groups (AASLD Practice Guideline 2005)<sup>[7]</sup>

Hepatitis B carriers	Non-hepatitis B carriers
Cirrhotic patients (incidence of HCC: 3%-5%/year)	Hepatitis C (incidence of HCC: 2%-8%/year)
Non-cirrhotic patients with high HBV DNA and hepatitis activity	Alcoholic cirrhosis
Positive family history of HCC	Genetic hemochromatosis
Asian males > 40 years (incidence of HCC: 0.4%-0.6%/year)	Primary biliary cirrhosis and autoimmune hepatitis
Asian females > 50 years (incidence of HCC: 0.2%/year)	$\alpha$ 1-antitrypsin deficiency
Africans > 20 years (incidence of HCC: > 0.2%/year)	Non-alcoholic steatohepatitis

lesions are less than 1 cm in diameter - merely 34%<sup>[10]</sup>.

Many epidemiological studies showed that only 50% of HCC lesions smaller than 1 cm in diameter are discovered during US examination. According to the Barcelona recommendations those lesions should be observed, or, more precisely, screened by US at a minimum of 6 mo intervals. When the tumor is growing and/or becomes larger than 1 cm in diameter on US, CT and/or MRI should be applied. It is recommended that focal liver changes > 1 cm but < 2 cm be subjected to histopathological verification. False negative results have been found in 40% of patients subjected to a targeted liver biopsy<sup>[11]</sup>, therefore exclusion of HCC in this way seems meaningless. If a tumor is > 2 cm in diameter with pathognomic arterial hypervascularity verified by other radiological methods, and there is a high level of total AFP serum concentration (> 400 ng/mL), then HCC can be diagnosed, according to the Barcelona criteria<sup>[12,13]</sup>.

At present more and more doubts have been raised about using AFP as a reliable HCC biomarker. For this reason, American hepatopathologists treat every tumor larger than 1 cm in diameter in cirrhotic liver as HCC, consequently ignoring AFP serology (very frequently false negative)<sup>[14]</sup>.

## HCC SPECIFIC BIOMARKERS

### AFP

AFP was discovered in 1956 by Bergstrand and Czar<sup>[15]</sup>, who used paper for electrophoretic separation of human fetoprotein in serum. The first reports on the usefulness of AFP as a diagnostic marker for HCC were presented

**Table 3** Diagnostic values of AFP as HCC biomarker<sup>[20,21]</sup>

Cut-off value (µg/L)	Sensitivity (%)	Specificity (%)	Ref.
20	55-60	88-90	[21,22]
50	47.0	96.0	[22]
100	31.2	98.8	[21]
200	22.4	99.4	[21]
400	17.1	99.4	[21]

AFP: α-fetoprotein.

in 1964 by Tatarinov and in 1968 by Abelev<sup>[16]</sup>. AFP is a glycoprotein with molecular weight of around 70 kDa, synthesized in the endodermal cells of the yolk sac during early fetal development, and then in embryonic hepatocytes<sup>[17]</sup>. It reaches a maximum serum concentration of 3 g/L in the 12-16th wk of fetal life and during the next 18 mo, AFP values decrease and normalize<sup>[18]</sup>. Its synthesis in adult life is repressed. Pathological elevation is seen in hepatocyte regeneration and hepatocarcinogenesis. Numerous data have proved that significantly higher AFP serum levels accompany various liver diseases (viral hepatitis, liver cirrhosis, liver tumors: primarily HCC and hepatoblastoma, also metastasis in 5%-10% cases), other neoplasms (mainly cancers of the digestive tract: pancreas -24%, stomach -15%, large intestine -3%, and gallbladder). Positive predictive values (PPV) for AFP are paradoxically significantly lower among patients with HCC viral etiology than non-viral (PPV: 70% *vs* 94%,  $P < 0.05$ )<sup>[19]</sup>. It has been confirmed on numerous occasions that AFP serum concentration increases in parallel with HCC tumor size. For this reason AFP has to be considered 'the golden standard' for HCC serum markers. However, the usefulness of AFP testing for the population at risk should be seriously questioned. AFP diagnostic values for this assay are undoubtedly poor. AFP specificity varies from about 76% to 96% and increases with elevated cut-off value. Simultaneous sensitivity decreases much more from about 25% for potentially resectable tumors of less than 3 cm in diameter to about 50% for lesions of > 3 cm in diameter (Table 3)<sup>[20,21]</sup>. 20%-30% AFP sensitivity coincides with cut-off values > 100 µg/L, which means that 70%-80% of the results - of a conventional test used as "a gold standard" for HCC diagnosis - are falsely negative. Based on these data 70%-80% of liver tumors, normally resectable are non-detectable. For this reason only one patient out of 5 receives potentially curative treatment. Unfortunately, the remaining majority unfortunately do not undergo treatment or are subjected to it too late.

Nowadays it is believed that early HCC diagnosis is presently considered feasible in 30%-60% of the cases in developed countries. Tumors smaller than 2 cm in diameter represented < 5% of cases in the 1990s in Europe, whereas now they represent up to 30% of cases in Japan<sup>[6]</sup>. Significantly more effective surveillance strategies in Japan lead to earlier HCC detection and earlier qualification for effectively curative radical surgery, with very good postoperative survival rates<sup>[22]</sup>. According to these assumptions, European and American experts have defined trends and

expected aims of surveillance policies in Western countries in 1980-2020. The applicability of potentially curative treatments have been divided into 3 periods: until 1990: 5%-10% of cases; 1990-2010: 30%-40% of cases; and 2010-2020: 40%-60% of cases<sup>[6]</sup>. Limitations of available therapies constitute a major challenge for diagnostic techniques, which are, most of all, modern visual methods and novel HCC specific biomarkers.

Numerous studies analyzing the chemical structure of AFP have shown that different sugar moieties of the bonds determine their binding capacity to lectin lens culinaris agglutinin (LCA)<sup>[23]</sup>. Taking those facts into consideration, Polish scientists, Breborowicz *et al*<sup>[24]</sup> identified in 1981 3 main glycoforms, namely AFP-L1, AFP-L2, AFP-L3. AFP-L1, the non-LCA-bound fraction, is the major AFP isoform in the serum of nonmalignant hepatopathy patients (chronic hepatitis, cirrhosis). AFP-L2 presents intermediate binding capability with its serum concentration increasing during pregnancy, and it is also present in cases of yolk sac tumors. AFP-L3, as the LCA-bound fraction, is the major glycoform in the serum of HCC patients<sup>[24-26]</sup>. It can be detected in 35% of patients with small HCC (< 3 cm). Some clinical studies have indicated that AFP-L3 can be detected 9-12 mo ahead of changes using visual techniques<sup>[27,28]</sup>. Sensitivities of AFP-L3 in detecting HCC range from 45% for lesions < 2 cm to > 90% for changes > 5 cm in diameter<sup>[20]</sup>. The specificity is more than 95%<sup>[26,29]</sup>. Fucosylation rate can be used in clinical practice (AFP-L3/AFP total). It has been confirmed that the ratio of more than 10% is closely associated with worse liver function and poorer tumor histology with implications such as larger tumor mass, a more invasive/malignant character, and earlier metastatic tendency<sup>[27,28]</sup>. Therefore AFP-L3 could be used as a reliable early HCC biomarker and a valuable indicator of poor prognosis. It is possible to achieve particularly accurate results for HCC screening with the use of AFP-L3 in combination with one of the 3 newly-discovered AFP glycoforms which can also be used as single tests, that is AFP-P4, AFP-P5 (E-PHA), and monosialylated AFP (IEF)<sup>[19]</sup>.

### Des-γ-carboxyprothrombin (DCP)

DCP, also known as PIVKA-II (protein induced by vitamin K absence or antagonist-II), is an abnormal, inactive prothrombin, lacking carboxylation of the 10 glutamic acid residues in the N-terminus, which is the result of an acquired post-translational defect of the prothrombin precursor in HCC cell lines. DCP was discovered in serum of patients during their anticoagulant therapy with a vitamin K antagonist. In 1984 Liebman *et al*<sup>[27]</sup> first described a higher DCP level both in patients with HCC and in cases of HCC recurrence after surgical resection, suggesting the usefulness of DCP as an HCC biomarker. It has been proved that significant concentrations of serum DCP are present in 50%-60% of all HCC patients, but in only 15%-30% of early HCC cases<sup>[31]</sup>. In the analyses of Nakagawa *et al*<sup>[32]</sup> the sensitivity of this test is 48%-62% and the specificity is 81%-98%. The diagnostic value of DCP as a biomarker is



**Table 4** Diagnostic values of HCC serum markers<sup>[34-36]</sup>

Type of test	Sensitivity (%)	Specificity (%)
AFP-L3	61.60 <sup>[34]</sup>	92.00 <sup>[35]</sup>
DCP	72.70 <sup>[34]</sup>	90.00 <sup>[35]</sup>
AFP	67.70 <sup>[34]</sup>	71.00 <sup>[35]</sup>
AFP-L3+DCP	84.80 <sup>[34]</sup>	97.80 <sup>[37]</sup>
AFP-L3+AFP	73.70 <sup>[34]</sup>	86.60 <sup>[35,36]</sup>
DCP+AFP	84.80 <sup>[34]</sup>	90.20 <sup>[35]</sup>
AFP-L3+DCP+AFP	85.90 <sup>[34]</sup>	59.00 <sup>[35]</sup>

DCP: Des- $\gamma$ -carboxyprothrombin.

approximately comparable with AFP. Grazi *et al*<sup>[33]</sup> proved that AFP and DCP are not correlated, so the combination of those markers significantly improves HCC detection: sensitivity 74.2%, specificity 87.2%. Carr *et al*<sup>[34]</sup> reported in 2007 interesting data based on prospective analyses of 99 patients with non-resectable HCC verified using liver biopsy (Table 4)<sup>[34-36]</sup>. Nowadays the best way to diagnose HCC is the use of AFP-L3 with DCP analyzed by immuno-enzymatic higher sensitivity methodology<sup>[33,37]</sup>.

#### **$\alpha$ -L-fucosidase (AFU)**

AFU is a normal lysosomal enzyme which hydrolyzes sugars containing L-fucose. In 1984 Deugnier *et al*<sup>[38]</sup> first reported that AFU is overexpressed in patients with HCC liver changes. It has been proved that the values of AFU serum concentration were not correlated with the tumor size and were frequent in early HCC cases<sup>[23]</sup>. Tangkijvanich *et al*<sup>[39]</sup> indicated that the sensitivity and specificity of AFU were about 80% and 70% respectively, in contrast with 40% and almost 100% for AFP. A simultaneous determination of both markers can improve the sensitivity to 82%<sup>[39]</sup>. This conclusion suggested that AFU could serve as a valuable supplement to AFP in early detection of HCC, similar to another popular serum enzyme -  $\gamma$ -glutamyl transferase.

#### **$\gamma$ -glutamyl transferase (GGT)**

GGT is a glycosylated membrane enzyme which activity is modulated in many physiological and pathological conditions, including differentiation and carcinogenesis<sup>[40]</sup>. It is mainly secreted by the hepatic Kupffer cell and endothelium of the bile duct. GGT is also overexpressed, similar to AFP, by fetal hepatoblasts and HCC cell lines<sup>[23]</sup>. The total serum GGT, a generally accepted cholestatic marker, has poor HCC specificity so can be useful only supplementary to AFP and other newer biomarkers for more effective HCC screening. In 1965, Polish scientists Kokot *et al*<sup>[41]</sup> separated the serum  $\gamma$ -glutamyl transferase into 3 to 4 bands by means of paper electrophoresis<sup>[38]</sup>. Since then, other methods have been used, that is, separation of GGT bands by means of starch gel (Orlowski *et al*<sup>[42]</sup>), cellulose acetate (Hitoi *et al*<sup>[43]</sup>), agarose gel (Hetland *et al*<sup>[44]</sup>), polyacrylamide gel electrophoresis (Kojima *et al*<sup>[45]</sup>, Suzuki *et al*<sup>[46]</sup>; Sawabu *et al*<sup>[47]</sup>; Kew *et al*<sup>[48]</sup>), and polyacrylamide stage gel plate (Xu *et al*<sup>[49]</sup>). Xu reported that they had fractionated 9 to

11 activity bands of GGT, in which GGT II was found in the sera of all patients with hepatoma. The positive rate of GGT was 90% and no correlation was observed with AFP<sup>[49]</sup> and DCP<sup>[50]</sup>. After 10 years of follow-up they reported that GGT II was positive in 90% of cases with HCC and negative in most patients with acute and chronic viral hepatitis, extrahepatic tumors, in pregnant women, and in healthy controls<sup>[51]</sup>.

#### **Glypican-3 (GPC-3)**

GPC-3 is an oncofetal protein being one of the members of heparan sulfate proteoglycans anchored to the plasma membrane through glycosylphosphatidylinositol<sup>[52]</sup>. GPC-3 is normally involved in the regulation of cell proliferation and survival during embryonic development and functions as a tumor suppressor. It has been reported to be downregulated in breast cancer, ovarian cancer and lung adenocarcinoma<sup>[53]</sup> but upregulated in HCC<sup>[54]</sup>. GPC-3 is absent in hepatocytes of healthy subjects and patients with nonmalignant hepatopathy, and can be detected in about 50% of HCC patients and 33% of HCC patients seronegative for both AFP and DCP. The specificity of GPC-3 is 100%<sup>[55]</sup>. Some clinical studies have indicated that the simultaneous determination of GPC-3 and AFP could significantly increase the sensitivity in HCC detection, without a reduction in the specificity<sup>[56]</sup>. More trials have confirmed the diagnostic value of 2 other, newly-discovered membranous proteins: Golgi protein 73 (GP73) and mucin 1 (MUC-1).

GP73 is a resident Golgi protein, shown to be upregulated in hepatocytes of patients with acute hepatitis<sup>[57]</sup> and cirrhosis<sup>[58]</sup> and in the sera of patients with HBV- and HCV-related HCC<sup>[59,60]</sup>. Marrero *et al*<sup>[60]</sup> reported a sensitivity of 69% and a specificity of 75% in HCC versus cirrhotic patients, indicating its superiority in comparison with AFP: sensitivity 30%, specificity 96%.

MUC-1 is a membrane protein expressed in many epithelial cells, but overexpressed in patients with breast cancer<sup>[61]</sup>, inflammatory lung diseases<sup>[62]</sup>, and HCC<sup>[63,64]</sup>. Moriyama *et al*<sup>[63]</sup> demonstrated expression of MUC-1 in HCC cells and in serum of patients with HCV-related HCC. Gad *et al*<sup>[64]</sup> reported specificity of 99%, sensitivity of 87% for combined MUC-1, DCP and AFP in Japanese and Egyptian patients with HCC.

#### **Squamous cell carcinoma antigen (SCCA)**

SCCA represents a family of serine proteases of high molecular weight, also known as serpins. There are 2 homologous genes: *SCCA1* and *SCCA2*, encoding 2 different SCCA isoforms, both expressed in many normal squamous epithelial cells. Increased SCCA levels have been detected in head and neck cancers and other epithelial malignancies, including cervix and lung. Recently Pontisso *et al*<sup>[65]</sup> first reported a high SCCA expression in HCC tissues, which seems very interesting, as liver does not possess squamous epithelial cells. Hepatocytes, however, share a common embryogenic origin. The sensitivity and specificity for SCCA in HCC diagnosis are 84% and 46% respectively. The complementary strengths

(high sensitivity/low specificity) and total AFP (low sensitivity/high specificity) suggest that the combination of the 2 markers should be of more value for screening, and in fact it leads to a diagnostic accuracy of 90%<sup>[66]</sup>.

### Markers (AFP, SCCA, DCP) in immunocomplexes with immunoglobulins of the IgM class (AFP-IgM IC, SCCA-IgM IC, DCP-IgM IC)

A new step for HCC testing is represented by forming known antigens (AFP, SCCA, DCP) into immunocomplexes (IC) with immunoglobulins of the IgM class. Monitoring of SCCA-IgM IC, AFP-IgM IC and DCP-IgM IC appears to be a much more advantageous approach for detecting patients with small HCC changes<sup>[67-70]</sup>. Giannelli *et al*<sup>[67]</sup> confirmed that the combined use of AFP IgM IC, SCCA and SCCA IgM IC in patients displaying low levels of AFP (< 20 IU/mL) identified 25.6% HCC. This study suggests that the use of a combination of all these markers in clinical practice provides a non-invasive and simple test that could increase the accuracy of HCC diagnosis. According to the results of Beneduce *et al*<sup>[68]</sup>: SCCA IgM IC significantly improves accuracy of HCC testing with sensitivity of 100%, specificity of 70%, PPV of 100%, and negative predictive value of 83%; AFP-IgM IC is a complementary serological marker to free AFP and the combination of these biomarkers may be useful in the diagnosis of liver cancer<sup>[69]</sup>; DCP-IgM IC in HCC patients was not associated with an increase in IgM concentration and was more frequently detected in HCC patients than DCP and AFP, strengthening the diagnostic role of IgM immune complexes in liver cancer<sup>[70]</sup>. The novel generation of HCC biomarkers seems very promising as it introduces new hope in supporting US for more accurate HCC screening.

## CONCLUSION

The distinction between early HCC changes and dysplastic nodules among cirrhotic patients is challenging even in expert hands. It frequently proves very difficult to characterize by available radiological and pathological examination. Serum biomarkers such as AFP, AFP-L3, DCP, AFU, GGT, GP-73, MUC-1, SCCA, GPC-3 and a new generation of IgM-immunocomplexes have significant diagnostic limitations, and in fact they are not particularly precise for the early diagnosis of HCC. Simultaneous determination of these markers in various combinations could improve the accuracy in differentiating HCC from nonmalignant hepatopathy, but there still exists the unresolved problem of tiny 'grey' nodules in the 'black and white' diagnostic perspective. The potential of gene-expression profiling as a novel tool to improve diagnostic and prognostic prediction is very exciting. The development and progression of HCC is known to be caused by an accumulation of genetic changes resulting in an expression of cancer-related genes: oncogenes, tumor suppressor genes, genes involved in many regulatory pathways, such as cell cycle control, apoptosis and angiogenesis. Modern technology enables investigators to measure the expression of thousands

of mRNA's simultaneously and therefore may provide comprehensive information for diagnosis and therapy of HCC. Currently there are many defined lists of genes selected for the HCC molecular index such as telomerase reverse transcriptase, topoisomerase II  $\alpha$ , heat shock protein 70, serin/threonine kinase 15), phospholipase A2, insulin-like growth factor 2, connexin 26, chemokine C-X-C motif ligand 12,  $\alpha$ -2-macroglobulin, plasminogen, thrombospondin 1, and platelet-derived growth factor receptor  $\alpha$ <sup>[71,72]</sup>. According to novel advancements in the management of HCC in 2008 by Llovet *et al*<sup>[73]</sup>, high accuracy rates are presented by a 3-gene set: glypican-3, LYVE1 (lymphatic vessel endothelial hyaluronan receptor-1), and survivin. However, more studies are needed to demonstrate its superiority, and presently this is not the first choice in research on early detection of HCC. Major limiting factors for routine use of molecular technology in a clinical setting at present are the cost and the access to them. Hopefully in the not so distant future the costs will decrease and this technology will become increasingly more popular and automated.

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## Colonoscopic perforation: Incidence, risk factors, management and outcome

Varut Lohsiriwat

Varut Lohsiriwat, Department of Surgery, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand  
**Author contributions:** Lohsiriwat V performed the literature review, acquisition and analysis of data, and manuscript preparation.

**Supported by** Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand

**Correspondence to:** Varut Lohsiriwat, MD, Department of Surgery, Faculty of Medicine, Siriraj Hospital, Mahidol University, Prannok Road, Bangkok 10700, Thailand. [bolloon@hotmail.com](mailto:bolloon@hotmail.com)

Telephone: +66-2419-8077 Fax: +66-2411-5009

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### Abstract

This review discusses the incidence, risk factors, management and outcome of colonoscopic perforation (CP). The incidence of CP ranges from 0.016% to 0.2% following diagnostic colonoscopies and could be up to 5% following some colonoscopic interventions. The perforations are frequently related to therapeutic colonoscopies and are associated with patients of advanced age or with multiple comorbidities. Management of CP is mainly based on patients' clinical grounds and their underlying colorectal diseases. Current therapeutic approaches include conservative management (bowel rest plus the administration of broad-spectrum antibiotics), endoscopic management, and operative management (open or laparoscopic approach). The applications of each treatment are discussed. Overall outcomes of patients with CP are also addressed.

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**Key words:** Colonoscopic perforation; Colonoscopy; Sigmoidoscopy; Complication; Postpolypectomy syndrome; Incidence; Risk factors; Treatment; Management; Outcome

### INTRODUCTION

Colonoscopic perforation (CP) is widely recognized as one of the most serious complications following lower gastrointestinal endoscopies. Although CP is a rare complication, it is associated with a high rate of morbidity and mortality<sup>[1-5]</sup>. This unpleasant complication could result in operation, stoma formation, intra-abdominal sepsis, prolonged hospital stay, and even death. This article describes an overview of incidence, risk factors, management and outcome of CP.

### INCIDENCE

The incidence of CP could be as low as 0.016% of all diagnostic colonoscopy procedures<sup>[6]</sup> and may be seen in up to 5% of therapeutic colonoscopies<sup>[7,8]</sup>. Meanwhile, the incidence of CP following flexible sigmoidoscopy varies from 0.027% to 0.088%<sup>[1,9-12]</sup>. Interestingly, rectal perforation during colonoscopic retroflexion was reported to be around 0.01%<sup>[13]</sup>. The incidences of CP in some larger series (sample size > 30 000 cases) published from 2000 onwards are shown in Table 1<sup>[2-4,9,14-18]</sup>. The most common site of colonic perforation is the rectosigmoid

**Table 1** Incidence of CP, management and outcomes from recent series with sample size > 30 000 cases

Author	Year	Number of patients	CP rate	Death rate in CP cases	CPT rate in CP cases	Surgical treatment (%)
Araghizadeh <i>et al</i> <sup>[14]</sup>	2001	34 620	0.090	3.2	NA	74
Gatto <i>et al</i> <sup>[9]</sup>	2003	74 584	0.145	5.6	NA	NA
Korman <i>et al</i> <sup>[17]</sup>	2003	116 000	0.032	0.0	NA	95
Cobb <i>et al</i> <sup>[16]</sup>	2004	43 609	0.032	0.0	21.4	93
Lüning <i>et al</i> <sup>[4]</sup>	2007	30 366	0.115	8.6	40.0	100
Rabeneck <i>et al</i> <sup>[18]</sup>	2008	97 091	0.085	NA	NA	NA
Iqbal <i>et al</i> <sup>[2]</sup>	2008	258 248	0.070	7.0	36.0	92
Teoh <i>et al</i> <sup>[3]</sup>	2009	37 971	0.113	25.6	48.7	91
Arora <i>et al</i> <sup>[15]</sup>	2009	277 434	0.082	NA	NA	NA

CP: Colonoscopic perforation; CPT: Complication; NA: Not available.

colon<sup>[1-4,17,19,20]</sup>. Several factors making this bowel segment vulnerable to being injured include a sharp angulation at either the rectosigmoid junction or the sigmoid-descending colon junction, and the great mobility of the sigmoid colon. A forceful insertion of an endoscope while having a sigmoid loop formation is the leading cause of anti-mesenteric bowel perforation due to an overextension of bowel by the shaft of the endoscope. Additionally, the sigmoid colon is commonly involved with diverticular formation<sup>[17,21]</sup>, and the muscular layer of the bowel wall may be thin or fragile due to previous inflammation (diverticulitis). Pelvic adhesions following previous pelvic operation or infection also contribute to a high incidence of sigmoid perforation<sup>[2,7]</sup>.

## RISK FACTORS

There has been convincing evidence that therapeutic colonoscopies have a significantly higher rate of CP than diagnostic colonoscopies<sup>[15,18,20,22]</sup>. The increased likelihood of CP in therapeutic endoscopy is because the perforation during therapeutic colonoscopy can occur not only through mechanisms that are similar to those seen for diagnostic colonoscopy (mechanical injury or barotrauma), but also through the fact that endoscopic interventions *per se* can cause perforation<sup>[20]</sup>. Several investigators have reported that some endoscopic interventions are associated with an increased CP rate, including polypectomy for polyps larger than 20 mm<sup>[23]</sup>, pneumatic dilatation for Crohn's stricture<sup>[24]</sup>, the use of argon plasma coagulation<sup>[25]</sup>, endoscopic mucosal resection and endoscopic submucosal dissection for colorectal neoplasia<sup>[8,26,27]</sup>.

Patients over 75 years of age also have an approximately 4-6 fold rise in the CP rate as opposed to younger patients<sup>[9,18,20,28]</sup>. Possible explanations for an increased rate of CP in patients with advanced age include the fact that the elderly have a declining colonic wall mechanical strength as recognized in colonic diverticular diseases, and they often have a greater frequency of abnormal colorectal findings which may require endoscopic intervention.

The risk of perforation from colonoscopy is 2-4 times greater than that from flexible sigmoidoscopy<sup>[4,9,20,29]</sup>. Pa-

tients with multiple comorbidities are also at greater risk of this perforation<sup>[9,15]</sup>. These comorbidities include diabetes mellitus, chronic pulmonary disease, congestive heart failure, myocardial infarction, cerebrovascular disease, peripheral vascular disease, renal insufficiency, liver disease and dementia<sup>[30-32]</sup>.

Other risk factors for CP reported in the literature include a history of diverticular disease<sup>[9]</sup> or previous intra-abdominal surgery<sup>[17]</sup>, colonic obstruction as an indication for colonoscopy<sup>[15]</sup>, and female gender<sup>[29]</sup>. The difference in anatomy of the large intestine between males and females was demonstrated by Saunders *et al*<sup>[33]</sup>. They found that women had a greater colonic length and a more mobile transverse colon, thus increasing the difficulty in performing colonoscopy in female patients.

## PRESENTATION AND DIAGNOSIS

The most common clinical feature of CP is the visualization of an extra-intestinal structure during the endoscopic examination<sup>[2]</sup>. However, CP patients could present with symptoms and signs of peritonitis (mainly abdominal pain and tenderness) within several hours after the completion of colonoscopy. Patients with CP from therapeutic colonoscopies tend to have a smaller size of the perforation and have a delay in presentation and diagnosis compared with diagnostic colonoscopies<sup>[3,4,17]</sup>. When perforation is suspected, a plain roentgenogram of the abdomen should be taken to rule out intraperitoneal air. Other sophisticated investigations, such as computed tomography (CT) scanning, and magnetic resonance imaging, are also of great help to identify the free gas<sup>[2]</sup>. Triple-contrast or double-contrast (intravenous and rectal) CT scanning is increasingly used in patients with a clinical suspicion of colonic perforation<sup>[34-36]</sup>, and in those with CP who are eligible for non-operative management<sup>[37]</sup>. Water-soluble contrast enema is seldom performed to detect the perforation, or to confirm a concealed perforation. Practically, patients can be diagnosed and treated for CP on the basis of generalized peritonitis without the radiologic evidence of perforation.

A perforated site is typically a large anti-mesenteric tear of colonic wall if it is caused by the shaft of the endoscope. Furthermore, a smaller perforation can be

found in an injury from the tip of the endoscope, or in those related to endoscopic interventions such as polypectomy. Although perforations usually occur during the colonoscopic examination or within 24 h after the procedure<sup>[1-3]</sup>, delayed perforation of the colon and rectum has been reported<sup>[38,39]</sup>. Physicians should therefore suspect a CP if a patient has fever, abdominal pain or distention following the colonoscopic examination, even if the patient presents with these symptoms several days after the procedure.

It is notable that postpolypectomy coagulation syndrome, also known as postpolypectomy syndrome or transmural burn syndrome, can mimic perforation by presenting with similar symptoms and signs<sup>[40]</sup>. Postpolypectomy syndrome occurs when there is a transmural injury of the bowel wall at the site of excised polyp, caused by an overt electrical current or thermal injury<sup>[41,42]</sup>. Without any obvious perforation, transmural bowel injury as well as serosal irritation results in a localized peritonitis, abdominal pain, fever and leukocytosis. Conventional radiography is often unremarkable in this setting. Meanwhile, CT scan may reveal focal mural thickening and pericolic fluid at the site of recent polypectomy as well as soft-tissue stranding of the pericolic fat, without any evidence of pneumoperitoneum or large hematoma<sup>[43,44]</sup>. Conservative management, as described in the following section, is generally successful with good outcomes<sup>[7,45]</sup>.

## MANAGEMENT

Management of CP remains a controversial issue as it can be effectively managed by both operative and non-operative strategies<sup>[37,46,47]</sup>. Although most patients with CP promptly require open surgery, there is an increasing use of non-operative or laparoscopic approaches in selected patients<sup>[48-55]</sup>. The viable options of CP management are discussed as follows.

### Conservative treatment

Clearly, the choice between conservative and surgical management depends on clinical grounds. Conservative management is reserved for CP patients in good general condition and without any sign of peritonitis. This approach involves intravenous fluids, absolute bowel rest and intravenous administration of broad-spectrum antibiotics. If the conservative treatment is successful, patient's clinical appearance should improve gradually within 24-48 h. If this is not the case, complicated intra-abdominal infections (such as fecal peritonitis or intra-abdominal abscess) should be considered, and thus further investigation and management are imperative. Patients must be prepared to proceed to surgical management if clinical improvement is not maintained or when progressive intra-abdominal sepsis occurs. Overall success rate of conservative treatment for CP varies from 33% to 73%<sup>[14,16,56]</sup>. A small perforation site caused by therapeutic colonoscopy has been shown to have a better success rate with medical treatment<sup>[56]</sup>. Colonic stricture following conservative treatment of a colonoscopic perforation has been reported in the literature<sup>[57]</sup>,

but this can be safely managed by either endoscopic dilatation or surgery.

### Endoscopic closure of the perforation

With recent advances in endoscopic technology (such as better optics, and availability of multichannel endoscopy and intraluminal endoclipping) as well as increasing experience of endoscopic interventions<sup>[58-60]</sup>, many endoscopists have been encouraged to perform the endoscopic closure of CP since the first successful endoscopic repair of CP was reported in 1997<sup>[61]</sup>. However, this approach requires not only high endoscopic skill but also appropriate endoscopic devices. In general, the size of the perforation suitable for endoscopic closure is less than 10 mm, but some reports showed successful endoscopic repairs of the perforation larger than 10 mm<sup>[52,62]</sup>. To overcome the problems of closing large defects, novel endoscopic closure devices have been designed such as detachable endoscopic snares and special metal rings in conjunction with endoscopic clips<sup>[63]</sup>.

Any endoscopic repair should be performed with as little air insufflation as possible because a distended lumen often makes it difficult to close the perforation site. Moreover, an extensive air insufflation not only leads to further fecal spillage into the intraperitoneal space but also causes massive pneumoperitoneum, which can compromise the cardiopulmonary system of CP patients<sup>[62]</sup>. After having endoscopic repair, patients should be given intravenous broad-spectrum antibiotics and a clear liquid diet until bowel movement returns and any evidence of peritonitis disappears. Intensive monitoring and serial abdominal examinations are also essential. A review of 75 reported cases of CP repaired by endoclipping, by Trecca *et al*<sup>[62]</sup> in 2008, showed a success rate of 69%-93%. Early recognition of the perforation, prompt complete endoscopic repair, and good bowel preparation are keys to the success of endoscopic treatment for CP.

### Operative treatment

Surgical management is recommended in those with diffuse peritonitis, those with clinical deterioration under non-surgical treatment, or those with a concomitant colonic pathology that requires surgery, such as colorectal cancer. A wide range of surgical options have been described to manage CP depending on the patient's condition, the size of the perforation, the underlying pathology of the large intestine, the quality of bowel preparation, the time between injury and diagnosis, and the surgeon's preference. Feasible choices of the operation are described as follows.

**Simple closure of the perforation:** This surgical approach is appropriate in the case of small colonic perforation (< 50% of bowel circumference), without significant fecal contamination and concomitant intestinal pathology requiring bowel resection. Oversewing of the perforation has been carried out in 25%-56% of immediate perforations, and the leakage rate following primary repair was extremely low<sup>[1-4,64]</sup>.

**Bowel resection with or without intestinal continuity:**

Bowel resection including the perforation site is required when the perforation site is large, or when primary closure of the perforation could compromise the lumen, or when there is concomitant colon pathology requiring bowel resection, such as severe colonic stricture, large sessile polyp or colorectal cancer. In the absence of significant intra-abdominal contamination, bowel resection and anastomosis can be performed with acceptable morbidity. However, when faced with extensive tissue inflammation or fecal peritonitis, bowel resection without anastomosis should be considered. An extensive study of 165 iatrogenic CP cases by Iqbal *et al*<sup>[2]</sup> in 2008 found that patients being diagnosed with CP within 24 h after the colonoscopic examination were more likely to have minimal peritoneal contamination and, thus, tended to undergo primary repair or resection with anastomosis. Conversely, patients presenting after 24 h were more likely to have feculent contamination and to receive a stoma formation. Furthermore, patients with blunt injuries were more likely to receive a stoma than those with polypectomy and thermal perforations.

Another issue under discussion is the role of laparoscopic surgery for CP<sup>[49,50,62,65,66]</sup>. With advanced laparoscopic techniques such as intracorporeal suturing, laparoscopic repair of CP is becoming widely practiced and acceptable. A small comparative study by Bleier *et al*<sup>[67]</sup> showed that a laparoscopic approach to CP resulted in less postoperative complications, decreased length of hospital stay, and a shorter incision length compared to an open method. However, an inability to laparoscopically localize the perforation site or doubt about the security of the repair should prompt conversion to laparotomy<sup>[50]</sup>.

**OUTCOME**

Patients with CP could have a remarkably high morbidity and mortality rate depending on their existing medical conditions, nature of the perforation, methods of CP management, experience of the care team and hospital setting. The 30-d morbidity and mortality rates are 21%-53% and 0%-26%, respectively<sup>[1-4,16]</sup>. The average length of hospital stay in CP patients is 1-3 wk<sup>[1,3,5,68]</sup>.

Surgical site infection is the most common complication, while cardiopulmonary complications and multiple organ failure are the leading causes of death<sup>[1,2]</sup>. Some investigators have suggested that predisposing factors for poor outcomes of CP patients include a large perforation site, a delayed diagnosis, extensive peritoneal contamination, poor bowel preparation, corticosteroid use, anticoagulants or anti-platelet therapy, prior hospitalization, advanced age of patients, and severe comorbid diseases<sup>[2,3,69,70]</sup>.

**CONCLUSION**

Colonoscopic perforation is a rare complication following lower gastrointestinal endoscopies; however, it is asso-

ciated with a high rate of morbidity and mortality. Special precautions should be taken during therapeutic endoscopy and while performing colonoscopic examination in patients with advanced age or those with several comorbidities. Management of patients with CP should be individualized based on patients' clinical grounds and their underlying diseases, nature of the perforation, and concomitant colorectal pathologies.

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## Hospitalized prevalence and 5-year mortality for IBD: Record linkage study

Lori A Button, Stephen E Roberts, Michael J Goldacre, Ashley Akbari, Sarah E Rodgers, John G Williams

Lori A Button, Stephen E Roberts, Ashley Akbari, Sarah E Rodgers, John G Williams, School of Medicine, Swansea University, Swansea SA2 8PP, United Kingdom

Michael J Goldacre, Unit of Health-Care Epidemiology, University of Oxford, Oxford OX2 7LF, United Kingdom

**Author contributions:** Roberts SE initiated the study; Roberts SE, Williams JG and Goldacre MJ designed the study; Roberts SE and Button LA reviewed the literature; Button LA and Akbari A undertook the analyses; Rodgers SE undertook the GIS analyses; Roberts SE, Button LA, Goldacre MJ and Williams JG interpreted the study findings and wrote the manuscript.

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**Correspondence to:** Dr. Stephen E Roberts, School of Medicine, Swansea University, Singleton Park, Swansea, SA2 8PP, United Kingdom. [stephen.e.roberts@swansea.ac.uk](mailto:stephen.e.roberts@swansea.ac.uk)  
Telephone: +44-1792-513426 Fax: +44-1792-513423

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ized prevalence of CD was slightly higher in the most deprived areas, but there was no association between social deprivation and hospitalized prevalence of UC. Mortality was 6.8% and 14.6% after 1 and 5 years follow-up for CD, and 9.2% and 20.8% after 1 and 5 years for UC. For both CD and UC, there was little discernible association between mortality and social deprivation, distance from hospital, urban/rural residence and geography.

**CONCLUSION:** CD and UC have distinct demographic profiles. The higher prevalence of hospitalized CD in more deprived areas may reflect higher prevalence and higher hospital dependency.

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**Key words:** Crohn's disease; Ulcerative colitis; Prevalence; Mortality; Record linkage

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### Abstract

**AIM:** To establish the hospitalized prevalence of severe Crohn's disease (CD) and ulcerative colitis (UC) in Wales from 1999 to 2007; and to investigate long-term mortality after hospitalization and associations with social deprivation and other socio-demographic factors.

**METHODS:** Record linkage of administrative inpatient and mortality data for 1467 and 1482 people hospitalised as emergencies for  $\geq 3$  d for CD and UC, respectively. The main outcome measures were hospitalized prevalence, mortality rates and standardized mortality ratios for up to 5 years follow-up after hospitalization.

**RESULTS:** Hospitalized prevalence was 50.1 per 100 000 population for CD and 50.6 for UC. The hospitalized prevalence of CD was significantly higher ( $P < 0.05$ ) in females (57.4) than in males (42.2), and was highest in people aged 16-29 years, but the prevalence of UC was similar in males (51.0) and females (50.1), and increased continuously with age. The hospital-

### INTRODUCTION

Inflammatory bowel disease (IBD), comprising mainly Crohn's disease (CD) and ulcerative colitis (UC), can be one of the most severe gastrointestinal disorders with a significant health and economic burden<sup>[1]</sup>. Presenting commonly in adolescence, IBD often results in debilitating morbidity and frequent relapses that impact heavily on

educational, social, professional and family life<sup>[1-3]</sup>.

CD has increased in many countries in the past few decades, with highest prevalence often reported in western regions such as Northern and Western Europe and North America<sup>[3-7]</sup>. In the United Kingdom, the incidence of UC has shown greater stability over time<sup>[6,8]</sup>, while CD has continued to rise in many but not in all regions<sup>[7,9-13]</sup>. Most studies have reported that CD is more common in females than in males<sup>[8,10,11,13-16]</sup>, whereas for UC, higher risks have been reported in females<sup>[3,17,18]</sup> or in males<sup>[2,19]</sup>, as well as similar risks for females and males<sup>[20,21]</sup>.

In the United Kingdom, the prevalence of IBD is not known accurately, although based on regional studies, it is thought to affect approximately 150 000 people<sup>[1]</sup>. Of these, the majority have active IBD, with many having severe IBD that may lead to substantial inpatient stays. Our study focused on severe IBD, which we define as an emergency admission to hospital with a length of stay of  $\geq 3$  d, because we wanted to study people who were acutely ill with severe IBD. Inclusion of all patients admitted to hospital, regardless of length of stay, would have covered a wide spectrum of disease severity, from elective day case investigation of IBD and admissions for elective surgery, to emergency care of patients who were severely ill with IBD.

Little is known about the prevalence of severe IBD in the United Kingdom, how it varies geographically, and its relationship with social deprivation. Mortality following hospitalization for IBD is significant<sup>[22]</sup>, but few studies have been based on long-term follow-up. Additionally, little is known about the effect on mortality of social deprivation and other socio-demographic factors such as area of residence and distance from hospital. These are important aspects of the epidemiology of IBD in the United Kingdom, which are important to understand in planning service provision and treatment.

The main objective of this study was to establish the prevalence of UC and CD that was severe enough to require admission to hospital for  $\geq 3$  d, and to report on subsequent mortality after up to 5 years follow-up. Wales has a population of 3 million, and a mixed urban and rural geography, with areas of significant social deprivation. Further objectives were to assess geographical variation in the hospitalized prevalence and mortality for CD and UC, and to investigate associations with social deprivation and other socio-demographic characteristics, including any association between mortality and distance of patients' residence from hospital and urban or rural residence.

## MATERIALS AND METHODS

For this study, we used hospital inpatient data from the Patient Episode Database for Wales (PEDW), which incorporates medical record linkage to mortality data from the National Health Service Administrative Register<sup>[23]</sup>. PEDW is a comprehensive administrative database that covers all inpatient and day-case admissions to every National Health Service (NHS) hospital in Wales, and has been used as the basis for previously published studies<sup>[24-27]</sup>.

It covers 17 district general or teaching hospitals in eight NHS hospital trusts across 22 health boards, and serves the Welsh population of 3 million. We used record linkage of inpatient and mortality data because this enabled all repeat hospital admissions to be identified for the same patients, and enabled deaths following discharge from hospital to be identified, as well as in-hospital deaths.

The International Classification of Diseases, 10th Edition (ICD-10) codes used were: K50 for CD and K51 for UC. Patients with an emergency hospital admission of three or more consecutive days were classified as severe cases and included in the study. We selected only those admissions for which CD or UC was recorded as the principal diagnosis on the discharge record, and selected only the first admissions for each patient that met the study inclusion criteria during the 8-year study period from 1999 to 2007. We excluded all elective admissions, all day cases and inpatient admissions lasting  $< 3$  d, all admissions subsequent to the first admission, and all admissions for which CD and UC were recorded on the discharge record as secondary diagnoses to other diseases such as colorectal malignancy.

Hospitalized prevalence rates for CD and UC were calculated per 100 000 resident population, using the numbers of severe hospitalized cases as the numerators and the Welsh residential population as the denominators. The direct method and the standard European population were used to standardize the prevalence rates. Mortality rates following hospital admission were calculated using the numbers of deaths (from all causes) as the numerators, and the total number of hospitalized cases, in the same time periods, as the denominators. Mortality rates were expressed as percentages and calculated at 1 and 5 years follow-up from the date of hospital admission.

To establish how mortality among the CD and UC patients compared with that in the general population, we used standardized mortality ratios (SMRs). These were calculated by applying the age- and sex-specific mortality rates in the general Welsh population to the numbers of study patients in the corresponding age- and sex-specific strata, to calculate the expected number of deaths in the study patients, and by comparing the observed deaths with the expected deaths.

Social deprivation scores were assigned to the patients' place of residence on each hospital record by using residential postcodes at the Lower Super Output Area (LSOA) and the Welsh Index of Multiple Deprivation (WIMD) 2005<sup>[28]</sup>. The average population size of the 1896 LSOAs in Wales in 2005 was 1560. The WIMD 2005 was based on seven domains of social deprivation: income, housing, employment, environment, health, access to services, and education. The LSOAs were ranked according to their WIMD 2005 score and grouped into quintiles, with quintile I representing the 20% of least deprived LSOAs and quintile V the 20% of most deprived LSOAs in Wales.

Urban/rural residences were measured using five categories, based on settlement sizes that ranged from  $< 2500$  to  $> 100\,000$  people. Distances from patients'



Table 1 Hospitalized prevalence of severe CD and ulcerative colitis across health boards, 1999-2007

Health board	Map <sup>1</sup>	No. of LSOAs	Deprivation score (mean $\pm$ SD)	CD			UC		
				Cases	Prevalence (per 100 000)	95% CI	Cases	Prevalence (per 100 000)	95% CI
Blaenau Gwent	1	47	33.8 $\pm$ 12.7	31	44.7	(30.4-63.5)	27	39.0	(25.7-56.7)
Bridgend	2	85	23.2 $\pm$ 13.0	41	31.5	(22.6-42.7)	62	47.6	(36.5-61.0)
Caerphilly	3	110	27.0 $\pm$ 14.1	97	57.0	(46.2-69.5)	91	53.5	(43.0-65.6)
Cardiff	4	203	21.3 $\pm$ 18.0	158	50.9	(43.3-59.5)	137	44.1	(37.1-52.2)
Carmarthenshire	5	112	22.0 $\pm$ 10.3	125	71.3	(59.3-84.9)	67	38.2	(29.6-48.5)
Ceredigion	6	47	15.0 $\pm$ 6.2	35	46.0	(32.0-63.9)	30	39.4	(26.6-56.2)
Conwy	7	71	18.0 $\pm$ 11.0	64	57.8	(44.5-73.8)	84	75.9	(60.6-94.0)
Denbighshire	8	58	20.4 $\pm$ 15.6	56	59.0	(44.6-76.7)	56	59.0	(44.6-76.7)
Flintshire	9	92	15.0 $\pm$ 10.2	74	49.6	(38.9-62.2)	77	51.6	(40.7-64.5)
Gwynedd	10	75	17.3 $\pm$ 7.4	63	53.7	(41.2-68.7)	70	59.6	(46.5-75.4)
Isle of Anglesey	11	44	20.7 $\pm$ 8.8	43	63.0	(45.6-84.9)	43	63.0	(45.6-84.9)
Merthyr Tydfil	12	36	37.2 $\pm$ 17.0	26	46.7	(30.5-68.4)	50	89.7	(66.6-118.3)
Monmouthshire	13	58	12.2 $\pm$ 6.0	37	42.9	(30.2-59.1)	44	51.0	(37.1-68.5)
Neath Port Talbot	14	91	28.2 $\pm$ 14.4	76	56.0	(44.1-70.0)	53	39.0	(29.2-51.0)
Newport	15	94	22.0 $\pm$ 15.6	73	52.4	(41.1-65.9)	71	51.0	(39.8-64.3)
Pembrokeshire	16	71	19.4 $\pm$ 8.9	44	38.0	(27.6-51.0)	47	40.6	(29.8-54.0)
Powys	17	80	14.3 $\pm$ 6.1	44	34.2	(24.9-46.0)	62	48.2	(37.0-61.8)
Rhondda Cynon Taff	18	152	29.2 $\pm$ 15.4	116	50.0	(41.3-60.0)	125	53.9	(44.9-64.2)
Swansea	19	147	22.6 $\pm$ 17.8	101	44.9	(36.6-54.6)	109	48.5	(39.8-58.5)
Torfaen	20	60	21.3 $\pm$ 10.8	46	50.6	(37.1-67.5)	51	56.1	(41.8-73.8)
Vale of Glamorgan	21	78	15.0 $\pm$ 10.5	49	40.7	(30.1-53.7)	74	61.4	(48.2-77.1)
Wrexham	22	85	19.9 $\pm$ 13.5	68	52.5	(40.8-66.5)	52	40.2	(30.0-52.6)
All Wales		1896	21.7 $\pm$ 14.3	1467	50.1	(47.5-52.7)	1482	50.6	(48.0-53.2)

<sup>1</sup>For geographical details of each health board, see Figure 1. CD: Crohn's disease; UC: Ulcerative colitis; LSOA: Lower Super Output Area.

residential LSOAs (geographical center points) to their hospital of admission were calculated using ArcGIS Geographical Information System (GIS) software and were measured in five categories that ranged from  $< 3$  to  $> 20$  km. Other methods used included logistic regression analysis,  $\chi^2$  tests, and Pearson's correlations.

## RESULTS

From 1999 to 2007, there were 1467 patients hospitalized for  $\geq 3$  d following emergency admission for CD (mean  $\pm$  SD, age: 45.8  $\pm$  20.4 years) and 1482 for UC (mean  $\pm$  SD, age: 52.0  $\pm$  21.0 years). All the results that follow refer to these patients who meet the definition of severe IBD, although, for brevity, we omitted the repeated use of the term severe.

### Hospitalized prevalence of CD and UC

The overall hospitalized prevalence rate per 100 000 population was 50.1 for CD and 50.6 for UC (Table 1). Geographically, across the 22 health boards in Wales (Table 1), the prevalence of Crohn's varied from 31.5 per 100 000 (95% CI: 22.6-42.7) to 71.3 (59.3-84.9), whereas for UC, it varied from 38.2 per 100 000 (29.6-48.5) to 89.7 (66.6-118.3).

For UC, there was a significant association between hospitalized prevalence and age ( $P < 0.05$ , Table 2). The hospitalized prevalence of UC increased with age, and was highest for males and females aged  $> 65$  years (103 and 93.4 per 100 000, respectively). There was no significant association between the hospitalized prevalence of CD

and age. The highest age-specific prevalence of CD was in the 16-29 years age group, for both males (61.4) and females (76.0). Overall, the hospitalized prevalence of CD was significantly higher ( $P < 0.05$ ) in females (57.4) than in males (42.2), whereas the hospitalized prevalence of UC was very similar in females (50.1) and males (51.0, Table 2).

Figure 1 shows the geographical distribution of the social deprivation quintiles to the 1896 LSOAs across Wales. The hospitalized prevalence of CD was associated significantly with social deprivation at the LSOA level (Pearson's correlation = 0.07,  $P = 0.004$ ), although the association was not strong. The prevalence of CD tended to be higher in more deprived areas, although there were not quite significant differences between the most and least affluent quintiles (Figure 2). For UC, there was no significant association between prevalence and social deprivation at the LSOA level, or across any of the five social deprivation quintiles (Figure 2).

### Mortality in CD and UC at 1 year

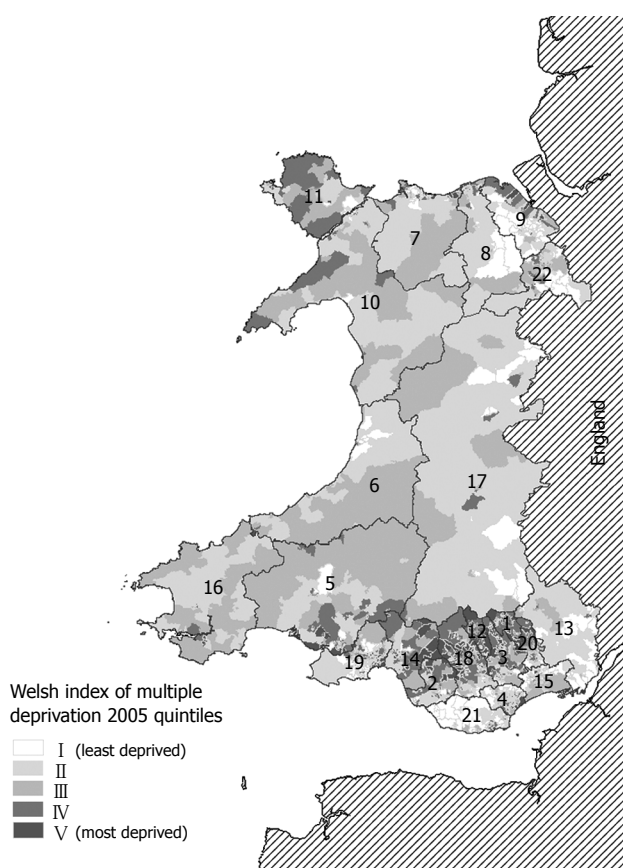
After 1 year follow-up, mortality was 6.8% for CD and 9.2% for UC (Table 3). Corresponding SMRs at 1 year were 5.1 (95% CI: 4.1-6.27; compared with mortality of 1.0 in the general population) for CD and 4.3 (95% CI: 3.6-5.1) for UC.

For CD and UC, mortality at 1 year increased sharply with age, and age-adjusted mortality was similar among males and females (Table 3). Very few deaths occurred among people aged  $< 45$  years. For CD and UC, there was no significant variation in mortality according to social deprivation, urban/rural residence, distance from

Table 2 Age- and sex-specific hospitalized prevalence rates for severe CD and UC, 1999-2007

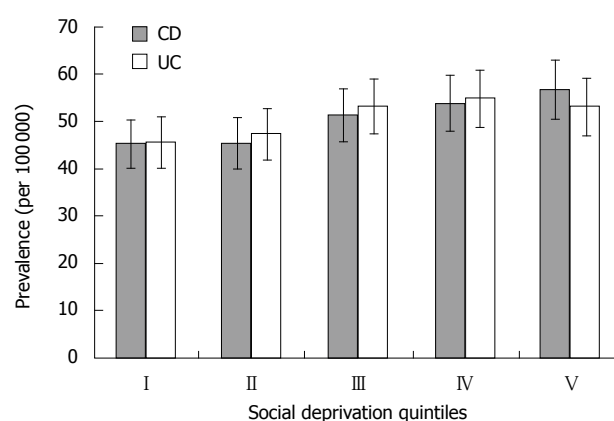
		CD			UC		
		Cases	Prevalence (per 100 000)	95% CI	Cases	Prevalence (per 100 000)	95% CI
Males	All	601	42.2	(38.9-45.8)	726	51.0	(47.4-54.9)
	0-15	25	8.4	(5.5-12.5)	20	6.7	(4.1-10.4)
	16-29	151	61.4	(52.0-72.0)	115	46.7	(38.6-56.1)
	30-44	141	47.6	(40.1-56.1)	168	56.7	(48.5-66.0)
	45-64	163	44.5	(38.0-51.9)	199	54.4	(47.1-62.5)
	65+	119	54.5	(45.2-65.3)	224	103	(89.7-117)
Females	All	866	57.4	(53.7-61.4)	756	50.1	(46.6-53.8)
	0-15	21	7.5	(4.6-11.4)	11	3.9	(2.0-7.0)
	16-29	185	76.0	(65.5-87.8)	114	46.9	(38.6-56.3)
	30-44	232	74.3	(65.1-84.5)	166	53.2	(45.4-61.9)
	45-64	222	58.9	(51.4-67.2)	189	50.1	(43.3-57.8)
	65+	205	69.4	(60.2-79.6)	276	93.4	(82.7-105)
All Wales		1467	50.1	(47.5-52.7)	1482	50.6	(48.0-53.2)

The ages of three Crohn's patients, two males and one female, was unknown.



**Figure 1** WIMD 2005 quintiles for the 22 health boards and the 1896 geographical LSOAs in Wales. The key to the health board numbering is as follow: 1 Blaenau Gwent; 2 Bridgend; 3 Caerphilly; 4 Cardiff; 5 Carmarthenshire; 6 Ceredigion; 7 Conwy; 8 Denbighshire; 9 Flintshire; 10 Gwynedd; 11 Isle of Anglesey; 12 Merthyr Tydfil; 13 Monmouthshire; 14 Neath Port Talbot; 15 Newport; 16 Pembrokeshire; 17 Powys; 18 Rhondda Cynon Taff; 19 Swansea; 20 Torfaen; 21 The Vale of Glamorgan; 22 Wrexham.

hospital, and whether or not an emergency colectomy was performed (Table 3). Although there was some variation in mortality across the 22 Welsh health boards, there were no significant differences between individual health boards.



**Figure 2** Hospitalized prevalence of severe Crohn's disease and ulcerative colitis according to social deprivation, 1999-2007. I: Least deprived social deprivation quintile; V: Most deprived. Vertical bars represent 95% CI. These prevalence rates are standardised for age group and gender.

### Mortality in CD and UC at 5 years

Mortality at 5 years was 14.6% for CD and 20.8% for UC (Table 4). SMRs at 5 years were 2.4 (95% CI: 1.9-2.9; compared with mortality of 1.0 in the general population) for CD and 1.9 (95% CI: 1.5-2.2) for UC.

For CD and UC, mortality at 5 years was comparable among females and males, and it increased sharply among older age groups: among men aged > 65 years and women aged > 60 years, mortality was 34% for CD and 49% for UC (Table 4). There were no significant differences in mortality at 5 years according to urban/rural residence, distance travelled to hospital, or whether an emergency colectomy was performed (Table 4). For CD, mortality was significantly higher in the most deprived quintile compared with the second most affluent quintile, although there were no other significant differences in mortality according to social deprivation.

## DISCUSSION

The main aims of this record linkage study were to

Table 3 Mortality at 1 year following hospitalization for severe CD and UC, 1999-2007

		CD				UC			
		Cases	No. of deaths	Mortality rate (%)	Adjusted OR <sup>1</sup> (95% CI)	Cases	No. of deaths	Mortality rate (%)	Adjusted OR <sup>1</sup> (95% CI)
All		1261	86	6.8		1300	119	9.2	
Age groups by gender									
Male	All	506	30	5.9		633	50	7.9	
	0-15	22	0	0.0		16	0	0.0	
	16-29	128	0	0.0		101	0	0.0	
	30-44	119	1	0.8		145	0	0.0	
	45-64	136	5	3.7		172	7	4.1	
	65+	99	24	24.2		199	43	21.6	
Female	All	755	56	7.4		667	69	10.3	
	0-15	19	0	0.0		8	0	0.0	
	16-29	165	2	1.2		96	0	0.0	
	30-44	202	5	2.5		151	3	2.0	
	45-64	189	7	3.7		169	8	4.7	
	65+	179	42	23.5		243	58	23.9	
Sex									
Male		504	30	6.0	Ref.	633	50	7.9	Ref.
Female		754	56	7.4	1.05 (0.63-1.74)	667	69	10.3	1.03 (0.67-1.57)
Colectomy performed									
Yes		193	9	4.7	Ref.	162	17	10.5	Ref.
No		1068	77	7.2	1.59 (0.74-3.41)	1138	102	9.0	0.57 (0.31-1.05)
Social deprivation									
I (least deprived)		229	16	7.0	0.99 (0.45-2.17)	234	20	8.5	0.83 (0.42-1.65)
II		236	10	4.2	0.49 (0.21-1.18)	245	21	8.6	0.89 (0.45-1.76)
III		257	23	8.9	1.23 (0.60-2.55)	282	27	9.6	0.86 (0.46-1.61)
IV		277	20	7.2	0.85 (0.41-1.77)	280	27	9.6	0.99 (0.52-1.86)
V (most deprived)		262	17	6.5	Ref.	259	24	9.3	Ref.
Urban/rural residence									
Under 2500 people		256	17	6.6	Ref.	277	22	7.9	Ref.
2500-9999		205	13	6.3	0.76 (0.33-1.76)	222	21	9.5	1.29 (0.64-2.59)
10000-24999		278	26	9.4	1.48 (0.72-3.06)	280	24	8.6	1.17 (0.60-2.30)
25000-99999		260	15	5.8	0.71 (0.32-1.60)	274	28	10.2	1.48 (0.77-2.85)
At least 100000		262	15	5.7	0.67 (0.29-1.54)	247	24	9.7	1.58 (0.80-3.14)
Distance from hospital									
< 3 km		187	14	7.5	Ref.	211	21	10.0	Ref.
3-4.99 km		145	6	4.1	0.55 (0.19-1.59)	159	14	8.8	0.97 (0.44-2.10)
5-9.99 km		269	18	6.7	1.03 (0.46-2.31)	285	25	8.8	0.91 (0.47-1.76)
10-19.99 km		318	22	6.9	1.14 (0.52-2.49)	303	30	9.9	0.94 (0.49-1.80)
≥ 20 km		207	16	7.7	1.14 (0.49-2.65)	206	19	9.2	1.03 (0.50-2.13)

<sup>1</sup>The OR for sex is adjusted for age group. The OR for social deprivation and colectomy was adjusted for age group and sex. The adjusted OR for urban/rural residence and distance from hospital was adjusted for age group, sex and social deprivation.

establish the extent and prognosis of severe CD and UC in Wales, and how they are associated with social deprivation, geography and other socio-demographic aspects. It is thought that IBD affects approximately 150 000 people in the United Kingdom, with corresponding prevalence rates of 55-140 per 100 000 for CD, 160-240 per 100 000 for UC, and approximately 13 300 combined new cases diagnosed each year<sup>[1]</sup>. We found a hospitalized prevalence rate of 50.1 per 100 000 population for severe CD and 50.6 for severe UC. If these prevalence rates for Wales were applied to the whole of the United Kingdom, this would suggest a total of approximately 62 000 people hospitalized with severe IBD over the 9-year study period (30 000 for CD and 32 000 for UC).

The main strength of this study was its size, which covered almost 3000 patients who were hospitalized for IBD in a geographically defined population of nearly 3 million over a 8-year period. Further strengths are that it

was based on systematic record linkage, which enabled long-term mortality follow-up of 5 years, by monitoring deaths that occurred in and following discharge from hospital. The study also used geographical measures of social deprivation to measure social inequality comprehensively on a national scale, as well as other novel prognostic/demographic risk factors, such as distance from home to hospital and urban or rural residences.

We defined severe IBD as emergency hospitalization for IBD, as the principal reason for admission, which lasted ≥ 3 d. This was to distinguish severe illness from relatively minor illness and from day-case investigations. We accept that the definition may also have included some patients who were admitted with long stays for other serious illnesses, for example, myocardial infarction or pneumonia, who also had IBD. However, we sought to minimize this by restricting case selection to people in whom IBD itself was coded as the principal reason for admission.

Table 4 Mortality at 5 years following hospitalization for severe Crohn's disease and ulcerative colitis, 1999-2007

		CD				UC			
		Cases <sup>2</sup>	No. of deaths	Mortality rate (%)	Adjusted OR <sup>1</sup> (95% CI)	Cases <sup>2</sup>	No. of deaths	Mortality rate (%)	Adjusted OR <sup>1</sup> (95% CI)
All		535	78	14.6		567	118	20.8	
Age groups by sex									
Male	All	209	28	13.4		275	55	20.0	
	0-15	10	0	0.0		5	0	0.0	
	16-29	50	0	0.0		43	1	2.3	
	30-44	52	2	3.8		73	0	0.0	
	45-64	58	8	13.8		63	4	6.3	
	65+	39	18	46.2		91	50	54.9	
Female	All	326	50	15.3		292	63	21.6	
	0-15	9	0	0.0		4	0	0.0	
	16-29	72	1	1.4		40	1	2.5	
	30-44	91	3	3.3		59	2	3.4	
	45-64	84	10	11.9		86	6	7.0	
	65+	70	36	51.4		103	54	52.4	
Sex									
Male		209	28	13.4	Ref.	275	55	20.0	Ref.
Female		326	50	15.3	0.96 (0.52-1.76)	292	63	21.6	0.76 (0.44-1.31)
Colectomy performed									
Yes		89	10	11.2	Ref.	72	15	20.8	Ref.
No		446	68	15.2	1.48 (0.63-3.52)	495	103	20.8	0.59 (0.26-1.33)
Social deprivation									
I (least deprived)		110	14	12.7	0.53 (0.20-1.39)	110	24	21.8	1.39 (0.61-3.19)
II		102	9	8.8	*0.23 (0.08-0.68)	110	19	17.3	0.85 (0.35-2.05)
III		112	16	14.3	0.56 (0.21-1.46)	114	31	27.2	1.62 (0.73-3.58)
IV		106	21	19.8	1.09 (0.45-2.68)	112	23	20.5	1.29 (0.55-3.02)
V (most deprived)		105	18	17.1	Ref.	121	21	17.4	Ref.
Urban/rural residence									
Under 2500 people		103	11	10.7	Ref.	124	28	22.6	Ref.
2500-9999		89	16	18.0	1.42 (0.49-4.12)	105	22	21.0	0.64 (0.26-1.53)
10000-24 999		124	21	16.9	1.32 (0.49-3.56)	109	18	16.5	0.47 (0.19-1.17)
25000-99 999		99	15	15.2	0.75 (0.26-2.14)	121	26	21.5	0.86 (0.37-1.97)
≥ 100000		120	15	12.5	0.89 (0.31-2.54)	108	24	22.2	0.84 (0.35-1.98)
Distance from hospital									
< 3 km		87	14	16.1	Ref.	101	24	23.8	Ref.
3-4.99 km		73	9	12.3	0.52 (0.16-1.63)	67	14	20.9	0.80 (0.31-2.09)
5-9.99 km		111	12	10.8	0.71 (0.25-2.01)	123	22	17.9	0.80 (0.35-1.85)
10-19.99 km		135	17	12.6	0.81 (0.30-2.19)	112	27	24.1	0.97 (0.41-2.29)
≥ 20 km		70	15	21.4	1.26 (0.42-3.78)	87	16	18.4	0.78 (0.31-2.00)

\* $P < 0.01$ . <sup>1</sup>The OR for sex was adjusted for age group. The OR for social deprivation and colectomy was adjusted for age group and sex. The adjusted OR for urban/rural residence and distance from hospital was adjusted for age group, sex and social deprivation; <sup>2</sup>Fewer people had 5 years than 1 year follow-up during the 9-year study period, therefore, the number of cases was lower in this Table than in Table 3.

The main limitation of record linkage studies such as this is the lack of detailed information about the history, severity and management of the illness. It was not possible to ascertain the extent of disease from endoscopic evidence or detailed pathology information from our data sources. Our hospitalized prevalence rates referred specifically to CD and UC patients with an inpatient stay of  $\geq 3$  d following emergency admission. Although length of stay is often affected by factors such as age, comorbidity, and effectiveness of self care and social support, we believe that a length of stay of  $\geq 3$  d after emergency admission usually indicates severe disease that requires intensive medical or surgical treatment rather than admission for diagnosis or assessment.

For both males and females, we found a continuous rise in the hospitalized prevalence of UC as age increased. For CD, we found highest prevalence among patients aged

16-29 years, with a second peak in prevalence in males aged  $> 65$  years and females aged  $\geq 60$  years. We also found a higher prevalence of CD in females than in males, but a similar prevalence of UC among males and females, which is consistent with other studies<sup>[8,10,11,13,14,16,19,29]</sup>.

IBD has been linked with higher socioeconomic groups in some studies, particularly for CD<sup>[30,31]</sup>, but also for UC<sup>[31]</sup>, although other studies have reported no association<sup>[32-34]</sup>. Instead, we found slightly higher hospitalized prevalence of CD in more socially deprived locations, although there were not quite significant differences across the social deprivation quintiles. As our study focused on more severe cases, possible reasons for this include higher levels of health-care dependency as well as higher levels of smoking and more comorbidity among patients from more deprived social backgrounds. For UC, we found no significant association between



hospitalized prevalence and social deprivation. The differing demographic profiles between CD and UC, and the stronger evidence of an association between CD and social deprivation suggests that environmental factors play a stronger part in the etiology of CD, but attempts to establish environmental factors have proved inconclusive and sometimes controversial in the past<sup>[31,33-37]</sup>.

Overall mortality was 6.8% at 1 year and 14.6% at 5 years for CD, compared with 9.2% at 1 year and 20.8% at 5 years for UC. At 5 years, mortality was high among older patients (49% and 54% for CD and UC, respectively, among patients aged  $\geq 65$  years). The higher mortality for UC is consistent with other studies<sup>[116,22,38]</sup>, although there is a lack of large studies with long-term follow-up for people hospitalized with IBD. Mortality at 1 year follow-up was five times higher than in the general population for CD and four times higher for UC. At 5 years, mortality was increased two fold for CD and UC. An earlier study has reported SMRs of 3.2 and 2.4 after 3 years follow-up for people hospitalized with severe CD and UC, respectively, in England<sup>[22]</sup>, and further demonstrates that people hospitalized with severe IBD are often at increased risk of mortality, which continues in the long term. Elective colectomy may well be the most effective means of reducing these excess risks of mortality<sup>[22]</sup>.

Although there was some variation in prognosis geographically across health boards, it was not significant for either CD or UC. We also found little discernible association between social deprivation quintiles, distance travelled to hospital and rural versus urban residence, and subsequent mortality at either 1 or 5 years. As IBD often relapses and requires regular monitoring and surveillance, it is possible that patients who live remotely from outpatient clinics and hospital care may have poorer outcomes. Our findings for mortality outcomes do not support such concerns, although recent health service developments in the United Kingdom that encourage greater management and treatment of IBD in primary care may partially offset the distances travelled by patients.

We found that severe CD and UC had quite distinct demographic profiles in Wales. CD and UC also showed slightly differing patterns of association with social deprivation. The higher prevalence of hospitalized CD in more deprived areas may reflect higher smoking rates, more comorbidity and higher levels of hospital dependency, as well as higher prevalence.

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## COMMENTS

### Background

The prevalence of Crohn's disease (CD) has increased in many countries in recent decades. Although inflammatory bowel disease (IBD) is thought to affect

about 150 000 people in the United Kingdom, the prevalence of severe IBD is not known. Mortality following hospitalization for IBD is significant but little has been reported on long-term follow-up.

### Research frontiers

This study determined the hospitalized prevalence of severe IBD and subsequent 5-year mortality in Wales, and investigates associations between severe IBD and social deprivation, distance travelled to hospital, and other socio-demographic characteristics.

### Innovations and breakthroughs

This study showed that the hospitalized prevalence of severe CD was slightly higher among the most deprived groups but there was no association between social deprivation and severe ulcerative colitis (UC). Five-year mortality was high for severe CD (14.6%) and UC (20.8%), but there was little association between mortality and social deprivation, distance from hospital, urban/rural residences or geography.

### Applications

The higher hospitalized prevalence of severe CD among the most deprived groups, and differing demographic profiles between CD and UC, suggest that environmental factors play a more significant role in the etiology of CD. The findings of this large population-based study on the prevalence and mortality of IBD are also important for service planning and provision.

### Terminology

Hospitalized prevalence of severe IBD in this study referred to the number of people (per 100 000 population) who were hospitalized as an emergency for  $\geq 3$  d for IBD, as the principal reason for admission, on at least one occasion during the 8-year study period. One and five-year mortality referred to the percentage of these people who died during the subsequent one and five-year follow-up period.

### Peer review

This was a well-conducted, large epidemiological study that provided interesting and novel findings. For example, the findings that distance travelled by patients to hospital did not affect long-term mortality for CD or UC has not been reported previously.

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## Clinical course of subepithelial lesions detected on upper gastrointestinal endoscopy

Yeun Jung Lim, Hee Jung Son, Jong-Soo Lee, Young Hye Byun, Hyun Joo Suh, Pool Lyul Rhee, Jae J Kim, Jong Chul Rhee

Yeun Jung Lim, Jong-Soo Lee, Young Hye Byun, Hyun Joo Suh, Center for Health Promotion, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50, Irwon-dong, Gangnam-gu, Seoul 135-710, South Korea

Hee Jung Son, Pool Lyul Rhee, Jae J Kim, Jong Chul Rhee, Division of Gastroenterology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50, Irwon-dong, Gangnam-gu, Seoul 135-710, South Korea

Author contributions: Son HJ, Lee JS and Rhee PL designed the research; Lim YJ, Byun YH, Suh HJ, Kim JJ and Rhee JC performed the research; Lim YJ and Son HJ performed the analysis; Lim YJ wrote the paper.

Correspondence to: Hee Jung Son, MD, Division of Gastroenterology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50, Irwon-dong, Gangnam-gu, Seoul 135-710,

South Korea. [hjls.son@samsung.com](mailto:hjls.son@samsung.com)

Telephone: +82-2-34100335 Fax: +82-2-34103849

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### Abstract

**AIM:** To evaluate the natural history of subepithelial lesions.

**METHODS:** We reviewed the medical records of 104159 patients who underwent upper gastrointestinal endoscopy at the Center for Health Promotion of Samsung Medical Center between 1996 and 2003. Subepithelial lesions were detected in 795 patients (0.76%); 252 patients were followed using upper gastrointestinal endoscopy for  $82.5 \pm 29.2$  mo (range, 12-160 mo; median, 84 mo; 1st quartile, 60 mo; 3rd quartile, 105 mo). The median interval of follow-up endoscopy was 12 mo (range, 6-105 mo; 1st quartile, 12 mo; 3rd quartile, 24 mo).

**RESULTS:** The mean patient age was 53 years (range, 22-80 years), and the male-to-female ratio was 2.36:1 (177/75). The lesion size at initial measurement averaged 8.9 mm (range, 2-25 mm; median, 8 mm; 1st quartile, 5 mm; 3rd quartile, 10 mm). Of the 252 lesions, 244 (96.8%) were unchanged and 8 (3.2%) were significantly increased in size (from  $12.9 \pm 6.0$  to  $21.2 \pm 12.2$  mm) after a mean interval of  $59.1 \pm 27.5$  mo (range, 12-86 mo). Surgical resection of lesions was performed when the lesions were  $\geq 3$  cm in diameter. Two lesions were diagnosed as gastrointestinal stromal tumors with an intermediate or high risk of malignancy and one lesion was classified as a schwannoma.

**CONCLUSION:** Most small subepithelial lesions do not change as shown by endoscopic examination, and regular follow-up with endoscopy may be considered in small, subepithelial lesions, especially lesions  $< 1$  cm in size.

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**Key words:** Subepithelial tumor; Ultrasonography; Gastrointestinal diseases; Gastrointestinal endoscopy; Time factors

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## INTRODUCTION

Upper endoscopy is commonly performed for the evaluation of symptoms, and for screening and surveillance of neoplasias. Subepithelial masses or bulges covered with normal-appearing mucosa are frequently encountered during endoscopy. Although a previous study has reported that the incidence of gastric subepithelial lesions is approximately 0.3%<sup>[1]</sup>, Kawanowa *et al.*<sup>[2]</sup> reported 50 microscopic gastrointestinal stromal tumors (GISTs) in 100 stomachs entirely resected from patients with gastric cancer. With more widespread use of endoscopy for screening, it is likely that subepithelial lesions will be detected more frequently. The optimal management of incidentally-detected, subepithelial lesions has not been determined.

When clinicians are faced with a subepithelial lesion, they must decide to remove or follow up the lesion. Endosonographic or endoscopic surveillance is common in patients with asymptomatic, subepithelial lesions without signs of malignancy, such as large size, rapid growth, or ulceration, although such an approach has not been formally validated<sup>[3]</sup>. Because little is known about the natural course of subepithelial lesions, the appropriate strategy for management is still controversial. This imposes a tremendous emotional burden on patients who can become preoccupied with the possibility that the tumor is malignant.

The aim of this study was to determine the natural history and provide a basis of surveillance of incidentally-detected, asymptomatic subepithelial lesions.

## MATERIALS AND METHODS

We used computerized medical records and a database of disease codes to study 104 159 patients who underwent upper gastrointestinal endoscopy at the Center for Health Promotion of Samsung Medical Center in Seoul, Korea between March 1996 and March 2003. A computer search over a 7-year period revealed 795 patients (0.76%) with the diagnostic code for submucosal tumors of the esophagus, stomach, or duodenum. Within this group, 252 patients had been followed with upper gastrointestinal endoscopy. Thirty-seven of 61 patients who had lesions > 1 cm in size were further evaluated with endoscopic ultrasonography (EUS). Seven of 8 lesions with a significant increase in size were evaluated using EUS. The duration of follow-up was determined by the last known visit for endoscopic examination at our hospital. All examinations were performed by experienced endoscopists (> 1000 endoscopic examinations each). Endoscopists approximated the size of lesions by using an open biopsy forceps for comparison (6 mm).

### Statistical analysis

Statistical evaluation was performed with SPSS (Statistical Program for the Social Sciences; SPSS, Inc., Chicago, IL, USA). Descriptive statistics were used when appropriate.

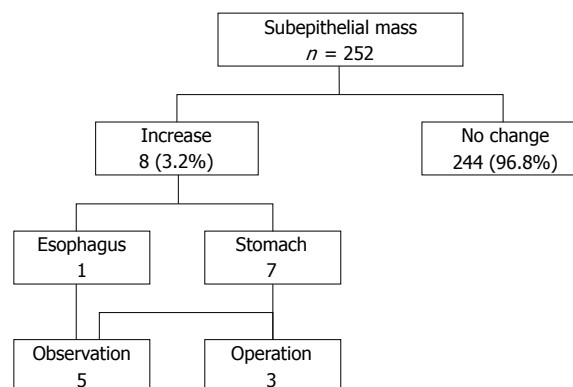


Figure 1 Clinical course of subepithelial masses.

## RESULTS

The mean age of the 252 patients with subepithelial lesions was 53 years (range, 22-80 years), and the male-to-female ratio was 2.36:1 (177/75). The mean lesion size was 8.9 mm (range, 2-25 mm; median, 8 mm; 1st quartile, 5 mm; 3rd quartile, 10 mm). The stomach [130 patients (51.6%)] was the most common site for a subepithelial lesion, followed by the esophagus [104 patients (41.3%)], and duodenum [18 patients (7.1%)].

Biopsies were obtained from 191 of the 252 patients at the time of the initial endoscopy. Endoscopy and biopsy were sufficient for diagnosis in 3 patients. Of these 3 patients, 1 had an esophageal leiomyoma, and 2 had Brunner gland hyperplasia of the duodenum.

Of 252 lesions, 244 (96.8%) were unchanged and 8 (3.2%) significantly increased in size (from  $12.9 \pm 6.0$  to  $21.2 \pm 12.2$  mm) during a mean interval of  $59.1 \pm 27.5$  mo (range, 12-86 mo, Figure 1, Table 1). In these 8 lesions, there was an increase of over 25% and more than 5 mm in diameter at surveillance. Six of the 8 lesions arose from the 4th layer, corresponding to the muscularis propria, and appeared hypoechoic; they were considered to be GISTs. One lesion arose from the 3rd layer and appeared hyperechoic; it was considered to be a lipoma. The other lesion was further evaluated by stomach computed tomography (CT) probably because of the patient's rejection of an EUS examination; however, the lesion was not observed on CT. Surgical resection was performed in 3 lesions  $\geq 3$  cm in size, which were diagnosed as GISTs with an intermediate and a high risk of malignancy, and a schwannoma (Figures 2-4). The 5 patients who did not undergo surgery were followed by means of upper gastrointestinal endoscopy or EUS. No further change was observed in size, shape and EUS finding such as echo pattern or regularity of the outer margin over a period of 1-5 years.

## DISCUSSION

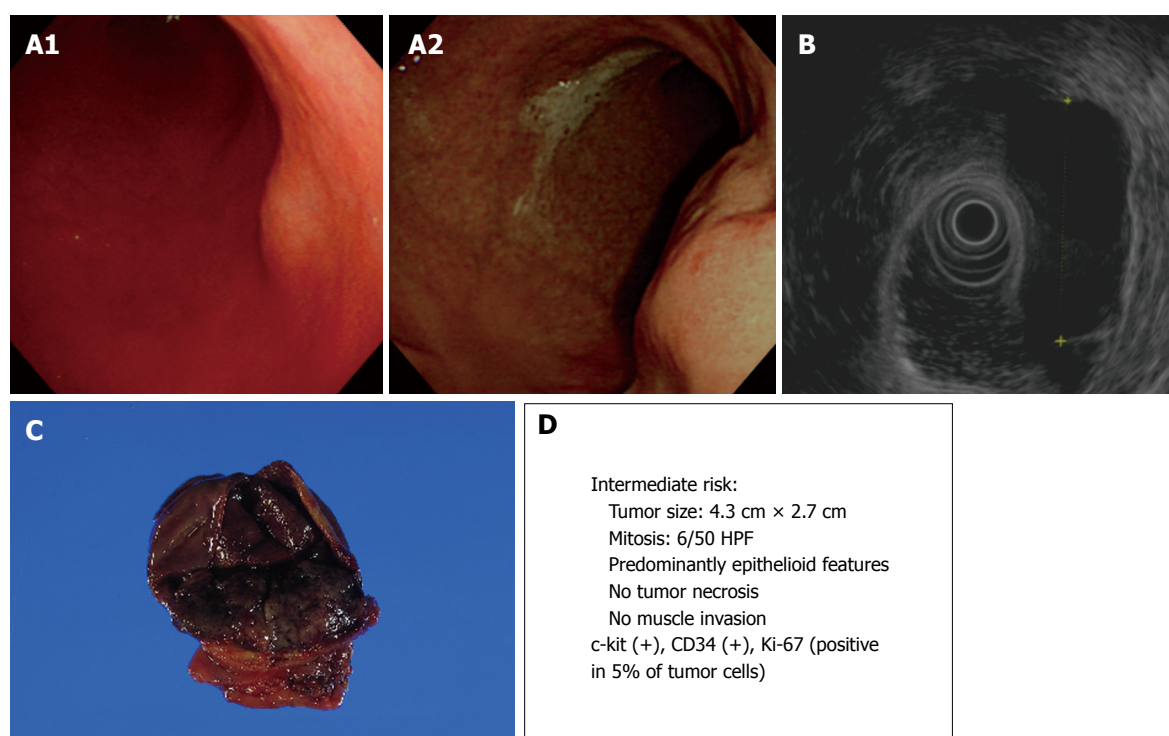
We demonstrated that during a mean period of  $82.5 \pm 29.2$  mo (range, 12-160 mo; median, 84 mo; 1st quartile, 60 mo; 3rd quartile, 105 mo), there was no significant



Table 1 Characteristics of 8 patients with increased subepithelial masses

Patients	Age (yr)	Gender	Location	Initial size (mm)	Increased size (mm)	Follow-up interval (mo)	EUS	Treatment	Surgical gross pathology
1	60	F	Stomach	22	30	40	GIST	Operation	Schwannoma
2	63	M	Stomach	20	30	86	GIST	Observation	
3	65	M	Stomach	15	40	12	GIST	Operation	GIST
4	37	F	Stomach	12	18	36	GIST	Observation	
5	53	F	Stomach	12	20	56	Lipoma	Observation	
6	44	F	Stomach	12	30	84	GIST	Operation	GIST
7	54	M	Stomach	4	10	81	Not performed	Observation	
8	37	F	Esophagus	8	15	78	GIST	Observation	

EUS: Endoscopic ultrasound; GIST: Gastrointestinal stromal tumor.

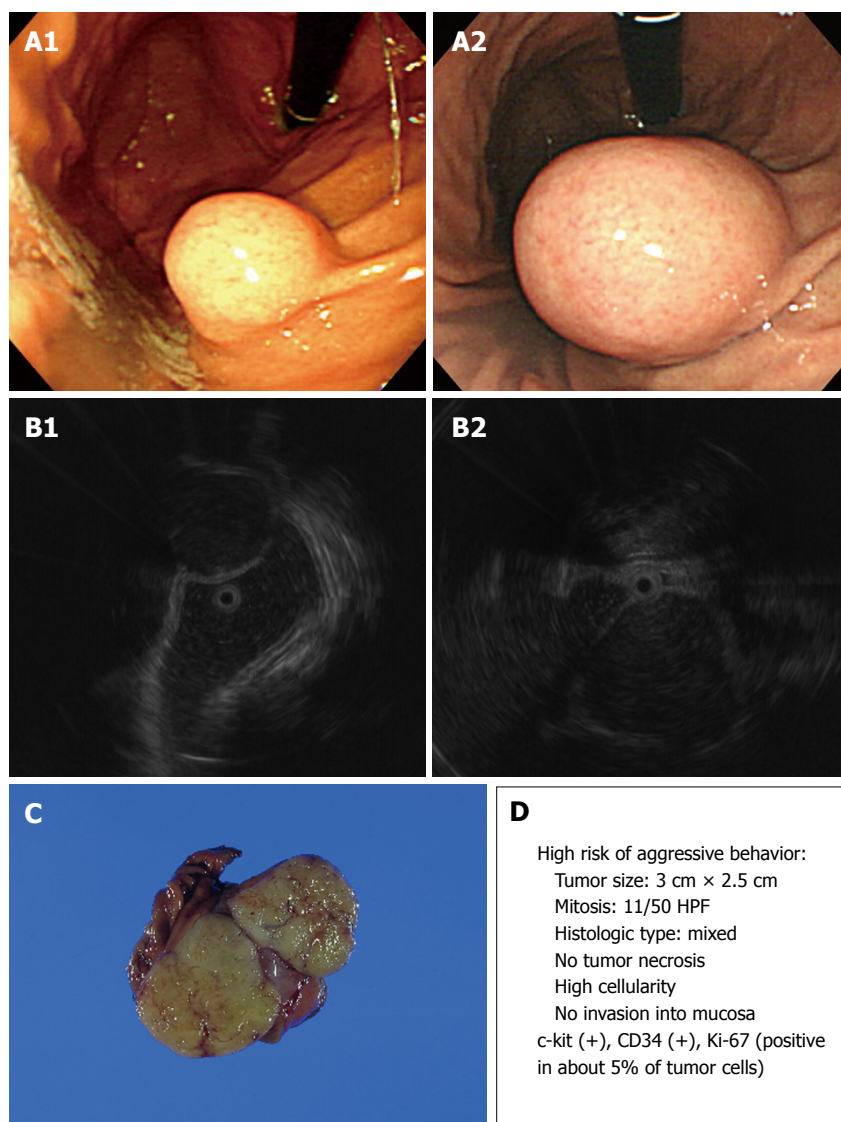


**Figure 2** Endoscopic, endoscopic ultrasonography (EUS), and gross findings of gastrointestinal stromal tumors (GISTs). A: Endoscopic view of a round subepithelial mass with a significant interval change; B: EUS shows an ovoid, homogeneous, hypoechoic mass in the fourth gastric wall layer; C: Gross findings of wedge resection reveal a soft, well-defined mass measuring 4.3 cm × 2.7 cm; D: Malignant potential.

change in the size of small (< 30 mm) subepithelial lesions detected incidentally during upper gastrointestinal endoscopy in 244 of 252 patients (96.8%). Eight lesions (3.2%) were significantly increased in size (from  $12.9 \pm 6.0$  to  $21.2 \pm 12.2$  mm) during a mean interval of  $59.1 \pm 27.5$  mo (range, 12-86 mo), four patients had subepithelial lesions  $\geq 3$  cm in size on follow-up endoscopic examination. Three patients of them underwent tumor resection and were diagnosed with intermediate or high risk GISTs and a schwannoma.

Our findings are consistent with prior studies of subepithelial lesions. Several prior studies have suggested that since most small subepithelial lesions do not exhibit changes that would raise the suspicion of malignant potential, a conservative policy (endoscopic follow-up without pathologic diagnosis) of surveillance is safe. Tio

*et al*<sup>[4]</sup> showed that the size and echo pattern of 21 small (< 3 cm) subepithelial lesions did not change over a period of 1-3 years. Melzer *et al*<sup>[5]</sup> also showed no changes in size or echo pattern in the small subepithelial lesions (< 4 cm) of 24 of 25 patients over a mean period of 19 mo. However, one gastric lesion enlarged from 30 to 38 mm and changed from a hypoechoic to a non-homogeneous pattern. The patient underwent resection of a stromal tumor with high malignant potential. Lee *et al*<sup>[6]</sup> followed patients with 16 esophageal tumors, 9 gastric tumors, and one benign duodenal mesenchymal tumor (< 3 cm) for a mean period of 47.4 mo, and noted no change in 25 of 26 patients during EUS surveys. However, one gastric lesion enlarged from 26 to 34 mm without a change in the echo pattern or regularity of the outer margin. The patient underwent resection of a leiomyoma.



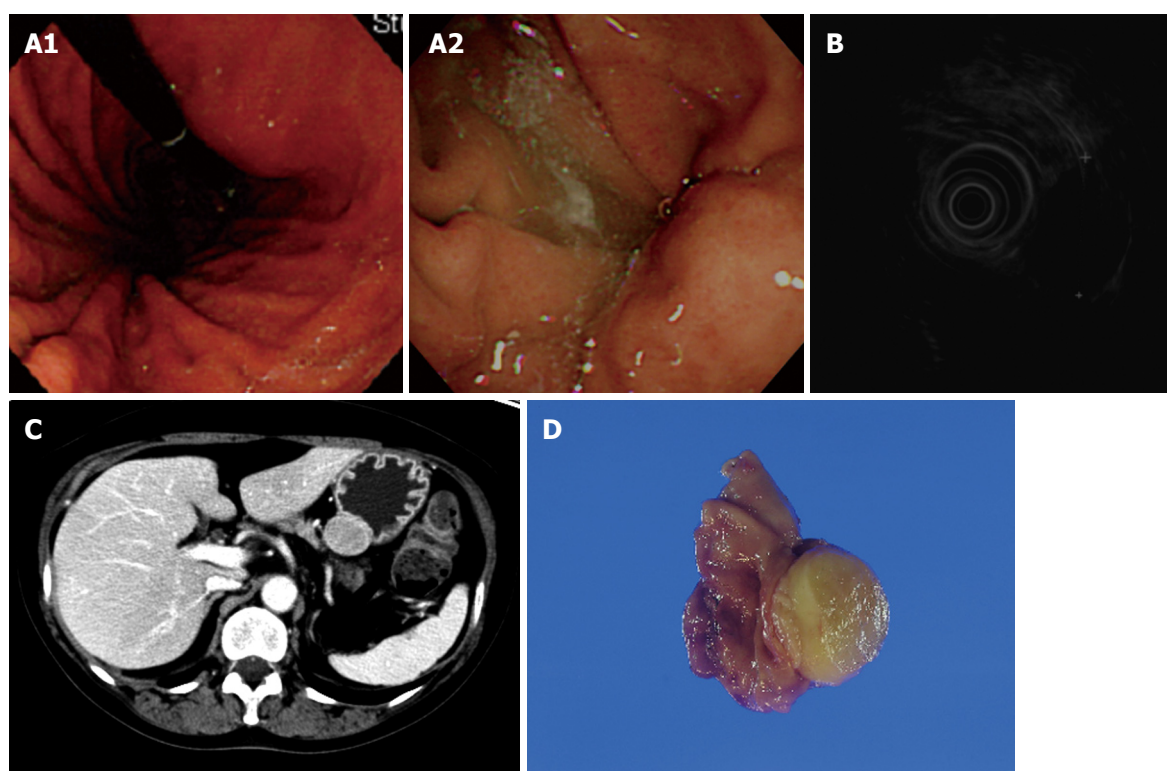
**Figure 3** Endoscopic, EUS, and gross findings of GISTs. A: Endoscopic view of a round subepithelial mass with a significant interval change; B: EUS shows an ovoid, homogeneous, hypoechoic mass in the fourth gastric wall layer; C: Gross findings of wedge resection reveal a soft, well-defined mass measuring 3.0 cm × 2.5 cm; D: Malignant potential.

Imaoka *et al*<sup>[7]</sup> followed 132 gastric subepithelial lesions for 5 years and found that only 2 lesions increased in size. These tumors were diagnosed as GISTs after surgical resection; one patient had liver metastasis. Lachter *et al*<sup>[8]</sup> found that the majority of small (< 17 mm) subepithelial tumors did not change in echogenicity or size during a median period of 5 years. The previous studies have been limited by small sample size and relatively short follow-up.

According to a stepwise approach to subepithelial tumors, EUS is recommended for subepithelial tumors > 1 cm in diameter, and histologic evaluation, such as EUS-guided fine needle aspiration biopsy (EUS-FNAB), is recommended for hypoechoic subepithelial tumors < 3 cm in diameter. Surgery is recommended for subepithelial tumors > 3 cm in diameter<sup>[9]</sup>. Although these procedures are helpful in categorizing a lesion, they cannot absolutely determine the type of lesion or determine if a lesion is benign or malignant<sup>[10,11]</sup>. Clinicians should consider if an invasive method, such as EUS-FNAB, is necessary or available. Furthermore, they should consider individual risk and patient preference.

The optimal management of subepithelial lesions remains controversial because the natural history of subepithelial lesions, such as GISTs, remains incompletely defined. GISTs are the most commonly identified intramural subepithelial tumors in the upper gastrointestinal tract<sup>[11]</sup>. Small GISTs (< 2 cm) have very low malignant potential according to the classification system proposed by the National Institutes of Health Consensus Conference<sup>[12]</sup>. The American Gastroenterological Association recommends periodic endoscopic or endosonographic follow-up or surgical resection for small, hypoechoic, 3rd- and 4th-layer (< 3 cm) masses, which are most likely GISTs<sup>[13]</sup>. Nishida *et al*<sup>[14]</sup> recommended that subepithelial tumors < 2 cm in size and without ulceration or surface depression can be followed with endoscopic examination once or twice per year.

Opinions concerning the duration of follow-up also vary. Brand *et al*<sup>[15]</sup> recommended follow-up for 6 mo after the initial diagnosis for subepithelial lesions with no EUS signs of malignancy. If there is no change during the initial follow-up period, annual follow-up is recommended. Hwang *et al*<sup>[16]</sup> suggested a 1-year follow-up interval



**Figure 4** Endoscopic, EUS, abdominal computed tomography (CT), and gross findings of a schwannoma. A: Endoscopic view of an ovoid subepithelial mass with a significant interval change; B: EUS shows a round, homogeneous, hypoechoic mass in the third gastric wall layer; C: CT scan shows a 2.8 cm × 2.2 cm homogeneous, well-defined, soft tissue mass on the upper body of the stomach; D: Gross findings of wedge resection reveal a 3 cm × 2.5 cm well-demarcated, round, firm, yellow mass.

and suggested that the interval between surveillance examinations be extended if the lesion remains unchanged for 2 consecutive follow-up examinations with EUS. Guidelines in Japan recommend endoscopic examination once or twice per year for subepithelial lesions < 2 cm in size<sup>[14]</sup>.

Our finding must be interpreted in the context of the strength and weakness of this study. The high number of patients and a long follow-up study is its strength. However, there are several limitations in this study. First, there is a lack of accuracy in estimation of size. The open-biopsy forceps technique can underestimate or overestimate the size of submucosal lesion and shows inter-observer variation, but it is convenient to use in clinical practice. In our study, 2 submucosal lesions were estimated as 2 mm in size and we could not exclude the possibility of an under-estimation of the size. Second, we could not analyze all the patients with the diagnostic code for submucosal tumors, because only a proportion of the patients were followed up and analysis was performed only for them.

In conclusion, although the management strategy for small subepithelial lesions is still controversial, regular follow-up with endoscopy or EUS may be considered in small, asymptomatic, subepithelial lesions. Endoscopic surveillance can be an appropriate strategy for lesions < 1 cm. Further prospective, multicenter studies with long-term follow-up would help to validate these surveillance programs.

## COMMENTS

### Background

The natural history of subepithelial lesions has not been clearly elucidated, and the appropriate management strategy for small subepithelial tumors is still controversial.

### Research frontiers

With more widespread use of endoscopy for screening, asymptomatic subepithelial lesions would be detected more frequently. However, the appropriate strategy for management is still controversial. In this study, the authors have determined the natural history and provided a basis for surveillance of incidentally-detected, asymptomatic subepithelial lesions.

### Innovations and breakthroughs

Although several studies pertaining to the natural history of subepithelial lesions, including gastrointestinal tumors, have been published, they have been limited by small sample size and relatively short follow-up. In Korea, many subjects have regular endoscopic examinations because of the high incidence of gastric cancer.

### Applications

The authors have provided the basis for surveillance of incidentally-detected, small subepithelial lesions by means of this study.

### Peer review

Lim *et al* performed a retrospective 3-year follow up study of subepithelial gastrointestinal lesions in 252 patients. This is a high number of patients and a long follow-up, which increases the value of the work.

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## Two stomach-originated *Lactobacillus* strains improve *Helicobacter pylori* infected murine gastritis

Yi Cui, Chun-Lian Wang, Xiao-Wei Liu, Xue-Hong Wang, Lin-Lin Chen, Xin Zhao, Nian Fu, Fang-Gen Lu

Yi Cui, Chun-Lian Wang, Xiao-Wei Liu, Xue-Hong Wang, Xin Zhao, Nian Fu, Fang-Gen Lu, Department of Gastroenterology, The Second Xiangya Hospital of Central South University, Changsha 410011, Hunan Province, China

Lin-Lin Chen, Department of Gastroenterology, Xiangya Hospital of Central South University, Changsha 410008, Hunan Province, China

Author contributions: Cui Y and Wang CL contributed equally to this work; Cui Y, Wang CL, Liu XW and Lu FG designed the research; Cui Y, Wang CL, Wang XH, Chen LL and Zhao X performed the research; Chen LL, Zhao X and Fu N analyzed and interpreted the data; Cui Y, Wang CL, Liu XW and Lu FG wrote the paper.

Correspondence to: Fang-Gen Lu, Professor, Department of Gastroenterology, The Second Xiangya Hospital of Central South University, Changsha 410011, Hunan Province, China. [lufanggenyao@163.com](mailto:lufanggenyao@163.com)

Telephone: +86-731-85295888 Fax: +86-731-84807725

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**RESULTS:** Histopathologic evaluation showed significant release of mucosal inflammation in gastric antrum and gastric body in *Lactobacillus* treated groups and triple treated group. *H. pylori* eradication rate in both *Lactobacillus* treated groups and triple treated group were higher than normal saline control group. *Lactobacillus* treated groups and triple treated group showed significant decrease of *H. pylori* bacterial density.

**CONCLUSION:** Both *Lactobacillus* strains have a significant anti-*H. pylori* activity; *L. fermenti* displays more efficient antagonistic activity *in vivo* against *H. pylori* infection.

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**Key words:** *Helicobacter pylori*; *Lactobacillus fermenti*; Murine gastritis; *Lactobacillus acidophilus*; Therapy

**Peer reviewer:** Yuan Yuan, Professor, Cancer Institute of China Medical University, 155 North Nanjing Street, Heping District, Shenyang 110001, Liaoning Province, China

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### Abstract

**AIM:** To investigate the potential anti-*Helicobacter pylori* (*H. pylori*) and anti-inflammation *in vivo* effects of two *Lactobacillus* strains from human stomach.

**METHODS:** Forty *H. pylori* infected Balb/c mice were randomly divided into 4 groups: proton pump inhibitor and antibiotics triple treated group, *Lactobacillus fermenti* (*L. fermenti*) treated group, *Lactobacillus acidophilus* treated group and normal saline control group. Ten uninfected mice were also included as blank control group. The infection of *H. pylori* was detected by rapid urease tests, Giemsa staining and bacterial culture. The colonization of *H. pylori* was assessed in bacterial density score and gastric inflammation was assessed in histological score. The colonization of *L. fermenti* was performed by fluorescent probe.

### INTRODUCTION

*Helicobacter pylori* (*H. pylori*) is a spiral-shaped, Gram-negative microaerophilic stomach rod that infects over 50% of the population around the world<sup>[1-3]</sup>, which is considered to be the most important etiological agent of chronic gastritis, peptic ulcer, gastric cancer and mucosa-associated lymphoid tissue<sup>[1,4,5]</sup>. So far, the eradication of

*H. pylori* depends on the combination of antibiotics and acid suppression drugs, with an efficiency of 80%-90%. Unexpectedly, symptoms of 10%-20% patients remain unimproved or reinfected due to the incomplete eradication or antibiotics resistance<sup>[5,6]</sup>. Moreover, the side effect of using multi-antibiotics is obvious, such as antibiotic associated diarrhea, enteric dysbacteriosis, pseudo-membranous colitis, and so on, which lower the curative effect and treatment compliance. Thus attention has been drawn to seek for any alternative method which can eradicate or inhibit *H. pylori* infection efficiently without antibiotics associated side effects.

As mentioned above, over 50% of the population are infected by *H. pylori*, while only a few of them suffer from *H. pylori* associated disease. Some published data showed that when the density of *H. pylori* in gastric antrum drops to less than  $10^5$ , it cannot lead to the formation of gastric and duodenum ulcer<sup>[7,8]</sup>. It is believed that the severity and activity of gastritis is related to the number of bacteria in stomach.

Recently, some reports have suggested that exogenous *lactobacilli* have some inhibitory effects on *H. pylori* infection<sup>[9,10]</sup>, and the colonization of *H. pylori* has a close relationship with the number of *lactobacilli*. It is also reported that *H. pylori* colonization can be inhibited in *lactobacillus* strain *L. salivarius*-fed mice<sup>[11,12]</sup>. Some studies showed that probiotics isolated from dairy products or human feces have suppressive effects on *H. pylori* infection<sup>[9,13]</sup>. Similar results from some clinical trials also support the inhibitory effects of some microbial ecological agents on *H. pylori* associated diseases<sup>[14,15]</sup>.

However, results of clinical trials from some separate groups did not make a definite conclusion. Some studies showed that it is effective to prevent and treat *H. pylori* infected diseases by administering *lactobacillus* or fermented dairy products<sup>[13,16,17]</sup>. Some believe that probiotics have no effect in treating *H. pylori* infected diseases because they are live microbes, and their living environment may change depending on what kinds of food the host takes<sup>[18]</sup>. The possibilities include inappropriate methods to assess the anti-*H. pylori* activity, different strains of probiotics and different origins of strains<sup>[12,19-21]</sup>. Therefore, more detailed studies on more stomach originated probiotic strains will help draw a more definite conclusion.

In the present study, we isolated two stomach originated *lactobacillus* strains, and screened their potential anti-*H. pylori* activity and anti-inflammatory effects on mouse model of *H. pylori*-associated Balb/c gastritis. We found that *lactobacillus* strain *Lactobacillus fermenti* (*L. fermenti*) could adhere to gastric epithelium and displayed antagonistic activity *in vivo* against *H. pylori* infection, thus significantly improving the *H. pylori*-associated Balb/c gastritis in mice.

## MATERIALS AND METHODS

### Bacterial stains and culture conditions

*H. pylori* standard strain SS1 (Sydney strain 1) was kindly

provided by the Infectious Disease Institute of the Chinese Center for Disease Control. The two *lactobacillus* strains *L. fermenti* (CCTCC M 206110) and *Lactobacillus acidophilus* (*L. acidophilus*) (strain LC) were isolated by us from gastric biopsy materials of patients who received endoscopic examination in the Gastroenterology Department of the Second Xiangya Hospital, Hunan, China.

*H. pylori* was resuscitated and inoculated into brain heart infusion broth containing 10% sheep blood, incubated under microaerophilic conditions (5% O<sub>2</sub>, 85% N<sub>2</sub>, 10% CO<sub>2</sub>) at 37°C for 3-5 d. Both *lactobacillus* strains were cultured in Man-Rogosa-Sharpe (MRS) broth, and incubated in anaerobic box at 37°C for 48 h. The concentration of bacteria was regulated to  $1 \times 10^9$  CFU/mL by turbidimetry.

### *H. pylori* infection of Balb/c mice

Sixty specific-pathogen-free 6-8-wk-old Balb/c mice (a male to female ratio of 1:1) were obtained from the Central Animal Facility of Wuhan. They were housed according to relevant Chinese national legislation, fed a commercial diet, and were given water and libitum, except as otherwise stated. *H. pylori* infections by the SS1 strain were induced as follows: freshly prepared aliquots (0.4 mL of  $1 \times 10^9$  CFU/mL) of *H. pylori* SS1 strain in brain heart infusion broth were administered to 50 mice *via* orogastric inoculation 3 times a week (days 1, 3 and 5) at a 1-d interval between inoculations for 2 wk. Accordingly, the other 10 non-infected control animals were inoculated with the same volume of plain brain heart infusion broth. At the end of the 1st and 2nd wk, 5 animals receiving *H. pylori* strain SS1 were sacrificed by carbon dioxide inhalation, and tests were made to confirm whether they were infected with SS1 strain successfully.

The following groups of animals were included in the study: *H. pylori*-infected mice treated by *L. fermenti* (*L. fermenti* group,  $n = 10$ ), *H. pylori*-infected mice treated by *L. acidophilus* (*L. acidophilus* group,  $n = 10$ ), *H. pylori*-infected mice treated by proton pump inhibitor (PPI) and antibiotics (triple group,  $n = 10$ ). The control groups were *H. pylori*-infected mice treated by normal saline solution (NS group,  $n = 10$ ). The non-infected mice (blank control group,  $n = 10$ ) were also included. The quantity of *lactobacillus* was regulated to 0.5 mL of  $1 \times 10^9$  CFU/mL per mouse for *L. fermenti* and *L. acidophilus* groups, and PPI and antibiotics were 0.25 mL of 0.4 mg/mL pantoprazole (Zhongmei East China Pharmaceutical Group Corporation in Hangzhou, 060923), 0.25 mL of 20 mg/mL clarithromycin (Huiren Pharmaceutical Group Corporation in Jiangxi, 0510019), and 0.1 mL of 50 mg/mL ampicillin (North China Pharmaceutical Group Corporation, 060307) per mouse for the triple group. The treatment lasted 10 d, and all animals were sacrificed by carbon dioxide inhalation 4 wk later.

All the methods for the assessment of *H. pylori* colonization and identification in gastric samples as well as evaluation of gastritis will be described in detail below.

### Assessment of *H. pylori* colonization and histopathologic analysis of gastric tissue samples

The stomach of each mouse was removed and dissected longitudinally, the antrum and body were divided and treated separately. One-third of each longitudinal strip was fixed in 10% formalin-buffered saline, embedded in paraffin, and processed for histopathologic analysis. The other two strips were used for rapid urease test and *H. pylori* culture, respectively. Four sections were cut from each paraffin block specimen. Two sections of them were stained with hematoxylin-eosin (HE) for evaluation of gastric inflammation, the other 2 sections were used for Giemsa staining for the assessment of *H. pylori* colonization. The bacterial density was scored from 0 to 4 as follows: Score 0, no bacteria; score 1, 1-2 bacteria on average/crypt; score 2, 3-10 bacteria on average/crypt; score 3, 11-20 bacteria on average/crypt; and score 4, > 20 bacteria on average/crypt<sup>[22]</sup>. The pathologic characteristics were graded for the degree of neutrophil and mononuclear cell infiltration in the antrum and body as follows: Score 1, mildly multifocal; score 2, mildly widespread or moderately multifocal; score 3, mildly widespread and moderately multifocal or severely multifocal; score 4, moderately widespread; score 5, moderately widespread and severely multifocal; and score 6, severely widespread<sup>[8]</sup>. Histopathologic evaluation was performed with no prior knowledge of the identity of the samples by the histopathologist.

### Determination of urease activity

Urease activity was determined by a method based on the commercial rapid urease test (Sanqiang Biochemical Industry Corporation in Fujian, China) with a sensitivity of  $10^2$  bacteria. Following the instruction of the product, each strip of stomach antrum and body was homogenized and placed in 1 mL of the reaction solution [1 g of urea/mL (wt/vol) containing 850 µg phenol red/mL (wt/vol) as a pH indicator]. The solution became pink or red or dark red within 5 min as positive result, still yellow as negative.

### *H. pylori* culture

Each sample of stomach antrum and body was homogenized and suspended in 500 µL brain heart infusion broth containing 10% sheep blood, and 100 µL was inoculated onto brain heart infusion agar plates and cultured under microaerophilic conditions at 37°C for 5-7 d. Gram's staining and urease activity were detected.

### Standard of *H. pylori* infection

When at least two of the three tests of rapid urease test, *H. pylori* culture and Giemsa staining for the assessment of *H. pylori* colonization, appeared positive, *H. pylori* infection could be diagnosed.

### Standard of *H. pylori* eradication

When the three tests of rapid urease test, *H. pylori* culture

and Giemsa staining of gastric mucosa samples were all negative, the eradication was confirmed to be successful.

### Labeling of bacteria with fluorescent probe

A 100 µmol/L stock solution of fluorescence-labeled molecular probe (cFDA-SE, Invitrogen Corporation) was prepared, which was first dissolved in 90 µL dimethyl sulfoxide and then further diluted in ethanol (1 mL; reagent grade). This solution was then filter sterilized (0.2-µm-pore-size Acrodisc filter; Gelman) before being aliquoted and stored at -20°C.

*L. fermenti* was grown overnight at 37°C in MRS broth. The bacterial culture was centrifuged at 3000 r/min for 10 min, and the pellet was washed twice in sterile phosphate-buffered saline (PBS). The concentration of bacteria was regulated to  $1 \times 10^{10}$  CFU/mL prior to labeling with 50 µm cFDA-SE at 37°C for 20 min. Fluorescent labeling was terminated by pelleting the bacteria, washing twice in PBS to remove excessive cFDA-SE, and resuspending the pellet in PBS.

### Animals

A group of 30 mice were administered orally with approximately  $10^9$  cFDA-SE-labeled *L. fermenti* by orogastric intubation. The other group of 6 mice that had been orally fed with sterile PBS served as controls. The food and water intake for the experimental and control mice was measured daily. Groups of 6 mice each were sacrificed at 2, 4, 8, 12 and 24 h after cFDA-SE. The stomach and duodenum of each mouse was divided and dissected longitudinally, any visible residual food particles were removed. Both specimens were examined for the adherence of cFDA-SE-labeled *L. fermenti*. PBS 150 µL was added to each 1.0 cm of the tissue and microbes from the mucosal surface were dislodged by a plunger from a syringe (1.0 mL), then fixed with formaldehyde (0.75%, vol/vol) prior to the detection under fluorescence microscope (Nikon E80, Japan).

### Fluorescent microscopic detection

Enumeration of cFDA-SE-labeled *L. fermenti* was conducted under a fluorescent microscope (Nikon E80, Japan) at a 488-nm excitation wavelength. Upon excitation at 488 nm under the fluorescent microscope, cFDA-SE gave a maximal emission signal in the green at 518 nm.

### Statistical analysis

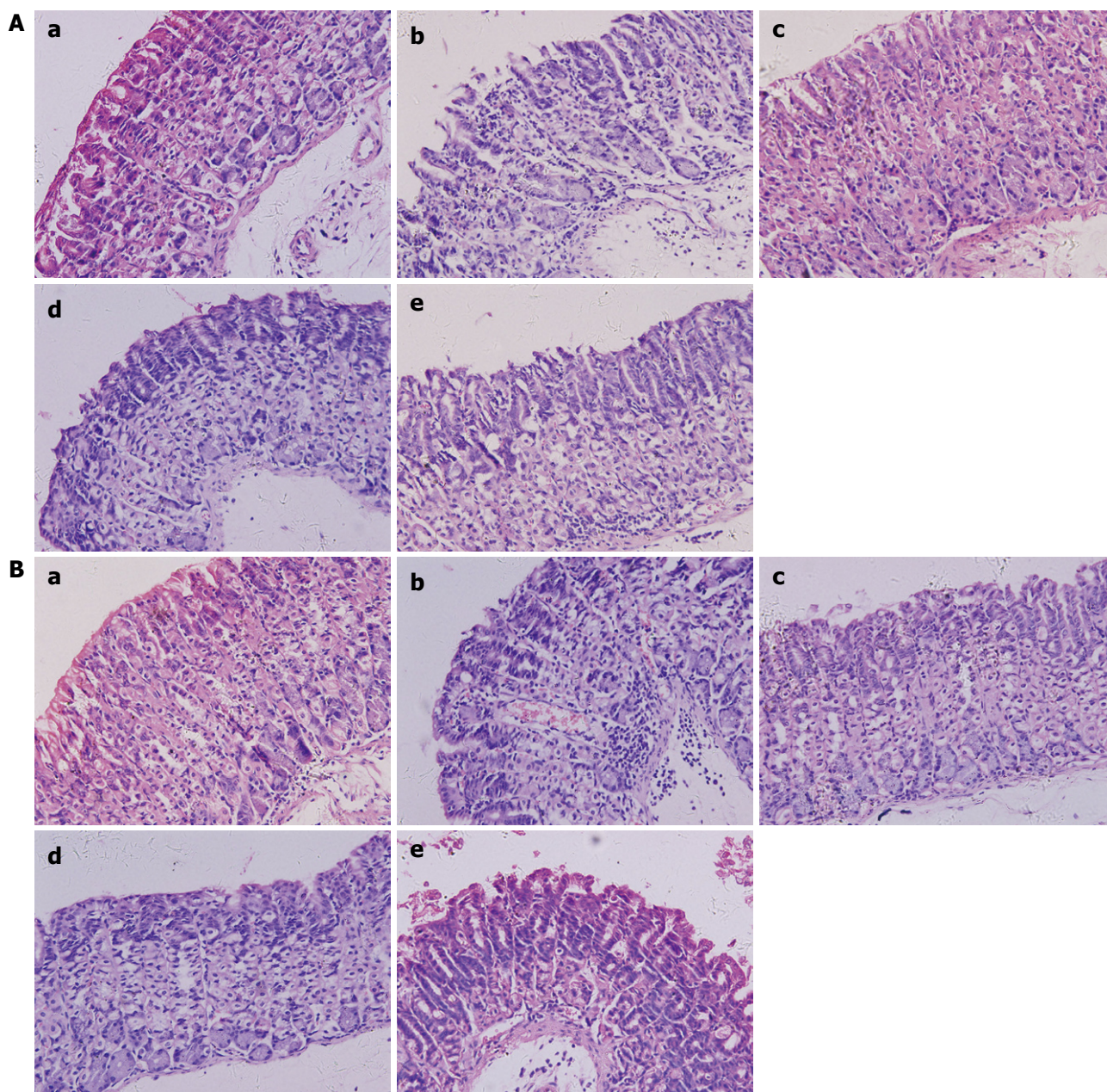
Statistical analyses were performed using SPSS for Windows, version 13.0. Results were expressed as measurement data and enumeration data. For statistical comparisons, *t* test and  $\chi^2$  test were performed and  $P < 0.05$  was considered statistically significant.

## RESULTS

### Evaluation of mucosal inflammation in stomach

Histopathologic evaluation revealed significant improvement of mucosal inflammation in gastric antrum and





**Figure 1** Histological image of gastric antrum and gastric body. A: Histological image of gastric antrum. a: Normal group, normal mucosa, no mucosal erosion; b: NS group, extensive inflammation in the mucosal layer, massive mixed cell infiltration (mainly mononuclear); c: Triple group, slight inflammatory cell infiltration; d: *L. fermenti* group, a few incomplete mucosa; e: *L. acidophilus* group, slight inflammation with moderate cell infiltration, (HE, light microscope,  $\times 200$ ); B: Histological image of gastric body. a: Normal group, normal mucosa, no mucosal erosion; b: NS group, extensive inflammation in mucosal layer, even in submucosal layer with massive mixed cell infiltration; c: Triple group, a few incomplete mucosa, slight inflammatory cell infiltration; d: *L. fermenti* group, almost normal mucosa, slight cell infiltration; e: *L. acidophilus* group, moderate inflammation with cell infiltration in submucosa (HE, light microscope,  $\times 200$ ).

gastric body both in *Lactobacillus* treated as well as triple treated groups. The score of pathologic characteristics of the gastric mucosa also showed obvious decrease. The score of *L. fermenti* treated group was significantly lower than normal saline control group, and almost similar as triple treated group and blank control group. The score of *L. acidophilus* treated group was also lower than normal saline control group and close to triple treated group. The score of *L. acidophilus* group in gastric body was similar to the blank control group, while it was higher in gastric antrum (Figures 1 and 2).

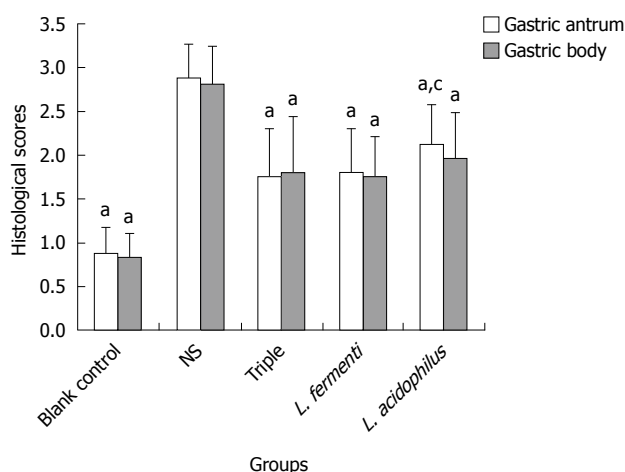
#### Effects of *Lactobacillus* on *H. pylori* eradication rate

The *H. pylori* infection rate in normal saline control group indicated that the model was made successfully while the rate of *H. pylori* infection in blank control group suggested that there was no pollution in environment and diet. We found a significant increase of *H. pylori* eradication rate in *Lactobacillus* treated groups and triple treated group. The eradication rate of *H. pylori* in *L. fermenti* treated group was higher than the normal saline control group and close to that in PPI triple treated group. The eradication rate of *H. pylori* in *L. acidophilus* treated group was higher than



**Table 1** Infection and eradication rate of *Helicobacter pylori* ( $n = 10$ ) (%)

Treatment	Infection rate		Eradication rate	
	Gastric antrum	Gastric body	Gastric antrum	Gastric body
Triple group	30.0 <sup>a</sup>	30.0 <sup>a</sup>	70.0 <sup>a</sup>	70.0 <sup>a</sup>
<i>L. fermenti</i> group	30.0 <sup>a</sup>	40.0 <sup>a</sup>	70.0 <sup>a</sup>	60.0 <sup>a</sup>
<i>L. acidophilus</i> group	50.0	50.0	50.0	50.0
NS group	80.0	80.0	20.0	20.0
Blank control group	0.0 <sup>a</sup>	0.0 <sup>a</sup>	0.0 <sup>a</sup>	0.0 <sup>a</sup>

<sup>a</sup> $P < 0.05$  vs normal saline group.**Figure 2** Histological score of all groups (mean ± SD,  $n = 10$ ). <sup>a</sup> $P < 0.05$  vs NS group. The highest score was  $2.88 \pm 0.38$  in gastric antrum and  $2.82 \pm 0.42$  in gastric body in NS group. <sup>c</sup> $P < 0.05$  vs blank control group.

the normal saline control group, but without significant statistical difference (Table 1).

### Effects of *Lactobacillus* on *H. pylori* colonization

*Lactobacillus* treated groups and triple treated group showed significant decrease of *H. pylori* bacterial density. In *L. fermenti* treated group, the bacterial density was lower than the normal saline control group and close to PPI triple treated group. The bacterial density in *L. acidophilus* treated group is lower than the normal saline control group, however it was statistically insignificant (Figure 3).

### Detection of *Lactobacillus L. fermenti* colonization

The fluorescence intensity indicated the colonization of *Lactobacillus* at various time points after orogastric intubation. The bacteria were not uniformly labeled by cFDA-SE, probably due to the physiological status of the bacteria at the time of incubation with cFDA-SE (Figure 4).

## DISCUSSION

*H. pylori* is a spiral-shaped, gram-negative organism that commonly causes chronic infections and is usually acquired in childhood<sup>[23]</sup>. Patients with complications (e.g.

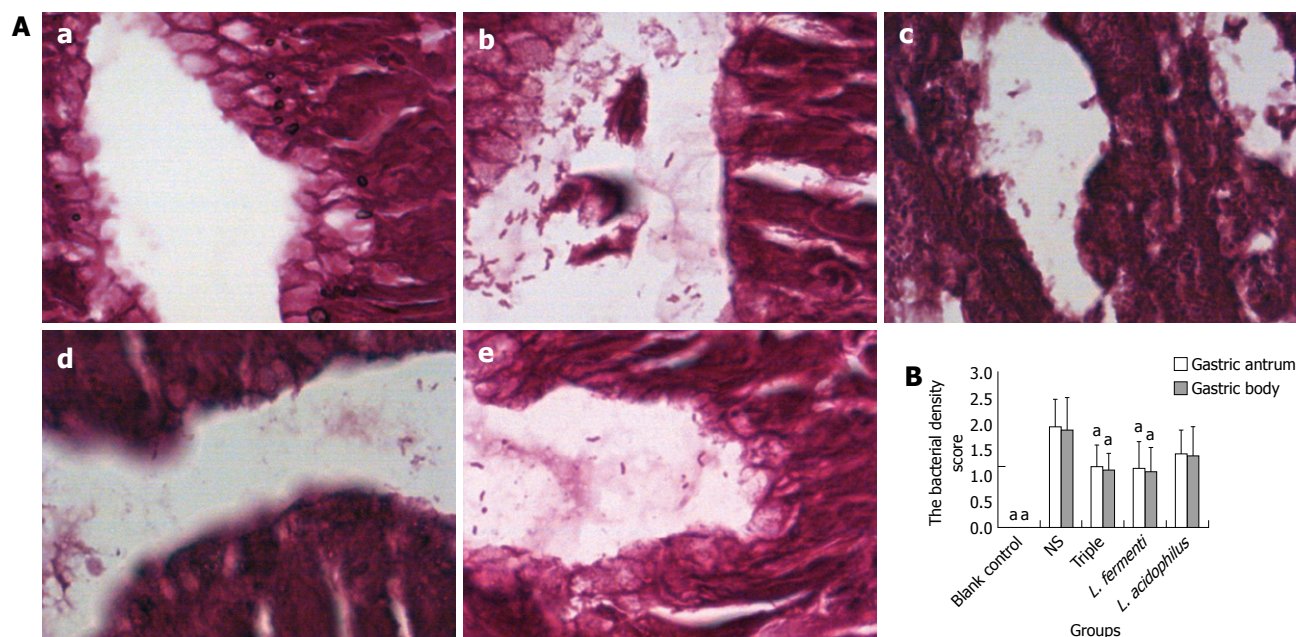
gastritis, ulcer and malignancy) should have the organism eradicated. So far, *H. pylori* can be typically eradicated using a combination of certain antibacterial agents and antacid treatment, which resulted in dramatic reduction in ulcer recurrence and associated complications. The efficacy of recommended eradication regimens is approximately 80%. In our study, standard *H. pylori* SS1 strain was used to colonize the stomach of Balb/c mouse, with an infection rate of 80.0% in gastric antrum and gastric body. This leads to the development of gastric mucosa inflammation closely mimicking human *H. pylori* associated gastritis. The eradication efficacy of *H. pylori* in our study was around 70.0% based on typical triple therapy, which proved that the mouse model of *H. pylori* infection was made successfully and could be established as an animal model to assess anti-*H. pylori* activities of potential therapeutic agents.

A recent clinical trials demonstrated that the addition of Will yogurt to triple therapy increased the *H. pylori* eradication rate by per-protocol (PP) analysis<sup>[14]</sup>. Another clinical study showed that some probiotics, such as *L. johnsonii* LA1, could be used as an adjuvant of antibiotic-antacid treatment to prevent the reemergence of *H. pylori* infection in humans<sup>[19]</sup>. It indicated that the addition of probiotics to PPI-based triple therapy could increase the likelihood of successful *H. pylori* eradication.

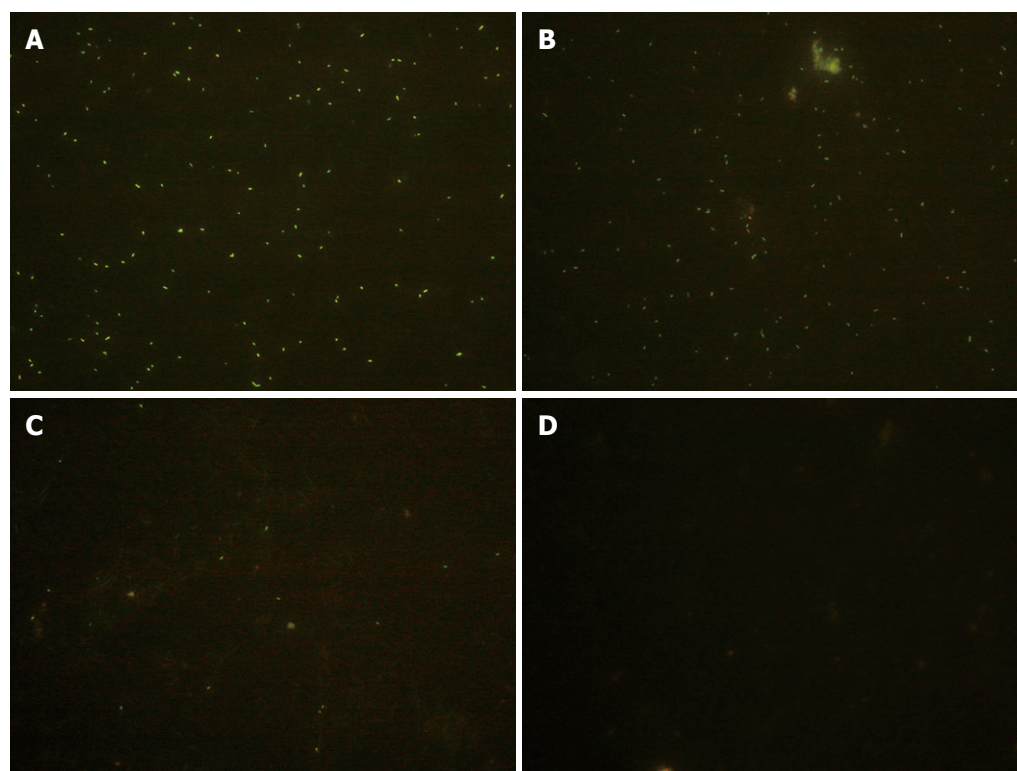
In our study, histopathologic changes on *H. pylori* SS1 mouse model showed a significant release of mucosal inflammation in both gastric antrum and gastric body in *Lactobacillus* strain *L. fermenti* and *L. acidophilus* treated groups and triple treated group. While the histological score of *L. acidophilus* treated group was higher than *L. fermenti* treated group and triple treated group. It showed that oral administration of *L. fermenti* strain could alleviate the gastric inflammation in *H. pylori*-infected Balb/c mouse model, with less significance in strain *L. acidophilus* treated group.

Accordingly, *Lactobacillus* treated groups and triple treated group showed a significant decrease of *H. pylori* bacterial density, with an eradication rate of *H. pylori* 70.0% in gastric antrum and 60.0% in gastric body in *L. fermenti* treated group, which was obviously higher than the normal saline control group and close to that in the triple treated group. The eradication rate of *H. pylori* in *L. acidophilus* treated group was 50.0% in gastric antrum and gastric body, being higher than the normal saline control group, but without statistical difference. It indicates that different *Lactobacillus* strains have various anti-*H. pylori* effects due to different *H. pylori* density and *H. pylori* eradication rate.

Our data from the SS1 mouse model with reference to *H. pylori* colonization were in agreement with the histopathological changes among different groups. *L. fermenti* can inhibit the colonization of *H. pylori* in gastric mucosa so as to improve its inflammatory injury. In the *L. fermenti* strain group, we observed a significant reduction of *H. pylori* colonization in the gastric mucosa



**Figure 3 Bacterial density and bacterial density score of *H. pylori*.** A: Bacterial density of *H. pylori*. a: Normal group, no detectable *H. pylori*; b: NS group, over 10 *H. pylori* were detected in one field of view; c: Triple group; d: *L. fermenti* group; e: *L. acidophilus* group, (Giemsa, light microscope,  $\times 1000$ ). B: Bacterial density score of *H. pylori*. mean  $\pm$  SD,  $n = 10$ .  $^aP < 0.05$  vs NS group. The highest score was  $1.95 \pm 0.52$  in gastric antrum and  $1.88 \pm 0.63$  in gastric body in NS group.



**Figure 4 Fluorescent microscopic detection of *L. fermenti* labeled with cFDA-SE.** A: 2 h, a diffusely distributed cFDA-SE labeled bacteria under fluorescent microscopy; B: 4 h, slightly reduced in comparison with 2 h; C: 8 h, cFDA-SE labeled bacteria reduced obviously; D: Blank control group, no cFDA-SE labeled bacteria was detected.

throughout the entire observation period. Continuous administration of *L. acidophilus* did not reduce the *H. pylori* colonizing numbers over this experimental period. These indicate that some strains of lactobacillus can

colonize in gastric mucosa and exhibit anti-*H. pylori* colonization. These strains due to their differences in species and specificity can lead to different anti-*H. pylori* activities. Detection of living bacteria under fluorescent



microscope provided evidence of colonization of the stomach and duodenum by *L. fermenti*. The appearance of *L. fermenti* supports the idea that they come from the supplement.

As it is known, lactobacilli are components of the normal intestinal flora of healthy humans which exert antagonistic activities against pathogens<sup>[7,9,10,16]</sup>. In particular, it has been reported that the primary microorganisms associated with the stomach belong to the genus *Lactobacillus*. *Lactobacillus* earn particular capacity to survive and develop in an acidic environment and live as an indigenous bacterium in gastric mucosa, which can effectively inhibit the colonization of *H. pylori*<sup>[11,12,15,21]</sup>. Our study also adds evidence that *H. pylori* can live together with other bacteria and maintain a dynamic equilibrium state. In another word, it is rational to prevent and control *H. pylori* infection by regulating the balance of flora in stomach. Thus *Lactobacillus* can be a choice to replace antibiotics or as an adjuvant to antibiotics in treating *H. pylori*-infected diseases.

In conclusion, both *Lactobacillus* strain *L. fermenti* and *L. acidophilus*, which are isolated from human gastric mucosa, showed significant anti-*H. pylori* activity, while strain *L. fermenti* displayed more efficient antagonistic activity *in vivo* whose efficacy is close to the standard triple therapy, thus significantly improving the *H. pylori*-associated Balb/c gastritis. It would be of great interest to further explore the role of such probiotic strains in the complex regulation of anti-*H. pylori* activities and screen for more efficient clinical potential agents.

## COMMENTS

### Background

*Helicobacter pylori* (*H. pylori*) are considered to be the most important etiological agents of chronic gastritis. The eradication of *H. pylori* depends on the combination of antibiotics and acid suppression drugs. Unfortunately, the side effects of antibiotics reduce the curative effect and treatment compliance. Probiotics provides an alternative method which can inhibit *H. pylori* infection efficiently without antibiotics associated side effects.

### Research frontiers

*Lactobacilli* earn particular capacity to survive and develop in an acidic environment and live as an indigenous bacterium in gastric mucosa, which can effectively inhibit the colonization of *H. pylori*. Different strains display different effects on *H. pylori* infected gastritis.

### Innovations and breakthroughs

The study showed that *H. pylori* has some interactions with other microbes in stomach. They live together and maintain a dynamic equilibrium state. Both the strains of *Lactobacillus* isolated from human gastric mucosa showed significant anti-*H. pylori* activity while *Lactobacillus fermenti* (*L. fermenti*) displayed more efficient antagonistic activity *in vivo*. This study also provides evidences of colonization of the stomach and duodenum by *L. fermenti*.

### Applications

The study provides a new clue for the therapy of *H. pylori* associated diseases, which could be prevented and treated by regulating the balance of flora in stomach. Thus *Lactobacillus* can be a choice to replace antibiotics or as an adjuvant to antibiotics in treating *H. pylori*-infected diseases.

### Peer review

In this paper, the authors investigated the potential effects of anti-*H. pylori* activity and anti-inflammation *in vivo* of two *Lactobacillus* strains from stomach. And they found that both *Lactobacillus* strains showed significant anti-*H. pylori*

activity, and *L. fermenti* displayed more efficient antagonistic activity *in vivo* against *H. pylori* infection. This paper gave us a new point of view on the interaction of gastric microbiology and also provided a new clue for *H. pylori* treatment.

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## Diagnostic utility of IgG and IgM immunohistochemistry in autoimmune liver disease

Roger Klein Moreira, Frank Revetta, Elizabeth Koehler, Mary Kay Washington

Roger Klein Moreira, Department of Pathology, Columbia University College of Physicians & Surgeons, New York, NY 10038, United States

Elizabeth Koehler, Department of Biostatistics, Vanderbilt University, Nashville, TN 37232, United States

Frank Revetta, Mary Kay Washington, Department of Pathology, Vanderbilt University, Nashville, TN 37232, United States

**Author contributions:** Moreira RK and Washington MK designed the study, selected patients, performed histopathologic and immunohistochemical analysis of slides, and wrote the manuscript; Koehler E was responsible for the statistical analysis of our data; Revetta F provided immunohistochemistry technical support and performed all immunohistochemistry stains used in this study.

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**Correspondence to:** Roger Klein Moreira, MD, Department of Pathology, Columbia University College of Physicians & Surgeons, 630 W168th Street VC14-238B, New York, NY 10032, United States. [rkm2124@columbia.edu](mailto:rkm2124@columbia.edu)

Telephone: +1-212-3429994 Fax: +1-212-3052301

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cases). The IgM /IgG ratio ( $< 1$  or  $\geq 1$ ) accurately distinguished PBC from AIH in 90.9% of cases (sensitivity = 92.3%, specificity = 90%), and PBC from either AIH or PSC in 87.8% of cases (sensitivity = 92.3%, specificity = 85.7%).

**CONCLUSION:** Plasmacytic infiltrates expressing predominantly IgM are characteristic of PBC, while other forms of liver disease analyzed in this study, including AIH, typically show an IgG-predominant plasma cell infiltrate. Our data indicate that IgM and IgG IHC may be a useful tool when PBC is a diagnostic consideration.

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**Key words:** Autoimmune hepatitis; Primary sclerosing cholangitis; Primary biliary cirrhosis; Immunoglobulin; Immunohistochemistry

**Peer reviewer:** Shmuel Odes, MD, Professor, Head of the Inflammatory Bowel Diseases Unit, Department of Gastroenterology and Hepatology, Soroka University Hospital, Faculty of Health Sciences, Ben Gurion University of the Negev, PO Box 151, Beer Sheva 84101, Israel

Moreira RK, Revetta F, Koehler E, Washington MK. Diagnostic utility of IgG and IgM immunohistochemistry in autoimmune liver disease. *World J Gastroenterol* 2010; 16(4): 453-457 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i4/453.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i4.453>

### Abstract

**AIM:** To assess the role of IgM and IgG immunohistochemistry (IHC) in the evaluation of autoimmune liver conditions - autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC).

**METHODS:** Forty one biopsies from untreated patients diagnosed with autoimmune liver disease (AIH,  $n = 20$ ; PBC,  $n = 13$ ; PSC,  $n = 8$ ) and fourteen biopsies of patients with chronic hepatitis C were selected. IgM and IgG-positive plasma cells were counted in each sample.

**RESULTS:** A predominance of IgG-positive plasma cells was seen in AIH (90% of cases), PSC (75% of cases), and chronic hepatitis C (100% of cases), while IgM-positive plasma cells predominated in PBC (92.8% of

### INTRODUCTION

Three major clinicopathologic entities are currently classified as autoimmune liver diseases: autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC). A combination of clinical, laboratory, and pathologic criteria are necessary for the diagnosis of these conditions. Each category of autoimmune liver disease has specific biologic,

prognostic, and therapeutic implications; therefore, an accurate diagnosis is essential.

While liver biopsies represent an important part of the diagnostic evaluation of these patients, significant histopathologic overlap exists among the different autoimmune liver diseases. An important histologic finding in liver biopsies, in this setting, is the presence of plasma cells, which are characteristically found in AIH, but are also commonly present in PBC and PSC, as well as in other forms of liver disease<sup>[1-6]</sup>. Previous studies have shown that assessment of immunoglobulin subclasses in plasma cells by immunohistochemistry (IHC) may be useful in distinguishing PBC from AIH, but the use of such ancillary studies has not yet gained wide acceptance as a diagnostic tool<sup>[7-9]</sup>. Moreover, limited data are available regarding the immunophenotype of plasma cells in PSC. In this study, we evaluate the predominant plasma cell immunoglobulin subclass present in liver biopsies of patients with well-established AIH, PBC, or PSC and assess the diagnostic utility of IgM and IgG IHC in this setting.

## MATERIALS AND METHODS

This study was reviewed and approved by our Institutional Review Board. Liver biopsies from untreated patients diagnosed with autoimmune liver disease at our institution from 1993 to 2006 were selected. Inclusion criteria for patients with AIH were: (1) clinical diagnosis of AIH, according to the international autoimmune hepatitis group scoring system<sup>[10]</sup>, including positive serology for AIH-related autoantibodies [antinuclear antibody (ANA), anti-smooth muscle antibody (ASMA), anti-liver-kidney microsomal antibody (anti-LKM-1), or soluble liver antigen antibody (anti-SLA)]; and (2) liver biopsy showing consistent histopathological findings. For PBC, inclusion criteria were: (1) positive anti-mitochondrial antibody (AMA) serology; (2) elevated alkaline phosphatase levels, and (3) consistent histopathological findings. For PSC, inclusion criteria were: (1) elevated alkaline phosphatase levels, and (2) characteristic cholangiographic findings by endoscopic retrograde cholangiopancreatography (ERCP) or magnetic resonance cholangiopancreatography (MRCP). Inclusion criteria for all groups also included availability of adequate paraffin-embedded tissue for immunohistochemical analysis. Exclusion criteria for all autoimmune liver disease groups included evidence of viral hepatitis, clinical suspicion for an alternative underlying etiology for the liver disease, and incompatible histopathological findings. For patients with AIH, PBC, and PSC, all biopsies included in this study were taken as part of the initial diagnostic workup. Patients who received any form of immunosuppressive medication (other than inflammatory bowel disease treatment in cases of PSC) or ursodeoxycholic acid previous to the biopsy procedure were excluded. Utilizing the above criteria, 41 patients were included in the study (AIH,  $n = 20$ ; PBC,  $n = 13$ ; and PSC,  $n = 8$ ). For comparison, we also studied 14 patients with chronic hepatitis C with at least focal plasma

cells identified on HE sections.

Biopsy samples were considered adequate for the purposes of this study if at least 3 complete portal tracts were present. For each biopsy, the length of each tissue fragment was multiplied by its average width (mm) and the total area of the sample (mm<sup>2</sup>) was calculated. Subsequently, all cells morphologically consistent with plasma cells showing unequivocal immunohistochemical expression of either IgM or IgG (Cell Marque, 1:20 000) were counted. The absolute number and the concentration of positive cells for each immunoglobulin subtype were assessed. IgM/IgG ratio was analyzed for sensitivity and specificity in the diagnosis of autoimmune liver diseases. Confidence intervals for these values are determined by a score interval<sup>[11]</sup>. For cases of AIH, PSC, and chronic hepatitis C, the degree of fibrosis was assessed in each case using the Batts-Ludwig staging system. Histological staging of PBC cases was performed using Ludwig's classification.

## RESULTS

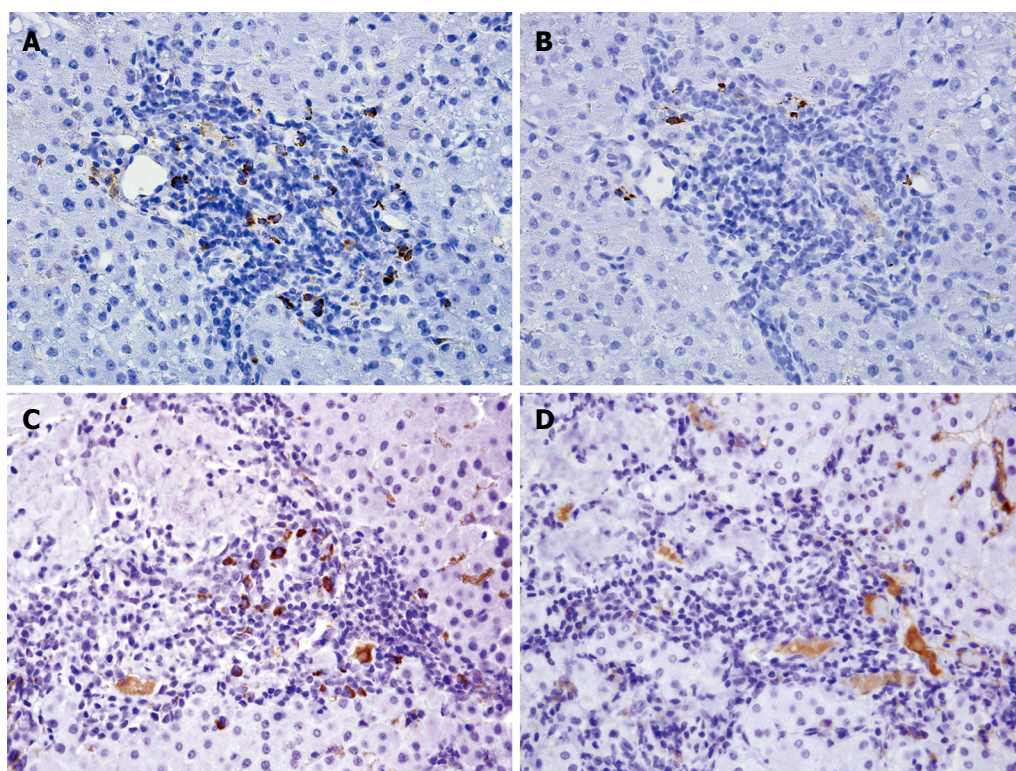
Twenty patients had a diagnosis of AIH (8 males, 12 females, mean age 32, range 4-63), all of whom had positive antinuclear antibody (ANA) serology. Two pediatric patients had positive anti-smooth muscle antibody serology in addition to a positive ANA. Associated autoimmune conditions included thyroid dysfunction (3 patients), rheumatoid arthritis (1 patient), vitiligo (1 patient), and alopecia (1 patient). One patient was diagnosed with drug-induced AIH while receiving minocycline treatment for acne. Fifty percent of AIH patients had early stage fibrosis (stages 0-2) and 50% had advanced fibrosis (stages 3 and 4).

Thirteen patients had a diagnosis of PBC (1 male, 12 females, mean age 52, range 43-71). Associated autoimmune conditions included sicca syndrome (26% of patients), and hypothyroidism (6% of patients). PBC patients were staged as following: stage 1 (3/13, 23%), stage 2 (7/13, 54%), stage 3 (3/13, 23%), stage 4 (1/13, 6.6%). Typical florid duct lesions were seen in 3/13 (23%) of PBC patients (stage 2, two patients; stage 3, one patient).

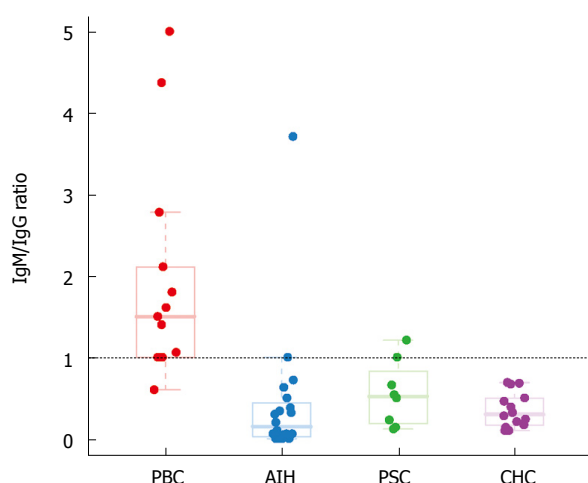
Eight patients had a diagnosis of PSC (6 males, 2 females, mean age 31, range 7-67), including characteristic findings on endoscopic retrograde cholangiopancreatography (ERCP) or magnetic resonance cholangiopancreatography (MRCP). Fifty percent of PSC patients had early stage fibrosis (stages 0-2), and 50% had advanced fibrosis (stages 3 and 4). Typical periductal fibrosis was seen in only 2 cases. Additional histologic findings included mild-moderate chronic lymphoplasmacytic portal inflammation, and bile ductular proliferation (8/10 patients). Associated conditions included ulcerative colitis (4/8, 50%), and Crohn's disease (2/8, 25%).

Fourteen patients with chronic hepatitis C infection were also included (9 males, 5 females, mean age 50, range 36-66). All patients in this group had documented





**Figure 1** Immunoglobulin expression in autoimmune hepatitis and primary biliary cirrhosis. IgG-positive plasma cells (A) outnumber IgM-positive plasma cells (B) in most cases of autoimmune hepatitis (AIH); In contrast, IgM-positive plasma (C) cells predominate over IgG-positive cells (D) in the majority of cases of primary biliary cirrhosis (PBC). IgG background staining within sinusoids and blood vessels is common due to the presence of serum IgG. (Immunohistochemistry, 400 × magnification).



**Figure 2** IgM/IgG ratio distinguishes the majority of cases of primary biliary cirrhosis (IgM/IgG ratio  $\geq 1$ ) from all other groups (IgM/IgG ratio  $< 1$ ). Single outlier in the AIH group was diagnosed with AIH-PBC sequential syndrome based on subsequent serology and pathological data (see discussion). Abbreviations: PBC: Primary biliary cirrhosis; AIH: Autoimmune hepatitis; PSC: Primary sclerosing cholangitis; CHC: Chronic hepatitis C.

chronic viral hepatitis C infection by serum polymerase chain reaction (PCR) (genotype 1,  $n = 11$ ; genotype 2,  $n = 2$ ; and genotype 3,  $n = 1$ ). Twelve patients underwent liver biopsy for grading and staging (native liver biopsies). Two patients had recurrent hepatitis C post liver transplantation. Interpretation of liver biopsy in all cases was consistent with hepatitis C (stage 0,  $n = 1$ ; stage 1,  $n = 2$ ; stage 2,  $n = 6$ ; stage 3,  $n = 3$ ; stage 4,  $n = 2$ ).

The combined number of IgG and IgM+ plasma cells in AIH (average = 9.75 cells/mm<sup>2</sup>) was higher than that seen in CHC, PBC or PSC (average = 6.90, 6.19

and 5.16 cells/mm<sup>2</sup>, respectively,  $P < 0.05$ ). In all cases, plasma cells were present predominantly within the portal tracts.

We found a predominance of IgG+ plasma cells in AIH (90% of cases), in PSC (75% of cases), and in chronic hepatitis C (100% of cases), while the majority of plasma cells in PBC were IgM positive (92.3% of cases) (Figures 1 and 2). An IgM/IgG ratio  $\geq 1$  showed high sensitivity and specificity for the distinction of PBC from other groups included in the study and accurately distinguished PBC from AIH in 90.9% (30/33) of cases, PBC from either AIH or PSC in 87.8% (36/41) of cases, and PBC from all other groups in 90.9% (Table 1). There was no correlation between age or gender and IgM/IgG ratio in any of the study groups.

## DISCUSSION

Typical cases of autoimmune liver diseases do not usually represent diagnostic dilemmas from a histopathologic standpoint. However, diagnostic findings are sometimes absent in liver biopsies of autoimmune liver diseases, and consideration of various entities has to be entertained in the differential diagnosis<sup>[12-14]</sup>.

Chronic inflammatory infiltrate, often plasma cell-rich, is a common feature in all autoimmune liver diseases<sup>[15]</sup>. There is evidence to suggest that the identification of the predominant immunoglobulin (Ig) subclasses within plasma cells in liver biopsies may be useful in the differential diagnosis of autoimmune liver diseases. van Spreuwel *et al.*<sup>[8]</sup>, in 1984, studied eight patients with PBC and 18 patients with “chronic hepatitis” (which included patients with hepatitis B, as well as patients diagnosed as

Table 1 IgM/IgG ratio in PBC compared to other autoimmune liver conditions

	Sensitivity (95% CI)	Specificity (95% CI)	Likelihood ratio - positive test (95% CI)	Likelihood ratio - negative test (95% CI)	Accuracy
PBC <i>vs</i> AIH	92.3% (66.7-98.6)	90.0% (69.9-97.2)	9.23 (2.45-34.69)	0.085 (0.012-0.56)	90.9% (30/33)
PBC <i>vs</i> AIH or PSC	92.3% (66.7-98.6)	85.7% (68.5-94.3)	6.46 (2.57-16.22)	0.089 (0.013-0.59)	87.8% (36/41)
PBC <i>vs</i> all others	92.3% (66.7-98.6)	90.5% (77.9-96.3)	9.69 (3.76-24.94)	0.085 (0.012-0.56)	90.9% (50/55)

PBC: Primary biliary cirrhosis; AIH: Autoimmune hepatitis; PSC: Primary sclerosing cholangitis; Positive test: IgM/IgG ratio  $\geq 1$ ; Negative test: IgM/IgG ratio  $< 1$ .

“chronic persistent hepatitis” and “chronic aggressive hepatitis”). The authors found a significantly higher absolute and relative number of IgM-positive plasma cells, by immunohistochemistry, in patients with PBC compared to patients with chronic hepatitis. The increased number of plasma cells correlated with increased serum levels of IgM in PBC patients.

Milne *et al*<sup>[7]</sup>, in 1990, studied 14 patients with PBC and 14 patients with “chronic active hepatitis” (which included patients with hepatitis B, ANA-positive hepatitis, and idiopathic chronic hepatitis). Using immunohistochemistry for IgA, IgM, and IgG, the authors found a predominance of IgA and IgG expression in plasma cells in “chronic active hepatitis”, while IgM and IgG represented the main Ig classes seen in cases of PBC. In both studies, all cases of PBC had positive AMA serology, increased alkaline phosphatase levels, and histopathological findings which were consistent with the diagnosis. However, the “chronic hepatitis” group was heterogeneous and included patients with hepatitis B, as well as other forms of chronic hepatitis, then classified as “chronic active hepatitis” and “chronic aggressive hepatitis”, which may have included cases of hepatitis C and autoimmune hepatitis by current diagnostic standards.

Recently, Daniels *et al*<sup>[9]</sup> specifically studied the immunophenotype of plasma cells in the differential diagnosis of PBC and AIH. Thirty eight patients with AIH and 18 patients with PBC were included. All cases of AIH showed an IgG-predominant plasma cell infiltrate, while IgM prevailed over IgG in 88% of PBC cases upon qualitative analysis of IHC slides (back-to-back comparison).

In the present study, we included untreated patients meeting strict clinical and pathologic diagnostic criteria for both AIH and PBC. In addition, we also included patients with PSC, which were not included in previous studies, as this condition may also be a diagnostic consideration in this setting. A group of hepatitis C patients was included for comparison.

Our results clearly show that most cases of PBC expressed a characteristic IgM/IgG immunophenotype. There was an obvious predominance of IgM expression in plasma cells of patients with PBC (92.3% of patients), compared to an IgG-predominant expression in the majority of plasma cells in patients with AIH (90% of cases), PSC (75% of cases), and CHC (100% of cases). Among the groups of patients included in this study, the differential diagnosis between AIH and PBC is likely the

most challenging from a histopathological perspective. The IgM/IgG ratio ( $< 1$  or  $\geq 1$ ) accurately distinguishes PBC from AIH in over 90% of cases (sensitivity = 92.3%, specificity = 90%). A high sensitivity and specificity (92.3% and 90.5%, respectively) was also found for the distinction between PBC and all other groups combined (Table 1).

Interestingly, in 1 case of autoimmune hepatitis which showed a very high IgM/IgG ratio, similar to that seen in the PBC group, a positive antimitochondrial antibody (titer  $> 1:160$ ) was detected on clinical follow-up (AMA serology was negative on initial evaluation). A second liver biopsy of this patient showed lymphoplasmacytic portal inflammation and associated prominent ductocentric granulomatous inflammation with significant bile duct destruction (florid duct lesion). A diagnosis of AIH-PBC sequential syndrome was rendered 3 years after the initial biopsy. This patient remained in the AIH group of this study because criteria for AIH were met at the time of initial biopsy. Although only 1 case of overlap syndrome was identified in our database, the fact that plasma cells predominantly expressed IgM in both the initial and the follow-up biopsies is intriguing. This raises the question of whether immunohistochemistry for specific immunoglobulins could provide additional information (i.e. identify a PBC-like pattern) in the setting of suspected overlap syndrome, or even in otherwise typical autoimmune hepatitis.

PSC cases showed a predominance of IgG+ plasma cells in most cases, although a significant number (25%) showed a predominance of IgM+ plasma cells. In most cases of PSC, however, the total number of plasma cells was low, and sampling error may have occurred during the evaluation of individual cases as IgM or IgG-predominant.

Among our chronic hepatitis C samples (15 cases selected from 30 consecutive cases of chronic hepatitis C), a plasmacytic component was present within the lymphocyte-predominant infiltrate in all cases. Because the cases were selected on the basis of presence of plasma cells, an obvious selection bias is present. However, since 15 of 30 consecutive cases were included, it is clear that plasma cells are common in our chronic hepatitis C population. All biopsies from hepatitis C patients showed predominantly IgG-producing plasma cells.

Overall, IgG was the predominant immunoglobulin subclass produced by plasma cells in all groups included in this study, except for the PBC group, in which an IgM-predominant plasma cell population is seen in the majority of cases. From a diagnostic standpoint, we believe



that plasma cell-rich infiltrates seen on liver biopsies with IgM/IgG ratio  $\geq 1$  should alert the pathologist to the possibility of PBC, while an IgM/IgG ratio  $< 1$  renders a diagnosis of PBC significantly less likely. IgG-predominant infiltrates, although characteristically present in AIH, are relatively nonspecific and may be seen in a variety of conditions.

In conclusion, the histological features of the different autoimmune liver diseases may overlap considerably in some cases. While the plasma cell-rich infiltrates seen in several forms of liver disease predominantly express IgG, PBC seems to be an exception, as most plasma cells in these patients express IgM. Our data indicates that a predominantly IgM+ plasma cell infiltrate, although not pathognomonic, should strongly support the diagnosis of PBC. The IgM/IgG ratio is particularly helpful distinguishing PBC from AIH.

## COMMENTS

### Background

Increased numbers of plasma cells may be present on liver biopsies of autoimmune hepatitis, primary biliary cirrhosis, and primary sclerosing cholangitis patients, and the histopathologic distinction of these entities may be difficult in some cases. Identification of characteristic immunohistochemical phenotypes of plasma cells in such cases could be useful in the differential diagnosis between the different entities in this group of diseases.

### Research frontiers

Previous studies have shown that assessment of immunoglobulin subclasses in plasma cells by immunohistochemistry may be useful in the histopathologic evaluation of autoimmune liver diseases. However, further studies are necessary in order to validate the diagnostic utility of IgM and IgG immunohistochemistry in this specific scenario.

### Innovations and breakthroughs

The histopathologic evaluation of autoimmune liver diseases has relied almost exclusively on morphologic features seen on routine stains. In this study, the authors describe the potential application of IgM and IgG immunohistochemistry in the setting of autoimmune liver diseases, especially in the differential diagnosis of PBC from other conditions. The authors have demonstrated that an IgM-predominant plasmacytic infiltrate is typical of PBC but uncommon in other forms of chronic liver disease.

### Applications

The characterization of plasma cells in liver biopsies of autoimmune liver disease patients by IgM and IgG immunohistochemistry may be very useful in selected cases and may serve as an adjunct method to conventional histopathologic evaluation.

### Terminology

Autoimmune liver disease refers to a group of chronic liver conditions that includes autoimmune hepatitis, primary biliary cirrhosis, and primary sclerosing cholangitis.

### Peer review

The authors have drawn attention to the preponderance of IgM-positive

plasma cells in liver biopsies of patients with PBC, thereby providing a tool for differentiating PBC from a series of other liver conditions.

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## Patient interest in video recording of colonoscopy: A survey

Meghana Raghavendra, Douglas K Rex

Meghana Raghavendra, Douglas K Rex, Division of Gastroenterology/Hepatology, Indiana University Medical Center, Indianapolis, IN 46202, United States

**Author contributions:** Raghavendra M conducted the survey. Raghavendra M and Rex DK analyzed the data and wrote the manuscript.

**Correspondence to:** Douglas K Rex, MD, Department of Medicine, Division of Gastroenterology, Indiana University School of Medicine, 550 N. University Boulevard UH 4100, Indianapolis, IN 46202, United States. [drex@iupui.edu](mailto:drex@iupui.edu)

Telephone: +1-317-2788741 Fax: +1-317-2745449

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cording of their procedure. Awareness of missing lesions during colonoscopy increased interest in having a videorecording.

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**Key words:** Colonoscopy; Video recording; Survey

**Peer reviewer:** Dr. Shinji Tanaka, Director, Department of Endoscopy, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan

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### Abstract

**AIM:** To find if patients are interested in obtaining a video recording of their colonoscopy procedure.

**METHODS:** We conducted a survey of outpatients presenting for colonoscopy regarding their interest in obtaining a video recording of their colonoscopy.

**RESULTS:** Two hundred and forty-eight patients (mean age 57.9 years; 57% male) were surveyed. Two hundred and one patients (81%) were interested in obtaining a video recording. No significant predictors of patients' interest in the video recording were identified. After reading a brief educational paragraph explaining missed lesions during colonoscopy, 135 patients (54%) were more interested in having a video recording, and none were less interested. One hundred and fifty-six patients (63%) were willing to pay for a video recording. In multivariable analyses, younger age was predictive of willingness to pay for a video recording. Prior history of colorectal cancer and a family history of colorectal cancer were predictive of willingness to pay a greater amount.

**CONCLUSION:** Patients undergoing colonoscopy expressed substantial interest in obtaining a videore-

### INTRODUCTION

There is increasing awareness that colonoscopy fails to prevent a substantial fraction of colorectal cancers<sup>[1-8]</sup> and that the performance of colonoscopy is highly operator dependent<sup>[9,10]</sup>. Some studies that have demonstrated imperfect protection<sup>[8]</sup> and operator dependency<sup>[9]</sup> have received considerable lay press attention. Recent studies have suggested that there is less protection against colorectal cancer by colonoscopy in the proximal compared to the distal colon<sup>[8,11]</sup>. Part of the cause of inadequate right colon protection might be exaggeration of cecal intubation rates by poorly trained colonoscopists<sup>[8]</sup>. Indeed, colonoscopies are often poorly documented with regard to cecal intubation, and reports claiming cecal intubation often fail to document cecal landmarks by notation or photography<sup>[12]</sup>.

We reasoned that as awareness of operator dependency of colonoscopy increases, some patients might be interested in obtaining a video recording of their colonoscopy, either as a way of ensuring that their examination was of high quality, or based on interest in viewing

the procedure, because patients are often not able to watch the examination in real-time.

Therefore, we conducted a survey of patients undergoing colonoscopy at Indiana University Hospital as to their interest in obtaining a video recording of their procedure and their willingness to pay for the recording.

## MATERIALS AND METHODS

The survey was conducted in 250 patients presenting to Indiana University Hospital or an ambulatory surgery center associated with the hospital. Permission to perform the survey was granted by the Institutional Review Board at Indiana University/Purdue University Indianapolis/Clarian Health Partners.

The survey identified age, gender, occupation, whether and how many prior colonoscopies the patient had undergone, any personal history of colorectal polyps or cancer, family history of colorectal cancer, and whether they owned a digital versatile disc (DVD) player. Patients were asked whether they would be interested in owning a video recording of their colonoscopy and were asked for an explanation of their response.

In the second part of the survey, there was a brief educational paragraph explaining miss rates during colonoscopy. Patients were asked if this explanation made them more or less likely to want a video recording. Patients were asked about their willingness to pay for the video recording and the amount they would pay. The paragraph read by the patients was as follows: "Colonoscopists have been shown to vary 4- to 10-fold in the number of precancerous polyps they detect and remove during colonoscopy. This means that some colonoscopists miss more than half of the precancerous polyps in the colon. Does this information make you: (A) Less likely to want a digital recording of your colonoscopy? (B) More likely to want a digital recording of your colonoscopy? (C) No change in your decision for a digital recording of your colonoscopy".

The surveys were conducted by a physician trained in internal medicine. The study was considered exploratory and the sample size was arbitrary. Patients were consecutive to the extent that the survey administrator was available and not already occupied with conducting a survey.

Chi square tests and logistic regression were used to determine whether the survey items were significant predictors of the desire to have a video recording of the colonoscopy and of willingness to pay for the video recording. Two-sample *t*-tests and correlation coefficients were used to determine how much patients were willing to pay. The amounts were analyzed two ways: (A) using only those patients who answered they were willing to pay and (B) using all patients, with those who answered they were unwilling to pay recorded as willing to pay zero dollars. To examine multiple-variable models, a backward elimination procedure was used, with all variables individually significant at  $P < 0.30$  included initially, and then removing variables one at a time until only variables with  $P$  values  $< 0.05$  remained.

**Table 1 Patient interest in owning a DVD of their colonoscopy**

		Interested	Not Interested	P
Age in years, mean (SD)		57.8 (12.8)	58.7 (12.8)	0.66
Gender	F	90 (85) <sup>1</sup>	16 (15) <sup>1</sup>	0.21
	M	110 (79) <sup>1</sup>	30 (21) <sup>1</sup>	
Own DVD player	Y	193 (81) <sup>1</sup>	44 (19) <sup>1</sup>	0.78
	N	7 (78) <sup>1</sup>	2 (22) <sup>1</sup>	
Prior Colonoscopy	Y	151 (83) <sup>1</sup>	31 (17) <sup>1</sup>	0.28
	N	50 (77) <sup>1</sup>	15 (23) <sup>1</sup>	
History of Polyps	Y	100 (82) <sup>1</sup>	22 (18) <sup>1</sup>	0.81
	N	101 (82) <sup>1</sup>	24 (19) <sup>1</sup>	
History of CRC	Y	9 (90) <sup>1</sup>	1 (10) <sup>1</sup>	0.48
	N	192 (81) <sup>1</sup>	45 (19) <sup>1</sup>	
FH of CRC	Y	47 (84) <sup>1</sup>	9 (16) <sup>1</sup>	0.57
	N	153 (81) <sup>1</sup>	37 (19) <sup>1</sup>	

SD: Standard deviation; F: Female; M: Male; Y: Yes; N: No; DVD: Digital video disc; FH: Family history; CRC: Colorectal cancer. <sup>1</sup>Number (percent).

## RESULTS

Two hundred and fifty patients were approached to answer the survey. One patient refused to participate and one was excluded due to inconsistent information. One patient did not answer whether they wanted a DVD, and a few data points were missing from other surveys (Tables 1 and 2). Thus, 248 patient surveys were available for analyses. Patients ranged in age from 19 to 87 years (mean 57.9 years). There were 238 patients (96%) who owned DVD players. There were 141 (57%) males, 183 (74%) had undergone a prior colonoscopy, and 123 (50%) had a prior history of polyps. Ten patients (4%) had a personal history of colorectal cancer, while 57 (23%) has a family history of colorectal cancer.

Among the 248 patients, 201 (81%) were interested in obtaining a video recording of their colonoscopy. Among the factors age, gender, prior colonoscopy, polyp history, history of colorectal cancer, and family history of colorectal cancer, none of these predicted desire to have a video recording in univariate analyses. Multivariate analysis confirmed that none of the factors was associated with an interest in obtaining a video recording (Table 1).

After a brief educational paragraph regarding polyp miss rates, interest in obtaining a video recording of colonoscopy was reassessed. One hundred thirty-five patients (54%) were more interested in video recording and none were less interested.

The most common reason for interest in having a video recording was "review" (68 patients, 27%), followed by "better records" (55 patients, 22%) and "better information" (43 patients, 17%) (Table 2). The most common reason for lack of interest in obtaining video recording was "no benefit over pictures" (Table 2).

There were 72 males (68%) and 84 females (60%) who stated they would be willing to pay for a video recording of their colonoscopy. A family history of colorectal cancer (odds ratio 2.09; 1.07-4.09) and younger age (odds ratio 1.39; 1.12-1.72 for each 10 year interval of decreasing

**Table 2** The reasons for interest or lack interest in having a videorecording *n* (%)

Reasons for interest in videorecording	
Review	68 (27)
Better records	55 (22)
Better information (more data, better image, better understanding)	43 (17)
Comparison	37 (15)
Follow-up (includes baseline information, follow changes, reference for next procedure)	24 (10)
Research/educational tool	16 (6)
Interesting	11 (4)
Second opinion	9 (4)
Medical-legal	1 (< 1)
Can take videorecording along if relocating	2 (1)
Reasons for lack of interest in videorecording	
No benefit over pictures, no benefit, not useful	32 (13)
Don't see need, trust physician expertise	7 (3)
No opinion	3 (1)

**Table 3** Predictors of stated willingness to pay for a video recording (univariate analysis)

		Willing to pay	Not willing to pay	<i>P</i>	Odds ratio (95% CI)
Gender	F	72 (68) <sup>1</sup>	34 (32) <sup>1</sup>	0.18	1.44 (0.85, 2.44)
	M	84 (60)	57 (40)		
DVD player	Yes	152 (64)	86 (36)	0.25	2.21 (0.58, 8.45)
	No	4 (44)	5 (56)		
Prior colonoscopy	Yes	116 (63)	67 (37)	0.96	1.01 (0.56, 1.82)
	No	41 (63)	24 (37)		
History of polyps	Yes	72 (59)	51 (41)	0.12	0.66 (0.4, 1.12)
	No	85 (68)	40 (32)		
History of CRC	Yes	9 (90)	1 (10)	0.11	5.47 (0.68, 43.92)
	No	148 (62)	90 (38)		
FH of CRC	Yes	43 (75)	14 (25)	0.03	2.09 (1.07, 4.09)
	No	113 (59)	77 (41)		
Age, mean (SD)		55.9 (12.7)	61.4 (13.9)	0.002	1.39 (1.12-1.72) <sup>2</sup>

<sup>1</sup>Number of patients (%); <sup>2</sup>Odds ratio for a 10-year decrease in age.

age) predicted willingness to pay for a video recording in univariate analyses (Table 3). In a multivariable analysis using a backward elimination procedure to remove non-significant factors, only younger age remained in the model, with a *P* value of 0.002.

With regard to the amount patients were willing to pay, univariate analyses showed that a prior history of colorectal cancer was the only predictor of willingness to pay more (mean \$354 *vs* \$65, *P* = 0.001), if only those willing to pay were considered (Table 4). If patients who were unwilling to pay for a video recording were assigned an amount of zero dollars, so that all patients interested in having a video recording were included, a prior history of colorectal cancer was the only predictor of the amount patients were willing to pay (\$319 *vs* \$40, *P* = 0.0001), though female gender approached significance (\$76 *vs* \$33, *P* = 0.06) (Table 4). In multivariable analysis, prior colorectal cancer (*P* = 0.0003) and a family history of colorectal cancer (*P* = 0.02) were both predictive of a higher amounts patients

**Table 4** Amounts patients were willing to pay for a video recording (univariate analysis)

		Amount willing to pay, considering only those willing to pay		Amount willing to pay, considering all patients	
		mean (\$) (SD)	<i>P</i>	mean (\$) (SD)	<i>P</i>
Gender	F	112.2 (288.7)	0.11	76.2 (243.2)	0.06
	M	55.4 (128.2)		32.9 (102.2)	
DVD player	Yes	83.4 (221.8)	0.63	53.1 (181.3)	0.51
	No	30 (13.5)		13.3 (17.9)	
Prior colonoscopy	Yes	89.2 (249.4)	0.46	56.5 (202.8)	0.46
	No	59.6 (74.6)		37.3 (65.5)	
History of polyps	Yes	90.3 (270.1)	0.65	52.6 (210.3)	0.93
	No	74.3 (164.6)		50.5 (139.9)	
History of CRC	Yes	353.9 (634.6)	0.001	318.5 (608.7)	0.0001
	No	64.9 (154)		40.3 (125.2)	
FH of CRC	Yes	92.3 (177.1)	0.72	69.6 (158.5)	0.39
	No	78.2 (233.7)		46.3 (183.7)	
Age	Correlation		<i>P</i>	Correlation	<i>P</i>
		0.07	0.40	0.01	0.92

EGC: EGD and colonoscopy.

were willing to pay, considering only those willing to pay. When all subjects were included, prior colorectal cancer (*P* = 0.0002) and a family history of colorectal cancer (*P* = 0.001) remained predictive of amount willing to pay for a video recording.

## DISCUSSION

In this study, we report interest among outpatients presenting to Indiana University Hospital and an ambulatory surgery center operated by our gastroenterology group, in obtaining a video recording of their colonoscopy. In our study, of the 248 patient surveys included, the majority (81%) were interested in having a video recording.

Sixty-three percent of patients said they were willing to pay for a video recording of their colonoscopy. Payment by patients could offset the costs of video recording. We found no significant predictors of desire to have a video recording, but a family history of colorectal cancer and younger patients' age predicted willingness to pay for a video recording.

Following the educational paragraph on missed lesions during colonoscopy, 54% of patients showed an increased interest in a video recording. Thus, increasing awareness of imperfect detection by colonoscopy increases interest in video recordings.

While video recordings can differentiate the quality of an individual colonoscopist's examination technique and time<sup>[13,14]</sup>, they are seldom obtained routinely during clinical practice. The impact of simply performing video recording during colonoscopy on quality and medical-legal risk is unknown.

A primary limitation of the study is that we did not actually test willingness to pay for video recordings by offering them for sale. Anecdotally, video recordings



of colonoscopies are rarely made available to patients routinely in the United States, either with or without payment by patients. Our gastroenterology group has not yet decided whether to pursue sale of video recordings as routine practice or to make systematic video recordings for inclusion in medical records. We found that younger age predicted stated willingness to pay for a video recording, and prior colorectal cancer and a family history of colorectal cancer predicted willingness to pay greater amounts.

In summary, a survey of 248 patients undergoing colonoscopy was conducted. The majority expressed interest in obtaining a video recording of their procedure. Awareness of missed lesions during colonoscopy increased patient interest in having a video recording. While there were no predictors of interest in having a video recording, younger patients were more willing to pay for a video recording. Prior colorectal cancer and family history of colorectal cancer predicted willingness to pay more for a video recording. We conclude that patient interest in having a video recording of their colonoscopy is substantial, and that awareness of missed lesions during colonoscopy increases interest in having a video recording. Payment by patients for video recordings is a potential mechanism of offsetting the cost of making video recordings.

## COMMENTS

### Background

Colonoscopy is operator-dependent and substantial numbers of pre-cancerous polyps are missed during colonoscopy. Colonoscopies are often poorly documented, with only a few still photographs taken of anatomic landmarks and abnormal findings.

### Research frontiers

Video recording is rarely used in colonoscopy except for teaching purposes; therefore, the potential impact of systematic video recording on the quality of colonoscopy is unknown. In this study the authors sought to understand patient interest in obtaining video recordings of their colonoscopies, and their willingness to pay for video recordings.

### Innovations and breakthroughs

No previous investigation of this issue has been made. Therefore the whole field of video recording of colonoscopies with regard to its effect on quality and cost, and medical-legal implications, is in its infancy.

### Applications

These results indicate that 81% of patients have interest in obtaining video recordings of their colonoscopy, and for a variety of reasons. Information regarding missed lesions during colonoscopy increases interest. There is some willingness to pay for the video recording, which could in the future help to cover the costs of systematic video recording.

### Terminology

Presently, colonoscopy is typically documented by still photography. Video recording in this paper refers to recording the entire colonoscopy or the withdrawal portion of the examination, and preserving the recording as a permanent record. Systematic digital recording of colonoscopy is becoming increasingly feasible.

### Peer review

The authors conducted a survey of outpatients presenting for colonoscopy of

their interest in obtaining a video recording of their colonoscopy. In my opinion, this paper is interesting.

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S- Editor Tian L L- Editor Stewart GJ E- Editor Ma WH

## Gender influence on defecographic abnormalities in patients with posterior pelvic floor disorders

Céline Savoye-Collet, Guillaume Savoye, Edith Koning, Anne-Marie Leroi, Jean-Nicolas Dacher

Céline Savoye-Collet, Edith Koning, Jean-Nicolas Dacher, Radiology Department, QUANT-IF LITIS EA 4108, Rouen University Hospital Charles Nicolle, 1 rue de Germont, F-76031 Rouen, France

Guillaume Savoye, Gastroenterology Department, ADEN EA 4311, Rouen University Hospital Charles Nicolle, 1 rue de Germont, F-76031 Rouen, France

Anne-Marie Leroi, Physiology Department, ADEN EA 4311, Rouen University Hospital Charles Nicolle, 1 rue de Germont, F-76031 Rouen, France

**Author contributions:** Savoye-Collet C designed the research, collected and analyzed the data; Savoye-Collet C and Koning E performed the procedures; Savoye-Collet C and Savoye G edited the paper; All authors cooperated in the final version of the manuscript.

**Correspondence to:** Céline Savoye-Collet, MD, Radiology Department, Rouen University Hospital Charles Nicolle, 1 rue de Germont, F-76031 Rouen, France. [savoyecel@hotmail.com](mailto:savoyecel@hotmail.com)  
Telephone: +33-2-32886496 Fax: +33-2-32888235

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### Abstract

**AIM:** To compare defecographic abnormalities in symptomatic men and women and to analyze differences between men and age- and symptom-matched women.

**METHODS:** Sixty-six men (mean age: 55.4 years, range: 20-81 years) who complained of constipation and/or fecal incontinence and/or pelvic pain underwent defecography after intake of a barium meal. Radiographs were analyzed for the diagnosis of rectocele, enterocele, intussusception and perineal descent. They were compared with age- and symptom-matched women ( $n = 198$ ) who underwent defecography during the same period.

**RESULTS:** Normal defecography was observed in 22.7% of men vs 5.5% of women ( $P < 0.001$ ). Defecography in men compared with women showed 4.5%

vs 44.4% ( $P < 0.001$ ) rectocele, and 10.6% vs 29.8% ( $P < 0.001$ ) enterocele, respectively. No difference was observed for the diagnosis of intussusception (57.6% vs 44.9%). Perineal descent at rest was more frequent in women ( $P < 0.005$ ).

**CONCLUSION:** For the same complaint, diagnosis of defecographic abnormalities was different in men than in women: rectocele, enterocele and perineal descent at rest were observed less frequently in men than in women.

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**Key words:** Fecal incontinence; Defecography; Rectocele; Hernia; Pelvic floor; Constipation

**Peer reviewer:** Reiji Sugita, MD, Department of Radiology, Sendai City Medical Center, 5-22-1, Tsurugaya, Miyagino-ku, Sendai 983-0824, Japan

Savoye-Collet C, Savoye G, Koning E, Leroi AM, Dacher JN. Gender influence on defecographic abnormalities in patients with posterior pelvic floor disorders. *World J Gastroenterol* 2010; 16(4): 462-466 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v16/i4/462.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i4.462>

### INTRODUCTION

Constipation, fecal incontinence and pelvic pain require meticulous evaluation because therapy can be effective and help patients to lead a fuller life. Radiographic dynamic rectal examination (defecography) is a valuable method to assess evacuation disorders<sup>[1-5]</sup>. This method provides precise information on anorectal and pelvic floor functions<sup>[6-8]</sup>. It can be used to evaluate efficiently defecation disorders after clinical examination for the exploration of constipation, pelvic pain and anal incontinence<sup>[9-13]</sup>.

Posterior pelvic floor disorders are common and well known in women, and some are related to obstetrical consequences. Little is known about gender influence in symptomatic patients investigated by defecography<sup>[14,15]</sup>. Men can also complain of constipation, fecal incontinence and pelvic pain, and these symptoms need the same careful evaluation because they may need specific therapy<sup>[16]</sup>.

The purpose of our study was to compare prevalence of defecographic abnormalities in men and age- and symptom-matched women.

## MATERIALS AND METHODS

### Patients

Over a 6-year period, 66 symptomatic aged 20-81 years (mean  $\pm$  SD: 55.4  $\pm$  14.9 years) addressed for defecography were evaluated consecutively in our institution. All patients were referred by a gastroenterologist or a digestive tract surgeon for the exploration of constipation, pelvic pain or fecal incontinence. Constipation was defined as less than two bowel movements per week. Fecal incontinence was defined by an uncontrolled loss of liquid or solid stools that corresponded to grade C and D of Park's classification<sup>[17]</sup>.

To compare defecographic abnormalities in men with women, a case-matched series was isolated from a prospective database of women ( $n = 700$ ) investigated by defecography in the same period with the same techniques. Three women for one man were isolated. A series of 198 age- and symptom-matched women was studied. A value of 0 (absence) or 1 (presence) was attributed to each of the three types of symptoms (constipation, anal incontinence or pelvic pain) for all the patients in the database. Several age groups (5-years periods) were isolated for both genders. In these groups of similar age, three women with the same combination of symptoms were associated with one man. The choice of the symptom-matched women was done blindly of the results of the defecography from a global database that contained all the symptomatic patients who underwent defecography during this period in our unit. For example, for a 63-year-old man with constipation and pelvic pain (values = 1/0/1), three women (aged 60-65 years) with the same combination (1/0/1) were isolated. The women were aged 20-81 years (mean: 55.1  $\pm$  14 years).

### Defecography

We applied a standardized protocol to perform and evaluate all defecography examinations. All patients received a barium meal 1.5 h before the examination to opacify the pelvic small bowel for the detection of enterocele. A lateral X-ray was first performed for bone and pelvic loop visualization. In women, a thick barium paste was injected into the vagina, to mark out the posterior vaginal wall. Then, 150 mL of thickened and viscous high-density barium contrast medium was injected in the rectum with the patient in the left lateral position.

Films were taken in a standing lateral position during the following maneuvers: at rest, at voluntary and maximal contraction of the sphincter and pelvic floor ("squeeze"), and at straining without defecation ("strain"). The pubococcygeal line was defined and the distance between this line and the anorectal junction was determined for the three positions. Finally, patients sat on an upright commode attached to the footboard of the fluoroscopy table (a modified toilet), and one frame per second films were taken during expulsion and after completion of defecation at maximum straining.

Precise explanations of the entire procedure were given by the radiologist prior to defecography. In young women, defecography was always performed during the first part of the cycle. Image analysis was done by one of the senior radiologists involved in this study (CSC or EK).

Pathological patterns were defined as follows. Pelvic floor descent was assessed from standing lateral views during maneuvers. For the diagnosis of perineal descent at rest, the distance in millimeters between the anorectal junction and the pubococcygeal line was noted. Perineal descent at rest was defined as a distance of  $> 30$  mm. For perineal descent at straining, the difference in millimeters between the anorectal junction position at straining and at rest was noted. Perineal descent at straining was defined as a difference of  $> 20$  mm between the two positions. The number of male and female patients with perineal descent at rest was compared. Anterior rectocele was defined as  $> 30$  mm outpouching of the anterior rectal wall. This outpouching should persist on incomplete evacuation. Intussusception was defined as an invagination of the rectal wall, either intrarectal, intra-anal, or an external prolapse of the whole circumference. Enterocele was defined as herniation of the small bowel between the vaginal posterior wall and the anterior rectal wall.

### Statistical analysis

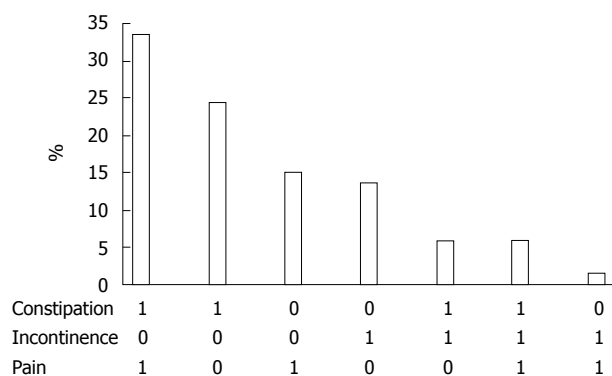
$\chi^2$  tests were used for the comparison of rectocele, enterocele, perineal descent and intussusception.  $P < 0.05$  was regarded as significant.

## RESULTS

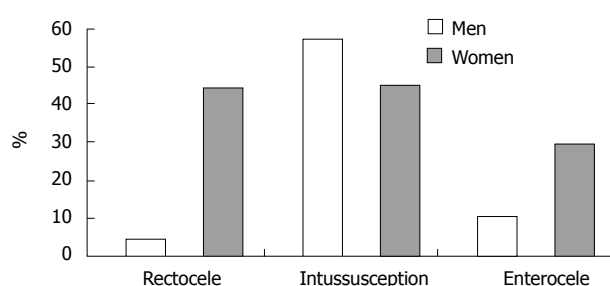
Constipation, fecal incontinence and pelvic pain were present in 69%, 27% and 56% of our male patients, respectively. These symptoms could be isolated (53%) or associated (Figure 1). The more frequent association was constipation and pelvic pain (33%).

Defecography could be satisfactorily performed in all patients and all examinations could be analyzed fully with the above-described criteria. In men, 22.7% of the defecography procedures were normal versus 5.5% in women ( $P < 0.001$ ).

Defecography in men showed intussusception in 38 patients (57.6%), enterocele in seven (10.6%), and rectocele in three (4.5%). Defecography in women showed intussusception in 89 patients (44.9%), enterocele



**Figure 1** Defecography in men: distribution with regard to the combination of symptoms (constipation and/or incontinence and/or pain). 0: Absence; 1: Presence.



**Figure 2** Results of defecography with regard to rectocele, intussusception and enterocoele in men and women. Rectocele:  $P < 0.001$ , Enterocoele:  $P < 0.005$ , Intussusception: NS.

in 59 (29.8%), and rectocele in 88 (44.4%). There was a significantly higher proportion of rectocele and enterocoele in matched women (Figures 2 and 3). There was no statistically significant difference for the diagnosis of intussusception.

Distance between the pubococcygeal line and the anorectal junction at the three positions (rest, squeeze and strain) was significantly different between men and women, with a lower position of the perineum in women regardless of position ( $P < 0.001$ ) (Figure 4). There was a greater incidence of perineal descent at rest in women compared with men ( $P < 0.005$ ) (Figure 5). There was no difference between men and women for the diagnosis of perineal descent at straining.

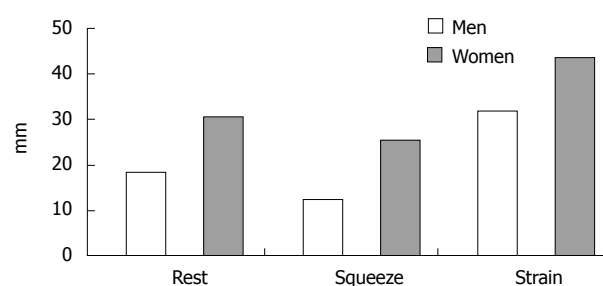
## DISCUSSION

Defecography is used commonly in women, but only a few procedures are carried out in men. In our department, men represented  $< 10\%$  of all defecography procedures. To the best of our knowledge, no specific study of defecography has been carried out to explore the gender difference in the diagnosis of defecographic abnormalities. The pairing of our case-matched study was done for age and symptoms, with one man for three matched women, as is usual in this type of study. It allowed us to establish any sex difference in the prevalence of defecographic abnormalities.

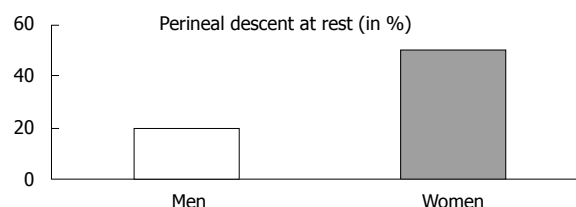
Intussusception is very common in both sexes



**Figure 3** Dynamic imaging during evacuation showed an enterocele (black arrow) associated with a rectocele (white arrow).



**Figure 4** Distance between the pubococcygeal line and the anorectal junction at rest, squeeze and strain in men and women.  $P < 0.001$ .



**Figure 5** Percentage of men and women with perineal descent at rest.  $P < 0.005$ .

(approximately half of patients undergoing defecography)<sup>[7,8]</sup>. We did not observe any sex difference in prevalence. Enterocoele and rectocele were more common in matched women. This could be explained by obstetrical anteriority (vaginal delivery or hysterectomy). The consequences of obstetric damage on the perineum are well known<sup>[18,19]</sup>. Enterocoele is also more frequent after hysterectomy<sup>[20]</sup>. Rectocele is diagnosed 10 times more frequently in women, and appears to be characteristic of the female population. Rectocele has been reported previously as being uncommon in men<sup>[15]</sup>. Chen *et al*<sup>[15]</sup> have reported a prevalence higher than ours (17% *vs* 4.4%), and a frequent association between rectocele and prostatectomy (40%). We did not assess the history of all patients, but this difference could be explained by the higher mean age of the patients in the study of Chen *et al*<sup>[15]</sup> (72.4 years) than in our study (55.4 years), because prostatectomy is more likely in older patients.

The distance between the pubococcygeal line and the



anorectal junction, which determines the position of the perineum, was greater in women for the three positions (rest, squeeze and strain). As a consequence, there was a gender difference for the diagnosis of perineal descent at rest. There was no such difference for perineal descent at straining. This raises the question that the normal level of the perineum could be different in both sexes. However, our study concerned only symptomatic patients and this needs to be investigated in control subjects. No sex-related definition of pelvic floor disorders was established, therefore, our diagnostic criteria were those commonly used, which means those used in women. However, new criteria for the diagnosis of perineal descent at rest in men could probably be defined. The same question can also be raised in elderly patients, for whom diagnostic criteria could also be adapted<sup>[21]</sup>.

Determination of the clinical value of defecographic abnormalities is also difficult and remains a subject of debate<sup>[22-26]</sup>. Defecographic abnormalities have been shown not to be symptom-related<sup>[12]</sup>. This study confirmed this finding, because there were gender differences among symptom-matched patients. Interpretation should be cautious because radiographic changes can be demonstrated in healthy subjects of various ages<sup>[27,28]</sup>. Interobserver agreement is good for rectocele and enterocele<sup>[29]</sup> but appears insufficient for perineal descent<sup>[25]</sup>. Different methods for measuring parameters, especially perineal descent, have been described, with controversy as to which is the most accurate, and with little agreement<sup>[26,30,31]</sup>. New examinations are also possible, such as magnetic resonance defecography, to assess pelvic floor disorders<sup>[32,33]</sup>.

In conclusion, defecography is a potential adjunct to clinical evaluation of men with constipation, fecal incontinence or pelvic pain. With symptoms equal, some defecographic abnormalities are sex-related: rectocele, enterocele and perineal descent at rest were observed less frequently in men.

## COMMENTS

### Background

Posterior pelvic floor disorders are caused by changes in the musculo-aponeurotic support of the pelvic floor. Defecography (radiographic dynamic rectal examination) provides precise information on anorectal and pelvic floor functions for patients with constipation, fecal incontinence and pelvic pain. These symptoms are more frequent in women, and some are related to obstetrical consequences. Little is known about the influence of gender in patients with pelvic floor disorders.

### Research frontiers

Radiological exploration of pelvic floor disorders is important to elucidate the pathophysiology and assist in deciding the therapeutic strategy. It is difficult to attribute a radiological abnormality as the direct cause of the symptoms. A better knowledge of gender difference in the prevalence of defecographic abnormalities appears to be useful.

### Innovations and breakthroughs

To the best of the authors' knowledge, no specific study of defecography has been carried out to explore the gender difference in the diagnosis of defecographic abnormalities. The strength of this study was that it assessed gender in the diagnosis of defecographic abnormalities.

### Applications

For the same complaint, defecographic abnormalities are different in men than

in women: rectocele, enterocele and perineal descent at rest were less frequent in men than in women. Discussion of sex differences in pelvic floor disorders could probably help to assess more precisely these nosological entities. New examinations are also possible, such as magnetic resonance defecography.

### Terminology

There are several defecographic abnormalities. Rectocele is an outpouching of the anterior rectal wall. Enterocele is a herniation of the small bowel between the vagina and rectum. Perineal descent is an abnormal position of the perineum under the pubococcygeal line.

### Peer review

The results of this study are interesting, although the method of this study (defecography) is relatively old and slightly controversial. The results of this study raise issue concerning gender differences in pelvic floor disorders, and may bring a new dimension.

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## Impact of endoscopy-based research on quality of life in healthy volunteers

Alexander Link, Gerhard Treiber, Brigitte Peters, Thomas Wex, Peter Malfertheiner

Alexander Link, Gerhard Treiber, Thomas Wex, Peter Malfertheiner, Department of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University, Leipziger St. 44, 39120 Magdeburg, Germany

Alexander Link, Department of Internal Medicine, Division of Gastroenterology, Baylor Research Institute, Baylor University Medical Center, 3500 Gaston Ave (H-250) Dallas, TX 75246, United States

Gerhard Treiber, Department of Gastroenterology, Oncology and General Internal Medicine, Zollernalb Clinic, Tuebingen St. 30, 72336 Balingen, Germany

Brigitte Peters, Institute for Biometry and Medical Informatics, Otto-von-Guericke University, Leipziger St. 44, 39120 Magdeburg, Germany

**Author contributions:** Link A and Treiber G contributed equally to this work; Link A, Treiber G, Wex T and Malfertheiner P designed the study; Treiber G performed endoscopic examinations; Link A and Peters B analyzed the data; Link A, Treiber G, Wex T and Malfertheiner P wrote the paper.

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**Correspondence to:** Dr. Peter Malfertheiner, Professor, Department of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University, Leipziger St. 44, 39120 Magdeburg, Germany. [peter.malfertheiner@med.ovgu.de](mailto:peter.malfertheiner@med.ovgu.de)

Telephone: +49-391-6713100 Fax: +49-391-6713105

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performed according to short- and long-term changes and compared to the control group.

**RESULTS:** All HV completed the study with duration of more than 6 mo. Initial quality of life score was comparable to a general population. Analyses of the SF-36 questionnaires showed no significant changes in physical, mental and total scores, either in a short-term perspective due to different medications, or to potentially endoscopic procedure-associated long-term cumulative changes. Analogous to SF-36, VAS revealed no significant changes in total scores for pathological abdominal symptoms and remained unchanged over the time course and when compared to the control population.

**CONCLUSION:** This study demonstrates that quality of life in HV is not significantly affected by a long-term endoscopy-based study with multiple endoscopic procedures.

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**Key words:** Endoscopy research; Ethics; Healthy volunteers; Quality of life

**Peer reviewers:** Ian C Roberts-Thomson, Professor, Department of Gastroenterology and Hepatology, The Queen Elizabeth Hospital, 28 Woodville Road, Woodville South 5011, Australia; William Dickey, Professor, Altnagelvin Hospital, Londonderry, BT47 6SB, Northern Ireland, United Kingdom

Link A, Treiber G, Peters B, Wex T, Malfertheiner P. Impact of endoscopy-based research on quality of life in healthy volunteers. *World J Gastroenterol* 2010; 16(4): 467-473 Available from: <http://www.wjgnet.com/1007-9327/full/v16/i4/467.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i4.467>

### Abstract

**AIM:** To study the impact of an endoscopy-based long-term study on the quality of life in healthy volunteers (HV).

**METHODS:** Ten HV were included into a long-term prospective endoscopy-based placebo-controlled trial with 15 endoscopic examinations per person in 5 different drug phases. Participants completed short form-36 (SF-36) and visual analog scale-based questionnaires (VAS) for different abdominal symptoms at days 0, 7 and 14 of each drug phase. Analyses were

### INTRODUCTION

Endoscopy is the main diagnostic tool for examination of the upper gastrointestinal tract. The main advantage

of endoscopy in comparison to other non-invasive procedures lies in its ability to obtain tissue biopsies, which permit histological evaluation of tissues for clinical as well as basic research<sup>[1]</sup>.

The majority of endoscopy-based pharmacological studies are performed in patients with or without a specific disease. Investigations surrounding this focused patient population yields answers for only a limited number of questions. Studies designed around healthy volunteers (HV) may resolve some of those issues (e.g. by reducing inter-individual differences); although at the same time may open a new series of questions. One of the main differences between patients and HV is hidden in the dilemma “treat the disease” in patients and “do no harm” in HV<sup>[2,3]</sup>. “Harm” can arise from a number of sources such as the study-related treatment, the endoscopy procedure itself, and/or other related discomfort (e.g. multiple blood samples, restriction of daily activities).

Upper GI-endoscopy is a relatively well-accepted procedure. The magnitude of discomfort to the examined person depends upon several factors such as previous contact with the examiner, appropriate elucidation of the procedure, sedation, investigator's experience, duration of examination and the expected benefit from the procedure<sup>[4]</sup>. Because of the potential therapeutic benefit, it is easier to justify the examination of the patients. Since in most cases, HV can not expect any health-related advantages from the procedure, the involvement of HV in endoscopy-based research is a matter of ethical concern<sup>[5]</sup>.

Updated recommendations on ethics in gastrointestinal endoscopy-based research were discussed and summarized in the recent Workshop of the European Society of Gastrointestinal Endoscopy (ESGE)<sup>[6]</sup>. The main ethical considerations were as follows: first, there must be a research issue that can not be adequately addressed by the involvement of patients; and second, the inherent risk for HV is regarded to be “acceptable”. However the term “acceptable” is not well defined and it is not uncommon that different scientific and ethical committees make different conclusions concerning endoscopy-based studies. Objective methods for ethical evaluation could potentially be helpful in narrowing such differences in opinions. Targeting the quality of life (QOL) in volunteers could potentially contribute to objectivity in ethical questions, however, there are no data regarding this issue.

For this reason we aimed to evaluate the QOL in HV during endoscopy-based research. To answer these questions, we used the previously well-validated short-form 36 (SF-36) and visual analog scale-based questionnaires (VAS). In this pilot study, we demonstrate that an endoscopy-based drug trial has no significant influence on the QOL in healthy participants even under rigorous conditions and a long-term protocol.

## MATERIALS AND METHODS

### Ethical approval and volunteer recruitment

The study design was approved by the local ethics committee of Magdeburg University Hospital and by

government authorities. HV, as defined below, were recruited for the study in accordance with the Declaration of Helsinki principles and recommendations of the ESGE-workshop on the ethics of gastrointestinal endoscopy-based research<sup>[6]</sup>. The recruitment of HV was performed through the distribution of brochures or information posters avoiding affiliation or dependency of HV to the investigators. The purpose, risks of the procedure and the drug-related side effects were fully explained to participants and included in the written informed consent (e.g. risk of bleeding due to platelet inhibitory drugs). HV were informed about the opportunity to revoke study consent and cancel participation at any time. At least 24 h consideration time was provided to the HV before all participants provided written informed consent. Due to the extensive study protocol, all HV were financially reimbursed for their time, effort and contribution to the research. Financial compensation was equal in all HV and was modestly calculated based on generally used factors including compensation for time (or possible income shortfall due to participation in the study), risk, discomfort and inconvenience.

### HV

Ten HV [8 men and 2 women, age 27.8 years (22-37) and body mass index 24.7 kg/m<sup>2</sup> (22.3-27.2)] were included in the study based on previously described inclusion/exclusion criteria<sup>[7]</sup>. Briefly, HV had no abnormality at physical examination or during routine laboratory examination, no history of relevant illnesses especially history of peptic ulceration, no pregnancy in women and were negative for *H. pylori* infection.

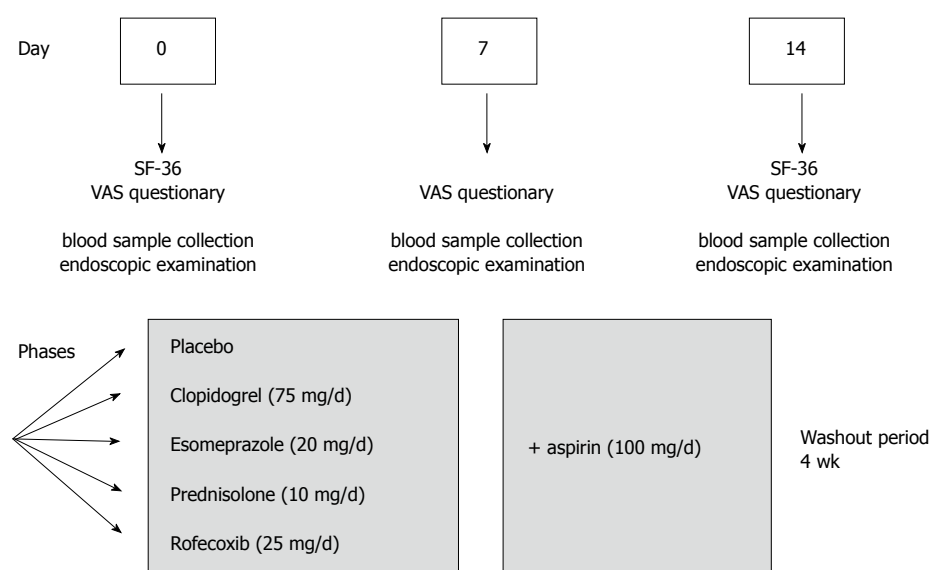
### Study design

The study was conducted as a prospective double-blind, placebo-controlled study with cross-over design. The main aim of the trial was to study the influence of several frequently used drugs on the healing of gastroduodenal lesions (preliminary clinical results were reported elsewhere)<sup>[8]</sup>. In addition, we aimed to evaluate the impact of the endoscopy-based trial on QOL in HV. All volunteers were asked to fill out the questionnaires on the day of each endoscopy, describing the subjective symptoms and QOL. Rofecoxib, clopidogrel, prednisolone, esomeprazole or placebo were given alone and/or together with acetylsalicylic acid (ASA). The endoscopic procedures were performed on days 0, 7 and 14 (Figure 1). The volunteers received one of the test drugs for one week and the same drug together with ASA for another week. Total duration of the study was over 6 mo including a 4-wk washout period between 5 different drug phases.

### Endoscopy

After overnight fasting, the endoscopic examination was performed by an experienced endoscopist (G.T.) using a standard endoscope (GIF 145, Olympus, Germany). All HV received topical lidocaine 2% for local anesthesia and 20 mg of butylscopolaminiumbromid intravenously





**Figure 1** Flow chart of the study. The multiple drug study with cross-over design was divided into 5 phases with test drug alone and in combination with low-dose aspirin during the second week. On days 0, 7 and 14 of each study, ten HV were asked to complete the questionnaires. Upper GI-endoscopy was performed on the same days with a 4-wk wash out period between the different phases.

(iv) to inhibit gastric and small bowel propagation. Additionally, midazolam 5 mg was given iv if requested by the participants. During the procedure, patients were monitored for heart rate and oxygen saturation. After a systematic examination of the stomach and duodenum, multiple biopsies (10 per region, a total of 30 per endoscopy) were taken using standard forceps (5 mm maximum open diameter) from the distal duodenal bulb, antrum and corpus.

#### Questionnaires: Visual analog scale and SF-36

To determine the QOL in HV, we used a validated German version of the SF-36 (short form) questionnaire which includes an 8-scale profile: physical functioning, role physical, bodily pain, general health, vitality, social functioning, emotional role and mental health. These scales further represent two distinct higher-ordered clusters due to physical and mental health variance. A lower score in SF-36 indicates a greater impairment and can range from 0 (worst health) to 100 (best health). To determine the abdominal symptoms of HV, we used a previously validated visual analog scale (VAS) based questionnaire<sup>[9]</sup> with the following dimensions of symptoms: abdominal pain, bloating, reflux, nausea, vomiting, diarrhea and loss of appetite. The summary symptom score (0-70) was calculated by adding up the values of the single scores. A single score value from 1-10 was defined as presence, and 0-1 as the absence of any symptoms. As shown in Figure 1, VAS questionnaires were filled out on days 0, 7 and 14 and the SF-36 questionnaires were filled out before and after each drug phase. The score parameters of HV were compared to a previously evaluated control population of 28 patients, who underwent a single endoscopic examination and in the absence of acid suppressive drugs no visible macroscopic or histological abnormalities were found<sup>[9]</sup>.

#### Statistical analysis

All data were analyzed using the SPSS 10 (Chicago, IL, USA) and Graph Pad Prism 4.0 (San Diego, CA, USA).

The differences between the groups were analyzed according to corresponding formula and rules, using where appropriate paired or unpaired *t*-test and Friedman's test (FT) for non-normal distributed data. For all comparisons, a *P*-value (two-sided) of < 0.05 was regarded as significant.

## RESULTS

#### Study participation

All 10 HV completed all 5 phases of the study with 15 endoscopies per HV. With the exception of 2 individuals who either experienced a prolonged bleeding after biopsies had been taken or melena (due to the combination of ASA and clopidogrel) without development of anemia, there were no further drug- or endoscopy-related complications. The VAS and SF-36 questionnaires were completed and returned in 99.5% and in 88% of all cases/time points, respectively. In 91% of endoscopies, HV declined to receive midazolam for sedation and received only topical anesthesia with lidocaine 2%.

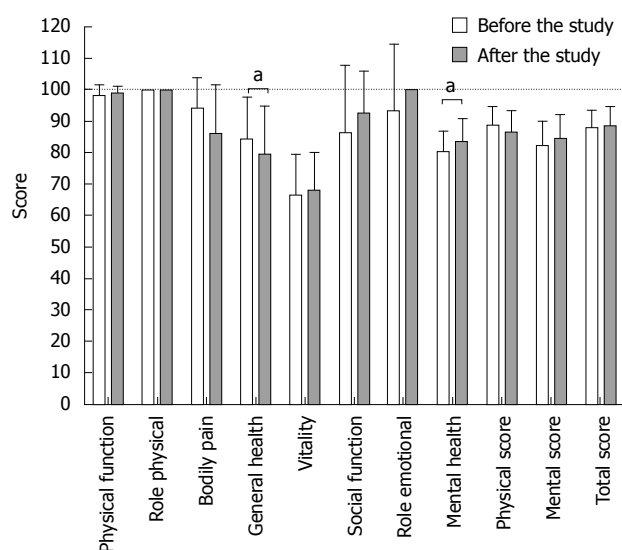
#### Analyses of the SF-36 questionnaires

SF-36 questionnaires were analyzed in regard to different aspects, such as influence of study drugs, different time points, as well as long term changes in QOL. The physical, mental and total scores, determined by the short form SF-36 questionnaire ranged between 65.6 to 97.0, 65.3 to 96.0 and 75.6 to 97.1, respectively. We found no significant changes in physical, mental or total scores either between different medications or due to participation in each drug phase (data not shown). Overall changes in physical, mental and total scores are shown in Table 1. For the long-term impact of the intensive endoscopy-based trial on the QOL in HV, the SF-36 assessed data were compared between baseline (day 0 during the first phase) and the last time point of the last phase more than 6 mo later. Remarkably, physical ( $88.4 \pm 6.0$  vs  $86.4 \pm 6.7$ ), mental ( $82.1 \pm 8.1$  vs  $84.6 \pm 7.4$ ) and total scores ( $88.0 \pm 5.7$  vs  $88.6 \pm 6.1$ ) showed no significant changes over the 6-mo period (Figure 2).

**Table 1** Changes in scores on the SF-36 questionnaires in HV according to different test drugs before and after endoscopic procedures

Test drug day	Physical score		<i>P</i>	Mental score		<i>P</i>	Total score		<i>P</i>
	0	14		0	14		0	14	
Placebo	88.7 ± 5.9	88.2 ± 4.1	NS	85.7 ± 8.5	86.0 ± 7.8	NS	89.9 ± 6.2	89.8 ± 4.8	NS
Esomeprazole	85.7 ± 7.7	84.1 ± 6.0	NS	83.3 ± 6.2	83.0 ± 6.0	NS	86.2 ± 7.2	87.4 ± 4.8	NS
Clopidogrel	86.6 ± 6.5	85.5 ± 6.7	NS	84.0 ± 7.3	83.8 ± 8.2	NS	88.1 ± 6.1	87.0 ± 6.3	NS
Prednisolone	85.1 ± 5.2	82.2 ± 9.6	NS	81.9 ± 7.0	82.6 ± 6.4	NS	87.0 ± 5.4	85.7 ± 6.7	NS
Rofecoxib	86.2 ± 8.2	86.4 ± 3.1	NS	85.0 ± 6.2	84.5 ± 4.7	NS	88.3 ± 6.5	88.4 ± 3.1	NS
Total Scores	86.4 ± 1.3	85.4 ± 2.0	NS	83.5 ± 1.3	84.2 ± 1.2	NS	87.8 ± 1.2	87.8 ± 1.5	NS

Test drugs were given for one week alone and for an additional week in combination with low-dose aspirin. Four-week washout periods were performed between the different drug phases. Results expressed in mean ± SD, paired *t*-test: *P* > 0.05 listed as NS. HV: Healthy volunteers.

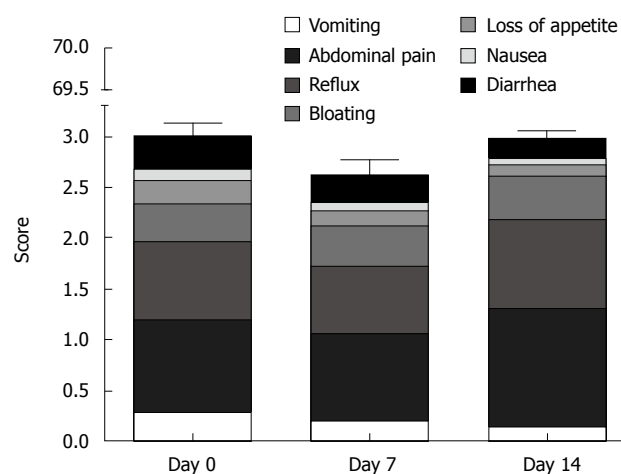


**Figure 2** Changes in different multiple dimensions and physical, mental and total scores between baseline and end of study determined by SF-36. During the 6-mo study with cross-over design, 10 HV underwent a total of 150 endoscopic examinations and 4500 biopsies from the antrum, corpus and duodenal bulb. The results are expressed in mean ± SD. Dotted line shows the theoretical max score from 100. Significant changes were observed (<sup>a</sup>) for general (84.4 ± 13.1 vs 79.4 ± 15.4, *P* = 0.023 paired *t*-test) and mental health (80.0 ± 6.8 vs 83.2 ± 7.5, *P* = 0.022 paired *t*-test). In the remaining dimensions, physical, mental and total scores *P* > 0.05.

Single score analysis revealed that only general health decreased from 84.4 ± 13.1 to 79.4 ± 15.4 (mean ± SD; paired *t*-test *P* = 0.023) and mental health increased from 80.0 ± 6.8 to 83.2 ± 7.5 (*P* = 0.022).

### Analyses of physical symptoms with visual analog scale (VAS) questionnaires

Physical complaints were assessed for different symptoms with VAS and analyzed in a similar manner to the SF-36 questionnaires. As anticipated, subjective physical and emotional perception even at baseline differed from one person to another (high inter-individual variability, data not shown). Almost 60% of all complaints were due to abdominal pain and reflux, but these scores showed no significant changes when compared with baseline (Figure 3). The rarest complaints with < 10% of the maximal score were nausea and loss of appetite. To



**Figure 3** Changes between different symptoms and summary scores due to the endoscopy-based study determined by visual analog scale (VAS) based questionnaires. Upper endoscopies were performed on days 0, 7 and 14 of each phase. During every medication phase, HV underwent 3 upper endoscopies with multiple biopsies from the antrum, corpus and duodenal bulb. Maximal complaint score for any symptom or summary scores are 10 and 70, respectively. The results are expressed as mean ± SE and analyzed using Friedman's test (*P* > 0.05 for physical, mental and total-scores).

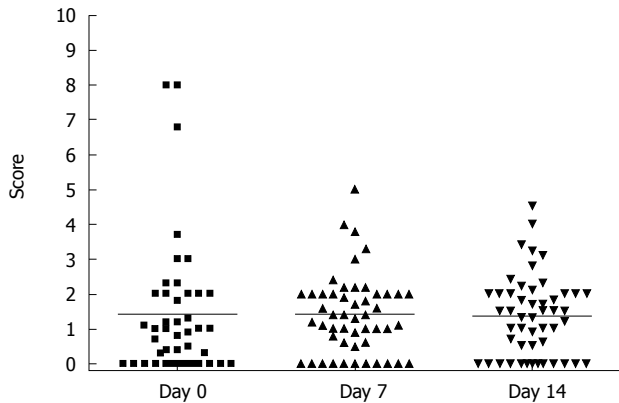
evaluate the global changes we constructed a summary score; values at baseline (3.2 ± 5.1) were comparable to scores at day 7 (2.6 ± 3.7) and day 14 (3.0 ± 3.4) and were less than 5% of the theoretical maximal score.

### Analyses of the impairment of endoscopic examination

To analyze the subjective endoscopy-related impairment in HV, we used VAS which allows the volunteers to score the procedure-oriented discomfort. In analogy to physical complaints VAS, we observed high inter-individual variability with the overall range varying from absence to 80% of maximum possible impairment during the endoscopy. As demonstrated in Figure 4, there was no significant difference between self-assessed scores for complaints about endoscopic procedures (1.4 ± 2.0, 1.4 ± 1.1 and 1.35 ± 1.1 for day 0, 7 and 14, RM ANOVA, *P* > 0.05).

### Difference between HV and controls

The VAS-scores from HV were compared with previously



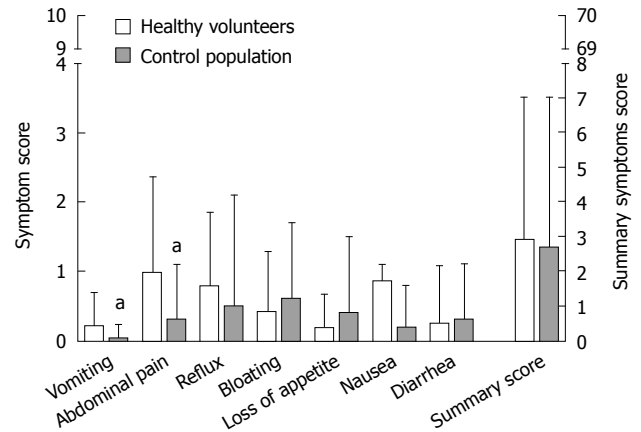
**Figure 4** Changes in compliance scores due to endoscopic procedures between different time points determined by visual analog scale (VAS) based questionnaire. Upper endoscopies were performed on days 0, 7 and 14 of each phase and 30 biopsies were taken during each procedure. Maximal complaint score is 10. The results are presented as scatter plots with means as the lines and were analyzed by ANOVA test ( $P > 0.05$ ).

published VAS-scores from a control population<sup>[9]</sup>. The overall mean for summary symptom scores of HV and controls were comparable (HV  $2.9 \pm 4$  and controls  $2.7 \pm 4.4$ ,  $t$ -test:  $P > 0.05$ ) and was lower than 5% of the theoretically possible value. We found significantly more complaints for abdominal pain ( $0.98 \pm 1.37$  vs  $0.3 \pm 0.8$ ,  $P = 0.012$ ) and vomiting ( $0.22 \pm 0.5$  vs  $0.03 \pm 0.2$ ,  $P = 0.046$ ) in the HV group (Figure 5), but due to the limited number of HV we could not perform age adjustment for the symptoms. The difference between both scores were below the defined cut-off value for the presence or absence of symptoms and were significantly lower than a symptomatic population as previously published<sup>[9]</sup>.

## DISCUSSION

QOL of HV is one of the major ethical concerns in endoscopy-based research. Due to the lack of objective data, the decision to include or not to include HV into a clinical trial is made rather arbitrarily and is mostly based on the individual judgment of clinicians and the opinion of expert committees. Therefore, there is a strong need for prospective data that would help to gain knowledge directly related to HV. To test if the endoscopy-based study impacted the QOL of HV, we prospectively evaluated QOL in HV under rigorous conditions. During the study of multiple potentially GI-harmful medications, HV underwent multiple (150) endoscopic examinations with over 4500 biopsies in 5 independent phases. We demonstrated that by accurate implementation of recommendations and guidelines, even a long-term endoscopy-based study has only a little or no impact on the QOL in HV.

Several different methods to measure QOL, for example Sickness Impact Profile, Medical Outcome Study Short-Form 36 (SF-36), Nottingham Health Profile, Quality of Well Being Scale and other disease-specific scales have been previously validated<sup>[10]</sup>. We



**Figure 5** Different symptoms and summary scores due to the endoscopy-based study compared in HV and the control group determined by visual analog scale (VAS) based questionnaires. Every HV completed 5 medication phases with 15 endoscopic examinations. Control population (data previously published in<sup>[9]</sup>, adopted with permission) underwent only one upper GI endoscopy without endoscopic or histological pathology. Maximal complaint score for any symptom or summary scores are 10 and 70, respectively. The absolute symptom value was below the cut-off for presence of the symptom.

decided to use the SF-36 questionnaire for its brevity and its comprehensiveness. SF-36 is one of the best validated and widely used questionnaires both in clinical practice and research work in gastroenterology<sup>[11]</sup>. To increase the reliability of the study, we also used VAS which was previously validated in dyspeptic patients by our group<sup>[9]</sup>. Both questionnaires are based on self-evaluation which minimizes the investigator-related impact, and further contributes to reliability of the outcome results.

In this pilot study, we found that the endoscopy-based trial was not associated with significant changes in QOL scores. These scores were comparable between different phases and time points as well as when compared to the general population<sup>[12]</sup> and to the control population<sup>[9]</sup>. Based on our results and previous knowledge, several conclusions can be made<sup>[6]</sup>. First, implementation of the current guidelines and recommendation are a reliable fundament for the successful realization of an endoscopy-based study. Second, careful selection of volunteers might be important, especially taking into account the motivation of the HV. Motivation is - at least in part - based on an idealistic character, even if financial compensation is provided. However, especially in developing countries, financial compensation may become the dominant stimulus for participation which may overcome the study-related discomfort<sup>[5,13]</sup>. In our study, inclusion of HV was based on a "first come, first served" principle, however, there is still a chance of creating a bias due to personal motivation, willingness to participate in the study or even financial interest<sup>[5,13]</sup>. In this regard it is important to mention that analogous to other studies, all the HV were reimbursed for their time, effort and contribution to the research, but financial compensation was modest at best. Since personal motivation may vary considerably, testing HV with higher and lower tolerance

to endoscopy-based studies, especially in relation to financial reward, could add interesting and valuable information and should be considered in future studies. Besides motivation, physician/clinician and nursing care (GCP) also plays an important role in subjective cognition of impaired QOL. All endoscopic examinations were performed by an experienced endoscopy team which could have influenced the satisfaction, and thereby tolerance to the procedure<sup>[14]</sup>.

To the best of our knowledge, the only comparable study that has addressed a similar issue was from Adachi *et al.*<sup>[15]</sup>. This group analyzed cardiac stress in HV without sedation, and showed that endoscopic examination of HV without sedation increased cardiac stress (without affecting cardiac output) by 66%<sup>[15]</sup>. Although evaluation of objective physiological parameters may add valuable information, especially if correlated to VAS or even SF-36, the correlation to personal impairment is still unknown. The VAS impairment score in our study was constant throughout the study, with mean impairment of 15% as a maximal value. Although premedication would improve tolerance to endoscopic procedures, premedication with midazolam was only used in 9% of procedures. From another perspective, the “social life” of HV may also have had an impact on the decision to use or not use premedication. Necessary daily activities like study, work or transportation could be significantly influenced by premedication, especially because the duration of this study was 6 mo. Furthermore, it is worth mentioning that none of the HV interrupted the study, and all of them declared that they would re-participate. Some of them did participate in a later endoscopy-based study with 4 endoscopic procedures within 2 h<sup>[7]</sup>.

It was not the aim of our study to clarify whether endoscopy-based research is ethical or not, but primarily to evaluate QOL in HV following the implementation of current recommendations and guidelines. Nevertheless, this information could indirectly contribute to the ethical considerations of endoscopy-based research, if the guidelines and recommendations are acceptable. Therefore, safety standards including experienced endoscopists are a pre-requisite in such studies. Endoscopy-based studies have some risks for complications, which in the worse case can be lethal, even if volunteers are young and healthy<sup>[16]</sup>. However, the risk is negligible if the procedure is carried out with maximum accuracy and according to the recommendations for standards of sedation and monitoring of patients during GI-endoscopy<sup>[3,17,18]</sup>.

In summary, in this pilot study we show for the first time that participation in long-term endoscopy-based trials is not necessarily associated with significant changes in QOL in HV. Evaluation of QOL might be a valuable tool regarding ethical questions in further studies. Larger studies are warranted to confirm the data and to further analyze the interaction between QOL and motivation of HV.

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## COMMENTS

### Background

Involvement of healthy volunteers (HV) is absolutely essential in gaining valuable knowledge in clinical and preclinical research. The involvement of HV (HV) has a long history, but many unresolved issues of ethical, methodological or even legal concerns in this regard still remain unanswered. The importance of this becomes even more pronounced when invasive procedures like GI endoscopy are undertaken. Existing ethical recommendations and guidelines on this topic are mostly based on the opinion of experts, but many of these questions have not been studied in a systematic manner.

### Research frontiers

Multiple studies have shown that the motivation of HV plays a key role in recruiting volunteers. Financial reward is one of the crucial motivating factors in HV. Participation in research studies might be associated with study-related risks, discomfort and inconvenience that might become even more relevant in endoscopy-based studies. Impairment in the quality of life in HV may play a crucial role in understanding these ethical concerns, however, so far no study has addressed the impact of endoscopy-based research on the quality of life of HV.

### Innovations and breakthroughs

In this pilot study the authors evaluate the changes in quality of life in HV in a long-term endoscopy-based drug study. The study was conducted according to existing ethical recommendation and guidelines. Using the well-validated SF-36 and visual analog scale-based questionnaires, they show that endoscopy-based research has no significant impact on quality of life in HV even under rigorous conditions.

### Applications

This study provides valuable and important information regarding the quality of life of HV who participate in endoscopy-based studies, and supports the existing recommendation and guidelines on this topic in Gastroenterology.

### Terminology

Healthy volunteer: is a person who voluntarily participates in a research study. Volunteer come from volunteering which means working, participating or being involved in something without being motivated by financial or material gain. The historical intention, such as to promote good, serve society and improve human quality of life show static movement in paid volunteerism. Endoscopy-based research: are studies or trials performed on individuals which involve endoscopic techniques. Short Form 36: is a well-validated and widely used short survey which includes 36 specific questions on health-related quality of life.

### Peer review

This study examines the impact of a demanding endoscopy protocol on gastrointestinal symptoms and quality of life in 10 human volunteers. The study involved 15 endoscopic procedures over a period of 6 mo.

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## Clinical relevance of *Helicobacter pylori* *babA2* and *babA2/B* in Costa Rica and Japan

Sergio A Con, Hiroaki Takeuchi, Mitsuaki Nishioka, Norihito Morimoto, Tetsuro Sugiura, Nobufumi Yasuda, Reinaldo Con-Wong

Sergio A Con, Reinaldo Con-Wong, Centro Digestivo Doctores Con-Mediplaza, 245-1200 San José, Costa Rica  
 Sergio A Con, Hiroaki Takeuchi, Mitsuaki Nishioka, Norihito Morimoto, Tetsuro Sugiura, Department of Clinical Laboratory Medicine, Kochi Medical School, Kochi University, Nankoku-city, Kochi 783-8505, Japan

Nobufumi Yasuda, Department of Public Health, Kochi Medical School, Kochi University, Nankoku-city, Kochi 783-8505, Japan

Author contributions: Con SA designed, performed the research and wrote the paper; Nishioka M, Con-Wong R, Morimoto N, and Sugiura T contributed reagents/analytic tools; Yasuda N analyzed the data; Takeuchi H revised the paper.

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Correspondence to: Sergio A Con, MD, PhD, Centro Digestivo Doctores Con-Mediplaza, 245-1200 San José, Costa Rica. [scon@gastrocolon.com](mailto:scon@gastrocolon.com)

Telephone: +506-2201-7028 Fax: +506-2201-7033

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*babB* and *babA2/B* genes. Statistical analysis was performed using the  $\chi^2$  test and the Fisher's exact probability test and multivariate analysis was performed by logistic regression adjusting for gender and age.  $P < 0.05$  was regarded as statistically significant.

**RESULTS:** The PCR-based genotyping of 95 Costa Rican and 95 Japanese isolates showed a higher prevalence of *babA2* in Japan (96.8%) than in Costa Rica (73.7%), while that of *babA2/B* was higher in Costa Rica (11.6%) than in Japan (1.1%). In Costa Rican isolates only, *babA2* was significantly associated with atrophic gastritis ( $P = 0.01$ ).

**CONCLUSION:** These results suggest that the status of *babA2* and *babA2/B* shows geographic differences, and that *babA2* has clinical relevance in Costa Rica.

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**Key words:** *babA2*; *babA2/B*; Costa Rica; *Helicobacter pylori*; Japan

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### Abstract

**AIM:** To evaluate the prevalence of *Helicobacter pylori* (*H. pylori*) *babA2*, *babB* and a recombinant gene between *babA2* and *babB* (*babA2/B*), and their role in the development of atrophic gastritis in Costa Rican and Japanese clinical isolates.

**METHODS:** A total of 95 continuous *H. pylori*-positive Costa Rican (41 males and 54 females; mean age, 50.65 years; SD,  $\pm 13.04$  years) and 95 continuous *H. pylori*-positive Japanese (50 males and 45 females; mean age, 63.43; SD,  $\pm 13.21$  years) patients underwent upper endoscopy from October 2005 to July 2006. They were enrolled for the polymerase chain reaction (PCR)-based genotyping of the *H. pylori* *babA2*,

### INTRODUCTION

*Helicobacter pylori* (*H. pylori*) infects the human stomach causing chronic inflammation, which can lead to peptic ulcer and gastric cancer<sup>[1,2]</sup>. The diverse clinical outcome of

gastric disease may involve differences in the prevalence or expression of bacterial virulence factors. *H. pylori* BabA is a blood-group antigen-binding adhesin encoded by the *babA2* gene, which has been shown to mediate adherence of *H. pylori* to human Lewis b blood-group (Le<sup>b</sup>) antigens<sup>[3,4]</sup>. Although three *bab* alleles have been identified (*babA1*, *babA2* and *babB*), only the *babA2* gene product is functional for Le<sup>b</sup> binding activity<sup>[5,6]</sup>. Studies in Western countries have disclosed associations between the presence of *babA2* gene and digestive diseases such as duodenal ulcer and gastric cancer<sup>[4]</sup>. However, in Asia, most of the *H. pylori* strains are *babA2*-positive, irrespective of clinical outcome<sup>[7,8]</sup>. Thus, conclusions about the relationship between *H. pylori* genotypes and clinical outcome derived from one geographic region may not be true for other geographic regions. Evidence concerning BabA adhesin-associated genes is insufficient in Costa Rica, where the incidence of gastric cancer is very high, similar to Japan<sup>[9]</sup>. The *babA2* gene, which encodes BabA, may play a role in the development of gastric cancer in the Costa Rican population. In order to investigate this hypothesis we aimed to correlate the status of *babA2* in Costa Rican clinical isolates with atrophic gastritis, a gastric premalignant lesion. In addition, because *H. pylori* populations are highly diverse and are constantly changing their genome by point mutations, substitutions, insertions, and/or deletions of their genome<sup>[10-12]</sup>, we decided to evaluate the prevalence of a recombinant gene between *babA2* and *babB* (*babA2/B*), already identified *in vitro*<sup>[13,14]</sup>, in Costa Rican as well as in Japanese clinical isolates, which were used also in this study for comparative purposes.

## MATERIALS AND METHODS

### Study population

Half of the patients in this study attended a digestive center in San Jose, Costa Rica and the other half attended a National University in Kochi, Japan. A total of 95 continuous *H. pylori*-positive Costa Rican (41 males and 54 females; mean age, 50.65 years; SD,  $\pm$  13.04 years) and 95 continuous *H. pylori*-positive Japanese (50 males and 45 females; mean age, 63.43; SD,  $\pm$  13.21 years) patients underwent upper endoscopy from October 2005 to July 2006. They were enrolled for the polymerase chain reaction (PCR)-based genotyping of *H. pylori* *babA2*, *babB* and *babA2/B* genes. Informed consent was obtained from each patient and the study was approved by the Ethics Committee of the institutions. Information was collected on age, gender, symptoms and medication. None of the participating patients had undergone *H. pylori* eradication therapy or gastric surgery. In addition, none of the patients had recent intake of proton pump inhibitors, antibiotics, or non-steroidal anti-inflammatory drugs. The patients were histopathologically classified into two groups; atrophic gastritis (AG) group (29 Costa Rican and 48 Japanese) and non-atrophic gastritis (NAG) group (66 Costa Rican and 47 Japanese) according to the updated Sydney System for the classification of gastritis<sup>[15]</sup>.

### Endoscopic and histological evaluations

Endoscopy was performed with Olympus EVIS EXERA I / II systems (Olympus America Inc., San Jose, CA, USA). From each participating subject, at least four biopsies (from the antrum, corpus and cisura angularis) were collected for histological examination. In addition, one antral biopsy was also taken to obtain the clinical isolates following bacterial culture.

The biopsy samples were conventionally fixed in 100 mL/L formaldehyde anidre and embedded in paraffin. Serial 3- to 4- $\mu$ m sections were stained with hematoxylin and eosin for histological observation. All biopsies were examined for the presence of glandular atrophy and were scored into four grades (0: none, 1: mild, 2: moderate and 3: marked) for both the antrum and the body of the stomach, according to the updated Sydney System of classification and grading of gastritis<sup>[15]</sup>. Gastric glandular atrophy was defined as the loss of gastric glands and its replacement with fibrosis or metaplastic epithelium.

### Determination of *H. pylori* infection

*H. pylori* infection was determined by either the rapid urease test (RUT) or histological examination in biopsy specimens obtained from the antrum, cisura angularis and body of the stomach. Patients were considered *H. pylori*-positive if either the biopsy specimen was positive for RUT or the bacterium was observed in any of the hematoxylin and eosin-stained sections.

### Isolation of *H. pylori* from biopsy specimens and DNA extraction

The homogenized biopsy specimens were placed on *H. pylori* selective agar plates (Helico VI agar, E-MS70, Eiken Chemical Co., Ltd., Japan) and cultured at 37°C under microaerobic conditions (100 mL/L CO<sub>2</sub>) for five to seven days. The presence of *H. pylori* colonies was confirmed by typical morphology, Gram staining and a positive urease test. Eventually, a total of 190 clinical isolates obtained from antrum specimens were subjected to genomic DNA (gDNA) extraction using a DNA kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions.

### Detection of *H. pylori* *babA2*, *babB* and *babA2/B* genes by PCR

The genomic DNAs were subjected to PCR-based genotyping of *babA2*, *babB* and *babA2/B* using two primer pairs including primers previously described<sup>[4,16]</sup> and new primers (Table 1) designed based on sequences of referential *H. pylori* strains 26 695 and J99. We used PCR conditions exactly matching those described<sup>[4,16]</sup> and the conditions for the new primers used in this study are shown in Table 1. Whenever necessary, in particular, to determine *babA2/B*, sequence analysis of the putative products was performed using Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and these sequences were compared with *babA* and *babB* genes of strains 26 695 (HP1243 and HP896, respectively) and J99 (jhp833 and jhp1164, respectively)

**Table 1** PCR primers and conditions for detection of *babA2*, *babB* and *babA2/B* genes

Region	Primer	Nucleotide sequence (5'-3')	PCR conditions	Ref.
<i>babA2</i>	<i>babA</i> -F	AATCCAAAAAGGAGAAAAAGTATGAAA		[4]
	<i>babA</i> -R	TGTTAG TGATTTCGGTGTAGGACA		
	<i>babA2</i> -Fnc1	GAAAAAACATGAAAAACACATCCTTTCAT		[16]
	<i>babA2</i> -Rmn2	TCTGGGTTAATGGCTTGCC		
<i>babB</i>	<i>babB</i> -Fnc1	CTCTCTCTCGTTTTTGCTCCA	96°C for 2 min, 30 cycles (96°C for 30 s, 48°C for 30 s, 72°C for 1 min)	This study
	<i>babB</i> -Rnc1	CTTCATAACACACCCTAAAGAGTC		
	<i>babB</i> -Fnc3	ATGAAAAAAACCCCTTTTACTC	96°C for 2 min, 30 cycles (96°C for 30 s, 46°C for 30 s, 72°C for 1 min)	This study
	<i>babB</i> -Rnc3	TGACCTGGATTGGTGCCCCCTACG		
<i>babA2/B</i>	<i>babA</i> -F	AATCCAAAAAGGAGAAAAAGTATGAAA		[4]
	<i>babB</i> -Rnc1	CTTCATAACACACCCTAAAGAGTC		
	<i>babA2</i> -Fnc1	GAAAAAACATGAAAAACACATCCTTTCAT		[16]
	<i>babB</i> -Rnc1	CTTCATAACACACCCTAAAGAGTC	96°C for 2 min, 30 cycles (96°C for 30 s, 62°C for 30 s, 72°C for 1 min)	This study
	<i>babB</i> -Rnc2 <sup>1</sup>	CTACGCTCACCCCTTGTTACTTC	96°C for 2 min, 30 cycles (96°C for 30 s, 63°C for 30 s, 72°C for 1 min)	This study
	<i>babB</i> -Rnc3 <sup>1</sup>	TGACCTGGATTGGTGCCCCCTACG	96°C for 2 min, 30 cycles (96°C for 30 s, 62°C for 30 s, 72°C for 1 min)	This study

<sup>1</sup>Used with *babA2*-Fnc1.**Table 2** Characteristics of Costa Rican and Japanese dyspeptic patients

	Costa Rican			Japanese		
	AG	NAG	P-value	AG	NAG	P-value
Patients number	29	66		48	47	
Sex (M/F)	15/14	26/40	0.264	24/24	26/21	0.604
mean age $\pm$ SD (yr)	54.8 $\pm$ 12.9	48.9 $\pm$ 12.8	0.041	68.4 $\pm$ 10.2	58.4 $\pm$ 14.1	< 0.001

AG: Atrophic gastritis; NAG: Non-atrophic gastritis; SD: Standard deviation.

using the BLAST 2 SEQUENCES system (<http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi>)<sup>[17]</sup>. When the putative recombinant gene was shown to be only homologous at the 5' and 3' positions of *babA2* gene open reading frame (ORF) and *babB* gene ORF, respectively, the gene was considered to be a recombinant *babA2/B* gene.

### Statistical analysis

Statistical analysis was performed using the  $\chi^2$  test and the Fisher's exact probability test [STATA SE (version 8) statistical software].  $P < 0.05$  was regarded as statistically significant. Multivariate analysis was performed by logistic regression [SPSS 13.0 Japanese version (SPSS Japan Inc., 2005)] adjusting for gender and age. Odds ratios with 95% confidence intervals were used to study the influence of these genes on the development of gastric atrophy.

## RESULTS

### Comparison of gender and age of patients between AG and NAG

There was no significant difference in gender between the AG group and NAG group from either Costa Rica or Japan (Table 2). However, mean age was significantly higher in the AG group than in the NAG group of both Costa Rican and Japanese patients.

### Prevalence of gastric atrophy in Costa Rican and Japanese patients

In Costa Rican patients, the prevalence of gastric atrophy was 30.5% (29/95) while that in Japanese patients was considerably higher (50.5%, 48/95).

### *H. pylori* *babA2*, *babB* and *babA2/B* genes in clinical isolates

In Costa Rican patients, the prevalence of *babA2* was 73.7% (70/95) and after gender and age adjustment, this gene was found to be significantly associated with AG in this population ( $P = 0.01$ ) (Table 3). The prevalence of *babB* and *babA2/B* was 81.1% (77/95) and 11.6% (11/95), respectively, and no significant differences were found between any of these genes and AG.

In Japanese patients, almost all patients were found to be *babA2*-positive (96.8%, 92/95), while only one patient had the *babA2/B* gene (98.9%). The prevalence of *babB* was 90.5% (86/95). After gender and age adjustment, no significant differences were found between any of these genes and AG in this population.

## DISCUSSION

The prevalence of *babA2* in Costa Rican isolates was 73.7%, which was higher than that shown in Western studies (38%-43%)<sup>[18-20]</sup>, but lower than that in Asian studies (80%-100%)<sup>[7,21-23]</sup>, including that in our Japanese



**Table 3** *H. pylori babA2, babB* and *babA2/B* genes according to atrophic gastritis in Costa Rican and Japanese patients

Gene	Costa Rican patients				Japanese patients			
	AG/NAG	P	OR	95% CI	AG/NAG	P	OR	95% CI
<i>babA2</i>								
Pos	27/43	0.01	7.80	1.63-37.40	46/46	0.88	0.82	0.06-10.70
Neg	2/23		1.00	(Reference)	2/1		1.00	(Ref.)
<i>babB</i>								
Pos	25/52	0.54	1.47	0.42-5.12	45/41	0.18	3.00	0.61-14.70
Neg	4/14		1.00	(Reference)	3/6		1.00	(Ref.)
<i>babA2/B</i>								
Pos	6/5	0.10	3.07	0.80-11.80	0/1	1.00	0.00	
Neg	23/61		1.00	(Reference)	48/46		1.00	(Ref.)

Pos: Positive; Neg: Negative; OR: Odds ratio; CI: Confidence intervals. Separate models were fitted to obtain an odds ratio for each gene with adjustment for gender and age in each country.  $P < 0.05$  was considered significant.

isolates (96.8%) (Table 3). It seems that the prevalence of *babA2* does not parallel the incidence rate of gastric cancer in those countries, since Costa Rica, has an incidence rate comparable to that of Japan and China<sup>[9]</sup>.

The *babA2* and *babB* genes exhibit extensive homologies at their 5' and 3' ends that should facilitate frequent recombination between them, suggesting that a recombination might interfere in the expression and functional activity of BabA. In this study, this recombination was found in 11 and 1 Costa Rican and Japanese (KMT28) isolates, respectively, irrespective of clinical outcome. However, in the Costa Rican strains, the *babA2* gene was found to be significantly associated with AG ( $P = 0.01$  and OR = 7.8) (Table 3). This association has been reported previously in Western studies<sup>[9]</sup>.

The PCR products of 12 *babA2/B* recombinant strains were employed for sequence analysis, revealing that several stop-codons in the amino acid sequence were found in all 11 Costa Rican strains, suggesting that these genes were non-functional. In contrast, since the Japanese strain KMT28 had complete in-frame sequence, the *babA2/B* gene was thought to be functional. In addition, reverse transcription-PCR (Toyobo Co., Ltd., Japan) using mRNA extracted from the KMT28 strain possessing the *babA2/B* gene with Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA), and sequence analysis were performed, demonstrating that the *babA2/B* transcript of KMT28 was definitely obtained and the sequence was identical to the *babA2/B* sequence (data not shown).

The relationship between *babA2*-positive *H. pylori* and an increased risk of developing clinical outcomes is controversial<sup>[7,18,20,24-26]</sup>, because the presence of *babA2* is not always to reflect the BabA binding activity due to regulation by the number of transcriptional start adenine [poly (A)] residues in the promoter region<sup>[5]</sup> and the presence of chimeric *babA/B* or *babB/A* genes<sup>[14,27]</sup>. Moreover, it is relatively difficult to detect the *babA2* gene by PCR with a single primer pair due to high homology between the sequences of *babA1* and *babA2*. Thus, to determine BabA binding activity and/or the presence of its transcript it was critical to consider the

functionality of BabA and its pathogenesis. Therefore we used at least two primer pairs to confirm the presence of *babA2* and recombinant *babA2/B* genes and investigated the relationship between the status of these genes and clinical outcomes.

Taken together, the status of *babA2* and *babA2/B* shows geographic differences, and *babA2* seems to have clinical relevance only in Costa Rica. A functional *babA2/B* was found in one Japanese isolate. However, we believe that a binding assay with Le<sup>b</sup> antigen is necessary to confirm whether the BabA is functional and/or the adhesive strength is regulated individually depending on an adaptation of the microorganism in the stomach involved in clinical manifestation.

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## COMMENTS

### Background

The clinical outcome of gastric disease may involve differences in the prevalence or expression of bacterial virulence factors. Contrary to Asian studies, western studies have disclosed associations between the presence of *babA2* gene and gastric cancer. Evidence concerning BabA adhesin-associated genes is insufficient in Costa Rica, where the incidence of gastric cancer is very high, similar to Japan. The *babA2* gene, which encodes BabA, may play a role in the development of gastric cancer in the Costa Rican population.

### Research frontiers

The research in this area is focused on the correlation between the status of *babA2* in Costa Rican clinical isolates and atrophic gastritis, a gastric premalignant lesion, and on the evaluation of the prevalence of a recombinant gene between *babA2* and *babB* (*babA2/B*), in Costa Rican and Japanese clinical isolates.

### Innovations and breakthroughs

The PCR-based genotyping of 95 Costa Rican and 95 Japanese isolates showed a higher prevalence of *babA2* in Japan (96.8%) than in Costa Rica (73.7%), while that of *babA2/B* was higher in Costa Rica (11.6%) than in Japan (1.1%). In Costa Rican isolates only, *babA2* was significantly associated with atrophic gastritis ( $P = 0.01$ ).

### Applications

These results suggest that the status of *babA2* and *babA2/B* shows geographic differences, and that *babA2* has clinical relevance in Costa Rica.

## Terminology

*Helicobacter pylori* (*H. pylori*) is a Gram-negative microaerobic bacterium that persistently colonizes the human gastric mucosa. *H. pylori* BabA is a blood-group antigen-binding adhesin encoded by the *babA2* gene, which has been shown to mediate adherence of *H. pylori* to human Lewis b blood-group antigens.

## Peer review

This paper has a correct design and is presented adequately. Title, results and discussion are clear and properly expressed. This topic is controversial, in some way, and this investigation constitutes an interesting contribution.

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## Lymphoid tyrosine phosphatase R620W variant and inflammatory bowel disease in Tunisia

Imen Sfar, Walid Ben Aleya, Leila Mouelhi, Houda Aouadi, Thouraya Ben Rhomdhane, Mouna Makhoulf, Salwa Ayed-Jendoubi, Houda Gargaoui, Taoufik Najjar, Taieb Ben Abdallah, Khaled Ayed, Youssr Gorgi

Imen Sfar, Walid Ben Aleya, Leila Mouelhi, Houda Aouadi, Thouraya Ben Rhomdhane, Mouna Makhoulf, Salwa Ayed-Jendoubi, Houda Gargaoui, Taoufik Najjar, Taieb Ben Abdallah, Khaled Ayed, Youssr Gorgi, Laboratory of Immunology, Charles Nicolle Hospital, Tunis 1006, Tunisia  
Mouelhi Leila, Najjar Taoufik, Department of Gastroenterology, Charles Nicolle Hospital, Tunis 1006, Tunisia

Author contributions: Ben Aleya W, Sfar I, Aouadi H, Makhoulf M, Ben Rhomdhane T and Ayed-Jendoubi S performed the majority of the experiments; Mouelhi L and Najjar T collected all the human material; Ben Abdallah T, Ayed K and Gorgi Y designed, supervised and provided financial support for this work; and Ben Aleya W wrote the manuscript.

Supported by Laboratory of Immunology, Charles Nicolle Hospital

Correspondence to: Dr. Ben Aleya Walid, Laboratory of Immunology, Charles Nicolle Hospital, Boulevard 9 Avril, Tunis 1006, Tunisia. [b\\_a\\_w@hotmail.fr](mailto:b_a_w@hotmail.fr)

Telephone: +216-98-478866 Fax: +216-71-561156

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### Abstract

**AIM:** To assess the possible association between *PTPN22* (R620W) gene polymorphism and inflammatory bowel disease (IBD).

**METHODS:** One hundred and sixty-four patients with IBD [105 Crohn's disease (CD) and 59 ulcerative colitis (UC)] and 100 healthy controls were recruited. Genotyping of the *PTPN22* gene 1858C→T polymorphism was performed by restriction fragment length polymorphism-polymerase chain reaction with *Rsa* I digestion.

**RESULTS:** The genotypic and allelic frequencies of (R620W) *PTPN22* gene polymorphism reveal a significant association of the *PTPN22* 620-W allele with IBD, compared to the healthy control group (OR: 17.81, 95% CI: 4.18-21.86,  $P = 0.00001$ ). Nevertheless, no

difference in this polymorphism was found between CD and UC patients. No significant association was found between the frequencies of genotypes of the *PTPN22* gene with either the clinical features such as sex, age, age at disease onset, and extent of colitis, or the production of serological markers (anti-Saccharomyces cerevisiae antibody in CD and perinuclear anti-neutrophil cytoplasmic antibody in UC).

**CONCLUSION:** These observations confirm the association of IBD susceptibility with the *PTPN22* 1858T (620-W) allele in Tunisian patients.

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**Key words:** Inflammatory bowel disease; *PTPN22*; Genetic polymorphism; Genetic susceptibility

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### INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic inflammatory bowel diseases (IBDs) that are characterized clinically by periods of well being punctuated by episodes of clinical disease activity that involve various sites in the gastrointestinal tract<sup>[1]</sup>. Several environmental, microbial, immunological, genetic, and life-style factors have been suggested to play a role in disease initiation<sup>[2]</sup>. Heterogeneity is observed in terms of disease location,

behavior, age of onset and serologic markers<sup>[3,4]</sup>. There is some support for the concept that this heterogeneity may be in large measure genetically determined<sup>[5]</sup>. Genome-wide scans performed in patients with IBD have failed to find a major unique susceptibility locus and have prompted the general agreement that these diseases are polygenic entities in which several genes may contribute to susceptibility<sup>[6-8]</sup>. Significant linkages in chromosomes 1, 3, 6, 7, 12, 14, 16, and 19 have been reported<sup>[9,10]</sup>.

The *PTPN22* gene, located on chromosome 1p13, encodes a lymphoid-specific protein tyrosine phosphatase (LYP), a member of a family of proteins involved in suppressing spontaneous T-cell activation via a negative regulatory C-terminal Src kinase (Csk)<sup>[11]</sup>. Indeed, LYP is implicated in maintaining the resting phenotype of lymphocytes and in controlling signals caused by an antigen, co-stimulation and cytokines<sup>[12]</sup>. A functional C1858T single nucleotide polymorphism (SNP), which encodes an arginine to tryptophan substitution at residue 620 (R620W), is located in the P1 proline-rich motif of *PTPN22*, which binds with high affinity to the Src homology 3 (SH3) domain of the tyrosine kinase<sup>[13,14]</sup>. Thus, it is plausible that this genetic discrepancy in *PTPN22* influences a range of diseases in which the phenotypic spectrum includes an aberrant or hyperactive immune response<sup>[14,15]</sup>. An association of *PTPN22* (R620W) polymorphism was reported first with type 1 diabetes<sup>[16,17]</sup> and later also with myasthenia gravis<sup>[18]</sup>, systemic lupus erythematosus<sup>[19]</sup>, and rheumatoid arthritis<sup>[14]</sup>. However, there are also some inflammatory diseases such as psoriasis, multiple sclerosis and Behcet's disease<sup>[20]</sup> without an association with this polymorphism.

In this study, we analyzed the *PTPN22* (R620W) polymorphism in Tunisian patients with CD and UC, to evaluate the contribution of the C1858T SNP to IBD susceptibility.

## MATERIALS AND METHODS

### Patients and controls

Blood samples were obtained from 164 subjects with IBD. There were 105 patients with CD (50 men, 55 women) with a mean age of 36.07 years (range: 23-60 years), and 59 patients with UC (17 men, 42 women) with a mean age of 37.89 years (range: 25-74 years). All subjects were unrelated Tunisians treated at the Department of Gastroenterology of Charles Nicolle and La Rabta Hospitals in Tunis. The diagnosis of CD and UC was determined in accordance with the standardized set of clinical, endoscopic and/or radiological and histological criteria<sup>[21]</sup> provided by International Organization for the study of IBD. Data obtained from each patient included age at diagnosis, disease location and extent, disease characteristics, and extra-intestinal manifestations (especially peripheral arthritis, ankylosing spondylitis in CD disease and primary sclerosing cholangitis in UC), which were used to group the patients according to the Vienna classification<sup>[22]</sup> (Table 1).

In the CD sample group, 47 sera out of 105 (44.8%)

**Table 1** Clinical characteristics and serological markers of CD and UC

CD patients	n = 105
Sex	55 men and 50 women
Mean age (yr)	36 ± 10.2
Age at diagnosis (yr)	31.2 ± 12.8
Disease location	
Ileitis (%)	27 (25.7)
Ileocolitis (%)	54 (51.5)
Colitis (%)	24 (22.8)
Anal involvement (%)	44 (42)
Extra-intestinal manifestations (%)	20 (19.04)
(peripheral arthritis and/or ankylosing spondylitis)	
ASCA (%)	47 (44.8)
UC patients	n = 59
Sex	17 men and 42 women
Mean age (yr)	38 ± 12.4
Age at diagnosis (yr)	36.3 ± 10.8
Disease extent	
Pancolitis (%)	38 (64.4)
Distal colitis (%)	21 (35.6)
Extra-intestinal manifestations (%)	3 (5.1)
Primary sclerosing cholangitis	
p-ANCA (%)	16 (27.1)

CD: Crohn's disease; UC: Ulcerative colitis; ASCA: Anti-Saccharomyces cerevisiae antibodies; p-ANCA: Perinuclear anti-neutrophil cytoplasmic antibodies.

were anti-Saccharomyces cerevisiae antibodies (ASCA)-positive, and in UC sample group, 16 sera out of 59 (27.1%) were perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA)-positive.

A total of 100 unrelated healthy subjects (52 men and 48 women) matched for age, sex and ethnic origin were used as the control population. None of the healthy controls had any evidence of autoimmune diseases such as IBD or diabetes.

All patients and controls gave informed consent to participate in this study, which was approved by the Ethics Committee of Charles Nicolle Hospital in Tunis.

### Methods

Genomic DNA isolated from EDTA-anticoagulated peripheral blood samples of unrelated healthy blood donors and IBD patients was extracted by a salting-out process.

Genotyping was performed using the restriction fragment length polymorphism-polymerase chain reaction method. The PCR reactions were performed in 10-μL final volume using 10 pmol of each primer: 5'-TGCCCATCCC ACACTTTAT-3', forward primer and 5'-ACCTCCTGGG TTTGTACCTTA-3', reverse primer, and contained 50 ng extracted DNA, 1 U Taq polymerase (Promega, Madison, WI, USA), 1.5 mmol/L MgCl<sub>2</sub> and 0.2 mmol/L dNTP. After an initial denaturing time of 15 min at 95°C, PCR reactions were run for 35 cycles including 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, with a final extension at 72°C for 10 min. The PCR product was digested by 1 U of the enzyme *Rsa* I (Promega) at 37°C for 1.5 h, subjected to electrophoresis in 3% agarose gel, and stained with



Table 2 Genotype and allele frequencies of *PTPN22* polymorphism in controls and patients

Groups	<i>n</i>	Genotype frequency			Allele frequency		<i>P</i>
		R/R	R/W	W/W	R	W	
Controls (%)	100	98 (98)	2 (2)	0 (0)	0.990	0.010	P/C: 0.00001 (OR: 17.81 CI 95%: 4.18-21.86)
IBD patients (%)	164	114 (69.5)	50 (30.5)	0 (0)	0.848	0.152	
CD (%)	105	68 (64.8)	37 (35.2)	0 (0)	0.823	0.177	CD/UC: NS
UC (%)	59	46 (78)	13 (22)	0 (0)	0.889	0.111	

P: Patients; C: Controls; NS: Not significant.

ethidium bromide. The PCR generated a 326-bp fragment that contained a restriction site for *Rsa* I, which permitted differentiation of the R620- allele (228 bp) and the 620-W allele (272 bp).

### Statistical analysis

All statistical analyses were performed with SPSS version 13.0 (Chicago, IL, USA). The Hardy-Weinberg equilibrium was assessed by the goodness-of fit test for biallelic markers. Calculation was done using internet programs from (<http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl>). Statistical power was calculated using a web power calculator (<http://calculators.stat.ucla.edu/powercalc/>). Allelic and genotypic frequencies were evaluated by direct counting. Statistical comparisons were performed, between patients and controls, by Pearson's  $\chi^2$  test calculated on  $2 \times 2$  contingency tables. Fisher's exact test was used when an expected cell value was  $< 5$ .  $P < 0.05$  was considered to be statistically significant. The strength of the association of *PTPN22* R620W genotypes as a genetic factor with the frequency of an IBD symptom was estimated by the calculation of OR ( $> 1$ : positive association;  $= 1$ : no influence;  $< 1$ : protective) and 95% CI, using the same software. For groups of equal variance, the correlation between age at onset of symptoms and *PTPN22* genotype was followed by two-sample Student's *t* test, to compare the means of the individual groups.

## RESULTS

The allele frequencies were in Hardy-Weinberg equilibrium both in the patients and controls. When comparing IBD patients with the control group, the frequency of the *PTPN22* 620-W allele was found to be significantly higher in IBD patients than in controls (Pc: 0.00001; OR: 17.81, 95% CI: 4.18-21.86). Nevertheless, no difference in this polymorphism was found between CD and UC patients (Table 2). Indeed, analysis of IBD patients according to clinical behavior revealed no difference between those carrying or not carrying the *PTPN22* (R620W) allele (data not shown), in both CD and UC patients.

When stratifying IBD patients according to the Vienna classification, we did not find a statistically significant association between the frequencies of genotypes of the *PTPN22* gene and the clinical features such as sex, age, age of disease onset, or extent of colitis. Moreover, no correlation was found between polymorphism studied

and the production of p-ANCA and ASCA in UC or CD patients, respectively.

## DISCUSSION

Autoimmune diseases represent a different set of associated phenotypes that are supposed to have common underlying mechanisms and thus, some degree of common genetic predisposition<sup>[23]</sup>. IBD is a chronic inflammatory condition of the gastrointestinal tract that manifests as UC or CD. Notwithstanding intensive research, the etiology of this condition remains unknown<sup>[24]</sup>. However, it is thought to result from a combination of genetic predisposition and environmental factors that may be channelled through an abnormality in gut-barrier function, with a loss of antigen tolerance. Some genetic markers that predispose to inflammatory disease have been identified (alleles DRB1\*0103, DRB\*12, and mutations in the *NOD2/CARD15* gene on chromosome 16). Nevertheless, the *CARD15* variations are not sufficient to explain the entire risk for predisposition to IBD<sup>[25]</sup>. Other polymorphisms in genes that code for proteins involved in the immune response also appear to exert an influence on the immunological mechanisms that lead to loss of tolerance to commensal microflora.

A specific role of *PTPN22* in T-cell regulation has been confirmed by the results of knocking out the murine homologue of *PTPN22*, which results in lowered thresholds for T-cell-receptor signaling in these animals. Recent findings have revealed that the *PTPN* risk-associated variant, W620, results in a gain of *PTPN22* phosphatase activity in T cells, which opens up new approaches for exploring disease mechanisms<sup>[26]</sup>.

Initially identified as a susceptibility allele for type 1 diabetes<sup>[17]</sup>, the *PTPN22* variant has now been implicated in the genetic etiology of rheumatoid arthritis<sup>[14]</sup>, Hashimoto thyroiditis, juvenile idiopathic arthritis<sup>[27]</sup>, Graves' disease<sup>[28]</sup> and most recently, systemic lupus erythematosus<sup>[19]</sup>. Despite the association of *PTPN22* C1858T SNP with several different autoimmune disorders, a role for this polymorphism in susceptibility to IBD does not appear so clear. Wagenleiter *et al.*<sup>[29]</sup> have revealed no association of the 620-W allele with CD in 146 patients of Northern German origin. Additionally, they have not found any difference in *PTPN22* allele frequencies between *CARD15*+ and *CARD15*- patients. These results are in agreement with previous studies of Criswell

*et al.*<sup>[30]</sup>, Martin *et al.*<sup>[31]</sup> in Spanish subjects, Prescott *et al.*<sup>[32]</sup> in British subjects, and van Oene *et al.*<sup>[33]</sup> in Canadian patients. However, given the relatively small sample size of the patient and control cohort in these studies, a false-negative result cannot be ruled out.

Our data showed the association of IBD susceptibility with the PTPN22 1858T (620-W) allele in Tunisian patients. Similar findings were detected in Takayasu's arteritis in which PTPN22 R620W polymorphism revealed a wide variation in allele frequencies among different populations; the polymorphic allele being present most in Scandinavia, but absent in Asian and African populations<sup>[34,35]</sup>.

No explanations have been presented for the discrepancy between positive findings, like ours, and the negative findings of others. The most plausible is the known genetic diversity of the different populations at the haplotype level. The reason for this divergence is not clear but might reflect an ethnic difference in the contribution of genetic factors.

Kyogoku *et al.*<sup>[19]</sup> have suggested that the PTPN22 C1858T variant predisposes persons to autoimmune diseases by assisting the production of certain disease-associated antibodies, thereby contributing to disease development. IBD is thought to be primarily a T-cell mediated disease, currently, a serology panel including p-ANCA and ASCA is used for auxiliary diagnosis of IBD. However, relying exclusively on serum antibodies for IBD diagnosis is not justified yet, because the available sero-immunological markers are not sensitive and specific enough, and their role in disease pathogenesis and progression is not well established<sup>[36]</sup>. In this study, we showed an association of IBD susceptibility with the PTPN22 1858T (620-W) allele in Tunisian patients but no significant association was found between the frequencies of genotypes of the *PTPN22* gene and the production of ASCA in CD or p-ANCA in UC.

*PTPN22* encodes LYP, which dephosphorylates the kinases LCK, Fyn and Zap-70, all known to be important in T-cell signaling<sup>[37]</sup>. An additional function of LYP is to downregulate activation of T cells by binding to Csk<sup>[14]</sup>, an important suppressor of kinases that mediate T-cell activation. Furthermore, LYP has been demonstrated to bind to the adaptor molecule growth factor receptor-bound protein 2, and this interaction is thought to play a negative regulatory role in T-cell signaling<sup>[38,39]</sup>. The PTPN22 1858 C/T SNP changes the amino acid at position 620 from an arginine (R) to a tryptophan (W), disturbs the interaction between LYP and Csk, which avoids formation of the complex, and therefore, the suppression of T-cell activation.

It remains to be determined precisely how the PTPN22 (620-W) allele influences the progression of IBD, especially since our study indicates that the analyzed polymorphism of the *PTPN22* gene do not appear to be involved in the severity of CD or UC, as defined by the need for surgery (data not shown).

In conclusion, our data showed the association of IBD susceptibility with the PTPN22 1858T (620-W) allele in Tunisian patients. However, no correlation was found between this *PTPN22* polymorphism and the clin-

ical or biological characteristics of CD or UC. Further studies are needed to confirm this association in more subjects and to determine the mechanisms by which this polymorphism affects the pathogenesis of this disease.

## COMMENTS

### Background

In recent years, a few studies have been published that have addressed the question of where and under which conditions PTPN22 is produced in the gut in the normal and neoplastic situation. Some of these studies have considerably influenced our view of the role of the PTPN22 system. That is why it has appeared necessary to analyze the PTPN22 1858 C/T SNP in unrelated Tunisian patients with Crohn's disease (CD) and ulcerative colitis (UC) to evaluate the contribution of the CD95 gene to genetic susceptibility to inflammatory bowel disease (IBD).

### Research frontiers

Recent findings have revealed that the PTPN risk-associated variant, W620, results in a gain of PTPN22 phosphatase activity in T cells, which has opened new approaches for exploring disease mechanisms.

### Innovations and breakthroughs

The relationship between PTPN22 polymorphism and IBD has not been reported yet. This is probably the first report on the association of PTPN22 polymorphisms in Tunisian IBD patients. However, this polymorphism was associated with the development of CD and UC, which provides strong support for an IBD susceptibility gene in the region surrounding PTPN22.

### Applications

By understanding how the PTPN22 polymorphism is associated with the development of CD and UC, this study may indicate a future strategy for therapeutic intervention in patients with IBD.

### Peer review

This clinical study focused upon the frequency of polymorphism in a specific gene in a small group of individuals with IBD. The results are of great interest and relevance to understanding the pathogenesis of IBD.

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## Shape of Barrett's epithelium is associated with prevalence of erosive esophagitis

Tomoyuki Akiyama, Masahiko Inamori, Hiroshi Iida, Hiroki Endo, Kunihiro Hosono, Yasunari Sakamoto, Koji Fujita, Masato Yoneda, Hirokazu Takahashi, Tomoko Koide, Chikako Tokoro, Ayumu Goto, Yasunobu Abe, Takeshi Shimamura, Noritoshi Kobayashi, Kensuke Kubota, Satoru Saito, Atsushi Nakajima

Tomoyuki Akiyama, Masahiko Inamori, Hiroshi Iida, Hiroki Endo, Kunihiro Hosono, Yasunari Sakamoto, Koji Fujita, Masato Yoneda, Hirokazu Takahashi, Tomoko Koide, Chikako Tokoro, Ayumu Goto, Yasunobu Abe, Takeshi Shimamura, Noritoshi Kobayashi, Kensuke Kubota, Satoru Saito, Atsushi Nakajima, Gastroenterology Division, Yokohama City University School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan

Author contributions: Akiyama T, Inamori M, Iida H, Endo H, Hosono K and Sakamoto Y designed the research; Akiyama T, Inamori M, Fujita K, Yoneda M, Takahashi H, Koide T and Tokoro C performed the research; Akiyama T, Inamori M, Goto A, Abe Y, Shimamura T, Kobayashi N and Kubota K analyzed the data; Akiyama T, Inamori M, Saito S and Nakajima A wrote the paper.

Correspondence to: Masahiko Inamori, MD, PhD, Gastroenterology Division, Yokohama City University School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan. [inamorim@med.yokohama-cu.ac.jp](mailto:inamorim@med.yokohama-cu.ac.jp)

Telephone: +81-45-7872640 Fax: +81-45-7843546

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**RESULTS:** Barrett's epithelium was diagnosed in 374 cases (43%). Most of these were diagnosed as short-segment Barrett's epithelium. The prevalence of erosive esophagitis was significantly higher in subjects with flame-like than lotus-like Barrett's epithelium, and in those with a C extent of  $\geq 2$  cm than  $< 2$  cm.

**CONCLUSION:** Flame-like rather than lotus-like Barrett's epithelium, and Barrett's epithelium with a longer segment were more strongly associated with erosive esophagitis.

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**Key words:** Barrett's epithelium; Esophagitis; Endoscopy

**Peer reviewer:** Helena Nordenstedt, MD, PhD, Upper Gastrointestinal Research, Department of Molecular Medicine and Surgery, Karolinska Institute, Stockholm 17176, Sweden

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### Abstract

**AIM:** To test the hypothesis that the shape and length of Barrett's epithelium are associated with prevalence of erosive esophagitis.

**METHODS:** A total study population comprised 869 patients who underwent endoscopy during a health check-up at our hospital. The presence and extent of Barrett's epithelium were diagnosed based on the Prague C & M Criteria. We originally classified cases of Barrett's epithelium into two types based on its shape, namely, flame-like and lotus-like Barrett's epithelium, and into two groups based on its length, its C extent  $< 2$  cm, and  $\geq 2$  cm. Correlation of shape and length of Barrett's epithelium with erosive esophagitis was examined.

### INTRODUCTION

In patients with Barrett's epithelium, the resulting replacement of normal squamous epithelium with columnar epithelium can be seen in the distal esophagus as a salmon-pink-colored area that is readily visible during endoscopic examination. Barrett's epithelium is recognized as a complication of erosive esophagitis and is the pre-malignant condition for adenocarcinoma



of the esophagus<sup>[1,2]</sup>. The incidence of esophageal adenocarcinoma is rapidly increasing in the United States and Europe<sup>[3-5]</sup>, and is reported to account for up to 50% of esophageal cancers seen in white males in the United States<sup>[5]</sup>. For esophageal carcinoma in Japan, however, the ratio of adenocarcinoma to squamous cell carcinoma is low, and no significant changes have been identified<sup>[6]</sup>. As the prevalence of erosive esophagitis is increasing, further observation of Barrett's epithelium and esophageal adenocarcinoma is required in Japan. However, the reasoning behind the recommendation for regular endoscopic screening and biopsies in patients with Barrett's epithelium in Japan is unclear, and whether all patients with Barrett's epithelium, or only a subgroup with risk factors for the development of adenocarcinoma should be screened, remains controversial.

It has been known for more than a century that chronic inflammation can contribute to cancer formation. Chronic inflammatory conditions of the gastrointestinal tract, such as ulcerative colitis and chronic pancreatitis, are well known to predispose patients to carcinogenesis. Lassen *et al.*<sup>[7]</sup> have reported that the risk of esophageal adenocarcinoma was fivefold greater among patients with diagnosed esophagitis, but most of these cancers seemed to be related to Barrett's esophagus. Several studies have indicated that a dose-response relationship exists between the severity of erosive esophagitis and the incidence of esophageal adenocarcinoma<sup>[8-11]</sup>. These findings suggest that the major risk factor for esophageal adenocarcinoma is the existence of Barrett's epithelium complicated with erosive esophagitis.

We hypothesized that some macroscopic features of Barrett's epithelium might be useful for identifying a subgroup with a high risk for the development of esophageal adenocarcinoma. Therefore, we conducted a retrospective cohort study to examine the correlation of the shape and length of Barrett's epithelium with erosive esophagitis.

## MATERIALS AND METHODS

### Patients

A total of 869 patients (463 men, 406 women; median age, 66 years; age range, 29-91 years) who had undergone an upper endoscopy at the Gastroenterology Division of Yokohama City University Hospital between August 2005 and July 2006 were enrolled in the study. The total study population consisted of consecutive patients who had undergone endoscopy for a health checkup in our hospital. The majority of the patients were outpatients. None of the patients had previously undergone an upper digestive tract operation. Ten patients were excluded, because their profiles were unsatisfactory or they refused to participate in the present study.

### Endoscopic findings

At the Yokohama City University Hospital, when endoscopically examining and photographing the esophageal

mucosa, the gastroesophageal junction (GEJ) is always prospectively photographed. Our hospital operates a digital filing system for endoscopic images. All digital endoscopic images were independently and retrospectively reviewed by two trained endoscopists to investigate the endoscopic findings, including gastric mucosal atrophy (GMA), hiatal hernia, erosive esophagitis, and Barrett's epithelium. If there was any inconsistency in the assessment of the digital endoscopic images, a final diagnosis was decided upon by a joint review of the digital endoscopic images.

### Barrett's epithelium

The presence and extent of Barrett's epithelium were diagnosed based on the Prague C & M Criteria<sup>[12]</sup>. According to these criteria, Barrett's epithelium is defined as the macroscopic identification, using a standard endoscopy examination, of abnormal columnar esophageal epithelium suggestive of columnar-lined distal esophagus. The length of Barrett's epithelium is measured (in cm) using the circumferential extent (the C extent) and the maximum extent (the M extent) above the GEJ, which is identified as the proximal margin of the gastric mucosal folds in centimeters<sup>[12]</sup>.

### Shape of Barrett's epithelium

We originally classified Barrett's epithelium into two types based on its shape. As shown in the Figure 1, we classified the shape of Barrett's epithelium as follows: (A) the L type, in which the difference between the C extent and M extent was  $< 2$  cm and the visible red columnar epithelium could be observed as a lotus-like shape; and (B) the F type, in which the difference was  $\geq 2$  cm and the columnar epithelium of Barrett's epithelium was observed as a flame-like shape.

### Length of Barrett's epithelium

We further classified Barrett's epithelium into two groups based on its length as follows: (1) Barrett's epithelium  $< 2$  cm, defined as a C extent of  $< 2$  cm in length; and (2) Barrett's epithelium  $\geq 2$  cm, defined as a C extent of  $\geq 2$  cm.

### Erosive esophagitis

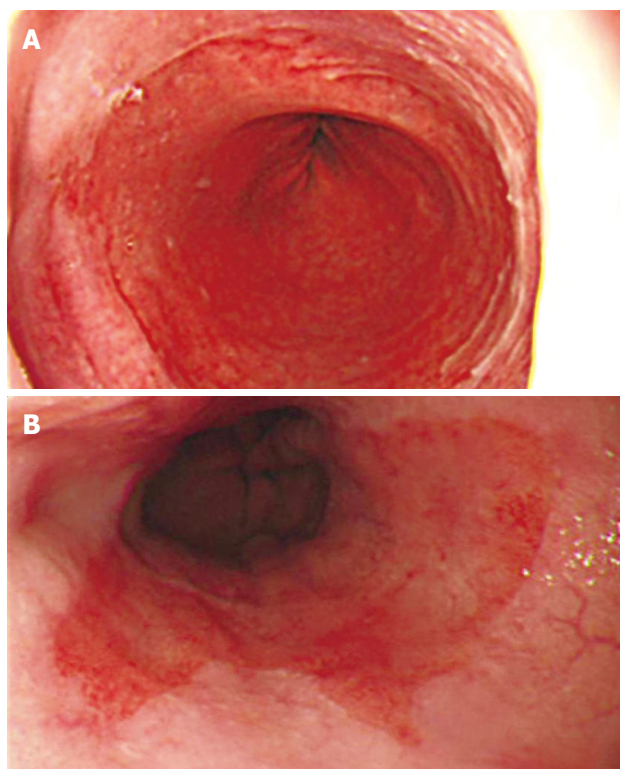
Erosive esophagitis was diagnosed based on the Los Angeles (LA) Classification<sup>[13]</sup> and was divided into three groups: none, mild (grades A and B), or severe (grades C and D).

### Hiatal hernia

Hiatal hernia was diagnosed when the distance between the GEJ and the diaphragmatic hiatus was  $\geq 2$  cm.

### GMA

The atrophic area of the stomach that can be visualized endoscopically is known to extend from the antrum to the body. Previously, Kimura and Takemoto endoscopically divided GMA into six groups (C1, C2, C3, O1, O2, and



**Figure 1 Shape of Barrett's epithelium.** We originally divided Barrett's epithelium into two types based on its shape. A: L type, in which the difference between the C extent and M extent was < 2 cm and the visible red columnar epithelium could be observed as a lotus-like shape; B: F type, in which the difference was  $\geq$  2 cm and the columnar epithelium of Barrett's epithelium was observed as a flame-like shape.

O3; C, closed; O, open)<sup>[14]</sup>. GMA has been shown to progress from C1 to O3 successively, and this classification correlates well with the histological features of GMA<sup>[14]</sup>. Gastric acid secretion in patients with open-type GMA has been reported to be lower than that in patients with closed-type GMA<sup>[15]</sup>. In the present study, we defined closed-type (C1-C3) cases as mild GMA and open-type (O1-O3) cases as severe GMA.

### Patient profiles

Complete patient information, including age, sex, body mass index (BMI), regular drinking habit and smoking habit, at the time of the initial diagnosis was obtained from the patient's medical records.

### Ethics

The study was conducted in accordance with the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of Yokohama City University Hospital. The patients gave their signed informed consent to be involved in the study.

### Statistical analysis

In our cohort study, to investigate the correlation between the shape and length of Barrett's epithelium and erosive esophagitis, the variables were compared between patients with different shapes and between

**Table 1 Clinical characteristics of patients enrolled in the present study**

Clinical characteristics	n (%)
Total patients	869
Patient profiles	
Age	
median; range (yr)	66; 29-91
Sex	
Female (%)	406 (46.7)
BMI	
median; range	22.2; 13.5-41.2
Regular drinking habit (%)	316 (36.4)
Smoking habit (%)	319 (36.7)
Endoscopic results	
Hiatal hernia (%)	266 (30.6)
GMA	
Open type (%)	466 (53.6)
Erosive esophagitis	
Total (%)	165 (19.0)
Mild (LA class A, B) (%)	152 (17.5)
Severe (LA class C, D) (%)	13 (1.5)
Barrett's epithelium	
Total (%)	374 (43.0)
SSBE (%)	370 (42.6)
LSBE (%)	4 (0.5)

GMA: Gastric mucosal atrophy; LA: Los Angeles; SSBE: Short-segment Barrett's epithelium; LSBE: Longer segment Barrett's epithelium.

patients with different lengths. The statistical analysis included a  $\chi^2$  test or a Fisher exact test to compare percentages, and a Mann-Whitney *U* test to compare continuous data. The level of significance was defined as  $P < 0.05$ . All statistical analyses were performed using Stat View software (SAS Institute, Cary, NC, USA).

## RESULTS

The baseline characteristics of the study population are summarized in Table 1. A total of 374 patients (43.0%) (211 men and 163 women; mean age, 68 years; range, 31-91 years) were diagnosed as having Barrett's epithelium based on the Prague C & M Criteria<sup>[10]</sup>. These consisted of 370 cases (42.6%) with short-segment Barrett's epithelium (SSBE), whose C extent was < 3 cm, and four cases (0.5%) with longer segment Barrett's epithelium (LSBE), whose C extent was  $\geq$  3 cm. A total of 165 cases (19.0%) had erosive esophagitis: 152 (17.5%) had mild esophagitis (LA grades A and B), and 13 (1.5%) had severe esophagitis (LA grades C and D).

Table 2 shows the clinical characteristics of the subjects according to the two types of Barrett's epithelium shape. No significant differences in age, sex, BMI, and hiatal hernia were observed between the subjects with F type and L type Barrett's epithelium. The subjects with F type Barrett's epithelium tended to have higher prevalence of regular drinking and smoking compared with those with L type Barrett's epithelium, but without statistical significance. The prevalence of open-type GMA was significantly lower in the subjects with F type than L type Barrett's epithelium. The prevalence of erosive

**Table 2** Clinical characteristics of patients according to the two shapes of Barrett's epithelium *n* (%)

	Shape of Barrett's epithelium		<i>P</i> value
	L type ( <i>n</i> = 353)	F type ( <i>n</i> = 21)	
Age			
Median; range (yr)	68; 31-91	68; 54-80	0.8532
Sex			
Female (%)	154 (43.6)	9 (42.9)	0.9450 <sup>1</sup>
BMI			
Median; range	22.5; 14.4-41.2	22.0; 18.1-30.5	0.8565
Regular drinking habit (%)	144 (40.8)	13 (61.9)	0.0568 <sup>1</sup>
Smoking habit (%)	160 (45.3)	14 (66.7)	0.0568 <sup>1</sup>
Hiatal hernia (%)	145 (41.1)	8 (38.1)	0.9669 <sup>2</sup>
GMA			
Open type (%)	184 (52.1)	5 (23.8)	0.0216 <sup>2</sup>
Erosive esophagitis (%)			
Total	112 (31.7)	13 (61.9)	0.0044 <sup>1</sup>
Mild	103 (29.2)	9 (42.9)	0.2787 <sup>2</sup>
Severe	9 (2.5)	4 (19.0)	0.0007 <sup>3</sup>

*P* value: Mann-Whitney *U* test; <sup>1</sup>:  $\chi^2$  test; <sup>2</sup>:  $\chi^2$  test (Yates' correction); <sup>3</sup>: Fisher's exact test.

esophagitis, especially severe esophagitis, was significantly higher in the subjects with F type than L type Barrett's epithelium.

Table 3 shows the clinical characteristics broken down by the two lengths of Barrett's epithelium. The prevalence of hiatal hernia and erosive esophagitis was significantly higher in subjects with Barrett's epithelium with a C extent of  $\geq 2$  cm.

## DISCUSSION

Chronic inflammation, such as ulcerative colitis and chronic pancreatitis, is a well known risk factor for cancer formation. As mentioned above, several clinical studies have suggested that the major risk factor for esophageal adenocarcinoma is Barrett's epithelium with complicated erosive esophagitis<sup>[7-11]</sup>. The histological evidence of moderate to severe inflammation, along with the expression of the pro-inflammatory cytokines interleukin (IL)-1 $\beta$ , IL-8, and nuclear factor (NF)- $\kappa$ B have been detected in biopsies of Barrett's epithelium<sup>[16,17]</sup>. Moreover, the infiltrating inflammatory cells are not the only source of pro-inflammatory cytokines, because Barrett's epithelial cells themselves have been found to express IL-8, IL-1 $\beta$ , and IL-10<sup>[16,18]</sup>. NF- $\kappa$ B activation and epithelial cell expression of tumor necrosis factor (TNF)- $\alpha$  and its receptor TNFR1 all have been found to increase as Barrett's epithelium develops dysplastic changes of progressive severity, which suggests that the inflammatory response might contribute to carcinogenesis<sup>[16,19]</sup>. Although elevated levels of IL-8 and IL-1 $\beta$  have not been found in dysplastic Barrett's epithelium, higher expression levels of both cytokines have been detected in esophageal adenocarcinoma<sup>[16]</sup>. These biohistochemical studies have demonstrated that chronic inflammation caused by

**Table 3** Clinical characteristics of patients according to the two lengths of Barrett's epithelium *n* (%)

	The C extent of Barrett's epithelium		<i>P</i> value
	< 2 cm ( <i>n</i> = 347)	$\geq 2$ cm ( <i>n</i> = 27)	
Age			
Median; range (yr)	68; 31-91	70; 42-86	0.4933
Sex			
Female (%)	149 (42.9)	14 (51.9)	0.3683 <sup>1</sup>
BMI			
Median; range	22.5; 14.4-41.2	22.3; 16.9-28.2	0.7199
Regular drinking habit (%)	143 (41.2)	14 (51.9)	0.2805 <sup>1</sup>
Smoking habit (%)	161 (46.4)	13 (48.1)	0.8606 <sup>1</sup>
Hiatal hernia (%)	136 (39.2)	17 (63.0)	0.0155 <sup>1</sup>
GMA			
Open type (%)	177 (51.0)	12 (44.4)	0.5111 <sup>1</sup>
Erosive esophagitis (%)			
Total	109 (31.4)	16 (59.3)	0.0031 <sup>1</sup>
Mild	97 (28.0)	15 (55.6)	0.0026 <sup>1</sup>
Severe	12 (3.5)	1 (3.7)	> 0.9999 <sup>2</sup>

*P* value: Mann-Whitney *U* test; <sup>1</sup>:  $\chi^2$  test; <sup>2</sup>: Fisher's exact test.

gastroesophageal reflux disease (GERD) is an important factor in the etiology of carcinogenesis in Barrett's epithelium, which suggests an increase in the malignant potential of Barrett's epithelium, especially when it is accompanied by erosive esophagitis. The identification of a subgroup with a high risk for the development of esophageal adenocarcinoma may be helpful in developing more efficient screening programs for patients with Barrett's epithelium.

We hypothesized that some macroscopic features of Barrett's epithelium might be useful for identifying a subgroup with a high risk for the development of esophageal adenocarcinoma. Therefore, we conducted the present study with the aim of examining the correlation of the shape and length of Barrett's epithelium with erosive esophagitis.

The present study demonstrated that 43.0% (SSBE, 42.6%; LSBE, 0.5%) of the study population were diagnosed as having Barrett's epithelium based on the Prague C & M Criteria<sup>[12]</sup>. These findings were consistent with those from other reports in Japan in which SSBE was frequent, whereas LSBE was rare compared with the United States and Western Europe<sup>[20,21]</sup>. The frequency of Barrett's epithelium might be affected by differences in its definition, with particular regard as to whether histological confirmation of specialized intestinal metaplasia is required. In western countries, several physicians think that confirmation of intestinal metaplasia with an esophageal biopsy is needed to identify correctly the pathology as Barrett's epithelium<sup>[22]</sup>, because it is considered a risk factor for esophageal adenocarcinoma<sup>[23]</sup>. In this regard, the cases of Barrett's epithelium in the present study based on the Prague C & M Criteria<sup>[12]</sup> were diagnosed endoscopically without histological confirmation, and were defined as endoscopic Barrett's epithelium.



We elucidated a significant correlation between the shape and length of Barrett's epithelium and prevalence of erosive esophagitis. The prevalence of erosive esophagitis, especially severe esophagitis, was significantly higher in the subjects with F type than L type Barrett's epithelium (Table 2). F type Barrett's epithelium might originate as a direct result of columnar replacement of areas damaged by erosive esophagitis. It was possible that F type Barrett's epithelium had more severe esophagitis because less had been transformed into Barrett's epithelium, and when the transformation to columnar epithelium had taken place, the previous area with erosive esophagitis would naturally decrease. Yamagishi *et al.*<sup>[24]</sup> have reported that the localization of Barrett's epithelium was similar to the localization of mild esophagitis, which was the most prevalent form of erosive esophagitis in Japan, which suggests that tongue-like Barrett's epithelium arises as a result of erosive esophagitis. The prevalence of erosive esophagitis was significantly higher in subjects with LSBE (Table 3), which was consistent with several studies that have shown that the extent of Barrett's epithelium is correlated with severe esophageal exposure to acid (an increased percentage of the time during which the esophagus is exposed to a pH below 4)<sup>[25-27]</sup>. These findings may explain partly why LSBE has a higher risk of the development of esophageal adenocarcinoma. Harle *et al.*<sup>[28]</sup> and Ransom *et al.*<sup>[29]</sup> have suggested a positive relationship between the extension of Barrett's epithelium and the risk of developing adenocarcinoma in the esophagus. The Rotterdam Esophageal Tumor Study Group has demonstrated that a doubling of the length of Barrett's epithelium increased the risk of adenocarcinoma by 1.7 times<sup>[30]</sup>.

The present study demonstrated that F type Barrett's epithelium and LSBE were significantly more strongly associated with erosive esophagitis, as an important factor in the development of esophageal adenocarcinoma, which suggests that patients with Barrett's epithelium with these macroscopic features are at higher risk for carcinogenesis compared to other types. The development of esophageal adenocarcinoma is usually seen as a sequence - GERD - erosive esophagitis - Barrett's epithelium - low-grade dysplasia - high-grade dysplasia - esophageal adenocarcinoma. Barrett's epithelium with these macroscopic features may have a risk of complication of low- or high-grade dysplasia and development of esophageal adenocarcinoma.

Our study had several limitations that may need to be considered. First, the data were collected from a review of endoscope images. The shape and length of Barrett's epithelium evaluated in a retrospective fashion were undoubtedly subject to some uncertainty. To minimize this limitation, we assessed the extent of Barrett's epithelium based on the Prague C & M Criteria, which consist of two indicators: the C extent and M extent, and defined F type Barrett's epithelium by the criterion in which the difference between the C extent and M extent should be  $\geq 2$  cm. Second, the present study might not have had a large enough population to

examine in detail the association between the shape and length of Barrett's epithelium and erosive esophagitis. Third, a disadvantage was the lack of histopathological examination of the samples to confirm the diagnosis, and also the ability to classify further into low- and high-grade dysplasia. Further large population-based cohort studies with histopathological examination of Barrett's esophagus to classify into low- or high-grade dysplasia are needed to confirm this assumption.

In conclusion, F type Barrett's epithelium and LSBE were significantly more strongly associated with erosive esophagitis, as an important factor in the development of esophageal adenocarcinoma. The identification of a subgroup with these macroscopic features of Barrett's epithelium may be helpful in developing more efficient screening programs for patients with Barrett's epithelium. Further prospective large population-based cohort studies are needed to confirm this assumption.

## COMMENTS

### Background

Barrett's epithelium is recognized as a complication of erosive esophagitis and is the pre-malignant condition for adenocarcinoma of the esophagus.

### Research frontiers

The authors hypothesized that some macroscopic features of Barrett's epithelium might be useful for identifying a subgroup with a high risk for the development of esophageal adenocarcinoma.

### Innovations and breakthroughs

Recent studies have suggested that the major risk factor for esophageal adenocarcinoma is the existence of Barrett's epithelium complicated with erosive esophagitis. The prevalence of erosive esophagitis was significantly higher in subjects with flame-like than with lotus-like Barrett's epithelium, and in those with a C extent of  $\geq 2$  cm than  $< 2$  cm.

### Applications

By understanding the shape of Barrett's epithelium, this study may represent a future strategy for intervention in the prevention of esophageal adenocarcinoma.

### Terminology

The development of esophageal adenocarcinoma is usually seen as a sequence - gastroesophageal reflux disease - erosive esophagitis - Barrett's epithelium - low-grade dysplasia - high-grade dysplasia - esophageal adenocarcinoma. Barrett's epithelium with these macroscopic features may have a risk of complication of low- or high-grade dysplasia and development of esophageal adenocarcinoma.

### Peer review

This is well-written paper on the important subject of trying to identify those subjects with Barrett's esophagus who might develop esophageal adenocarcinoma. The structure is clear and the presentation is good.

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## Endoscopic retrograde cholangiopancreatography in pancreatic and biliary tract disease in Korean children

Joo Young Jang, Chong Hyun Yoon, Kyung Mo Kim

Joo Young Jang, Department of Pediatrics, Asan Medical Center Children's Hospital, University of Ulsan College of Medicine, Seoul 138-736, South Korea; Department of Pediatrics, Ajou University School of Medicine, Suwon 443-721, South Korea  
Chong Hyun Yoon, Department of Pediatric Radiology, Asan Medical Center Children's Hospital, University of Ulsan College of Medicine, Seoul 138-736, South Korea  
Kyung Mo Kim, Department of Pediatrics, Asan Medical Center Children's Hospital, University of Ulsan College of Medicine, Seoul 138-736, South Korea

Author contributions: Jang JY analyzed the data and wrote the paper; Kim KM designed the study, analyzed the data, wrote the paper, and performed the ERCP; Yoon CH provided the radiologic interpretation and confirmation of the results of ERCP.

Correspondence to: Kyung Mo Kim, MD, Department of Pediatrics, Asan Medical Center Children's Hospital, University of Ulsan, 388-1 Pungnap-Dong, Songpa-Gu, Seoul 138-736, South Korea. [kmkim@amc.seoul.kr](mailto:kmkim@amc.seoul.kr)

Telephone: +82-2-30103380 Fax: +82-2-4733725

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### Abstract

**AIM:** To assess the indications, findings, therapeutic procedures, safety, and complications of endoscopic retrograde cholangiopancreatography (ERCP) performed in Korean children.

**METHODS:** The demographic characteristics, indications for ERCP, findings, therapeutic procedures, and complications of 122 pediatric patients who underwent 245 ERCPs in the Asan Medical Center between June 1994 and March 2008 were investigated.

**RESULTS:** The mean age of the 122 patients was  $8.0 \pm 4.2$  years. Indications were biliary pathology in 78 (64.0%), pancreatic pathology in 43 (35.2%), and chronic abdominal pain in one. Biliary indications included choledochal cysts in 40, choledocholithiasis in 24, suspected sclerosing cholangitis in 8, trauma in 2, and other conditions in 4. Pancreatic indications included

acute pancreatitis in 7, acute recurrent pancreatitis in 11, chronic pancreatitis in 20, trauma in 3, and pancreatic mass in 2. Of the 245 ERCPs, success rate was 98.4% and 190 (77.6%) were for therapeutic purposes, including endoscopic nasal drainage (51.8%), biliary sphincterotomy (38.0%), pancreatic sphincterotomy (23.3%), stent insertion (15.1%), stone extraction (18.8%), and balloon dilatation (11.0%). Complications were post-ERCP pancreatitis in 16 (6.5%), ileus in 23 (9.4%), hemorrhage in 2 (0.8%), perforation in 2 (0.8%), sepsis in 1 (0.4%), and impacted basket in 1 (0.4%). There were no procedure-related deaths, and most complications improved under supportive care.

**CONCLUSION:** This study showed that there is a high incidence of choledochal cyst and diagnostic and therapeutic ERCP for the management of various biliary and pancreatic diseases was safe and effective in Korean children.

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**Key words:** Endoscopic retrograde cholangiopancreatography; Pancreatic diseases; Biliary tract diseases; Choledochal cyst; Pancreatitis; Pediatrics

**Peer reviewers:** De Aretxabala Xabier, Professor of Surgery, Universidad de Chile, Santos Dumont 999, Santiago, 8380000, Chile; Jose Sahel, Professor, Hepato-gastroenterology, Hospital Sainti Marevenite, 1270 Boulevard AE Sainti Margrenise, Marseille 13009, France

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### INTRODUCTION

Endoscopic retrograde cholangiography (ERCP) is

a standard diagnostic and therapeutic modality for pancreaticobiliary diseases in adults<sup>[1]</sup>. Its standard use in children, however, has been limited by technical difficulties, low incidence of pancreaticobiliary disease, and lack of knowledge of ERCP by pediatric surgeons and pediatricians<sup>[2]</sup>. Pediatric ERCP is used as a therapeutic tool in addition to its diagnostic applications<sup>[3,4]</sup>. Regional differences in indications have been observed for pediatric ERCP<sup>[3-7]</sup>. Although the frequency of choledochal cysts is higher in Asian than in Western populations, there have only been a few studies of pediatric ERCP in Asian countries, with each involving small numbers of patients<sup>[5,7]</sup>. We therefore describe our experience in performing 245 ERCPs in 122 Korean children with pancreaticobiliary diseases, focusing on the presenting diseases, diagnostic and therapeutic efficacy, and safety.

## MATERIALS AND METHODS

Between June 1994 and March 2008, 122 children (mean age,  $8.0 \pm 4.2$  years; range, 1 month to 16 years) underwent 245 ERCPs at the Asan Medical Center, Seoul, Korea. Of these 122 patients, 52 were boys and 70 were girls. We retrospectively reviewed the computerized hospital records of all patients to determine indications for ERCP, success of the procedure, diagnostic findings, therapeutic procedures, and complications. We also reviewed the basic characteristics of patients and the types of sedation used. Indications for ERCPs included biliary disease, pancreatic disease, and recurrent abdominal pain. ERCP was performed by standard techniques, using either a conventional adult duodenoscope (JF: Olympus America Inc.), a therapeutic duodenoscope (TJF: Olympus America Inc.), Pediatric duodenoscope (PJF-7.5E: Olympus America Inc.). The first few ERCPs were supervised by an adult gastroenterologist, with most subsequent procedures were performed by a pediatrician alone, except for some complicated cases. Informed, written consent was obtained from the parents of all patients, and this study was approved by our Internal Review Board. All ERCPs were performed under general anesthesia or deep sedation (midazolam, ketamine, and fentanyl). Successful ERCP was defined as cannulation of the bile duct or pancreatic duct along with completion of any planned diagnostic study or therapeutic procedure. Post-ERCP pancreatitis was defined as pancreatic abdominal pain with serum amylase/lipase elevated to over 3 times the upper normal limit.

## RESULTS

During the 14-year time period, we performed 245 ERCPs, including 55 (22.4%) diagnostic and 190 (77.6%) therapeutic procedures, on 122 children. Sixty-seven ERCPs (27.3%) were performed under general anesthesia and 178 (72.7%) under deep sedation. Of these 245 procedures, 241 (98.4%) resulted in successful cannulation. There were 78 (64.0%) patients with biliary indications,

Table 1 Indications for ERCP in 122 children *n* (%)

Indications	No. of patients	No. of procedures
Biliary indication	78 (64.0)	103 (42.0)
Choledochal cyst	40 (32.8)	60 (24.5)
Choledocholithiasis	24 (19.7)	27 (11.0)
Suspected sclerosing cholangitis	8 (6.6)	8 (3.3)
Trauma	2 (1.6)	4 (1.6)
Biliary complication after liver transplantation	2 (1.6)	2 (0.8)
Biliary stenosis after operation	1 (0.8)	1 (0.4)
Biliary atresia	1 (0.8)	1 (0.4)
Pancreatic indication	43 (35.2)	141 (57.6)
Acute pancreatitis	7 (5.7)	10 (4.1)
Acute recurrent pancreatitis	11 (19.7)	23 (9.4)
Chronic pancreatitis	20 (16.4)	102 (41.6)
Pancreatic mass	2 (1.6)	2 (0.8)
Trauma	3 (2.4)	4 (1.6)
Chronic abdominal pain	1 (0.8)	1 (0.4)
Total	122	245

ERCP: Endoscopic retrograde cholangiopancreatography.

43 (35.2%) with pancreatic indications, and one (0.8%) with chronic abdominal pain (Table 1).

### Biliary indications

The most common biliary indication was choledochal cyst (CC), also known as congenital biliary dilatation. Table 2 shows the diagnostic findings in 78 patients with biliary indications. Forty patients with choledochal cysts underwent 60 ERCPs. Choledochal cysts were divided to 4 types using the Todani classification<sup>[8]</sup>. There were 14 patients with type I c, 2 with type III, and 24 with type IV CC. Thirty of these 40 patients had bile duct stones (choledocholithiasis) and 9 had common bile duct (CBD) strictures. Anomalous union of the pancreaticobiliary duct (AUPBD) was detected in 25 patients with CC. Endoscopic sphincterotomy was performed on 2 CC type III patients without recurrence. Patients with acute biliary pancreatitis and choledocholithiasis underwent various procedures, including endoscopic sphincterotomy, endoscopic nasal biliary drainage, and stone extraction. ERCPs were performed for preoperative treatment or evaluation, except for one patient who underwent ERCP after surgery for removal of remnant stones. Two patients (one type Ic and one type IVa), who showed much improved bile duct dilatation after ERCP, underwent laparoscopic cholecystectomy. Eight type Ic patients and 18 type IVa patients underwent Roux-en-Y hepaticojejunostomy after acute pancreatitis or acute biliary colic had resolved. One type IVa patient showed improved bile duct dilatation and cholestasis after ERCP, but died of an underlying disease, acute myeloid leukemia. Ten patients, 6 of type Ic and 4 of type IVa, are well, with mild dilatation of the CBD; surgery is planned for each. Two patients with type I CC showed improved dilatation, with the disappearance of CC confirmed by ultrasound.

Of the 24 patients with choledocholithiasis, 19

**Table 2** Diagnostic findings in 78 patients with biliary indications

Diagnosis (number of patients)	ERCP findings							
	Bile duct stone	CBD dilatation	CBD stricture	SC	Bile leak	AUPBD	Normal	Failure
Choledochal cyst ( <i>n</i> = 40)	30	38	9			25		1
Choledocholithiasis ( <i>n</i> = 24)	24	19	4			6		
Suspected SC ( <i>n</i> = 8)				4			4	
Trauma ( <i>n</i> = 2)			1		1			
Biliary complication after liver transplantation ( <i>n</i> = 2)					1		1	
Biliary stenosis after operation ( <i>n</i> = 1)			1					1
Biliary atresia ( <i>n</i> = 1)								1

CPD: Common bile duct; SC: Sclerosing cholangitis; AUPBD: Anomalous union of the pancreaticobiliary duct.

**Table 3** Diagnostic findings in 43 patients with pancreatic indications

Diagnosis (number of patients)	ERCP findings									
	PD dilatation	PD stenosis	Pancreaticolith	PD anomaly	AUPBD	Pancreas divisum	Trans-section of pancreas	Other	Normal	Failure
Acute pancreatitis ( <i>n</i> = 7)		1	1					1 CBD stenosis	4	
Acute recurrent pancreatitis ( <i>n</i> = 11)		2		2	2	2			3	
Chronic pancreatitis ( <i>n</i> = 20)	16	11	6	1	1	8				1
Pancreatic mass ( <i>n</i> = 2)	1							1 SPPN		
Trauma ( <i>n</i> = 3)							3			

SPPN: Solid pseudopapillary neoplasm; PD: Pancreatic duct.

showed CBD dilatation, however they did not show the evidence of CC and 4 had CBD stricture. AUPBD was found in 6 patients. Only 2 patients had specific hematologic diseases, one each with hereditary spherocytosis and glucose-6-phosphate dehydrogenase (G6PD) deficiency. Eight patients underwent ERCP for suspected sclerosing cholangitis, but only 4 showed evidence of sclerosing cholangitis, with the other 4 showing no abnormalities. Two patients who experienced blunt trauma underwent ERCP; one had intrahepatic bile duct leakage and one had a benign CBD stricture. Two patients were suspected to have biliary complications after liver transplantation; one showed a bile leak and the other showed no abnormal findings. One patient had a CBD stricture after resection of a duodenal web. Cannulation was unsuccessful in one patient with biliary atresia.

### Pancreatic indications

The most common pancreatic indication was chronic pancreatitis, observed in 20 patients. Other pancreatic indications included acute pancreatitis (7 patients), acute recurrent pancreatitis (11 patients), pancreatic mass (2 patients), and trauma (3 patients). Table 3 shows the diagnostic findings in 43 patients with pancreatic indications. Pancreas divisum was observed in 10 patients, 7 of whom underwent repeated endoscopic minor pancreatic sphincterotomy because of minor duct stenosis. Of the 8 patients with acute pancreatitis, 4 showed normal findings, and one each showed evidence of pancreatic duct stenosis, pancreaticoliths, and CBD stenosis. ERCP on a boy aged 9 years, with pancreatitis and a suspected

intraductal papillary mucinous neoplasm, showed a bulge of the ampulla of Vater and large amounts of sticky mucinous materials, but histology after surgery showed only chronic pancreatitis. Of the 11 patients with acute recurrent pancreatitis, 2 showed pancreatic duct stenosis, 2 showed pancreatic duct anomalies, 2 had AUPBD, 2 had pancreas divisum, and 3 showed normal findings. Preoperative ERCP on 2 patients with pancreatic masses showed that one had a retroperitoneal teratoma with extrinsic distal CBD compression, and the other had a solid pseudopapillary neoplasm in the tail of the pancreas with abrupt interruption of the distal pancreatic duct. Three patients with traumatic pancreatitis showed transection of the pancreas.

### Abdominal pain

ERCP showed that one patient with recurrent abdominal pain had a normal anatomy of the pancreaticobiliary system. We did not perform the sphincter of Oddi manometry because of its technical problems and difficulties of translation with no definite normal range in children with ERCP.

### Therapeutic procedures

Of the 245 ERCPs, 127 (51.8%) were for endoscopic nasal drainage, 93 (38.0%) were for biliary sphincterotomy, and 57 (23.3%) were for pancreatic sphincterotomy. Stents were inserted during 37 procedures (15.1%), stone extraction was achieved during 46 (18.8%), balloon dilatation of a strictured CBD or pancreatic duct was performed in 27 (11.0%). Endoscopic drainage of a



Table 4 Therapeutic procedures during 245 ERCPs

Type of endoscopic therapy	Number of procedures (% of 245 cases)
Endoscopic nasal drainage	127 (51.8)
Biliary sphincterotomy	93 (38.0)
Pancreatic sphincterotomy	57 (23.3)
Stone extraction	46 (18.8)
Stent insertion	37 (15.1)
Balloon dilatation	27 (11.0)
Endoscopic drainage of pseudocyst	2 (0.8)

pseudocyst complicating acute pancreatitis was performed in 2 patients (Table 4). One had a pneumoperitoneum, which was improved by supportive care with nil by mouth. In early times, we placed the nasobiliary drain in patients but nowadays we prefer to insert 5 to 7 Fr plastic stents depending on age for biliary obstruction or septic cholangitis.

### Complications

Post-ERCP pancreatitis was observed after 16 procedures (6.5%), and post-ERCP ileus after 23 (9.4%). In 16 cases of post-procedure pancreatitis, there were 4 mild, 10 moderate, 2 severe cases and all cases developed early (a few hours) after ERCP by Cotton's definition<sup>[9]</sup>. Two patients had major episodes of hemorrhage at the sphincterotomy site, requiring red blood cell transfusion. These patients underwent additional ERCPs and epinephrine injections. Intestinal perforation developed in 2 patients. One, with perforation of the CC wall and bile leakage, underwent an emergency operation, and the other, with perforation of the posterior duodenal wall, improved following supportive care. Culture-proven sepsis was observed in one patient and an impacted basket in one. In the latter, the basket was broken during the removal of a large, firm, pancreatic duct stone, which was being managed by extracorporeal shock wave lithotripsy followed by endoscopic removal of the remaining basket with stones. There was no procedure-related mortality in our patients (Table 5).

## DISCUSSION

ERCP has become a new diagnostic and therapeutic modality in children with pancreaticobiliary disease<sup>[3,10]</sup>. Almost all studies, however, have been performed on Western children, with few in Asian children. The most common indications for ERCP in Western children are choledocholithiasis and pancreatitis<sup>[3,10]</sup>. Indications differ, however, in Asian countries<sup>[5,7,11]</sup>. For example, the most common indication in Saudi Arabia was choledocholithiasis in patients with sickle-cell anemia<sup>[11]</sup>, whereas the most frequent indication in Japan and India was CC<sup>[5,7]</sup>. As in Japan, the most common indication in our group of 122 Korean children was CC. Almost all patients with CC had acute biliary pancreatitis or obstructive jaundice with stone/sludge; these patients were treated by endoscopic sphincterotomy, stone removal, endoscopic nasal biliary

Table 5 Incidence of complications following 245 ERCPs

Type of complications	Number of episodes (% of 245 cases)
Pancreatitis	16 (6.5)
Ileus	23 (9.4)
Hemorrhage	2 (0.8)
Perforation	2 (0.8)
Sepsis	1 (0.4)
Impacted basket	1 (0.4)

drainage (ENBD), or stent insertion. We previously reported that biliary pancreatitis is a common cause of acute pancreatitis in Korean children and that intervention with ERCP may be a useful treatment modality<sup>[12]</sup>. We found that ERCP helped to improve pancreatitis and associated inflammation, and offered relief of acute symptoms. For this purpose, we performed ENBD or indwelling stent implantation prior to operation of CC. ERCP-guided interventions in patients with complicated CCs, combined with AUPBD, have been reported to be helpful in refractory cases and to optimize patient condition prior to definitive surgery<sup>[13-16]</sup>. In addition to symptomatic relief, preoperative ERCPs also may provide surgeons with more precise anatomical knowledge of the bile duct and the pancreatic duct, which may help during total surgical excision, the optimal treatment for CC. Surgeons require information on the length of the distal narrow portion of the dilated bile duct and clear visualization of its confluence with the pancreatic duct<sup>[7]</sup>. In addition, ERCP has been reported to be superior to magnetic resonance cholangiopancreatography (MRCP) in evaluation of minor ductal anomalies<sup>[17]</sup> although MRCP is a noninvasive, safe technique. Endoscopy is the treatment of choice for uncomplicated choledochocoele (type III)<sup>[18]</sup>, and we treated two such patients with endoscopic sphincterotomy.

A total of 33 patients had AUPBD, including 25 with CC, 6 with choledocholithiasis, and 2 with acute recurrent pancreatitis. Thirty patients underwent endoscopic sphincterotomy and drainage. The incidence of AUPBD in Asian patients undergoing ERCP has been reported to be 1.5%-2.6%<sup>[19]</sup>. These patients require surgical treatment, because biliary cancer can arise from chronic inflammation of the bile duct because of the reciprocal regurgitation of pancreatic and bile juice reflux, although endoscopic sphincterotomy can be considered for most symptomatic patients<sup>[16]</sup>. Using an AUPBD puppy model, sphincteroplasty was shown to be effective in reducing bile duct dilatation and mucosal hyperplasia<sup>[20]</sup>. Prophylactic cholecystectomy is considered the best treatment option for patients with AUPBD but without bile duct dilatation<sup>[21]</sup>. We found that bile duct dilatation improved in 2 such patients, both of whom underwent laparoscopic cholecystectomy.

Choledocholithiasis is the second most frequent biliary indication for ERCP. Such patients were treated by endoscopic sphincterotomy and stone removal with basket/balloon extraction. Only 2 patients had hematologic diseases: hereditary spherocytosis and G6PD deficiency.

ERCP is considered the treatment of choice in children with CBD stones<sup>[11]</sup>. One patient with both CBD and gallbladder stones underwent endoscopic CBD stone removal and subsequent laparoscopic cholecystectomy for gallbladder stones or sludges. Therefore, surgery was minimally invasive.

The most common pancreatic indication in our study was chronic pancreatitis, observed in 20 patients who underwent 102 ERCPs (40.8%; mean 5.2 ERCPs per patient). These patients underwent repeated sphincterotomy with nasal drainage, and stents were placed in 9 patients.

Pancreas divisum was observed in 10 patients with pancreatic indications, 8 with chronic pancreatitis and 2 with acute recurrent pancreatitis. ERCP is a useful diagnostic and therapeutic procedure in patients with pancreas divisum<sup>[22]</sup>. Minor papilla sphincterotomy and stent insertion can decrease the rate of recurrent acute pancreatitis<sup>[23]</sup>. We performed minor duct sphincterotomy and inserted stents in 7 of these 10 patients, without specific complications.

Other pancreatic indications included suspected intraductal mucinous pancreatic adenoma, which was revealed to be chronic pancreatitis, pancreatic duct lesions related to a pancreatic mass, and transected pancreas arising from trauma. Although recurrent abdominal pain has been reported to be a common indication for ERCP<sup>[3]</sup>, we found that only one patient underwent ERCP for suspected pancreatic abdominal pain and that, on ERCP, this patient showed normal findings.

Among the ERCP-associated procedures now becoming therapeutic options in children with pancreaticobiliary disease are sphincterotomy, balloon dilatation, stone extraction, stent insertion, and nasal drainage<sup>[2,3,6,10,11]</sup>. We also performed various therapeutic procedures (Table 4), almost all of which relieved acute symptoms, as well as revealing more exact anatomy to the pediatric surgeons.

ERCPs performed by expert endoscopists in children have been reported to have similar success rates and lower complication rates compared with ERCPs in adult patients<sup>[4]</sup>. Although we experienced some complications, there was no procedure-related mortality. Post-ERCP pancreatitis occurred after 16 ERCPs (6.5%) however all showed improvement with supportive care. The incidence of pancreatitis following ERCPs in adults has been reported to be 2.6%–13.1%<sup>[24–26]</sup>.

In conclusion, diagnostic and therapeutic ERCPs were performed safely and effectively in Korean children for the management of various biliary and pancreatic diseases. Pediatricians and pediatric surgeons, especially those working in Asian countries, should become more familiar with ERCP as a diagnostic and therapeutic modality, as Asia has a high incidence of CCs and anomalous union of the pancreaticobiliary duct.

## COMMENTS

### Background

Endoscopic retrograde cholangiography (ERCP) is a standard diagnostic and therapeutic modality for pancreaticobiliary disease. Recent reports showed the

application of ERCP in therapeutic purposes besides diagnostic evaluations in pediatric patients.

### Research frontiers

This study is the first report on ERCP in a large number of Korean pediatric patients.

### Innovations and breakthroughs

The authors demonstrated that diagnostic and therapeutic ERCPs were performed safely and effectively for managements of various biliary and pancreatic diseases.

### Applications

ERCP can be considered as an effective and safe modality in children with pancreatobiliary disease.

### Peer review

In this study, ERCPs were performed in a large number of Korean children with various biliary and pancreatic diseases safely and effectively. Pediatricians and pediatric surgeons, especially those working in Asian countries, should become more familiar with ERCP as a diagnostic and therapeutic modality, as Asia has a high incidence of CCs and anomalous union of the pancreaticobiliary duct.

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## Mucosal patterns of *Helicobacter pylori*-related gastritis without atrophy in the gastric corpus using standard endoscopy

Sheng-Lei Yan, Shwu-Tzy Wu, Chien-Hua Chen, Yeh-Huang Hung, Tsung-Hsun Yang, Yun-Siew Pang, Yung-Hsiang Yeh

Sheng-Lei Yan, Yeh-Huang Hung, Tsung-Hsun Yang, Yun-Siew Pang, Yung-Hsiang Yeh, Division of Gastroenterology, Department of Internal Medicine, Chang Bing Show-Chwan Memorial Hospital, Changhua County 505, Taiwan, China  
Sheng-Lei Yan, Shwu-Tzy Wu, Department and Graduate Program of Bioindustrial Technology, Dayeh University, Changhua County 51591, Taiwan, China

Chien-Hua Chen, Division of Gastroenterology, Department of Internal Medicine, Show-Chwan Memorial Hospital, Changhua City 500, Taiwan, China

Author contributions: Yan SL drafted the article; Wu ST critically reviewed the content of the article; Hung YH, Yang TH and Pang VS performed endoscopic image evaluation in reproducibility study; Chen CH contributed to analysis and interpretation of the data; Yeh YH finally approved the publication of the article.

Correspondence to: Yung-Hsiang Yeh, MD, Division of Gastroenterology, Department of Internal Medicine, Chang Bing Show-Chwan Memorial Hospital, No. 6, Lugong Rd., Lugang Township, Changhua County 505, Taiwan, China. [yslendo@yahoo.com](mailto:yslendo@yahoo.com)

Telephone: +886-4-7073237 Fax: +886-4-7073226

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were categorized into 4 types. Type 1 pattern was defined as cleft-like appearance, type 2 as regular arrangement of red dots, type 3 pattern as the mosaic mucosal pattern and type 4 pattern as the mosaic pattern with a focal area of hyperemia. Type 1 and type 2 mucosal patterns were statistically significant in predicting *H. pylori*-negative status as compared with other mucosal types ( $\chi^2 = 12.79$  and  $61.25$  respectively,  $P < 0.01$ ). Type 3 and type 4 mucosal patterns were statistically significant in predicting a *H. pylori*-positive status as compared with other mucosal types ( $\chi^2 = 21.22$  and  $11.02$  respectively,  $P < 0.01$ ). Furthermore, the sensitivity, specificity, positive and negative predictive values of type 3 plus type 4 patterns for predicting *H. pylori*-positive gastric mucosa were 100%, 86%, 94%, and 100%, respectively. The mean  $\kappa$  values for inter- and intra-observer agreement in assessing the various endoscopic patterns were 0.808 (95% CI, 0.678-0.938) and 0.826 (95% CI, 0.727-0.925) respectively.

**CONCLUSION:** Our study suggests that mucosal patterns in *H. pylori*-infected gastric mucosa without atrophy can be reliably identified using standard endoscopy in the gastric corpus.

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### Abstract

**AIM:** To identify the mucosal patterns of *Helicobacter pylori* (*H. pylori*)-related gastritis in the gastric corpus using standard endoscopy and to evaluate their reproducibility.

**METHODS:** A total of 112 consecutive patients underwent upper gastrointestinal endoscopy. The endoscopists classified the endoscopic findings into 4 patterns. In the second part of the study, 90 images were shown to 3 endoscopists in order to evaluate the inter-observer and intra-observer variability in image assessment.

**RESULTS:** The mucosal patterns of the gastric body

**Key words:** Gastric mucosa; *Helicobacter pylori*; Gastritis; Digestive system diagnostic techniques; Endoscopy

**Peer reviewer:** Joseph J Cullen, MD, Professor, Department of Surgery, University of Iowa Carver College of Medicine, 4605 JCP, University of Iowa Hospitals and Clinics, 200 Hawkins Drive, Iowa City, IA 52242, United States

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## INTRODUCTION

After the discovery of *Helicobacter pylori* (*H. pylori*) in 1983, strong evidence has indicated that *H. pylori* infection plays an important role in the pathogenesis of chronic gastritis, peptic ulcer and gastric carcinoma<sup>[1,2]</sup>. *H. pylori* infection can be diagnosed by invasive and noninvasive techniques; however, at least 2 different tests are necessary to make the diagnosis of infection according to the European guidelines<sup>[3]</sup>. Although endoscopic features of *H. pylori* have been reported in the literature, there is still some debate over whether *H. pylori*-related gastritis can be diagnosed *via* endoscopic features alone. Most studies concluded that it is not possible to diagnose *H. pylori*-related gastritis on the basis of endoscopic findings<sup>[4-7]</sup>. Recent publications suggest that high resolution magnification endoscopy has been proved to be useful in the identification of normal gastric mucosa and *H. pylori*-related gastritis<sup>[8-10]</sup>. However, practicing high resolution magnification endoscopy in daily endoscopy examinations seems not to be feasible, because it takes more examination time and needs more training and experience. If specific mucosal patterns of *H. pylori*-related gastritis can be identified using standard endoscopy, they may be applicable to targeted biopsy of suspected *H. pylori* infection in daily practice. Up to the present, there have been no reports regarding specific mucosal patterns of *H. pylori*-related gastritis in the gastric corpus using standard endoscopy.

The aim of this study was to classify the mucosal patterns of *H. pylori*-related gastritis in the gastric corpus using standard endoscopy and to evaluate their reproducibility.

## MATERIALS AND METHODS

A pilot phase of this study was conducted from May 2007 to July 2007, in which 2 experienced endoscopists observed mucosal morphology of the gastric body, and agreed on the classification of mucosal patterns. From August 2007 to February 2008, a total of 112 consecutive patients who underwent upper gastrointestinal endoscopy for the investigation of dyspeptic symptoms were enrolled in the study. The exclusion criteria were the following: anemia; history of cirrhosis; use of certain drugs, including non-steroidal antiinflammatory drugs, proton pump inhibitors, and H2-receptor antagonists; bleeding tendency; a history of gastric surgery; and a history of eradication of *H. pylori*. The study was performed in accordance with the principles of the Declaration of Helsinki, and approved by the audit department of our institution.

### Endoscopic findings

Premedication was the same as for conventional upper gastrointestinal (GI) endoscopic examination. The endoscopic procedures were performed using an upper GI videoendoscope (Fujinon Corporation, Saitama, Japan). The whole stomach was examined first with conventional

endoscopy. The gastric corpus was chosen for observation according to previous studies using magnification endoscopy<sup>[8-10]</sup>. The observed mucosal morphology of the gastric body was classified into 4 patterns. Type 1 was defined as cleft-like appearance mainly extending along the longitudinal axis of gastric body (Figure 1A). Type 2 was defined as regular arrangement of red dots (Figure 1B). Type 3 was defined as the mosaic mucosal pattern without a focal area of hyperemia (Figure 1C). Type 4 was defined as the mosaic pattern with a focal area of hyperemia (Figure 1D). Four biopsy samples were taken directly from the observation sites as shown in Figure 1. Two samples were sent for histological analysis and 2 for a rapid urease test (Hpfast, GI Supply, Camp Hill, PA, USA). If a gastric ulcer or gastric atrophy was present, the nearby non-atrophic mucosa was chosen for observation and biopsy sampling. All endoscopies were performed by 2 experienced endoscopists (Yan SL and Chen CH), who were unaware of the results of histology and rapid urease tests before determining the mucosal types of examined patients. The sensitivity, specificity, positive and negative predictive values of various mucosal types were calculated.

### Histological analysis

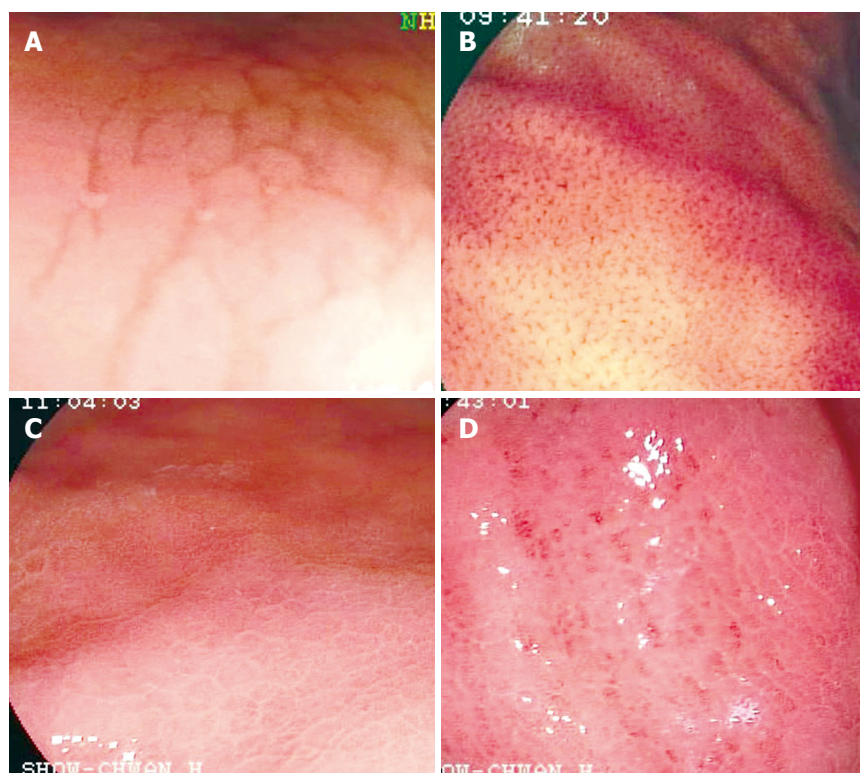
Specimens for histological analysis were placed in 10% formalin solution and routinely processed. The hematoxylin and eosin stain and Giemsa stain were used for identification of *H. pylori*. The pathologist was blinded to the clinical and endoscopic findings but was aware of the region in the stomach where each biopsy specimen had been obtained.

### Diagnosis of *H. pylori* infection

A diagnosis of *H. pylori* infection was made if *H. pylori* were seen on histopathological examination and the rapid urease test was positive. Patients with negative results in both examinations were considered to be *H. pylori*-negative. According to the European guidelines for the diagnosis of *H. pylori* infection<sup>[3]</sup>, patients were excluded from the study when they had only one positive result of the rapid urease test or histological examination.

### Image evaluation

All endoscopic examinations were digitally recorded and still images of observation site were captured for use in the reproducibility study. The selected images were transferred to a software program without distorting brightness, contrast or color balance. Three endoscopists (Hung YH, Yang TH and Pang VS) who had performed over 2000 upper endoscopies were invited to review these still images and were asked to classify them into type 1 to type 4 mucosal patterns as described above. All endoscopists were blinded to the result of *H. pylori* status and histology before reviewing the slides. A total of 110 images were selected and 20 of these were shown to endoscopists as a reference guide to the 4 types of mucosal pattern. The remaining 90 images (3 type 1, 19 type 2, 50 type 3, 18 type 4) were shown to each endoscopist independently. One week after initial



**Figure 1** Mucosal patterns. A: Type 1 mucosal pattern showing cleft-like appearance; B: Type 2 mucosal pattern showing regular arrangement of red dots; C: Type 3 mucosal pattern showing mosaic appearance; D: Type 4 mucosal pattern showing mosaic appearance with focal area of hyperemia.

**Table 1** Correlation between *H. pylori* infection and the observed mucosal patterns

Pattern	<i>H. pylori</i> (+)	<i>H. pylori</i> (-)	Total
Type 1	0	7	7
Type 2	0	24	24
Type 3	54	5	59
Type 4	22	0	22
Total	76	36	112

assessment, all endoscopists had to reassess the same images in a random sequence. No time limit for reviewing the slide was imposed. The endoscopists recorded their results on a preprinted form. Data obtained were used for calculation of inter- and intra-observer variabilities.

### Statistical analysis

Statistical analysis was performed using Minitab 14.1 (Minitab Incorporated, Pennsylvania, USA). Inter-group differences were evaluated by the  $\chi^2$  test. A *P* value < 0.05 was considered to be statistically significant. The sensitivity, specificity, positive and negative predictive values of the various mucosal patterns were calculated. To examine the chance-adjusted agreement, the  $\kappa$  value was calculated for inter- and intra-observer variabilities. Inter-observer variation was calculated from the results of the first reading, with 3 pairs in all. Intra-observer variation was determined by comparing the first and second assessment for each endoscopist, with 3 pairs in all.  $\kappa$  values below 0.4 indicated poor agreement, values between 0.4 and 0.8 represent moderate agreement, values between 0.6 and 0.8 represented substantial agreement,

and values greater than 0.8 corresponded to excellent agreement.

## RESULTS

A total of 112 consecutive patients (59 men, 53 women; mean age 47.0 years, range 17-91 years) were enrolled in the study from August 2007 to February 2008. Of the 112 patients included, 7 patients showed type 1 pattern, 24 patients showed type 2, 59 patients showed type 3, and 22 patients showed type 4 (Table 1). *H. pylori* infection was demonstrated by a positive result of the rapid urease test and histological examination in 76 patients (68%).

### Type 1 findings

All 7 patients with a type 1 mucosal pattern corresponded to an *H. pylori*-negative stomach (type 1 *vs* other mucosal types,  $\chi^2 = 12.79$ , *P* < 0.01). The sensitivity, specificity, positive and negative predictive values of the type 1 pattern for predicting *H. pylori*-negative gastric mucosa were 20%, 100%, 100%, and 72%, respectively (Table 2).

### Type 2 findings

All 24 patients with a type 2 mucosal pattern corresponded to an *H. pylori*-negative stomach (type 2 *vs* other mucosal types,  $\chi^2 = 61.25$ , *P* < 0.01). The sensitivity, specificity, positive and negative predictive values of the type 2 pattern for predicting *H. pylori*-negative gastric mucosa were 67%, 100%, 100%, and 86%, respectively (Table 2).

### Type 3 findings

Fifty four out of 59 patients with a type 3 mucosal pattern

**Table 2** The value of various mucosal patterns in predicting negative and positive *H. pylori* status (%)

Pattern predicting	Sensitivity	Specificity	Positive predictive value	Negative predictive value
<i>H. pylori</i> (-)				
Type 1	20	100	100	72
Type 2	67	100	100	86
Type 3	14	29	8	42
<i>H. pylori</i> (+)				
Type 3	71	86	92	58
Type 4	29	100	100	40
Type 3+4	100	86	94	100

corresponded to an *H. pylori*-positive stomach (type 3 *vs* other mucosal types,  $\chi^2 = 21.22$ ,  $P < 0.01$ ). The sensitivity, specificity, positive and negative predictive values of type 3 pattern for predicting *H. pylori*-positive gastric mucosa were 71%, 86%, 92%, and 58%, respectively (Table 2).

#### Type 4 findings

All 22 patients with a type 4 pattern corresponded to an *H. pylori*-positive stomach (type 4 *vs* other mucosal types,  $\chi^2 = 11.02$ ,  $P < 0.01$ ). The sensitivity, specificity, positive and negative predictive values of type 4 pattern for predicting *H. pylori*-positive gastric mucosa were 29%, 100%, 100%, and 40%, respectively (Table 2).

Type 3 and type 4 patterns were combined for analysis because these 2 mucosal types were generally mosaic in appearance. A combination of these 2 mucosal types was statistically significant in predicting *H. pylori*-positive status as compared with other mucosal types ( $\chi^2 = 82.80$ ,  $P < 0.01$ ). The sensitivity, specificity, positive and negative predictive values of type 3 plus type 4 patterns for predicting *H. pylori*-positive gastric mucosa were 100%, 86%, 94%, and 100%, respectively (Table 2).

#### Inter- and intra-observer variability assessment

The mean  $\kappa$  values for inter- and intra-observer agreement in assessing the various endoscopic patterns were 0.808 (95% CI: 0.678-0.938) and 0.826 (95% CI: 0.727-0.925), respectively.

## DISCUSSION

Few studies have addressed the endoscopic features of *Helicobacter*-related gastritis and most of these studies concluded that *H. pylori* infection cannot be diagnosed based on endoscopic findings alone<sup>[4-7]</sup>. Recently, Yagi *et al*<sup>[9]</sup> first described the characteristic magnification endoscopic findings of the *H. pylori*-negative stomach. Further, Anagnostopoulos *et al*<sup>[8]</sup> demonstrated the usefulness of magnifying endoscopy in the identification of *H. pylori*-associated gastritis in a Western population. However, practicing magnification endoscopy takes more examination time and needs more training and experience. It seems not to be feasible to practice magnification endoscopy in daily endoscopy examinations.

In our study, mucosal patterns of the gastric corpus were classified into 4 patterns using standard endoscopy. The type 1 pattern corresponded to *H. pylori*-negative gastric mucosa with a specificity of 100% but a rather low sensitivity of 20%. The type 2 pattern corresponded to *H. pylori*-negative gastric mucosa with a sensitivity of 67% and a specificity of 100%. Type 1 and type 2 mucosal patterns were both statistically significant in predicting *H. pylori*-negative status compared to other mucosal types ( $P < 0.01$ ). The red-dot appearance of the type 2 mucosal pattern represented the regular arrangement of collecting venules (RAC) of the gastric corpus under magnification endoscopy. Yagi *et al*<sup>[9]</sup> first proposed this magnification endoscopic finding as the normal gastric mucosa. In their study, RAC had a sensitivity of 93.8% and a specificity of 96.2% as an indicator of the *H. pylori*-negative stomach. Nakagawa *et al*<sup>[11]</sup> further classified the morphology of collecting venules into 3 magnification endoscopic patterns: regular (R), irregular (I), and obscure (O). The R pattern corresponded to *H. pylori*-negative gastric mucosa with a sensitivity of 66.7% and a specificity of 100% in the greater curvature of the gastric body.

Type 3 and type 4 mucosal patterns were both statistically significant in predicting *H. pylori*-positive status as compared with other mucosal types ( $P < 0.01$ ). Because type 3 and type 4 were generally mosaic in appearance, these 2 mucosal patterns were further combined for analysis, yielding a higher sensitivity of 100%, and a specificity of 86%. The positive and negative predictive values for predicting *H. pylori*-positive gastric mucosa were 94%, and 100%, respectively. The mosaic mucosa pattern in our study was similar to prominent areae gastricae, which has been shown to be a characteristic double contrast radiological finding in gastritis caused by *H. pylori* infection<sup>[12]</sup>. Furthermore, the prominent areae gastricae observed in our study is also similar to the mosaic mucosal pattern in patients with portal hypertension<sup>[13]</sup>. However, patients with cirrhosis were excluded in our study. Therefore, the mosaic mucosal pattern in our study seems to be a good indicator in predicting *H. pylori*-positive gastric mucosa in the gastric corpus.

The reproducibility of a classification system is very important in clinical practice. The  $\kappa$  values below 0.4 correspond to poor agreement, values between 0.4 and 0.8 indicate moderate agreement, values between 0.6 and 0.8 represent substantial agreement, and values greater than 0.8 correspond to excellent agreement. In our reproducibility study, the mean  $\kappa$  values for inter- and intra-observer agreement in assessing the various endoscopic patterns were 0.808 (95% CI: 0.678-0.938) and 0.826 (95% CI: 0.727-0.925) respectively. Therefore, our classification system is good to excellent.

In conclusion, our study suggests that mucosal patterns in *H. pylori*-infected gastric mucosa without atrophy can be reliably identified using standard endoscopy in the gastric corpus.



## COMMENTS

### Background

Although endoscopic features of *Helicobacter pylori* (*H. pylori*) infection have been reported in the literature, most studies concluded that it is not possible to diagnose *H. pylori*-related gastritis on the basis of endoscopic findings alone. Recent studies suggest that high resolution magnification endoscopy has been proved to be useful in the identifying of normal gastric mucosa and *H. pylori*-related gastritis. However, there have been no reports regarding specific mucosal patterns of *H. pylori*-related gastritis in the gastric corpus using standard endoscope.

### Research frontiers

Although high resolution magnification endoscopy has been proved to be useful in the identifying of normal gastric mucosa and *H. pylori*-related gastritis, practicing high resolution magnification endoscopy in daily endoscopy examinations takes more examination time and needs more training and experience. If specific mucosal patterns of *H. pylori*-related gastritis can be identified using standard endoscope, they may be applicable to targeted biopsy of suspected *H. pylori* infection in daily endoscopy examinations.

### Innovations and breakthroughs

In this study, endoscopic findings in the gastric corpus without atrophy were classified into 4 patterns. Type 3 and type 4 patterns were generally mosaic in appearance. The sensitivity, specificity, positive and negative predictive values of type 3 plus type 4 patterns for predicting *H. pylori*-positive gastric mucosa were 100%, 86%, 94%, and 100%, respectively.

### Applications

The result of this study suggests that the mosaic mucosal pattern in the gastric corpus seems to be a good indicator in predicting *H. pylori*-positive gastric mucosa and may guide endoscopists to targeted biopsy of suspected *H. pylori* infection.

### Peer review

This is an interesting paper that may be very beneficial for the gastroenterologist who performs significant numbers of endoscopy and treats patients with *H. pylori* infection.

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## Staging of liver fibrosis in chronic hepatitis B patients with a composite predictive model: A comparative study

Sheng-Di Wu, Ji-Yao Wang, Lei Li

Sheng-Di Wu, Ji-Yao Wang, Lei Li, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Zhongshan Hospital, Fudan University, Shanghai 200032, China

Author contributions: Wang JY designed and conducted the study, accessed all the data and took the full responsibility for the data analysis; Wu SD and Li L collected and analyzed the data; Wu SD wrote the manuscript; Wang JY revised the paper and improved the English; all authors participated in reviewing and revising the manuscript.

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Correspondence to: Ji-Yao Wang, Professor, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Zhongshan Hospital, Fudan University, Shanghai 200032, China. [wang.jiyao@gmail.com](mailto:wang.jiyao@gmail.com)

Telephone: +86-21-64041990 Fax: +86-21-64432583

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### Abstract

**AIM:** To evaluate the efficacy of 6 noninvasive liver fibrosis models and to identify the most valuable model for the prediction of liver fibrosis stage in chronic hepatitis B (CHB) patients.

**METHODS:** Seventy-eight CHB patients were consecutively enrolled in this study. Liver biopsy was performed and blood serum was obtained at admission. Histological diagnosis was made according to the METAVIR system. Significant fibrosis was defined as stage score  $\geq 2$ , severe fibrosis as stage score  $\geq 3$ . The diagnostic accuracy of 6 noninvasive liver fibrosis models, including serum aspartate aminotransferase (AST) to platelet ratio index (APRI), FIB-4, Forn's index, Fibrometer, Hepascore, and Shanghai Liver Fibrosis Group's index (SLFG), was investigated.

**RESULTS:** The APRI, FIB-4 and Forn's index under receiver operating characteristic curve (AUROC) for sig-

nificant fibrosis were 0.71, 0.75 and 0.79, respectively, with a diagnosis accuracy of 67%, 77% and 80%, respectively, and 0.80, 0.87 and 0.86, respectively, under the AUROC for severe fibrosis. The Hepascore, SLFG, and Fibrometer were 0.80, 0.83 and 0.85, respectively under the AUROC for significant fibrosis ( $P < 0.01$ ). The diagnosis accuracy of Hepascore and SLFG was 86% and 88%, respectively. The Hepascore, SLFG, and Fibrometer were 0.95, 0.93, and 0.94, respectively, under the AUROC for severe fibrosis ( $P < 0.01$ ).

**CONCLUSION:** The models containing direct serum markers have a better diagnostic value than those not containing direct serum markers.

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**Key words:** Chronic hepatitis B; Liver fibrosis; Serum marker; Noninvasive model; Receiver operating curve

**Peer reviewers:** Damiao Carlos Moraes Santos, DCM, PhD, Bio-Manguinhos, Fundação Oswaldo Cruz, Avenida Brasil 4365, Manguinhos, Rio de Janeiro, 21040360, Brazil; Roberto J Carvalho-Filho, MD, PhD, Hepatitis Section, Division of Gastroenterology, Federal University of Sao Paulo, Rua Botucatu, 740, 2.o andar, Vila Clementino, State of Sao Paulo, 04023-060, Brazil

Wu SD, Wang JY, Li L. Staging of liver fibrosis in chronic hepatitis B patients with a composite predictive model: A comparative study. *World J Gastroenterol* 2010; 16(4): 501-507 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i4/501.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i4.501>

### INTRODUCTION

Chronic hepatitis B virus (HBV) infection affects 350 million individuals worldwide. At least one million people chronically infected with HBV would die of chronic liver diseases each year<sup>[1]</sup>. Thus, it is important to prevent the progression of early liver fibrosis to

cirrhosis<sup>[2]</sup>. Although liver biopsy is the gold standard for the assessment of fibrosis, it has several disadvantages, such as poor patient compliance, sampling error, limited usefulness for dynamic surveillance, and poor intra- and inter-observation concordance<sup>[3-5]</sup>. Considering these limitations, noninvasive histology predictors are urgently needed<sup>[6]</sup>.

Since single fibrosis surrogate cannot measure fibrosis, an alternative approach combined with a number of parameters can generate algorithms capable of evaluating fibrosis. A number of noninvasive models containing serum markers, such as serum aspartate aminotransferase (AST) to platelet ratio index (APRI), FIB-4, Forn's index, Fibrometer, Hepascore, Shanghai Liver Fibrosis Group's index (SLFG) have been studied worldwide<sup>[7-12]</sup>. Additionally, except for SLFG, little has been known about the role of these models in predicting fibrosis stage of chronic hepatitis B (CHB) because most studies were performed in chronic hepatitis C (CHC). China has a high prevalence of CHB, and most hepatocellular carcinomas result from chronic HBV infection. Therefore, we carried out this study to identify the best practical noninvasive model of liver fibrosis in CHB.

## MATERIALS AND METHODS

### Patients

Seventy-eight consecutive eligible CHB patients who underwent a liver biopsy in March 2006-August 2008 at Zhongshan Hospital, Fudan University, Shanghai, China, were included in this study. Blood serum was collected and stored at -80°C for further test. Chronic HBV infection was diagnosed based on positive surface antigen of HBV (HBsAg) and fluctuated alanine aminotransferase. Exclusion criteria included chronic liver disease due to other causes or co-infection with hepatitis D, clinically overt cirrhosis, previous or concomitant anti-HBV therapy, alcohol consumption exceeding 20 g/d in men and exceeding 10 g/d in women. Data were retrospectively analyzed. The study protocol was approved by the Institutional Review Board in our hospital. Written informed consent was obtained from each patient.

### Liver histology and quantification of fibrosis

Liver tissue was obtained by sono-guided percutaneous biopsy (Bard®, Magnum®, 18G, USA) and stained with hematoxylin-eosin-safran and Masson's trichrome. Fibrosis staging (F) and inflammatory activity (A) were decided according to the METAVIR system. Fibrosis staging was divided into F0-F4 (F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = periportal fibrosis with few septa, F3 = septal fibrosis with many septa, and F4 = cirrhosis). Inflammatory activity was divided into A0-A3 (A0 = no histologic necroinflammatory activity, A1 = minimal activity, A2 = moderate activity, A3 = severe activity). The activity was assessed by integrating the severity and intensity of piecemeal (periportal) and lobular necrosis<sup>[13]</sup>. According to the American Association for the Study of Liver Disease Practice

Guidelines, we defined significant fibrosis as METAVIR fibrosis with a score  $\geq 2$  (F2, 3, 4) and severe liver fibrosis as METAVIR fibrosis with a score  $\geq 3$  (F3, 4)<sup>[14]</sup>.

### Serum parameters

Following parameters, including AST, alanine amino-transferase (ALT),  $\gamma$ -glutamyl transpeptidase (GGT), bilirubin, total cholesterol, urea, prothrombin time (PT), prothrombin index (PI), hemoglobin and platelet count (PLT) were assayed. AST, ALT, GGT, bilirubin, total cholesterol, urea were tested with Hitachi 7600, Japan. PT and PI were tested with Sysmex CA7000, Japan. Hemoglobin and PLT was tested with Sysmex routine blood test pipeline, Japan. The reference value was 0-75 IU/L for ALT and AST. Serum  $\alpha_2$ -macroglobulin (A<sub>2</sub>M) (GenWay Biotech, San Diego, USA) and hyaluronic acid (HA) (Shanghai High Medical Biotech, Shanghai, China) concentrations were measured by enzyme linked immunosorbent assay.

APRI, FIB-4, Forn's index, SLFG, Hepascore, and Fibrometer were detected according to the following formulas:  $APRI = [AST \text{ (U/LN)}/PLT \text{ (10}^9\text{/L)}] \times 100$ ,  $FIB-4 = [age \text{ (yr)} \times AST \text{ (U/L)}]/\{[PLT \text{ (10}^9\text{/L)}] \times [ALT \text{ (U/L)}]^{1/2}\}$ ,  $Forn's \text{ index} = 7.811 - 3.131 \times \ln [PLT \text{ (10}^9\text{/L)}] + 0.781 \times \ln [GGT \text{ (U/L)}] + 3.467 \times \ln [age \text{ (yr)}] - 0.014 \times [cholesterol \text{ (g/L)}]$ ,  $SLFG = 10 \times e^Y/(1+e^Y)$ ,  $Y = -13.995 + 3.220 \times \lg [A_2M \text{ (g/L)}] + 3.096 \times \lg [age \text{ (yr)}] + 2.254 \times \lg [GGT \text{ (U/L)}] + 2.437 \times \lg [HA \text{ (ng/mL)}]$ ,  $Hepascore = e^Y/(1+e^Y)$ ,  $Y = -4.185818 - [0.0249 \times age \text{ (yr)}] + [0.7464 \times sex \text{ (male = 1, female = 0)}] + [1.0039 \times A_2M \text{ (g/L)}] + [0.0302 \times HA \text{ (ng/mL)}] + [0.0691 \times TB \text{ (}\mu\text{mol/L)}] - [0.0012 \times GGT \text{ (U/L)}]$ ;  $Fibrometer = -0.007 \times PLT \text{ (10}^9\text{/L)} - 0.049 \times PI \text{ (\%)} + 0.012 \times AST \text{ (U/L)} + 0.005 \times A_2M \text{ (g/L)} + 0.021 \times HA \text{ (ng/mL)} - 0.270 \times urea \text{ (mmol/L)} + 0.027 \times Age \text{ (yr)} + 3.718$ .

### Statistical analysis

Statistical analysis was performed using Spearman's two-tail test and univariate analysis.  $P < 0.05$  was considered statistically significant. Sensitivity, specificity, positive and negative predictive values (NPV and PPV) were calculated by using cutoffs according to the original studies. The overall diagnostic performance of scores was evaluated by area under ROC curves (AUROCs). We used DANA method, which was developed by Poynard *et al.*<sup>[15,16]</sup>, to adjust the observed AUROCs in our study. All the AUROCs were adjusted to a standard DANA of 2.5 using the formula: Adjusted AUROC (AdAUC) = Observed AUROC + 0.1056  $\times$  (2.5 - Observed DANA). The AUROCs were compared with the method of Hanley-McNeil<sup>[17]</sup>.

## RESULTS

### Patient characteristics

The mean age of the 78 patients (66 males, 12 females) was  $32.6 \pm 12.3$  years. The mean length of liver biopsies was  $18.2 \pm 3.4$  mm, and the liver specimen length was longer than 15 mm. Significant fibrosis was found in 32

Table 1 Main characteristics of patients studied

	Patients (n = 78)	FOF1 (n = 46)	F2F3F4 (n = 32)	P value (FOF1 vs F2F3F4)
Age (mean ± SD, yr)	32.6 ± 12.3	29.6 ± 12.0	36.9 ± 11.4	0.009
Men (n, %)	66 (84.6)	38 (82.6)	28 (87.5)	0.113
CHB family history (n, %)	29 (37.2)	18 (39.1)	11 (34.4)	0.104
WBC (mean ± SD, 10 <sup>9</sup> /L)	5.3 ± 1.4	5.5 ± 1.2	4.9 ± 1.6	0.060
Hb (mean ± SD, g/L)	142.6 ± 15.7	145.6 ± 13.4	138.3 ± 17.8	0.044
PLT (mean ± SD, 10 <sup>9</sup> /L)	170.2 ± 51.5	185.9 ± 40.7	147.6 ± 57.3	0.002
TB [median (interquartile range), μmol/L]	15.4 (11.5-20.6)	14.7 (10.6-19.1)	16.7 (12.1-24.1)	0.087
CB [median (interquartile range), μmol/L]	5.7 (4.0-7.8)	5.5 (3.9-7.1)	6.4 (4.5-10)	0.057
ALT [interquartile median (range), U/L]	115 (55-241)	93.5 (32-240)	132 (76-263)	0.165
AST [interquartile median (range), U/L]	67.5 (38-121)	56 (30-95)	86.5 (41-152)	0.042
GGT [interquartile median (range), U/L]	52.5 (27-76)	36.5 (21-59)	66.5 (46-94)	0.006
Alb (mean ± SD, g/L)	42.4 ± 5.1	44.0 ± 4.8	40.2 ± 4.6	0.001
PT (mean ± SD, s)	12.0 ± 1.1	11.5 ± 0.9	12.6 ± 0.9	< 0.001
PI (mean ± SD, s)	1.00 ± 0.08	1.03 ± 0.07	0.95 ± 0.06	< 0.001
TC (mean ± SD, mmol/L)	3.8 ± 0.8	3.8 ± 0.8	3.7 ± 0.9	0.135
HA [interquartile median (range), ng/mL]	125 (75-224)	88 (49-129)	167 (116-382)	< 0.001
A2M (mean ± SD, g/L)	2.96 ± 0.58	2.73 ± 0.48	3.28 ± 0.55	< 0.001
Lg HBV-DNA (mean ± SD)	6.0 ± 1.9	5.9 ± 2.0	6.1 ± 2.1	0.314
HBeAg positive (n, %)	55 (70.5)	32 (69.6)	23 (71.9)	0.223
Liver specimen length (mean ± SD, mm)	18.2 ± 3.4	18.4 ± 3.3	17.9 ± 3.6	0.254
METAVIR A stage (n, %)				
A0	4 (5.1)			
A1	41 (52.5)			
A2	32 (41.1)			
A3	1 (1.3)			
METAVIR F stage (n, %)				
F0	13 (16.7)			
F1	33 (42.3)			
F2	13 (16.7)			
F3	10 (12.8)			
F4	9 (11.5)			

SD: Standard deviation; WBC: Leucocyte; Hb: Hemoglobin; TB: Total bilirubin; CB: Conjugated bilirubin; Alb: Albumin; TC: Total cholesterol.

patients (41.0%), severe fibrosis in 19 patients (24.4%), and early cirrhosis in 9 patients (11.5%), respectively. Main features of the patients are summarized in Table 1.

### Correlation between non-invasive model and fibrosis stage

METAVIR fibrosis stages were significantly correlated with APRI, FIB-4, Forn's index, Fibrometer, Hepascore and SLFG. A better correlation was observed between Fibrometer ( $r = 0.69$ ), SLFG ( $r = 0.68$ ) and Hepascore ( $r = 0.62$ ) ( $P < 0.001$ ). The box-plots of fibrosis scores are shown in Figure 1. A correlation was also found between scores and histological activity, especially between SLFG ( $r = 0.55$ ), Fibrometer ( $r = 0.54$ ) and APRI ( $r = 0.51$ ) ( $P < 0.001$ ).

### Overall diagnostic performance of serum markers

The mean levels of AST, GGT, PT, PI, A2M and HA were higher in patients with F2-F4 fibrosis than in those with F0-F1 fibrosis ( $P < 0.01$ ). The mean levels of hemoglobin, PLT and albumin were lower in patients with F2-F4 fibrosis than in those with F0-F1 fibrosis ( $P < 0.01$ ). Multiple regression analysis showed that A2M and HA were the independent factors for significant fibrosis (A2M, OR = 5.36, 95% CI: 1.58-18.13,  $P = 0.007$ ; HA, OR = 1.01, 95% CI: 1.00-1.02,  $P = 0.007$ ). AUROC

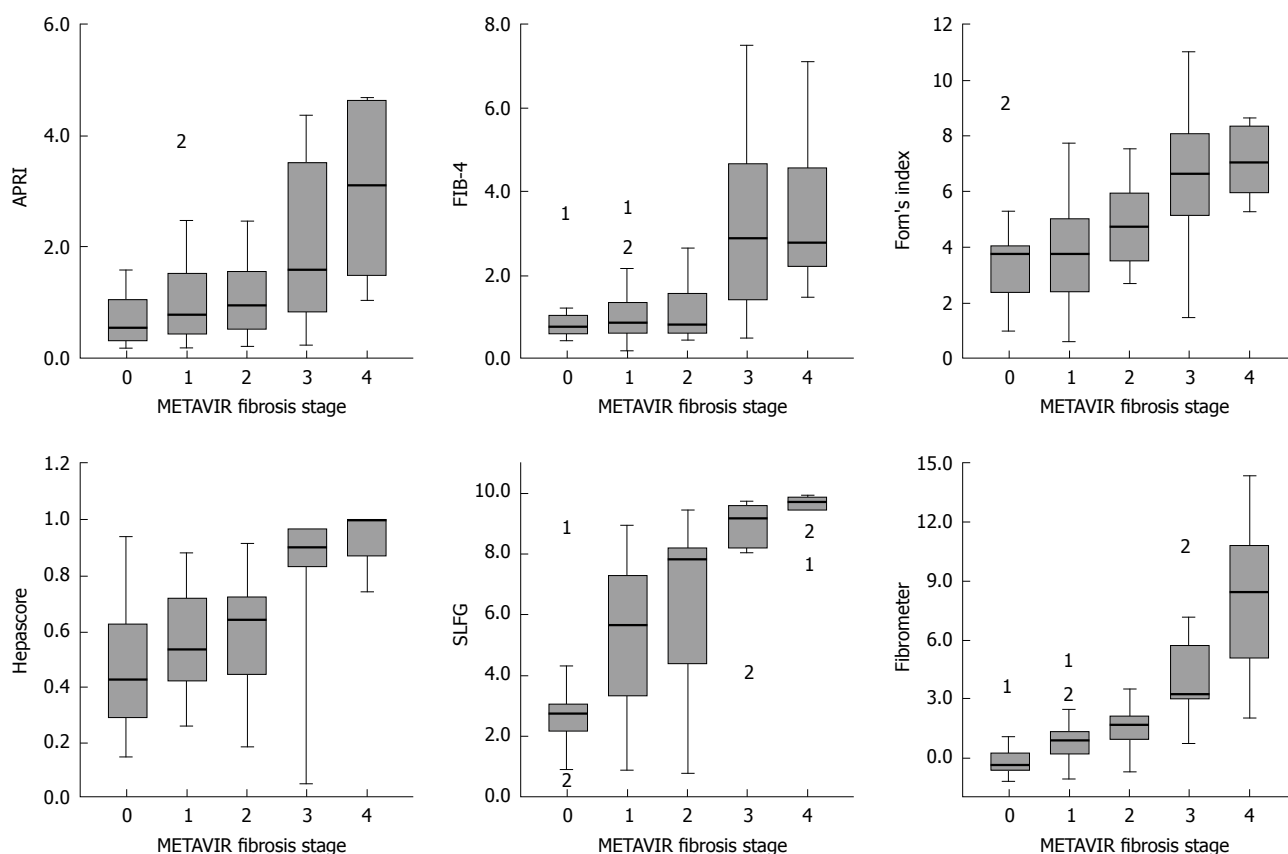
was used to evaluate the overall diagnostic performance of scores (Table 2).

The APRI, FIB-4, Forn's index, Hepascore, SLFG and Fibrometer were 0.71, 0.75, 0.79, 0.80, 0.83, and 0.85 respectively under the AUROC for F0-F4 (Figure 2A), and 0.75, 0.79, 0.83, 0.84, 0.86, and 0.88 respectively under the adjusted AUROC for F0-F4 with DANA method. The AUROC for Fibrometer, SLFG and Hepascore was better than that for APRI and FIB-4 ( $P < 0.01$ ).

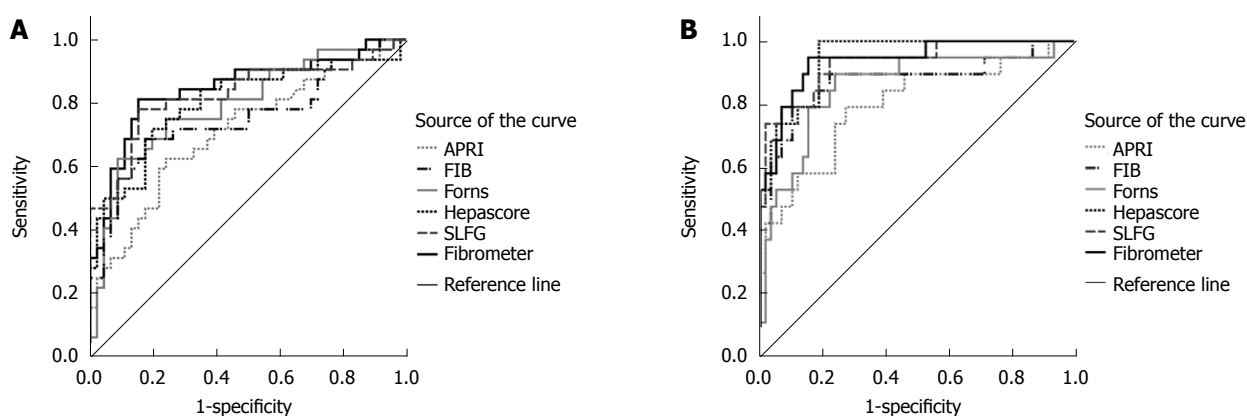
The APRI, FIB-4, Forn's index, Hepascore, SLFG and Fibrometer were 0.80, 0.87, 0.86, 0.95, 0.93, and 0.94 under the AUROC for F0-F4 (Figure 2B). The Hepascore, Fibrometer and SLFG levels were significantly higher than the APRI level under the AUROC for F0-F4 ( $P < 0.01$ ).

### Sensitivity, specificity, positive and negative predictive values

NPV and PPV for the diagnosis of significant fibrosis are presented in Table 3. Cutoffs were chosen for each model as previously described. This analysis could not be performed for Fibrometer since no cutoff was provided in the study by Calès *et al.*<sup>[10]</sup>. When different cutoffs were used for each model, the percentage of classifiable subjects was 45%-78%, with a diagnostic accuracy of



**Figure 1** Model values according to METAVIR fibrosis stages. The top and bottom of each box are the 25th and 75th centile interval, the line through the box is the median and the error bars are the 5th and 95th centile interval. 1 and 2 indicate the extreme values.



**Figure 2** ROC curves for the 6 fibrosis models to discriminate METAVIR fibrosis stages F0-1 from F2-4 (A) and F0-2 from F3-4 (B).

67%-86% (Table 4). Significant fibrosis (F2-4) was predicted in 13%-32% of patients with their PPV ranged 62%-88%. Since lower cutoffs were originally described to rule out significant fibrosis, attention must be paid to NPV ranging 76%-85%. The best positive predictive value (PPV = 0.88) for significant fibrosis was observed when SLFG > 8.7.

## DISCUSSION

Noninvasive models have been proposed for the assessment of liver fibrosis. The diagnostic performance of

APRI, FIB-4, Forn's index, Fibrometer, Hepascore and SLFG was evaluated for the assessment of liver fibrosis in CHB patients. These models are mainly based on two kinds of serum markers, direct and indirect. Direct serum markers are directly linked to the modifications in extracellular matrix (ECM) metabolism. Indirect serum markers have no direct link with liver fibrosis but reflect liver dysfunction or other phenomena caused by fibrosis. We focused on the serum markers of fibrosis. The main end-point of our study was to evaluate the global diagnostic performance of models by comparing their AUROCs. Our study indicated that models containing



Table 2 AUROC for FOF1 *vs* F2-4 and F0-2 *vs* F3-4

	F0-1 <i>vs</i> F2-4				F0-2 <i>vs</i> F3-4		
	AUROC	SD	95% CI	AdAUC	AUROC	SD	95% CI
APRI	0.71	0.06	0.59-0.83	0.75	0.80	0.06	0.67-0.92
FIB-4	0.75	0.06	0.63-0.87	0.79	0.87	0.06	0.76-0.99
Forn's index	0.79	0.05	0.69-0.90	0.83	0.86	0.06	0.75-0.96
Hepascore	0.80	0.05	0.70-0.91	0.84	0.95	0.02	0.90-0.99
SLFG	0.83	0.05	0.73-0.93	0.86	0.93	0.03	0.87-0.99
Fibrometer	0.85	0.05	0.75-0.94	0.88	0.94	0.03	0.88-0.99

Table 3 Sensitivity, specificity, predictive values and likelihood ratios of scores according to different cutoffs for the diagnosis of significant fibrosis

Score	Cutoff	%	Significant fibrosis (F2-4)					
			Sen	Spe	PPV	NPV	+LR	-LR
APRI	< 0.50	27	0.84	0.35	0.47	0.76	1.29	0.46
	> 1.50	32	0.47	0.80	0.62	0.69	2.35	0.66
FIB-4	< 1.45	65	0.63	0.85	0.74	0.76	4.20	0.44
	> 3.25	13	0.25	0.96	0.80	0.65	6.25	0.78
Forn's index	< 4.20	46	0.75	0.63	0.59	0.78	2.03	0.40
	> 6.90	18	0.34	0.96	0.85	0.68	8.50	0.69
Hepascore	< 0.50	23	0.88	0.50	0.55	0.85	1.74	0.26
	> 0.84	27	0.50	0.91	0.80	0.72	5.56	0.55
SLFG	< 3.00	23	0.91	0.33	0.48	0.83	1.36	0.27
	> 8.70	22	0.47	0.96	0.88	0.72	11.75	0.55

Sen: Sensitivity; Spe: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; +LR: Positive likelihood ratio; -LR: Negative likelihood ratio.

Table 4 Percentage of classifiable subjects, correct prediction, diagnostic accuracy and biopsies that could be avoided *n* (%)

Models	Cut-offs	Classifiable subjects	Correct prediction	Diagnostic accuracy	Biopsy avoided
APRI	< 0.50	21 (27)	16 (76)	67%	31 (40)
	> 1.50	25 (32)	15 (62)		
FIB-4	< 1.45	51 (65)	39 (76)	77%	47 (60)
	> 3.25	10 (13)	8 (80)		
Forn's index	< 4.20	36 (46)	28 (78)	80%	40 (51)
	> 6.90	14 (18)	12 (85)		
Hepascore	< 0.50	18 (23)	15 (85)	82%	32 (41)
	> 0.84	21 (27)	17 (80)		
SLFG	< 3.00	18 (23)	15 (83)	86%	30 (38)
	> 8.70	17 (22)	15 (88)		

direct serum markers (Fibrometer, SLFG, Hepascore) performed much more accurately than models containing only indirect serum markers (APRI, FIB-4, Forn's index).

Direct serum markers are useful for assessing the speed of liver fibrogenesis. HA, a component of ECM, is a glycosaminoglycan synthesized by hepatic stellate cells and degraded by liver sinusoidal cells<sup>[18]</sup>. A<sub>2</sub>M is a protease inhibitor with its concentration increased due to stellate cell activation and liver fibrosis<sup>[19]</sup>. Studies have demonstrated that HA and A<sub>2</sub>M levels are correlated with hepatic fibrosis in patients with CHB or CHC<sup>[11,20-22]</sup>, which is consistent with the findings in our study. Multiple

regression analysis in our study showed that HA and A<sub>2</sub>M were the independent factor for significant fibrosis and had a better diagnostic accuracy.

It has been reported that APRI under the AUROC for significant fibrosis is 0.76 (95% CI: 0.74-0.79)<sup>[23]</sup>. It has been shown that the accuracy of APRI under the AUROC for significant fibrosis is 0.63 and 0.72 in CHB patients<sup>[24,25]</sup>. Zhang *et al*<sup>[26]</sup> showed that APRI has low diagnostic accuracy of liver fibrosis and APRI combined HA can achieve a better diagnostic accuracy of liver fibrosis. FIB-4 has a high diagnostic accuracy of severe fibrosis<sup>[27]</sup>. Mallet *et al*<sup>[28]</sup> reported that FIB-4 is 0.81 under the AUROC for severe fibrosis. In our study, the FIB-4 was 0.87 under the AUROC for severe fibrosis. The reported Forn's index is 0.76 under the AUROC for significant fibrosis<sup>[29]</sup>. In our study, the Forn's index was 0.79 under the AUROC for significant fibrosis. The diagnostic value of APRI, FIB-4 and Forn's index was much lower in CHB patients than in CHC patients.

Calès *et al*<sup>[10]</sup> have developed Fibrometer for the diagnosis of significant fibrosis and found that its diagnostic performance is stable in patients with different chronic liver diseases<sup>[30,31]</sup>. Hepascore has been used in diagnosis of significant and severe fibrosis<sup>[12]</sup>. In our study, the Hepascore was 0.80 and 0.95 under the AUROC for significant and severe fibrosis. SLFG is the first developed model in CHB patients. In our study, the NPV and PPV of SLFG under AUROC are similar to the reported

data<sup>[11]</sup>. These results indicate that Fibrometer, SLFG and Hepascore can be used in diagnosis of liver fibrosis. However, these noninvasive models should be validated in a larger number of CHB patients.

Broadly speaking, no true noninvasive model could exactly reflect liver fibrosis. Transient elastography (fibroScan) is another noninvasive method to detect the mean liver stiffness for diagnosing fibrosis. However, it is expensive and may be limited in those with narrow intercostal spaces, morbid obesity or significant ascites<sup>[32,33]</sup>. These noninvasive models can be used in clinical management of CHB by offering an attractive alternative to liver biopsy.

In our study, since the sample size was small, further study is needed before these models are used in clinical practice. Validating against not only histological stage scores but also digital image analysis and clinical outcomes may also be a better choice.

In conclusion, serologic models containing direct serum markers of Hepascore, SLFG, and Fibrometer have better diagnostic values in CHB patients than those containing only indirect serum markers of APRI, FIB-4, Forns' index.

## COMMENTS

### Background

Clinically, developing noninvasive methods for diagnosing liver fibrosis is important. Noninvasive models have been established most in hepatitis C patients.

### Research frontiers

Noninvasive models have been proposed for the assessment of liver fibrosis. These models have been evaluated at many medical centers in chronic hepatitis C (CHC), but few in chronic hepatitis B (CHB). In this study, the efficacy of 6 noninvasive models was evaluated and the more valuable models were identified for predicting liver fibrosis in CHB.

### Innovations and breakthroughs

The efficacy of 6 noninvasive models was evaluated in a cohort of Chinese patients with CHB. The results indicate that their efficacy is not influenced by ethnic and virus factors. This study also showed that the serologic models containing direct serum markers, such as hyaluronic acid (HA) and  $\alpha_2$ -macroglobulin (A<sub>2</sub>M) have better diagnostic values in CHB patients than in those not containing direct serum markers.

### Applications

Noninvasive models can be used in diagnosis of liver fibrosis in patients with CHB in China or in Asia.

### Terminology

DANA method: A method used to adjust the differences caused by the prevalence of fibrosis stages. Standard prevalence is defined as a prevalence of 0.20 for each of the five stages.

### Peer review

The study adds information that helps establishment of strategies against liver fibrosis diagnosis using noninvasive methods. The study was scientifically designed. The manuscript is logical and readable.

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## Application of contrast-enhanced intraoperative ultrasonography in the decision-making about hepatocellular carcinoma operation

Hong Wu, Qiang Lu, Yan Luo, Xian-Lu He, Yong Zeng

Hong Wu, Xian-Lu He, Yong Zeng, Department of Hepato-Biliary-Pancreatic surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Qiang Lu, Yan Luo, Department of sonography, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

**Author contributions:** Wu H and Luo Y designed the research; Wu H, He XL, Zeng Y performed the surgery; Lu Q and Luo Y did the ultrasound scans; Wu H and Lu Q wrote the paper.

**Correspondence to:** Yong Zeng, Professor, Department of Hepato-Biliary-Pancreatic surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China. [zengyong@medmail.com](mailto:zengyong@medmail.com)

Telephone: +86-28-85422868 Fax: +86-28-85422475

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### Abstract

**AIM:** To evaluate the detection and differentiation ability of contrast-enhanced intraoperative ultrasonography (CE-IOUS) in hepatocellular carcinoma (HCC) operations.

**METHODS:** Clinical data of 50 HCC patients were retrospective analyzed. The sensitivity, specificity, false negative and false positive rates of contrast enhanced magnetic resonance imaging (CE-MRI), IIOUS and CE-IOUS were calculated and compared. Surgical strategy changes due to CE-IOUS were analyzed.

**RESULTS:** Lesions detected by CE-MRI, IIOUS and CE-IOUS were 60, 97 and 85 respectively. The sensitivity, specificity, false negative rate, false positive rate of CE-MRI were 98.2%, 98.6%, 98.6%, 60.0%, respectively; for IIOUS were 50.0%, 90.9%, 1.8%, 1.4%, respectively; and for CE-IOUS were 1.4%, 40.0%, 50.0%, 9.1%, respectively. The operation strategy of 9 (9/50, 18.0%) cases was changed according to the results of CE-IOUS.

**CONCLUSION:** Compared with CE-MRI, CE-IOUS performs better in detection and differentiation of small metastasis and regenerative nodules. It plays an important role in the decision-making of HCC operation.

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**Key words:** Hepatocellular carcinoma; Liver resection; Contrast enhanced magnetic resonance imaging; Intraoperative ultrasonography; Contrast-enhanced intraoperative ultrasonography

**Peer reviewer:** Brian Kim Poh Goh, MBBS, MMed, MSc, FRCS, FAMS, Department of Surgery, Singapore General Hospital, Outram Road, Singapore 169608, Singapore

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### INTRODUCTION

The incidence rate of hepatocellular carcinoma (HCC) and cirrhosis in the hepatic B infection population is high in China, about 53.8%-85.9%, and more than 95% in some reports. There are several stages of hepatocarcinogenesis, from regenerative nodule, to degenerative nodule and to HCC. While comparing preoperative imaging results and pathological results after operation, the sensitivity of contrast enhanced magnetic resonance imaging (CE-MRI) is not satisfactory and can hardly detect some lesions<sup>[1,2]</sup>. It has been shown that intraoperative ultrasound (IOUS) is the most accurate diagnostic technique for detecting focal liver lesions (FLL) and has a great impact on the surgical approach to liver



tumors<sup>[3,4]</sup>. However, in cirrhotic patients with HCC, not all nodules detected by IOUS are neoplastic<sup>[5]</sup>. How to differentiate small HCC from the nodules detected by IOUS poses a big challenge for surgeons. The application of intravenous ultrasound contrast agents during transcutaneous ultrasonography of the liver has been shown to improve nodule characterization in comparison with unenhanced ultrasound<sup>[6-10]</sup>. Therefore, we investigated whether the application of contrast-enhanced ultrasound examination intraoperatively could solve the aforementioned deficiencies of IOUS during liver exploration.

## MATERIALS AND METHODS

### Common materials

The data from 50 HCC patients, including 38 males, 12 females, mean age 45 years (range, 19-67 years) was retrospectively analyzed. Thirty nine cases had a history of hepatitis B infection and 2 of hepatitis C infection; nine had no hepatitis history. Three cases had undergone surgical resection for HCC before. Preoperative MRI, IOUS and contrast-enhanced intraoperative ultrasonography (CE-IOUS) were performed in 395 liver segments of 50 patients.

### CE-MRI

CE-MRI examinations were performed with a 1.5 T imaging system (Gyrosan Intera, Philips Medical Systems Best, Netherlands), using a breathhold 3D gradient echo sequence with fat saturation sequence, following an iv bolus of 0.1 mmol gadobenate dimeglumine (MultiHance, Bracco SpA, Milan, Italy) per kg of body weight at a rate of 2 mL/s. Data was acquired in the hepatic arterial, portal venous, and equilibrium phases.

### IOUS

IOUS scans were done by a VIVID4 (GE, US) ultrasound system with I-shaped 10-4 MHz intraoperative probe. After mobilization of the liver, IOUS was performed to search for nodules. Suspected lesions were counted and mapped.

### Contrast-enhanced intraoperative ultrasonography (CE-IOUS)

CE-IOUS scans were carried out both for lesion characterization and new nodule detection. Since no specific intraoperative probe is available for contrast study, we used the IU22 unit (Philips, USA) equipped with a 5-2 MHz convex transducer and a 9-3 MHz linear transducer instead. Both of the probes have the capacity for contrast enhanced ultrasound studies. The contrast agent was SonoVue (Bracco Imaging, Milan, Italy) which consists of sulphur hexafluoride microbubbles stabilized by a phospholipid shell; 4.8 mL of SonoVue per exploration was injected intravenously through a peripheral vein. A low mechanical index (MI) < 0.1 mode was used. All phases of contrast enhancement, including arterial (10-20 s to 25-35 s after injection),

portal (30-45 s to 120 s) and late parenchymal (> 120 s) phases were recorded and analyzed.

### Imaging analysis

In the CE-IOUS study, HCC is characterized by arterial phase hyper-enhancing and wash out of microbubbles during the portal and late phase, while benign solid lesions are characterized by persistence of contrast enhancement during the portal and late phase.

### Surgery and follow up

Lesions considered malignant were removed surgically. Ultrasound-guided biopsy and ethanol ablation would be an alternative if the lesion can not be removed surgically. Nodules regarded as benign were removed only in cases located close to the main lesion and others were followed by examinations of  $\alpha$ -fetoprotein (AFP) level and ultrasound and/or CE-MRI every 3 mo for more than 6 mo.

### Golden standard

Pathologic examination was taken as the golden standard. Those unresected lesions with negative findings during 3 mo follow-up were regarded benign.

### Statistical analysis

$\chi^2$  tests were used to analyze the data,  $P < 0.05$  was considered to be statistically significant.

## RESULTS

### Imaging results

Preoperative MRI detected 60 lesions in total and IOUS found 96 nodules in 50 patients (Figure 1A-C). A total of 85 lesions were detected by CE-IOUS, among them, 73 HCC (Figure 2A and C) and 7 benign nodules (Figure 2B) were proved by pathology; another 5 lesions which were considered benign were not removed. Follow-up ultrasound showed no sign of malignancy with normal AFP levels after 6-15 mo in 4 patients and the size of the lesion in the other patient increased during 3 mo follow up. Further surgery was performed in this patient and proved to be HCC at histology.

Malignant and benign lesions detected by CE-MRI, IOUS and CE-IOUS were listed in Table 1.

The sensitivity, specificity, false negative ratio and false positive ratio of CE-MRI, IOUS and CE-IOUS, respectively, were shown in Table 2.

Particularly, one isoechoic HCC nodule was missed by IOUS, but showed a typical contrast agent wash-out pattern on CE-IOUS late parenchymal phase (Figure 2D). Another hypo-enhanced nodule was diagnosed as malignant by CE-IOUS but proved to be a necrotic nodule at histology.

### Operation

Among 18 additional malignant lesions detected by CE-IOUS, 1 patient had 3 lesions, 4 patients had 2 lesions, and 7 patients had 1 lesion. The size of lesions was 5-20

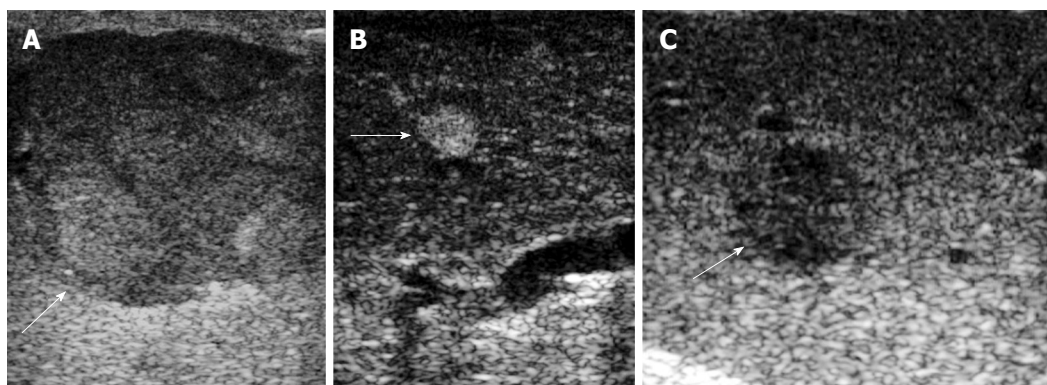


Figure 1 IOUS showing typical sonographic appearance of HCC nodules with mosaic pattern (A, arrow) and hyperechoic regenerative nodules (B, arrow); It is hard for IOUS to accurately diagnose a hypoechoic nodule (C, arrow) which could be a regenerative nodule or a small HCC nodule or focal fatty sparing nodule.

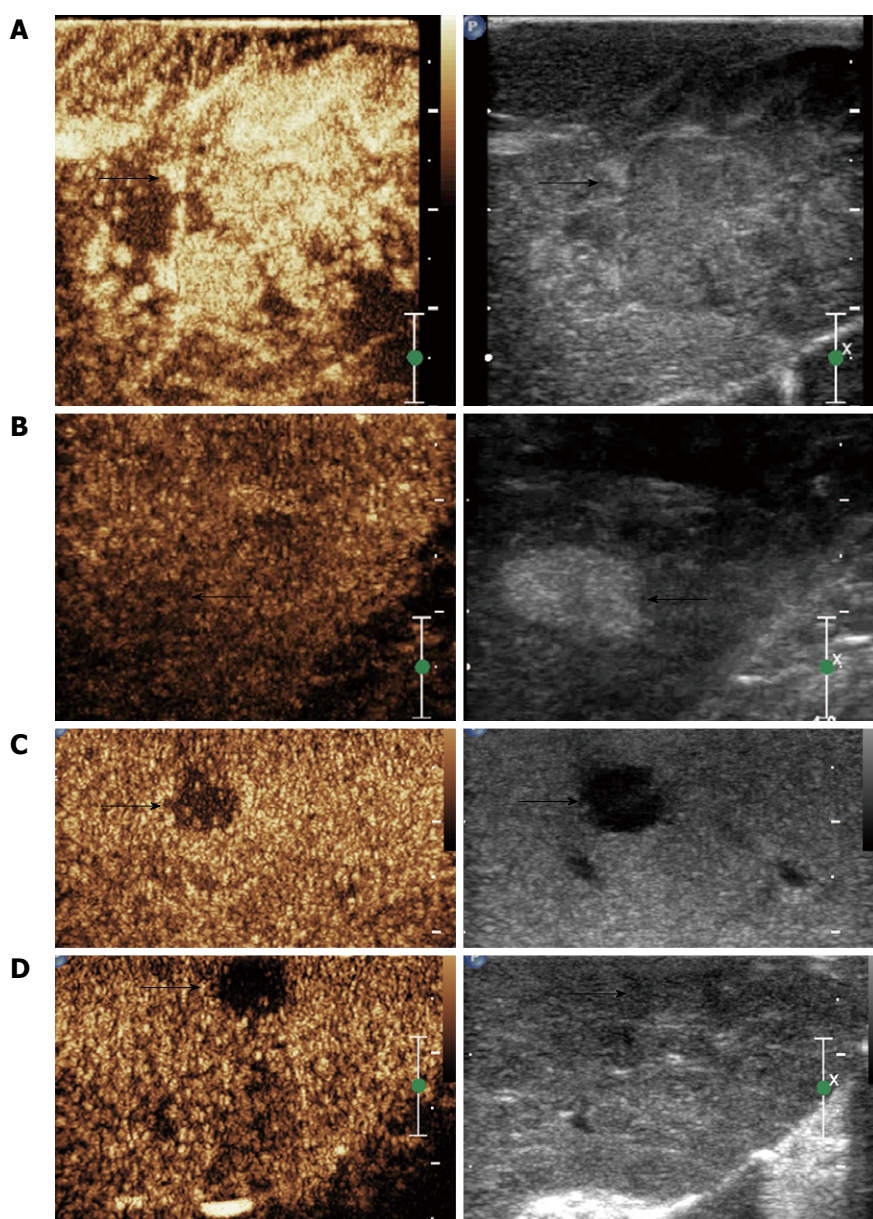


Figure 2 CE-IUS showing an HCC nodule with hyperenhancement in arterial phase (A, arrow) while a regenerative hyperechoic nodule shows isoenhancement on CE-IUS (B, arrow); Hypoechoic intrahepatic metastatic nodule showing wash out of contrast agent on late phase (C, arrow); Isoechoic nodule missed on IOUS showing a clear margin on CE-IUS (D, arrow).

mm (mean 12 mm). Surgical strategies of 9 patients (18.0%, 9/50) were changed because newly detected lesions were not in the same segment as the old ones (Table 3).

## DISCUSSION

Recently, the incidence of HCC has had a tendency to

**Table 1** Malignant and benign lesions detected by CE-MRI, IOUS and CE-IOUS

	Malignant lesions	Pathology	Benign lesions	Pathology + follow up
CE-MRI	56	54	4	3
IOUS	89	72	8	7
CE-IOUS	74	73	11	10

CE-MRI: Contrast enhanced magnetic resonance imaging; IOUS: Intraoperative ultrasonography; CE-IOUS: Contrast-enhanced intraoperative ultrasonography.

**Table 2** Sensitivity, Specificity, False negative rate and False positive rate of CE-MRI, IOUS and CE-IOUS (%)

	Sensitivity	Specificity	Accuracy	False negative rate	False positive rate
CE-MRI	98.2	60	95	1.8	40
IOUS	98.6	50	81.4	1.4	50
CE-IOUS	98.6 <sup>a</sup>	90.9 <sup>c</sup>	97.6	1.4 <sup>e</sup>	9.1 <sup>f</sup>

<sup>a</sup> $P > 0.05$  compared with CE-MRI and IOUS, <sup>c</sup> $P < 0.05$  compared with CE-MRI and IOUS, <sup>e</sup> $P > 0.05$  compared with CE-MRI and IOUS, <sup>f</sup> $P < 0.05$  compared with CE-MRI and IOUS.

**Table 3** Operation changed according to the newly detected lesions by CE-IOUS

	Operation (liver resection)								Liver transplantation	Liver resection + ethanol injection
	1 segment	2 segments	3 segments	Right liver	Left liver	Right 3 segment	Left 3 segment			
Pre-operation	13	21	7	4	3	1	1	0		0
Intra-operation	9	18	8	6	4	2	1	1 <sup>1</sup>		1

<sup>1</sup>This patient accepted right lobe of liver resection and ethanol injection in the I stage, then accepted liver transplantation 4 mo later.

increase and radical resection is considered to be the most effective therapy<sup>[11,12]</sup>. The rate of HCC with cirrhosis is high in China; therefore, differentiation of regenerative nodules from malignant ones plays an important role in the decision-making about surgery. However, the performance of preoperative CE-MRI is not satisfactory in detection of small lesions and unenhanced IOUS can hardly make the differential diagnosis of regenerative nodules from malignant ones in cirrhotic patients. The purpose of our research is to find a better imaging method with high sensitivity and specificity.

CE-IOUS is a real time gray scale imaging with low mechanical index (MI), which can clearly show microcirculation and perfusion of a tumor. HCC is a typical hyper-vascular tumor with the majority of blood contained in microvessels which can be demonstrated by CE-IOUS. The contrast agent we used in our research is SonoVue which consists of sulphur hexafluoride microbubbles stabilized by a phospholipid shell. Microbubbles of SonoVue can stay in blood for about 8 min which makes it possible for us to observe dynamic changes of liver enhancement. Pulse inversion harmonic technology can use non-linear signals in low acoustic pressure while restraining the linear signals from liver parenchyma<sup>[13]</sup>, so it has the high sensitivity of harmonic signal detection.

Sensitivity of CE-MRI (98.2%), IOUS (98.6%) and CE-IOUS (98.6%) were high, but compared with CE-IOUS, the specificity of CE-MRI and IOUS were fairly poor. In our study, 85 lesions were diagnosed in 50 patients finally, preoperative CE-MRI only detected 60 lesions, indicating the diagnosis rate of CE-MRI for micro lesions is poor. In the 97 lesions that were detected by IOUS, only 74 malignant lesions were finally diagnosed, and the false positive rate of IOUS is too

high compared with CE-IOUS. Consequently, we believe that CE-IOUS is an ideal diagnostic method in the decision-making about hepatocellular carcinoma surgery. Among 85 lesions detected by CE-IOUS, we had one false negative and one false positive case. The reason for the false negative case is that the minor lesion was located under the right diaphragm, and the probe we used for CE-IOUS was too big to thoroughly scan that area. The reason for the false positive case is that the minor nodule was a necrosis nodule. Parenchymal phase was used to search for malignant nodules during the CE-IOUS study and both necrotic nodules and small HCC shows hypoenhancement in parenchymal phase.

CE-MRI had 19 false negative cases (27%), with size 5-20 mm (mean 12 mm). Among these, 3 HCCs and 5 metastatic nodules had typical hyper-enhancement in artery phase and hypo-enhancement in portal phase in CE-IOUS. All patients accepted surgery and malignancy was proved by pathological results. Research found that CE-IOUS with low MI is more sensitive than CE-MRI in revealing artery perfusion of liver tumors. Time resolution of CE-MRI is relatively low, so it can not observe the dynamic enhancement of lesions, which is very important in differential diagnosis of HCC. Therefore, CE-IOUS is proved to be another sensitive imaging method with high diagnostic value<sup>[14]</sup>.

CE-IOUS is of vital importance in detection and differentiation of lesions which were not detected pre-operatively with other imaging methods. Newly found tumors in pre-considered normal liver segments may lead to expanded resection, or combination treatment with radiofrequency ablation and ethanol injection. Sometimes, surgeons were obliged to give up or change their operation strategy. It had been reported that IOUS changed 18%-51% of operation strategies in liver



metastasis patients with rectal cancer<sup>[15-19]</sup>. Eighteen newly detected malignant liver nodules by CE-IOUS in all 50 patients were proved by histology. The primary operation strategy was changed in 9 cases but not in another 3 cases. Two cases proved to have micro-metastatic lesions in another lobe of the liver during operation which can not be radical cured. One had half liver resection and ethanol injection of another nodule. Another patient had right part of liver resection and ethanol injection of another nodule. After operation, the patient's AFP level decreased to normal and gradually increased over 2 and 4 mo later, he received cadaveric liver transplantation.

In our preliminary study, CE-IOUS is proved to be better than CE-MRI and IOUS in detecting and differentiating micro-metastatic liver lesions and hyperplastic nodules, which helped decision-making about surgical strategy. However its impact on increasing the long-term survival rate need further follow-up.

## COMMENTS

### Background

In cirrhotic patients with hepatocellular carcinoma (HCC), preoperative imaging was unsatisfactory in detection of some lesions. Intraoperative ultrasonography (IOUS) was sensitive in finding small lesions but not all nodules detected by IOUS are neoplastic. How to differentiate small HCC from the nodules detected by IOUS poses a big challenge for surgeons. Contrast enhanced ultrasound of the liver has been shown to improve nodule characterization. Therefore, the authors investigated if the application of contrast-enhanced ultrasound examination intraoperatively could solve the aforementioned problems during liver exploration.

### Research frontiers

Radical resection is the goal of surgery for HCC and the key is to find all the malignant lesions.

### Innovations and breakthroughs

CE-IOUS proved to be better than CE-MRI and IOUS in detecting and differentiating micro-metastatic liver lesions and hyperplastic nodules, which helped decision-making about operation strategy.

### Applications

The application of CE-IOUS in surgery for HCC has a positive impact on the decision making about surgical strategy.

### Terminology

CE-IOUS is an ultrasound exam performed by applying contrast agent during surgery. Besides the high sensitivity of detecting focal liver lesion, it shows tumor vascularity and tissue microcirculation, thus helping differentiate malignant nodules from benign ones.

### Peer review

This article aims to determine the role of CE-IOUS vs IOUS vs CE-MRI in the detection and characterization of suspicious nodules in cirrhotic patients.

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## Percutaneous catheter drainage in combination with choledochoscope-guided debridement in treatment of peripancreatic infection

Li-Jun Tang, Tao Wang, Jian-Feng Cui, Bing-Yin Zhang, Shi Li, Dong-Xuan Li, Shu Zhou

Li-Jun Tang, Tao Wang, Jian-Feng Cui, Bing-Yin Zhang, Shi Li, Dong-Xuan Li, Shu Zhou, Center of General Surgery, Chengdu General Hospital of Chengdu Military Area Command, Chengdu 610083, Sichuan Province, China

Author contributions: Tang LJ, Wang T and Cui JF performed the majority of operation and wrote the manuscript; Zhang BY, Li S and Li DX provided the operative tools and observed the patients after operation; Zhou S collected the data of patients.

Correspondence to: Li-Jun Tang, MD, Center of General Surgery, Chengdu General Hospital of Chengdu Military Area Command, Chengdu 610083, Sichuan Province, China. whjtlj@yahoo.com.cn

Telephone: +86-28-86570524 Fax: +86-28-86570620

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**CONCLUSION:** Percutaneous catheter drainage in combination with choledochoscope-guided debridement is a simple, safe and reliable treatment procedure for peripancreatic infections secondary to SAP.

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**Key words:** Severe acute pancreatitis; Peripancreatic infection; Percutaneous catheter drainage; Choledochoscope; Debridement

**Peer reviewer:** Naoaki Sakata, MD, PhD, Division of Hepato-Biliary Pancreatic Surgery, Tohoku University Graduate School of Medicine, 1-1 Seiryomachi, Aoba-ku, Sendai, Miyagi 980-8574, Japan

### Abstract

**AIM:** To introduce and evaluate the new method used in treatment of pancreatic and peripancreatic infections secondary to severe acute pancreatitis (SAP).

**METHODS:** A total of 42 SAP patients initially underwent ultrasound-guided percutaneous puncture and catheterization. An 8-Fr drainage catheter was used to drain the infected peripancreatic necrotic foci for 3-5 d. The sinus tract of the drainage catheter was expanded gradually with a skin expander, and the 8-Fr drainage catheter was replaced with a 22-Fr drainage tube after 7-10 d. Choledochoscope-guided debridement was performed repeatedly until the infected peripancreatic tissue was effectively removed through the drainage sinus tract.

**RESULTS:** Among the 42 patients, the infected peripancreatic tissue or abscess was completely removed from 38 patients and elective cyst-jejunum anastomosis was performed in 4 patients due to formation of pancreatic pseudocysts. No death and complication occurred during the procedure.

Tang LJ, Wang T, Cui JF, Zhang BY, Li S, Li DX, Zhou S. Percutaneous catheter drainage in combination with choledochoscope-guided debridement in treatment of peripancreatic infection. *World J Gastroenterol* 2010; 16(4): 513-517 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i4/513.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i4.513>

### INTRODUCTION

Severe acute pancreatitis (SAP) is an extremely dangerous and refractory disease characterized by complicated pathogenesis and high mortality<sup>[1]</sup>. Secondary peripancreatic infection is a severe complication of SAP, which is one of the main causes for death of SAP patients<sup>[2]</sup>. Clinically, peripancreatic infection can be divided into infected effusion containing rich pancreatin around the pancreas occurring in early SAP, and infected pancreatic and peripancreatic necrosis, peripancreatic abscess usually observed during the course of SAP progress. In addition to the clinical signs and symptoms of bacterial infection, SAP patients have a large amount of necrotic peripancreatic tissue and fluid which can be

found by imaging analysis, computed tomography (CT) or ultrasonography<sup>[3,4]</sup>.

Open surgical debridement is a traditional procedure for SAP patients with infected peripancreatic foci<sup>[5-7]</sup>. However, it should not be performed in early SAP<sup>[2,7-9]</sup>. Since the infected peripancreatic foci in early SAP are not well encapsulated by fibrous connective tissue and no visible distinct boundary can be observed between infected necrotic tissue and living pancreas or peripancreatic tissue, it is difficult to completely remove the necrotic tissues, with a re-operation rate of 18%-68%<sup>[10]</sup>. In addition, the function of vital organs, including lung and kidney, is often insufficient in early SAP patients, and is further deteriorated by open-abdominal surgery, leading to multiple vital organ failure and death. Therefore, it is generally agreed that surgical debridement should be delayed until the infected peripancreatic foci are well demarcated<sup>[2,7]</sup>.

If debridement could not be performed in early SAP, the infected peripancreatic necrotic tissue and effusion would become the source of infection in other organs. In addition, peripancreatic effusion containing enzyme-enriched pancreatic juice can further corrode the peripancreatic tissue, leading to more severe peripancreatic necrosis. Furthermore, retroperitoneal necrotic tissue and effusion may stimulate celiac nerves that can induce enteroparalysis and exacerbate abdominal distension, leading to respiratory and cardiac failure in severe cases. Thus, it is necessary to remove the infected peripancreatic tissue and effusion promptly and effectively.

In this retrospective study, early SAP patients were treated with ultrasound-guided puncture and catheter drainage in combination with early debridement under choledochoscope.

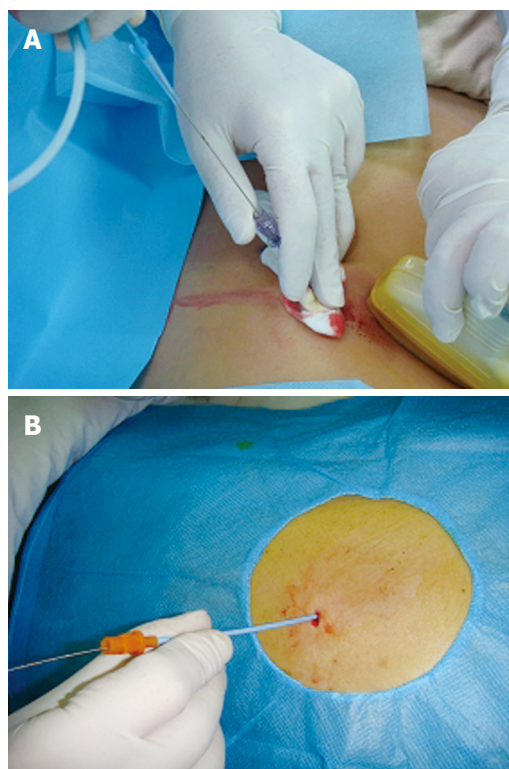
## MATERIALS AND METHODS

### Patients

A total of 42 patients (25 males and 17 females), at the age of 24-67 years, were diagnosed as SAP according to the "Atlanta classification of acute pancreatitis" in 2001-July 2008 in our hospital<sup>[11]</sup>. The disease was due to alcohol drinking, stones in the common bile duct, and unknown etiology in 9, 8, and 25 patients, respectively. Physical examination revealed abdominal tenderness, rebound pain, distention, and hypoactive or absent bowel sounds, one or more organ failure, pulmonary insufficiency, renal failure, systemic complications, low calcium, presence of infected necrotic tissue and fluid surrounding the pancreas. Patients received relevant treatment to maintain their vital organ functions. However, infected peripancreatic foci still remained after treatment. Once the infected foci were localized, patients should be immediately treated with ultrasound-guided percutaneous puncture and catheter drainage.

### Ultrasound-guided percutaneous puncture and catheter drainage

Patients underwent abdominal ultrasound examination at



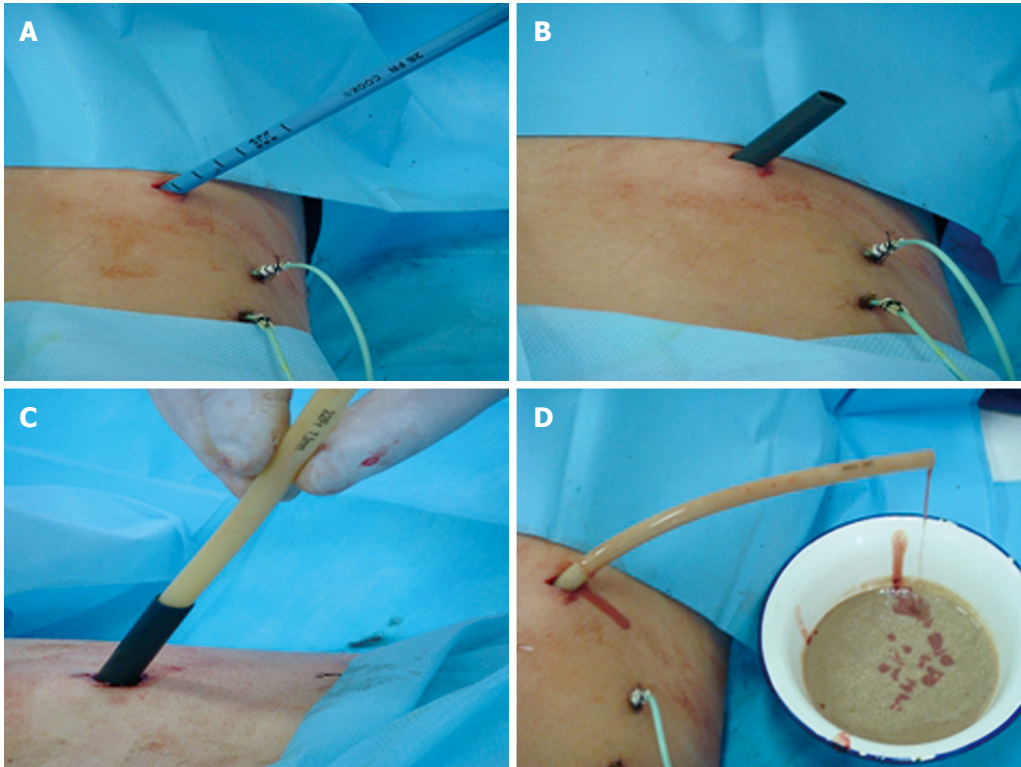
**Figure 1** Ultrasound-guided percutaneous puncture and catheter drainage. A: An 18-G puncture needle is inserted into the peripancreatic focus under the guidance of ultrasound with a guidewire placed in the focus through the puncture needle lumen; B: An 8-Fr drainage catheter is immediately delivered into the focus along the guidewire after adequate expansion of abdominal wall layers.

a supine position to determine the safe site and direction of percutaneous puncture. The selected puncture site was close to the focus. Under the guidance of ultrasound, an 18-G puncture needle (Promex Company, USA) was inserted into the focus through the abdominal wall and the needle core was then pulled out. Pus and necrotic tissue fragments were drawn out from the focus. A guidewire was then placed in the deepest location of the focus *via* the puncture needle lumen under ultrasonic monitoring (Figure 1A). After the puncture needle was slowly removed, an 8-Fr skin expander (Cook Corporation, USA) was inserted along with the guidewire. Once the abdominal wall layers were properly expanded, the skin expander was removed and an 8-Fr drainage catheter (Hakko International Trading Co., Ltd.) was immediately inserted into the focus along with the guidewire (Figure 1B). Finally, the guidewire was pulled out with the drainage catheter fixed on the abdominal wall.

### Replacement of drainage catheter with a larger drainage tube

The infected peripancreatic fluid was drained through the 8-Fr drainage catheter for 3-5 d, and then the drainage catheter was replaced with a large drainage tube. First, a guidewire was inserted into the focus through the lumen of the 8-Fr drainage catheter which was then removed. In order to apply a larger tube, patients were given local anesthesia with 0.5% procaine and a 1 cm-deep small





**Figure 2 Replacement of the drainage catheter with a larger-diameter drainage tube.** A: Gradual expansion of the drainage catheter sinus tract using different Fr skin expanders along with the guidewire; B: Pulling out the expander with its sheath left in the focus after insertion of a sheath matching the 24-Fr skin expander into the focus; C: Insertion of a 22-Fr drainage tube into the focus through the sheath lumen of a skin expander; D: Left in the focus.



**Figure 3 Debridement guided by choledochoscope.** Formation of sinus tract around the drainage tube (A) with a choledochoscope slowly inserted into the focus through the sinus tract (B) 7 d after drainage with a large drainage tube, flushing of accumulated pus and necrotic tissue fragments from the foci of SAP by injecting a large amount of sterile saline and 0.5% metronidazole into the infected foci via the water inlet of choledochoscope (C).

incision was made through the skin and subcutis, and then the 8-Fr skin expander was inserted along with the guidewire to expand the sinus tract made initially by the drainage catheter. Second, the 8-Fr skin expander was pulled out, and 12-, 16-, 20-, and 24-Fr skin expanders with a diameter of 8 mm were inserted to gradually expand the sinus tract (Figure 2A). A sheath matching the 24-Fr skin expander was inserted and left in the focus (Figure 2B), and the expander was then removed. Finally, a 22-Fr drainage tube was inserted into the focus through the sheath lumen of the 24-Fr skin expander (Figure 2C and D).

#### **Debridement under the guidance of choledochoscope**

A sinus tract around the drainage tube was firmly

formed 7 d after drainage with the larger drainage tube. Meanwhile, the peripancreatic focus was also encapsulated with fibrous connective tissue, and then debridement was performed under choledochoscope (Pentax Co. Japan). The large drainage tube was removed, and a choledochoscope was slowly inserted into the peripancreatic focus through the sinus tract of the drainage tube (Figure 3A and B). Sterile saline was rapidly injected into the focus through the water inlet of choledochoscope. After a certain amount of saline was injected into the focus, the choledochoscope was removed with pus and necrotic tissue fragments flushed out. During the procedure, bigger pieces of necrotic tissue were removed with biopsy forceps under choledochoscope.

The necrotic tissue fragments were irrigated until the effluent became clear (Figure 3C). The focus was finally flushed with 0.5% metronidazole. A drainage tube was placed into the focus through the original sinus tract and left there for next debridement.

## RESULTS

The time from the onset of pancreatitis to drainage was 4-11 d (mean 5.3 d). The number of 8-Fr drainage catheters used for external drainage was 1-5 (mean 2.2 catheters) depending on the number and locations of infected peripancreatic foci. Before the sinus tract was expanded, the external drainage was maintained for 3-5 d (mean 3.6 d). A large 22-Fr drainage tube was used for 7-10 d (mean 8.2 d).

Based on the amount of infected peripancreatic necrotic tissue and pus, the presence of systematic infection symptoms, each of the 42 patients underwent 5-14 times of choledochoscope-guided debridement (mean 8.5 times) at an interval of 2-7 d (mean 4.5 d).

After the infected peripancreatic foci were removed, the infection of patients was well controlled with decreased body temperature and normal blood leukocyte count, and the overall conditions of patients were significantly improved. CT or ultrasonic scanning showed that their infected peripancreatic necrotic tissue and fluid were significantly diminished or completely disappeared. Thirty eight out of the 42 patients were discharged from the hospital with a cure rate of 90.5%. The remaining 4 patients underwent elective cyst-jejunum anastomosis due to formation of pancreatic pseudocysts. The hospital stay time of the patients was 1-3 mo (mean 1.5 mo) depending on the severity of SAP. No complications such as hemorrhage or intestinal leakage occurred.

## DISCUSSION

Death of SAP patients may occur due to multiple organ dysfunction and severe peripancreatic infection secondary to SAP. In recent years, most SAP patients can survive after proper treatment. Since secondary peripancreatic infection may become the most refractory cause for death of SAP patients, its effective treatment is an urgent challenge for surgeons. In fact, peripancreatic effusion and infection have been existed in the early pathological process of SAP. As early as 1980s, the risk of early peripancreatic infection was recognized, and early surgical debridement in combination with postoperative drainage has become the effective procedure since then. However, it is difficult for the procedure to completely remove the infected peripancreatic necrosis tissue, and the procedure itself may result in multiple complications with high mortality. Therefore, it is generally recommended that surgical debridement should be delayed until the infected peripancreatic focus is localized and encapsulated. However, if the infected peripancreatic focus is not removed or drained, it can further exacerbate peripancreatic infection and SAP symptoms.

Some minimal invasive treatment modalities are available for pancreatic and peripancreatic infection, including CT-guided percutaneous catheter drainage<sup>[12,13]</sup>, retroperitoneal debridement and drainage<sup>[14,15]</sup>, and laparoscopic-assisted percutaneous drainage<sup>[16,17]</sup>. These procedures can resolve peripancreatic fluid collection and reduce peripancreatic infection-associated mortality. However, they are insufficient to treat peripancreatic infection secondary to SAP. Segal *et al*<sup>[15]</sup> used CT-guided percutaneous puncture and catheterization to drain the infected peripancreatic effusion and found that only slender drainage catheters can be placed in the infected focus and the infected effusion secondary to SAP is mixed with necrotic pancreatic and peripancreatic tissue fragments, which in turn block the slender drainage catheter and make it difficult to drain the infected effusion.

In the present study, an 8-Fr drainage catheter was placed into the focus under the guidance of ultrasound for a few days, and the sinus tract was expanded using a skin expander, and then a large drainage tube was used to drain the effusion. Since more and more necrotic tissue and pus are accumulated in the peripancreatic focus due to the pathological features of SAP itself, it is almost impossible to completely drain the infected necrotic tissue fragments with only one or several drainage tubes. Accordingly, debridement followed by postoperative drainage appears to be the only effective procedure. However, it causes additional traumas and postoperative complications, such as hemorrhage and intestinal leakage. Even though the infected focus is localized or well encapsulated, it is also difficult to completely remove the infected peripancreatic necrotic foci during an open-abdominal operation.

Choledochoscope is widely used in biliary surgery because of its easy manipulation and good flexibility. Flexible choledochoscope can reach various locations of the infected foci, thus guiding the flushing and debridement with minimal invasive intervention but no general anesthesia. In our study, choledochoscope was used to assist the debridement and flushing after the infected peripancreatic focus was drained with a large drainage tube for a few days when a fibrous wall was formed around the sinus tract. In this way, continuously generated necrotic tissue can be completely discarded. As a result, 38 out of the 42 SAP patients completely recovered without any complications with cure rate of 90.5% and no death occurred after treatment with the procedure, indicating that this procedure is safe, reliable and efficient to treat peripancreatic infection.

The following points should be addressed. First, in order to avoid damage to adjacent blood vessels and vital organs during puncture prior to catheterization, the puncture site should be carefully located under the guidance of ultrasound and the needle should be close to the focus. Second, the choledochoscope should not be inserted arbitrarily as it may prick the blood vessels in the focus leading to bleeding. Third, since smaller fragments of removable necrotic tissue in the focus can be cleaned by flushing with a large amount of sterile saline through



the water inlet of choledochoscope, and larger removable necrotic tissue pieces can be gently removed with a biopsy forceps. However, adhered necrotic tissue pieces should not be fiercely removed with the forceps since they will gradually become loose during the pathological process of SAP and can be removed in the next debridement under choledochoscope.

In summary, peripancreatic infection secondary to SAP can be treated promptly and efficiently with ultrasound-guided puncture and percutaneous catheter drainage followed by multiple debridement under choledochoscope. This procedure, in particular, can be performed in early SAP. Since this procedure does not require general anesthesia and open the peritoneal cavity with minimal surgical traumas, it can be safely performed for critically ill patients or for those who are unfit to undergo conventional surgical debridement. Percutaneous catheter drainage combined with choledochoscope-guided debridement can serve as an alternative to surgical debridement. We believe that this procedure is a novel and effective approach to the treatment of peripancreatic infection secondary to SAP.

## COMMENTS

### Background

Peripancreatic infection is a severe complication of severe acute pancreatitis (SAP) with a high mortality. Surgical debridement is necessary to remove infected tissue but it should be delayed till the peripancreatic infected tissue is demarcated. However, the presence of infected peripancreatic tissue without prompt debridement can further deteriorate the clinic condition of SAP patients. Thus, removal of the infected peripancreatic tissue timely and effectively becomes an urgent challenge for treatment of SAP patients.

### Research frontiers

Peripancreatic infection is one of the main causes of death of SAP patients. Open surgical debridement is a traditional treatment for peripancreatic infection, but it is believed that open surgical debridement can not completely remove necrotic tissues and further lead to multiple vital organ failure and death. Thus, it is necessary to remove infected peripancreatic tissue and effusion with minimal invasive approaches promptly and effectively.

### Innovations and breakthroughs

The results of this study have provided the evidence of successful treatment for early SAP patients using the ultrasound-guided puncture and catheter drainage combined with repeated debridement under choledochoscope, which can achieve satisfactory outcome.

### Applications

The procedure introduced in this study can be used as an alternative to the conventional open-abdominal surgical or laparoscopic debridement in treatment of peripancreatic infection of early SAP patients.

### Peer review

The study is interesting and impressive. The authors revealed the effectiveness

and safety of the new procedure they used. Although no control group was provided, the results can be accepted.

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## Appendicular tuberculosis: The resurgence of an old disease with difficult diagnosis

Francesco Barbagallo, Saverio Latteri, Maria Sofia, Agostino Ricotta, Giorgio Castello, Andrea Chisari, Valentina Randazzo, Gaetano La Greca

Francesco Barbagallo, Saverio Latteri, Maria Sofia, Agostino Ricotta, Giorgio Castello, Andrea Chisari, Valentina Randazzo, Gaetano La Greca, Department of Surgical Sciences, Transplantation and New Technologies, University of Catania, Via Messina 354, 95126 Catania, Italy

**Author contributions:** Barbagallo F and La Greca G contributed equally to this work; Barbagallo F, La Greca G and Latteri S designed the research; Barbagallo F, La Greca G, Latteri S, Ricotta A and Castello G performed the research; Chisari A acquired and elaborated the images; Barbagallo F and La Greca G, Randazzo V and Sofia M analyzed the data; Barbagallo F and La Greca G wrote the paper.

**Correspondence to:** Francesco Barbagallo, MD, Department of Surgical Sciences, Transplantation and New Technologies, University of Catania, Via Messina 354, 95126 Catania, Italy. [barbaf74@tiscali.it](mailto:barbaf74@tiscali.it)

Telephone: +39-347-3095821 Fax: +39-95-7122221

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discuss the difficulty in its diagnosis.

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**Peer reviewer:** Gianlorenzo Dionigi, MD, FACS, ESES, ETA, Associate Professor of Surgery, Department of Surgical Sciences, University of Insubria, Ospedale di Circolo, v. guicciardini, 21100 Varese, Italy

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### Abstract

Gastrointestinal tuberculosis (TB) is quite rare, representing only 3% of all extra-pulmonary cases. Blind gut and ileum are the most common gastrointestinal localizations, while appendix involvement is infrequent. Appendix involvement is usually related to symptoms of acute appendicitis since the caseous necrosis may lead to adhesions and surgical complications such as perforation. For this reason patients with suspected appendicular TB usually undergo surgery even without a secure diagnosis. In these cases, due to the absence of specific symptoms and signs, the diagnosis is delayed after surgery, thus resulting in a high percentage of important, and sometimes lethal, complications. Histopathological examination is often the only way to reach a diagnosis and to establish specific antibiotic therapy, while an early diagnosis could avoid surgical treatment. We report a case of appendicular TB not only for its rarity but also to

### INTRODUCTION

Although tuberculosis (TB) can affect all human organs, the most commonly affected region is the lung<sup>[1]</sup>. Among the extra-pulmonary patterns, bones and the urinary system are the most frequently affected (30% and 24%, respectively), followed by lymph nodes (13%) and other localizations<sup>[1]</sup>. It is estimated that 1.3 million cases of TB and 450 000 deaths due to TB occur worldwide annually<sup>[1]</sup>.

Intestinal TB is rare in Western countries<sup>[2]</sup>, with incidence rates of 35.7 and 0.43 per 100 000 per year for the immigrant and native populations, respectively<sup>[3]</sup>. Despite a clear increase in the frequency of extra-pulmonary TB in immuno-suppressed patients, the clinical features of intestinal TB are rarely seen<sup>[3]</sup>. Blind gut and ileum are the most common gastrointestinal localizations, while appendix involvement is infrequent. Prompt diagnosis is dependent on a high index of suspicion as clinical signs may be non-specific and microbiological confirmation is difficult.

Furthermore, the tuberculin skin test and chest radiograph may initially be negative in as many as 50% of patients<sup>[4]</sup>. We report a case of appendicular TB and the difficulty in its diagnosis.

## CASE REPORT

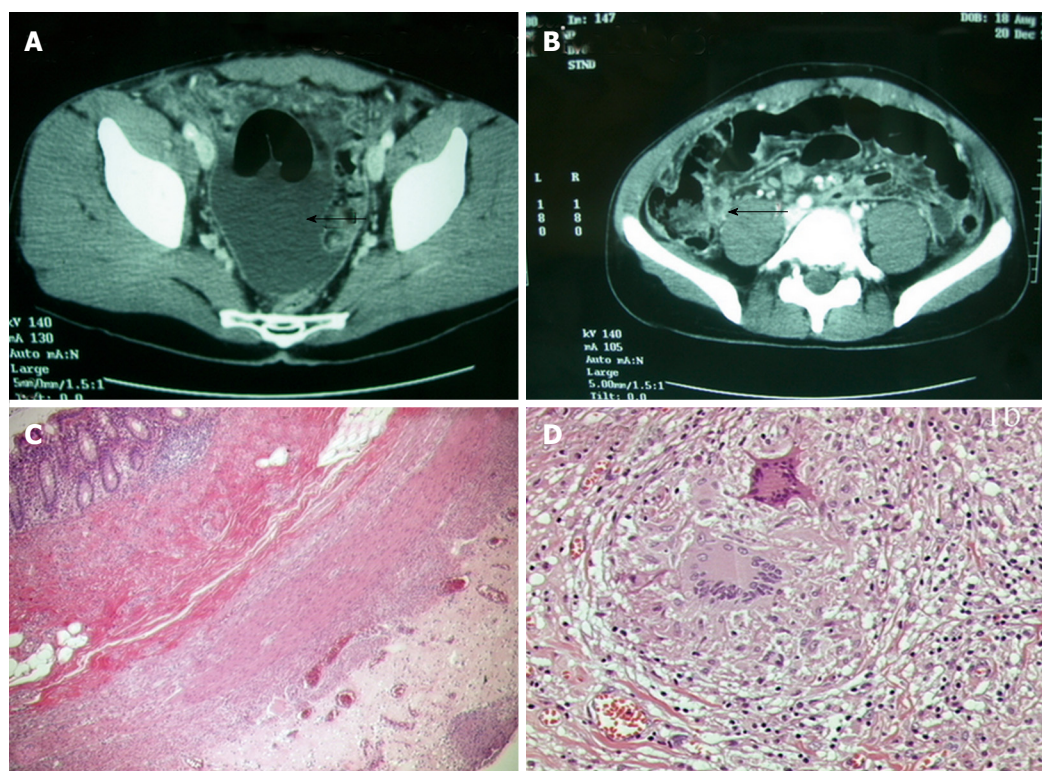
A 25-year-old male was admitted to the Infective Diseases Department of our hospital with asthenia and a light evening temperature since about a month. Backache and earache were also present. The patient was an Italian white man who had not traveled to foreign countries in the recent past. During the last two weeks the patient had a high temperature (up to 40°C), together with nausea and vomiting. The physical examination showed signs of bilateral pleural deposit, globoid abdomen, tense and hardly manageable but still canalized to gas and feces. Clinical examination only showed enhancement of the sedimentation rate. After a first assessment with thoracic X-ray and abdominal ultrasound (US) examination which demonstrated the presence of thoracic and modest abdominal fluid deposit, the patient underwent a thoracic and abdominal computed tomography (CT)-scan which confirmed the presence of a light bilateral pleural deposit, heavier on the right side, ascites and an unexpected appreciable peritoneal deposit (Figure 1A). This was associated with a solid-liquid bilobate formation of about 8 cm in diameter, with a thickened wall and peripheral enhancement (Figure 1B). The CT-scan did not describe or identify the origin of the formation from the ileum or the appendix and the patient was promptly transferred to our department and submitted to surgery, which consisted of an explorative laparoscopy soon converted to laparotomy with appendectomy and omentectomy. At a first view after laparotomy, the abdomen seemed to be affected by a diffuse peritoneal carcinosis (Figure 2A), however, on closer inspection showed the presence of a diffuse micronodular aspect involving the bowels and omentum, and tenacious adhesions especially in the appendix-ileum-blind gut region were identified (Figure 2B). The aspect was diffusely inflammatory (Figure 2C), hyperemic and edematous and was soon defined as “military” in the operative theater. A diagnosis of TB was suspected and was confirmed by definitive histopathological examination, which described the presence of “granulomatous nodular epithelioid appendicitis and mesenteritis with gigantic and Langhans cells and diffuse caseous necrosis” (Figure 1C-D). The patient was started on specific antibiotic therapy for two months consisting of daily doses of isoniazid 400 mg, rifampicin 600 mg and pirazinamide 2 g, followed by four months of isoniazid and rifampicin therapy; the treatment lasted six months and the patient made a complete recovery without complications. One, three, six and twelve months after antibiotic therapy, the patient showed no signs of recurrence on thoracic X-ray and expectorate culture.

## DISCUSSION

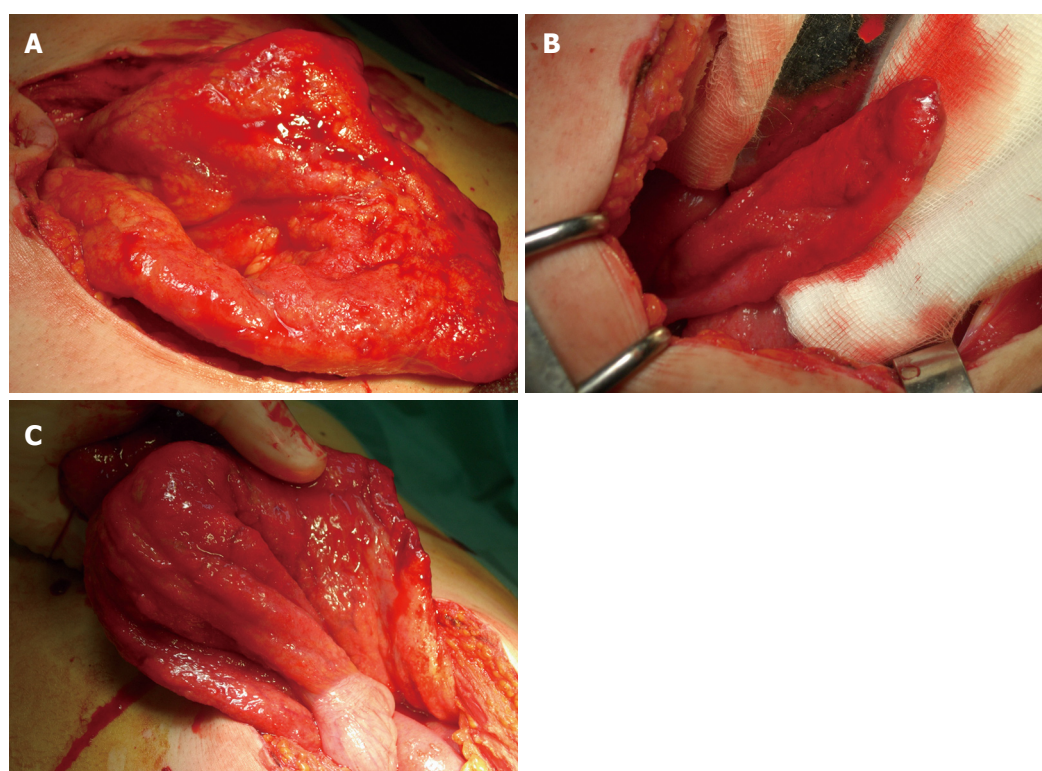
According to the WHO Report of 2009 (WHO/HTM/

TB/2009.411) an estimated one third of the world's population is infected with *Mycobacterium tuberculosis*, and nearly 9 million people develop disease caused by *M. tuberculosis* each year. This resurgence is accompanied by a rise in multidrug-resistant TB (MDR TB), which is resistant to the two most effective first-line therapeutic drugs, isoniazid and rifampicin. In addition, virtually untreatable strains of *M. tuberculosis* are emerging globally. Extensively drug-resistant TB (XDR TB) is MDR TB that is also resistant to the most effective second-line therapeutic drugs used commonly to treat MDR TB; these drugs are fluoroquinolones and at least one of three injectable second-line drugs used to treat TB (amikacin, kanamycin, or capreomycin). XDR TB has been identified in all regions of the world. Because of the limited responsiveness of XDR TB to available antibiotics, mortality rates among patients with XDR TB are similar to those of TB patients in the pre-antibiotic era<sup>[5]</sup>. The peritoneum is one of the most common extra-pulmonary sites of TB infection. Peritoneal TB remains a significant problem in parts of the world where TB is prevalent. Increasing population migration, the use of more potent immuno-suppressant therapies and the acquired immunodeficiency syndrome epidemic have contributed to a resurgence of this disease in regions where it had previously been largely controlled<sup>[6]</sup>. Gastrointestinal TB can occur as a primary or secondary form: the first form is due to a primary infection of the intestinal mucosa by *Mycobacterium bovis*; the second form is usually a consequence and complication of primary pulmonary TB by *M. tuberculosis*. It may involve any part of the intestinal tract but the most common localizations are the ileum, the blind gut and the ascending colon. Appendicular TB is rare and of uncertain physiopathological etiology. Several authors agree that the appendix as a secondary localization by a more frequent ileum, cecal or genital infection due to blood contamination<sup>[7]</sup>. In our case, no signs of TB involvement were detected other than in the appendix itself, and all the other CT aspects were compatible with reactive signs and referable to the appendicular infection. This led us to consider it as a primary localization of TB since there was no other way to distinguish it from a secondary infection in the absence of other demonstrable localizations. Histopathological examination distinguishes three types of intestinal TB: the ulcerative form, which represents 60% of all cases and the hypertrophic and ulcer-hypertrophic forms, which represent 10% and 30%, respectively, of the remaining cases. Clinical presentation is usually dominated by ascites (100%), fever (76.2%) and abdominal pain (73.8%)<sup>[8]</sup>. Signs and symptoms of abdominal TB are nonspecific and similar to those of several other chronic abdominal diseases such as Crohn's syndrome; otherwise it may simulate an acute appendicitis such as the present case. It is very important to make a correct differential diagnosis, but in most cases this is not possible until histopathological examination is carried out, which is almost always after surgery. The diagnosis of a secondary localization by a pulmonary infection is usually more simple since the radiological aspects of pulmonary TB are often characteristic and a secondary interest in the





**Figure 1** CT scan pre-operative images and histopathological images. A: CT-scan image showing relevant amount of fluid in the Douglas; B: CT-scan image showing clear thickening of the appendicular wall (arrows) and "target" image as an evident sign of appendicitis; C, D: Histopathological aspects of the biptic and resected samples.



**Figure 2** Specimen after laparotomy. A: Diffuse military aspect, simulating a peritoneal carcinosis; B: Tenacious adhesions in the appendix-ileum-blind gut region; C: Diffuse inflammatory, hyperemic and edematous aspects of the intestine.

bowel become at least assumable. In the case of a primary abdominal infection there are no characteristic radiological or serological signs and a presumptive diagnosis is really difficult to make. In these cases, great attention should be paid to the history of the patients and their contact with active TB<sup>[9]</sup>. Other than surgical diagnosis, when TB is first suspected a tuberculin skin test (TST) should be carried out although the positivity has been reported to be

only 18%-27%<sup>[10-12]</sup> in several studies. The literature also shows that culture positivity of peritoneal fluid is rarely seen<sup>[11,13-15]</sup>. High levels of adenosine deaminase (ADA) in the ascitic fluid have been shown to be compatible with the diagnosis of TB with high sensitivity (100%) and specificity (97%), but the analysis of ADA activity may not be available everywhere<sup>[15-17]</sup>. US can also be useful in detecting ascites and, in very expert hands, the caseous necrosis



causing hypoechogenic centers of the bloated lymph nodes<sup>[9,15]</sup>. The most accurate diagnostic alternative to surgery may be endoscopic biopsy of the lesions, but this still depends on the localization of the lesion itself (impossible in a case such as ours) and on the gravity of the case, possibly requiring urgent surgery. Serum tests are usually negative, other than nonspecific inflammatory signs, similar to radiological signs, which are evident but not specific for suspected TB such as mucosal wall thickening, distortions of the intestinal crests, ulcers and stenosis. During laparoscopy or open surgery, the intestinal wall appear thicker and it is usually possible to identify an inflammatory mass near the ileum-cecal region; mesenteric lymph nodes are frequently bloated and hardened with a caseous necrosis; the mucosa is often hyperemic, edematous and sometimes infected. Cytology shows the presence of Langhans cells. With or without surgical intervention, antibiotic therapy is always necessary and is similar to that of the pulmonary forms, consisting of isoniazid 300 mg/d, etambutol 15 mg/kg per day, and rifampicin 600 mg/d. Many authors suggest combining corticosteroid administration with specific antibiotic treatment to reduce the complications of abdominal TB<sup>[18-20]</sup>.

In conclusion, appendicular tuberculosis is a very rare occurrence but still a significant cause of morbidity and mortality worldwide. In the absence of specific symptoms and signs, its diagnosis is delayed, thus resulting in a high percentage of important, and sometimes lethal, complications. An assumed diagnosis of TB should be made in all patients presenting with ascites not liver related, accompanied by fever and abdominal pain. Appendicular TB is more frequent in younger patients and especially in these cases, when surgery is not yet necessary, TST, ADA peritoneal activity, culture of peritoneal fluid or endoscopic biopsy should be performed. Moreover, in patients with features similar to peritoneal carcinosis seen during surgery, TB in its military form should always be suspected. Histopathological examination after surgery for acute appendicitis often remains the only way to reach a secure diagnosis and to establish the best therapy, since surgery is often necessary but specific antibiotic therapy must be started as soon as possible. We are all seeing a resurgence of this old disease even in developed countries where it was thought to have disappeared. This is probably due to the migration of a great, and not always controlled, number of people from countries where TB still has a high incidence. The only way to resolve this problem is prophylaxis and prevention worldwide, but in the meanwhile a renewed acquaintance with this old disease is imperative.

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## A perivascular epithelioid cell tumor of the stomach: An unsuspected diagnosis

Cristina Aparecida Troques da Silveira Mitteldorf, Dario Birolini, Luis Heraldo da Camara-Lopes

Cristina Aparecida Troques da Silveira Mitteldorf, Luis Heraldo da Camara-Lopes, Laboratory of Surgical and Molecular Pathology, Hospital Sírío Libanês, rua Dona Adma Jafet 91, São Paulo, SP 01308-050, Brazil

Dario Birolini, Department of Surgery of the University of São Paulo Medical School, av. Dr. Enéas de Carvalho Aguiar 255, 05403-000, São Paulo, SP, Brazil, and Hospital Sírío Libanês, rua Dona Adma Jafet 91, São Paulo, SP 01308-050, Brazil

Luis Heraldo da Camara-Lopes, Genoa Biotechnology S.A., Alameda Ministro Rocha Azevedo, 346, 1st floor, 01410-000, São Paulo, SP 01308-050, Brazil

Author contributions: Mitteldorf CATS, Camara-Lopes LH and Birolini D contributed equally to this work; Birolini D is the surgeon responsible for the patient; Mitteldorf CATS reviewed the literature and wrote the manuscript advised by Camara-Lopes LH.

Correspondence to: Cristina Aparecida Troques da Silveira Mitteldorf, MD, PhD, Senior Pathologist, Laboratory of Surgical and Molecular Pathology, Hospital Sírío Libanês, rua Dona Adma Jafet 91, São Paulo, SP 01308-050, Brazil. [crismitteldorf@ig.com.br](mailto:crismitteldorf@ig.com.br)

Telephone: +55-11-31550556 Fax: +55-11-32312249

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tration was seen. Tumor cells were uniformly positive for vimentin, smooth muscle actin, desmin and melan A. Although unusual, PEComa should be considered in the differential diagnosis of gastric neoplasia with characteristic epithelioid and oncocyctic features and prominent vasculature.

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**Key words:** Perivascular epithelioid cell tumor; Stomach; Gastrointestinal hemorrhage

**Peer reviewer:** Jae J Kim, MD, PhD, Associate Professor, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50, Irwon-dong, Gangnam-gu, Seoul 135-710, South Korea

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### Abstract

Perivascular epithelioid cell tumor (PEComa) is a rare mesenchymal neoplasia and currently well recognized as a distinct entity with characteristic morphological, immunohistochemical and molecular findings. We report a case of PEComa arising in the antrum of a 71-year-old female with melena. The tumor, located predominantly in the submucosa as a well delimited nodule, measured 3.0 cm in diameter and was completely resected, with no evidence of the disease elsewhere. Histologically, it was composed predominantly of eosinophilic epithelioid cells arranged in small nests commonly related to variably sized vessels, with abundant extracellular material, moderate nuclear variation and discrete mitotic activity. No necrosis, angiolymphatic invasion or perineural infil-

### INTRODUCTION

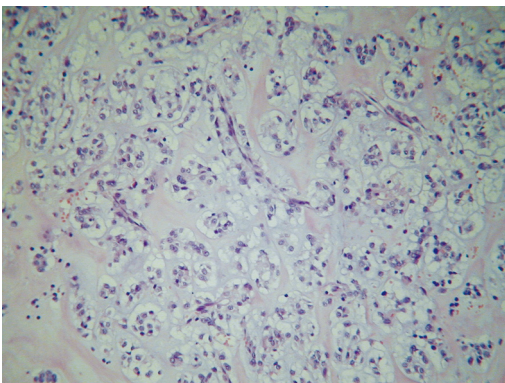
Perivascular epithelioid cell tumor (PEComa) is defined by the World Health Organization (WHO) as a mesenchymal neoplasia composed of perivascular epithelioid cells, with characteristic morphologic and immunohistochemical distinctive features<sup>[1]</sup>. It belongs to a heterogeneous family of tumors and genetic alterations have been related to cases associated with tuberous sclerosis complex (TSC)<sup>[1-3]</sup>. PEComa has been described in numerous locations, especially digestive system and uterus<sup>[1-3]</sup>, but rarely in gastrointestinal tract<sup>[4-13]</sup>. We present a case of PEComa of the stomach, a site of origin not previously reported.

### CASE REPORT

A 71-year-old female was admitted to our emergency



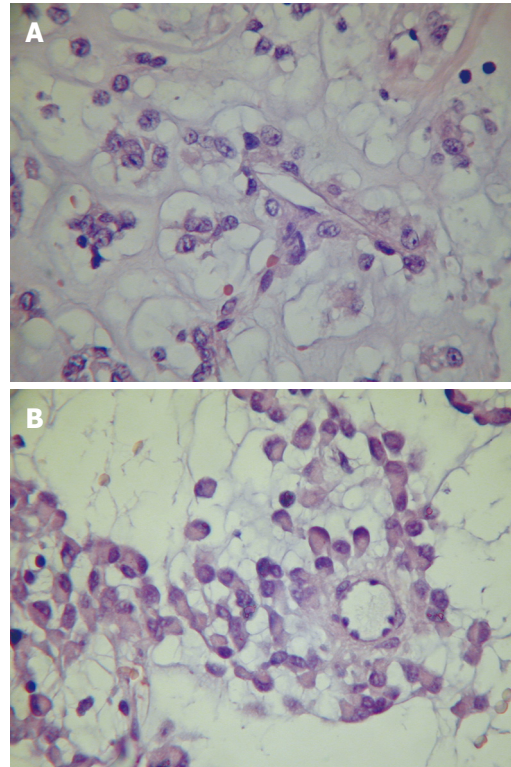
**Figure 1** Low power view showing the tumor predominantly located in the submucosa with focal ulceration of the adjacent mucosa.



**Figure 2** Microscopy showing the tumor cells mostly arranged as small nests commonly related to variably sized vessels, with abundant extracellular material and mucinous or collagenous characteristics.

room due to a recent episode of melena, referring previous similar occurrences. She had a past history of multiple laparotomies for colonic diverticulitis, acute cholecystitis and associated complications. On admission, an upper endoscopy revealed elevated gastric antral mucosa with ulceration, probably due to a submucosal lesion. A biopsy did not represent the tumor. A colonoscopy showed only diverticular disease without signs of hemorrhage or inflammation. The patient underwent a partial gastrectomy with complete resection of the lesion, during which no lymph node or peritoneal metastasis was found. After histopathological diagnosis, the patient was scanned for systemic disease, including a positron emission tomography-computed tomography (PET-CT), but nothing was found. She recovered well and was discharged 7 d after operation.

Specimen showed a predominant submucosal nodule, with focal extension to the muscularis propria. The lesion caused a discrete elevation of the adjacent mucosa with focal ulceration (Figure 1). The tumor measured 3.0 cm × 2.8 cm × 1.6 cm. Microscopic examination revealed a well-circumscribed but not encapsulated lesion. The cells were mostly arranged as small nests commonly related to variably sized vessels, with abundant extracellular material and mucinous or collagenous characteristics (Figure 2). Pseudoalveolar and focal fusocellular pattern was



**Figure 3** Neoplastic cells showing eosinophilic and clear epithelioid characteristics and perivascular arrangement (A) and focal rhabdoid features (B).

observed in some areas. The neoplasia was predominantly composed of eosinophilic and clear epithelioid cells (Figure 3A), also presenting focal rhabdoid features (Figure 3B). Moderate nuclear variation and discrete mitotic activity (1 mitosis/50 HPF) were observed, but there were no necrosis, angiolymphatic invasion or perineural infiltration. Immunohistochemically, the tumor cells were positive for vimentin, smooth muscle actin, desmin, melan A, and for CD56, but negative for pan-cytokeratin AE1/AE3, chromogranin A, CD34, CD31, CD117, S100 protein, muscle specific actin HHF-35 and HMB-45.

## DISCUSSION

PEComa is a rare tumor, resulting from the proliferation of PEC. There is a female predominance with a wide age distribution. The tumor has been described in numerous locations outside the kidney, but not in the stomach. It was reported that the findings of a conventional angiomyolipoma of the stomach, presented as a submucosal lesion, are similar to those of its renal counterpart<sup>[14]</sup>. Only 13 cases of PEComa, primarily located in the gastrointestinal tract, have been reported, including 3 in the small bowel, 5 in the colon, 1 in the appendix and 4 in the rectum<sup>[1-13]</sup>. All these cases were females, except for a 12-year-old boy with a past history of cervical neuroblastoma, probably related to TSC<sup>[10]</sup>. The age of these patients ranged 6-63 years, with an average age of 29 years, and the greatest tumor



size varied from 1.3 to 10.0 cm. Interestingly, all the four reported cases of gastrointestinal angiomyolipoma were males, with their age ranged 55-67 years (average age of 59 years), and smaller tumors (2 in the colon, 1 in the stomach, and 1 in the rectum, measuring 1.0-3.0 cm in diameter<sup>[14-17]</sup>.

PEComa is characterized by perivascular location, and the tumor cells have mostly epithelioid and spindle appearance, with clear to lightly granular eosinophilic cytoplasm. The PEC is positive for melanocytic and muscle markers<sup>[1]</sup>. The pathological findings in our case were very consistent with those of PEComa and immunohistochemistry confirmed the diagnosis.

In PEComa, the most sensitive melanocytic markers are HMB-45, Melan-A and microphthalmia transcription factor<sup>[1]</sup>. Although our experience is limited, in part due to the rarity of the disease, we have seen other cases which were weakly and even dubiously positive for HMB-45, but strongly and uniformly positive for melan A. Now, we routinely include a panel of at least three melanocytic markers to better characterize this entity.

The main differential diagnosis includes gastrointestinal stromal tumor (GIST), smooth muscle tumor, metastatic melanoma and endocrine neoplasia. GIST is the most common primary mesenchymal neoplasm of the gastrointestinal (GI) tract, but rarely shows oncocytic or rhabdoid morphology, clear cell features or pleomorphism. On the other hand, PEComa may mimic GIST when it contains fusocellular arrangement or expressing CD117. The morphology of leiomyoma and leiomyosarcoma may be very similar to that of PEComa. Some of the cases diagnosed in the past as smooth muscle tumor may indeed represent PEComas. Immunohistochemical analysis is the only way to establish the diagnosis of PEComa. Metastatic melanoma should always be considered, especially with diffuse expression of melanocytic markers. Our case was negative for S100 protein and HMB-45, but strongly and uniformly positive for smooth muscle actin and desmin. Some cases, however, may not present with such a characteristic profile. The distribution of these positive substances may be useful in the differential diagnosis. Most gastric endocrine neoplasms are represented by well-differentiated tumors or carcinoids, but small and large cell neuroendocrine carcinomas may infrequently occur. Adenocarcinomas with neuroendocrine differentiation, and mixed tumors with glandular and endocrine features have been reported. We included epithelial and neuroendocrine markers in our panel to consider these possibilities.

Ultrastructural findings, like abundant cytoplasmic glycogen, pre-melanosomes, thin filaments with occasional dense bodies, hemidesmosomes and poorly-formed intercellular junctions<sup>[1]</sup>, would help to define the lineage or origin of these peculiar cells, but we do not routinely perform ultrastructural analysis in our laboratory.

The criteria of malignancy for PEComa have not been well established. WHO guidelines based on the data from well-documented malignant PEComas, suggest

that PEComas should be regarded as malignant when they display infiltrative growth, marked hypercellularity, nuclear enlargement, hyperchromasia, high mitotic activity, atypical mitotic figures and coagulative necrosis<sup>[1]</sup>. More recently it has been reported that tumor size over 5 cm, infiltrative growth pattern, high nuclear grade, necrosis and mitotic activity over 1/50 HPF is significantly associated with aggressive clinical behavior of PEComas of soft tissue and gynecologic origin<sup>[3,7,13]</sup>.

Optimal treatment of PEComas is not well established. Currently, surgery is the main treatment modality for primary tumor, local recurrence and metastasis<sup>[18]</sup>. It is obviously difficult to perform therapeutic trials, but perhaps in the near future new specific targeted therapy may be used in patients with locally advanced or metastatic disease, when chemotherapy and immunotherapy are considered. The present case was treated exclusively with surgical resection and the patient was well, free of disease after 19 mo.

In conclusion, gastric PEComa, as an isolated lesion, is presented with gastrointestinal hemorrhage, without evidence of the disease elsewhere. Although unusual, PEComa should be considered in the differential diagnosis of gastric neoplasia with characteristic epithelioid and oncocytic features and prominent vasculature.

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## A case of laparoscopic hepatectomy for recurrent hepatocellular carcinoma

Tan To Cheung, Kelvin Kwok-chai Ng, Ronnie Tung-ping Poon, See Ching Chan, Chung Mau Lo, Sheung Tat Fan

Tan To Cheung, Kelvin Kwok-chai Ng, Ronnie Tung-ping Poon, See Ching Chan, Chung Mau Lo, Sheung Tat Fan, Department of Surgery, The University of Hong Kong, 102 Pok Fu Lam Road, Queen Mary Hospital, Hong Kong, China

Author contributions: Poon RT designed the work; Cheung TT performed the work, analyzed the data and wrote the paper; Ng KK and Chan SC revised the paper; Lo CM and Fan ST supervised the study.

Correspondence to: Tan To Cheung, Associate Consultant, Department of Surgery, The University of Hong Kong, 102 Pok Fu Lam Road, Queen Mary Hospital, Hong Kong, China. [tantocheung@hotmail.com](mailto:tantocheung@hotmail.com)

Telephone: +852-28553025 Fax: +852-28165284

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### Abstract

Conventional hepatectomy is an effective way to treat hepatocellular carcinoma. However, it is invasive and stressful. The use of laparoscopy in hepatectomy, while technically demanding, reduces surgical invasiveness and stressfulness but still achieves complete resection with adequate margins. Compared with conventional hepatectomy, laparoscopic hepatectomy provides a better chance and situation for further surgery in the case of recurrence of hepatocellular carcinoma. Even aged patients can successfully endure repeated hepatectomy using laparoscopy, as shown in the present report. This report presents a case of repeated laparoscopic hepatectomy treating hepatocellular carcinoma and its recurrence in an aged patient having cirrhosis, a disease causing extra difficulty for performing laparoscopic hepatectomy. The report also describes techniques of the operation and displays characteristic results of laparoscopic hepatectomy such as smaller wounds, less blood loss, less pain, less scars and adhesion, shorter postoperative hospital stay, and faster recovery.

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### INTRODUCTION

Laparoscopic surgery, a kind of minimally invasive surgery, has recently gained considerable advances. Nonetheless, laparoscopic hepatectomy still presents various kinds of difficulties to surgeons. Initial laparoscopic approaches in hepatic surgery were first limited to staging procedures, then extended to non-anatomical resection for hepatic lesions such as cyst, adenoma, hemangioma, and solitary liver metastasis<sup>[1-3]</sup>. Challenges of liver manipulation, parenchymal transection and hemostasis were hurdles in the progress of laparoscopic hepatectomy. Hazards such as gas embolism and difficulty in controlling bleeding discouraged many surgeons. However, with the accumulation of experience, laparoscopic hepatectomy, albeit technically demanding, is being employed to treat hepatocellular carcinoma (HCC), and reports on its safety, efficacy and reproducibility are emerging.

Like conventional hepatectomy, laparoscopic hepatectomy allows complete resection of tumors with adequate margins; but on top of this, it has a number of advantages over the former. It causes smaller wounds, less blood loss, less pain, less scars and adhesion, and smaller chance of incisional hernia. These advantages provide a better chance and situation for further surgery

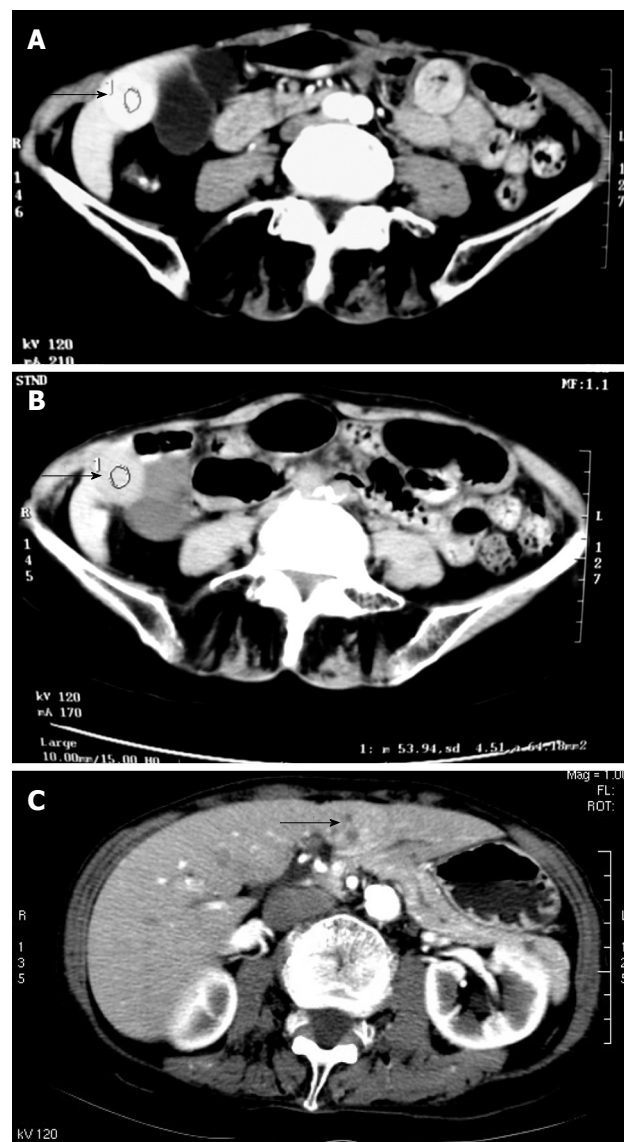
in the case of HCC recurrence which is common and hence anticipated<sup>[4]</sup>. Moreover, they render the operation less invasive and less stressful, and so make it a good treatment option for aged patients. Patients also need shorter postoperative hospital stay and have faster recovery. In the past, laparoscopic hepatectomy was not recommended for cirrhotic livers because of their high tendency towards bleeding during operation, but with improved magnification and illumination in laparoscopy today, the risk of bleeding has lessened and laparoscopic hepatectomy is no longer precluded by cirrhosis<sup>[5-7]</sup>.

This report presents a case of repeated laparoscopic hepatectomy treating HCC and its recurrence in an aged cirrhotic patient and describes the surgical techniques therein.

## CASE REPORT

An 85-year-old woman who had left radical mastectomy performed 30 years ago for carcinoma of the left breast was referred to us because of deranged liver function associated with elevated serum alpha-fetoprotein level. An ultrasound study was carried out and revealed a 2.7-cm hypoechoic lesion at the inferior tip of the right hepatic lobe. She had a serum alpha-fetoprotein level of 80 ng/mL and  $\gamma$ -glutamyl transferase level of 226 U/L. Her platelet count was  $132 \times 10^9$ /L. She was negative for both hepatitis B virus and hepatitis C virus, and her liver function was classified as Child-Pugh class A. Her indocyanine green retention rate at 15 min was 12.7%. Computed tomography (CT) scan with contrast revealed a 2.5-cm hypervascular tumor with early washout of contrast in segment 5 of the liver (Figure 1A and B). The overall picture was indicative of HCC, and laparoscopic wedge resection was planned.

The operation was performed under general anesthesia. The patient was placed in a supine position with a 30-degree Trendelenburg adjustment. The surgeon and an assistant stood at the right side of the patient. A 12-mm port was created using the open method. Pneumoperitoneum was introduced by insufflation of CO<sub>2</sub>, and intra-abdominal pressure was maintained at 12 cmH<sub>2</sub>O. A second 12-mm port was created 2 cm below the left costal margin along the anterior axillary line. A third 12-mm port was made at the right iliac fossa. Three trocars were positioned along a semicircular line, with the concavity facing the right subcostal margin. Finally, a 5-mm port was made in the subxiphoid region (Figure 2). Standard diagnostic and staging laparoscopy was performed, and the liver was examined using laparoscopic ultrasound (Aloka, Tokyo, Japan) to confirm the extension of the tumor and its relationship to the vasculature (Figure 3). The area to be transected was marked by diathermy. Resection was performed with the no-touch technique, and the liver parenchyma was transected using electrocautery supplemented by 6 cycles of radiofrequency ablation using a 3-cm single cooled-tip electrode. Each cycle of radiofrequency ablation lasted for 2 min. Hemostasis was achieved using metallic clips and by argon beam coagulation. A 1-cm resection margin was



**Figure 1** Contrast CT scan of the abdomen. A, B: CT scan of the abdomen showing a 2.5-cm tumor in segment 5 of the liver (arrow); C: CT scan of the abdomen showing a 3-cm tumor in the left lateral segment of the liver (arrow).

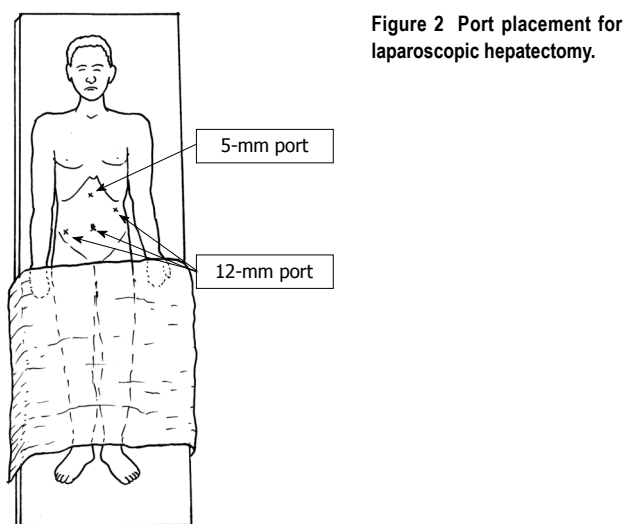
achieved. The operation lasted 180 min. No complication occurred during the operation. Blood loss of 120 mL was recorded and no blood transfusion was needed. Pringle maneuver was not applied in the operation.

Feeding started on the second day of the operation. Liver function returned to preoperative level on the fifth day. The patient was discharged home on the sixth day.

Pathology confirmed the presence of moderately differentiated HCC measuring 2.5 cm, 8 mm from the resection margin (Figure 4).

The patient was regularly followed up with CT scan and serum alpha-fetoprotein check every three months. Her serum alpha-fetoprotein level returned to 50 ng/mL three months after surgery and remained static in subsequent follow-ups, until the end of the first year when it rose to 3489 ng/mL.

CT scan with contrast was done and revealed an arterial enhancing mass measuring 3 cm  $\times$  1.8 cm  $\times$  3 cm in



**Figure 2** Port placement for laparoscopic hepatectomy.



**Figure 3** Intraoperative ultrasound scan of the liver showing a tumor measuring 23.9 mm × 21.4 mm in segment 5.

segment 2 and segment 3 of the liver, which suggested HCC (Figure 1C). Repeated laparoscopic hepatectomy was to be performed.

The patient was placed in the Lloyd-Davis position under general anesthesia. The surgeon stood between the legs with one assistant on each side. Ports were re-created at sites as in the previous operation, and pneumoperitoneum was performed. The ports measured between 5 mm and 12 mm, allowing easy change of instruments, offering flexibility in the use of ultrasonic dissector with both hands, and facilitating the introduction of endo-staplers. The falciform ligament was divided and laparoscopic ultrasound was applied to identify the vasculature of the liver. The left lateral segment was mobilized and parenchymal transection was performed with a Cavitron ultrasonic surgical aspirator (CUSA). The left lateral segment pedicle was divided using an endovascular stapler. The liver was delivered through a 6-cm left subcostal incision. The operation lasted 250 min. No adhesion was encountered and no complication occurred. Blood loss of 200 mL was recorded and no blood transfusion was needed. Pringle maneuver was not applied in the operation.

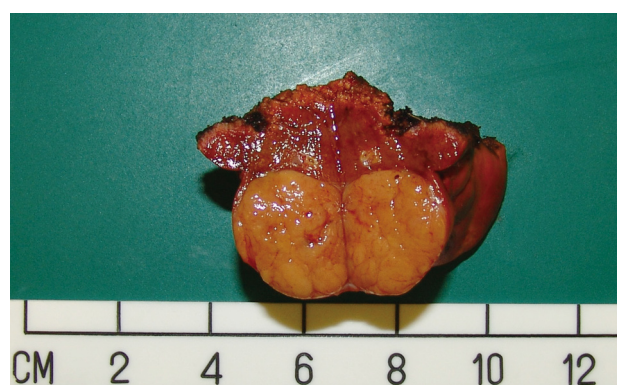
The patient was monitored in the intensive care unit for one day, and was transferred to the general surgical ward on the second day when feeding was resumed. Her liver function returned to preoperative level on the fifth day. She was discharged home on the ninth day.

Pathology confirmed the presence of moderately differentiated HCC measuring 7 cm, 7 mm from the resection margin.

The patient was faring well and remained disease-free at follow-up 18 mo later.

## DISCUSSION

Different new techniques for hepatic resection have arisen, but they remain controversial. Amongst these new techniques, the minimally invasive approach using laparoscopy has been drawing much attention<sup>[8-11]</sup>. Since the introduction of laparoscopic cholecystectomy in 1987, the laparoscopic approach has been developed for differ-



**Figure 4** The 2.5-cm resected tumor that originated in segment 5 of the liver after laparoscopic wedge excision.

ent kinds of abdominal surgery, such as gastrointestinal treatment. As to hepatic treatment, initial laparoscopic procedures in hepatectomy were limited to biopsy, tumor staging, fenestration of liver cysts, and resection of benign liver tumors. Currently, there are a few reported series on laparoscopic resection of HCC in patients with cirrhosis<sup>[6,7,12-15]</sup>. Experience of laparoscopic hepatectomy is still scarce, and laparoscopic resection of cirrhotic liver is still considered technically difficult, especially in patients with deranged liver function.

Lesurtel *et al.*<sup>[16]</sup> reported the results of 16 laparoscopic left lateral segmentectomies for benign liver diseases, HCC and metastasis. Compared with laparotomy, laparoscopic left lateral lobectomy displayed longer operation time (202 min *vs* 145 min,  $P < 0.01$ ) and longer portal triad clamping time (39 min *vs* 23 min,  $P < 0.05$ ), but less blood loss (236 mL *vs* 429 mL,  $P < 0.05$ ). The morbidity rate of the laparoscopic group was 11% and that of the open group was 15%. No death occurred. No specific complication of hepatic resection (hemorrhage, subphrenic collection, biliary leak) was observed after the laparoscopic operation, but in the open group there were some such complications.

Wounds created by laparoscopic surgery are generally smaller than those made by open surgery. In laparoscopic hepatic surgery, 4 to 5 ports are used, each measuring



5 mm to 12 mm only, so the wounds are smaller than those in traditional hepatectomy, resulting in less severe pain and smaller dosage of postoperative narcotics. The risk of incisional hernia after laparoscopic liver resection is also lower. Moreover, shorter hospital stay is observed. In most studies, the mean hospital stay after laparoscopic hepatectomy was 2 d shorter than that after open hepatectomy<sup>[16-18]</sup>.

Another generally observed advantage of laparoscopic surgery over open resection is that it causes less scarring and less adhesion. Future hepatectomy and repetition of laparoscopic resection for recurrent HCC thus become feasible. Abdominal postsurgical adhesions develop following mesothelial trauma, which can be caused by surgical handling and contact of instruments and foreign materials such as sutures and glove dusting powder, as well as by desiccation and overheating. Postoperative adhesions occur after most surgical procedures and can result in serious complications including intestinal obstruction, infertility and pain. This is a long-term unpredictable problem and impacts surgical workload and hospital resources, resulting in considerable health-care expenditure<sup>[19-21]</sup>.

Adhesions result from normal peritoneal wound-healing response and develop in the first five to seven days after injury. Adhesion formation and adhesion-free re-epithelialization are the two alternative pathways, and both begin with coagulation which initiates a cascade of events resulting in the build-up of fibrin gel matrix. If not removed, the fibrin gel matrix may become the progenitor of adhesion by forming a band or bridge when two peritoneal surfaces coated with it are apposed. The band or bridge is the basis for organization of adhesion. Protective fibrinolytic enzyme systems of the peritoneum, such as the plasmin system, can remove the fibrin gel matrix. However, surgery dramatically diminishes fibrinolytic activity. The apposition of two damaged surfaces and the extent of fibrinolysis are pivotal in determining whether the pathway taken is adhesion formation or adhesion-free re-epithelialization<sup>[20,22]</sup>.

Oncologic clearance is an important issue in laparoscopic liver resection. Risks of tumor-cell seeding and port-site metastasis are of great concern. In the early era of laparoscopic surgery in the management of malignant diseases, it was particularly worrying that pneumoperitoneum would increase tumor seeding and promote peritoneal or even intraportal spread<sup>[23]</sup>. In the first operation of the present case, the tumor to be resected was maintained intact and put in a plastic specimen bag before removal through the subumbilical port<sup>[24,25]</sup>. The tumor resection margin was 1 cm. This was to minimize the risk of tumor seeding during laparoscopic handling of the malignant tissue. In the second operation, the laparoscopic approach was again adopted for resection of the recurrent tumor because it was well localized, without invasion to any major vessel or organ, and free of adhesion. It was proven technically feasible to resect the tumor with a clear margin.

Elderly patients and patients having multiple diseases are prone to morbidity and mortality instigated by open hepatectomy. Choices of surgical management are relatively limited when the possibility of complications is taken into consideration. Minimally invasive surgery provides a chance of complete surgical resection of recurrent tumors for these patients. In the present case, the patient underwent two hepatic resections completed in the laparoscopic approach under general anesthesia within 13 mo and recovered well without complications. This is, to our knowledge, the first report regarding repeated laparoscopic hepatectomy in an elderly HCC patient with cirrhosis.

Laparoscopic hepatectomy demands a high level of technical skill, especially when bleeding is anticipated. Control of major vessel bleeding is difficult and there may be indications of conversion. With recent advancement in laparoscopic instruments, laparoscopic surgical difficulties are decreasing. Various instruments have been designed for safe transection of the liver. In the present case, we adopted a combined use of radiofrequency ablation, ultrasonic dissector, endoscopic CUSA, and endovascular stapler. We completed the liver transection by applying the endovascular stapler to the Glisson's pedicle of the left lateral segment. This stapling technique makes it fast and safe to control hepatic pedicles with an outside chance of complications, and is widely employed in both the open approach and the laparoscopic approach because it reduces the risk of bleeding and shortens operation time<sup>[8,9,12,26,27]</sup>.

Owing to anticipated technical difficulties and the possibility of bleeding during surgery, laparoscopic hepatectomy is still not a common practice amongst most hepatic surgeons. However, it is given recognition for causing less intraoperative blood loss, intractable ascites, encephalopathy and other complications of decompensation of cirrhosis. These advantages can partly be attributed to good illumination and magnification in laparoscopy. Magnification in laparoscopy can be increased up to five times without compromise of the visual field and allows even small vessels to be clearly identified, which helps in decreasing blood loss. The performance of diagnostic ultrasonographic assessment of laparoscopy can avoid unnecessary laparotomy for tumors that are too advanced.

In conclusion, laparoscopic hepatectomy for HCC is a good alternative to open hepatectomy because it has advantages including causing less adhesion after surgery, which renders re-operation much easier in cases of recurrence. It is a comparatively less invasive procedure that offers a chance of surgical clearance of tumors. Further studies with greater numbers of patients and longer follow-up are needed for a better perspective regarding the role of laparoscopic hepatectomy in the management of HCC.

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**Vito Annese, MD**, Department of Internal Medicine, Unit of Gastroenterology, Hospital, Viale Cappuccini, 1, San Giovanni Rotondo 71013, Italy

**Ramsey Chi-man Cheung, MD, Professor**, Division of GI & Hepatology, VAPAHCS(154C), 3801 Miranda Ave, Stanford University School of Medicine, Palo Alto, CA 94304, United States

**Diego Garcia-Compean, MD, Professor**, Faculty of Medicine, University Hospital, Department of Gastroenterology, Autonomous University of Nuevo Leon, Ave Madero y Gonzalitos, 64700 Monterrey, NL, México

**Ajay Goel, PhD**, Department of Internal Medicine, Division of Gastroenterology, Baylor University Medical Center and Charles A Sammons Cancer Center, 3500 Gaston Avenue, Suite H-250, Dallas, TX 75246, United States

**Marek Hartleb, Professor**, Department of Gastroenterology, Silesian Medical School, ul. Medyków 14, Katowice 40-752, Poland

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**Luis Rodrigo, Professor**, Gastroenterology Service, Hospital Central de Asturias, c/ Celestino Villamil, s.n., Oviedo 33.006, Spain

**Tor C Savidge, PhD, Associate Professor**, Department of Gastroenterology & Hepatology, Galveston, TX 77555, United States

**Eldon Shaffer, Professor** of Medicine, Division of Gastroenterology, Department of Medicine, Health Science Centre, University of Calgary, 3330 Hospital Dr N.W., Calgary, AB, T2N4N1, Canada

**Ned Snyder, MD, FACP, AGAF, Professor of Medicine, Chief** of Clinical Gastroenterology and Hepatology, Department of Internal Medicine, The University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555-0764, United States

**Scott Steele, MD, FACS, FASCRS, Chief**, Colon & Rectal Surgery, Dept of Surgery, Madigan Army Medical Center, Fort Lewis, WA 98431, United States

**Ulrike S Stein, PhD, Assistant Professor**, Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Straße 10, 13125 Berlin, Germany

**Luca Stocchi, MD**, Desk A 30, Department of Colorectal Surgery, Digestive Disease Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, United States

**Qin Su, Professor**, Department of Pathology, Cancer Hospital and Cancer Institute, Chinese Academy of Medical Sciences and Peking Medical College, PO Box 2258, Beijing 100021, China

**Rakesh Kumar Tandon, Professor**, Pushpawati Singhan Research Institute for Liver, Renal and Digestive Diseases, Sheikh Sarai- Phase II, New Delhi 110017, India

**Mitsuyoshi Urashima, MD, PhD, MPH**, Division of Molecular Epidemiology, Jikei University School of Medicine, 3-25-8 Nishi-shimbashi, Minato-ku, Tokyo 105-8461, Japan

**Robert Christiaan Verdonk, MD, PhD**, Department of Gastroenterology and Hepatology, University Medical Centre Groningen, Hanzeplein 1, Groningen, 9700 RB, The Netherlands

**Nathalie Wong, PhD, BSc(hons), Professor**, Department of Anatomical and Cellular Pathology, The Chinese University of Hong Kong, Shatin NT, Hong Kong, China



## Meetings

### Events Calendar 2010

January 25-26  
Tamilnadu, India  
International Conference on Medical  
Negligence and Litigation in Medical  
Practice

January 25-29  
Waikoloa, HI, United States  
Selected Topics in Internal Medicine

January 26-27  
Dubai, United Arab Emirates  
2nd Middle East Gastroenterology  
Conference

January 28-30  
Hong Kong, China  
The 1st International Congress on  
Abdominal Obesity

February 11-13  
Fort Lauderdale, FL, United States  
21th Annual International Colorectal  
Disease Symposium

February 26-28  
Carolina, United States  
First Symposium of GI Oncology at  
The Caribbean

March 04-06  
Bethesda, MD, United States  
8th International Symposium on  
Targeted Anticancer Therapies

March 05-07  
Peshawar, Pakistan  
26th Pakistan Society of  
Gastroenterology & Endoscopy  
Meeting

March 09-12  
Brussels, Belgium  
30th International Symposium on  
Intensive Care and Emergency  
Medicine

March 12-14  
Bhubaneswar, India  
18th Annual Meeting of Indian  
National Association for Study of  
the Liver

March 23-26  
Cairo, Egypt  
14th Pan Arab Conference on  
Diabetes PACD14

March 25-28  
Beijing, China  
The 20th Conference of the Asian

Pacific Association for the Study of  
the Liver

March 27-28  
San Diego, California, United States  
25th Annual New Treatments in  
Chronic Liver Disease

April 07-09  
Dubai, United Arab Emirates  
The 6th Emirates Gastroenterology  
and Hepatology Conference, EGHG  
2010

April 14-17  
Landover, Maryland, United States  
12th World Congress of Endoscopic  
Surgery

April 14-18  
Vienna, Austria  
The International Liver Congress™  
2010

April 28-May 01  
Dubrovnik, Croatia  
3rd Central European Congress  
of surgery and the 5th Croatian  
Congress of Surgery

May 01-05  
New Orleans, LA, United States  
Digestive Disease Week Annual  
Meeting

May 06-08  
Munich, Germany  
The Power of Programming:  
International Conference on  
Developmental Origins of Health  
and Disease

May 15-19  
Minneapolis, MN, United States  
American Society of Colon and  
Rectal Surgeons Annual Meeting

June 04-06  
Chicago, IL, United States  
American Society of Clinical  
Oncologists Annual Meeting

June 09-12  
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Emergency Medicine

June 14  
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Gastro-intestinal Models in  
the Research of Probiotics and  
Prebiotics-Scientific Symposium

June 16-19  
Hong Kong, China  
ILTS: International Liver  
Transplantation Society ILTS Annual  
International Congress

June 20-23  
Mannheim, Germany  
16th World Congress for  
Bronchoesophagology-WCBE

June 25-29  
Orlando, FL, United States  
70th ADA Diabetes Scientific  
Sessions

August 28-31  
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Diseases of the Oesophagus 2010

September 10-12  
Montreal, Canada  
International Liver Association's  
Fourth Annual Conference

September 11-12  
La Jolla, CA, United States  
New Advances in Inflammatory  
Bowel Disease

September 12-15  
Boston, MA, United States  
ICAAC: Interscience Conference  
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Meeting

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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]

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- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua 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 1, 274 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. *HRSA 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. Wiczorek 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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## AIM AND SCOPE

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### EDITING

Editorial Board of *World Journal of Gastroenterology*, Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China  
Telephone: +86-10-5908-0039  
Fax: +86-10-8538-1893  
E-mail: [wjg@wjgnet.com](mailto:wjg@wjgnet.com)  
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Telephone: +86-10-8538-1892  
Fax: +86-10-8538-1893  
E-mail: [baishideng@wjgnet.com](mailto:baishideng@wjgnet.com)  
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## MicroRNAs, development of Barrett's esophagus, and progression to esophageal adenocarcinoma

Cameron M Smith, David I Watson, Michael Z Michael, Damian J Hussey

Cameron M Smith, David I Watson, Damian J Hussey, Department of Surgery, Flinders University, Room 3D211, Flinders Medical Centre, Bedford Park, South Australia 5042, Australia  
Michael Z Michael, Department of Gastroenterology & Hepatology, Flinders University, Bedford Park, South Australia 5042, Australia

**Author contributions:** Smith CM reviewed and summarized aspects of the literature that formed the basis of the manuscript; Smith CM, Watson DI, Michael MZ, Hussey DJ (all authors) contributed to conceptual design of the manuscript and data interpretation; all authors also contributed to drafting of the article and provided critical analysis of the discussed literature; all authors gave final approval of the submitted manuscript.

**Correspondence to:** David I Watson, Professor, MBBS, MD, FRACS, Head, Department of Surgery, Flinders University, Room 3D211, Flinders Medical Centre, Bedford Park, South Australia 5042, Australia. [david.watson@flinders.edu.au](mailto:david.watson@flinders.edu.au)

Telephone: +61-8-82046086 Fax: +61-8-82046130

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### INTRODUCTION

Barrett's esophagus is characterized by a metaplastic transition whereby a columnar-lined epithelium with intestinal metaplasia arises within the squamous epithelium of the distal esophagus<sup>[1,2]</sup>. The development of Barrett's esophagus appears to be a protective action, initiated in response to the esophagus being continually exposed to chronic gastroesophageal reflux<sup>[3,4]</sup>. The clinical significance of Barrett's esophagus lies with the increased risk for cancer development, as it can progress through varying grades of dysplasia to esophageal adenocarcinoma. Barrett's esophagus is the principle identifiable precursor to esophageal adenocarcinoma. The prevalence of esophageal adenocarcinoma has been increasing significantly in Western countries, and it has a poor prognosis, with the overall 5-year survival remaining less than 20%<sup>[5,6]</sup>.

Due to the increased prevalence of esophageal adenocarcinoma, the precursor lesion, Barrett's esophagus, has come under the spotlight. Although a number of alterations in gene expression have been identified in its progression to cancer, the current sequence of molecular events that drive the development of Barrett's esophagus, and its subsequent progression to cancer, remains un-

### Abstract

Barrett's esophagus is a premalignant condition caused by gastroesophageal reflux. Once developed, it can progress through varying grades of dysplasia to esophageal adenocarcinoma. Whilst it is well accepted that Barrett's esophagus is caused by gastroesophageal reflux, the molecular mechanisms of its pathogenesis and progression to cancer remain unclear. MicroRNAs (miRNAs) are short segments of RNA that have been shown to control the expression of many human genes. They have been implicated in most cellular processes, and the role of miRNAs in disease development is becoming increasingly evident. Understanding altered miRNA expression is likely to help unravel the molecular mechanisms that underpin the development of Barrett's esophagus and its progression to cancer.

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clear<sup>[4]</sup>. Great interest surrounds the identification of early molecular changes which contribute to the pathogenesis of Barrett's esophagus, as these may present opportunities for therapeutic development, or new strategies for the prevention of esophageal adenocarcinoma. Of particular interest is the relatively newly identified class of molecules known as microRNAs (miRNAs), and the potential roles they play in the development of Barrett's esophagus and adenocarcinoma.

## miRNAs

miRNAs are approximately 21 nucleotide long, non-coding segments of RNA that act to regulate gene expression<sup>[7]</sup>. Since their initial discovery in *Caenorhabditis elegans* in 1993, an enormous body of research has been published, implicating miRNAs in almost every cellular process investigated<sup>[7,8]</sup>. Both miRNA biogenesis and miRNA regulation of protein synthesis have been reviewed extensively<sup>[7,9-12]</sup>. In short, miRNA biogenesis begins with primary miRNAs (pri-miRs, inactive form), which are either transcribed by RNA polymerase II or are excised as portions of introns<sup>[9,12]</sup>. Pri-miRs are processed in the nucleus by Drosha ribonuclease and the resultant precursor-miRNA (pre-miR) is then exported to the cytoplasm<sup>[9,12,13]</sup>. In the cytoplasm, Dicer ribonuclease processes the pre-miR and a single RNA strand is transferred to an argonaute protein within the RNA-induced silencing complex (RISC) complex<sup>[9,12]</sup>. The mature (active) miRNA-RISC complex targets complementary mRNA transcripts to repress translation<sup>[9,10]</sup>.

## THE ROLE OF miRNAs IN BARRETT'S ESOPHAGUS DEVELOPMENT

Key areas of interest for miRNAs in Barrett's esophagus include their involvement in transdifferentiation and its links with the development of columnar metaplasia, and also cancer development within Barrett's esophagus. miRNAs are useful biomarkers for tumor classification, and their expression pattern may have a role to play in the early detection of cancer development<sup>[14,15]</sup>.

### Can the developing esophagus provide insight?

To understand where miRNAs may fit into the development of Barrett's esophagus, it is important to consider how the normal esophagus develops and also how developmental mechanisms may drive pathogenesis. The esophagus begins to form during week four of embryonic development, with the formation of the foregut<sup>[16]</sup>. However, it is not until week five that the foregut can be visually divided into esophagus, stomach and duodenum<sup>[17]</sup>. Development of the esophageal lumen begins in weeks seven and eight, when the epithelium begins to proliferate, and by week 10 the lumen is enclosed and lined with a ciliated epithelium<sup>[16]</sup>. In the fourth month of gestation, the ciliated epithelium begins to be

replaced by a squamous epithelium. Residual islands of ciliated epithelia remain, and these give rise to esophageal glands<sup>[16]</sup>.

It is interesting that the human embryonic esophagus is initially lined with a columnar epithelium, which is then replaced by a stratified squamous epithelium<sup>[16]</sup>. A reversal of normal developmental mechanisms is observed in Barrett's esophagus where a columnar epithelium with intestinal metaplasia arises in the squamous-lined esophagus. Transdifferentiation involves a change from one differentiated cell type to another, occurring as metaplasia, resulting in a change in cell fate or a switch in phenotype<sup>[18]</sup>. The columnar-squamous transition that occurs in the developing esophagus is likely to result from a transdifferentiation event, as it is not dependent on cell division. In addition, some epithelial cells express both squamous and columnar markers during the transition period<sup>[19]</sup>. Although these two criteria are not a requirement for transdifferentiation, they do suggest that the epithelium is not being replaced with new epigenetically distinct cells. Therefore, gene expression profiles can be initiated to drive a squamous or columnar phenotype in the esophagus. As miRNAs function by regulating gene expression<sup>[20]</sup>, it is likely that miRNAs are involved in directing gene expression in esophageal development, and they may contribute to the development of the columnar-lined epithelium which colonizes the luminal surface of the esophagus in Barrett's esophagus.

### miRNAs can directly regulate transdifferentiation

What drives the formation of a columnar-lined epithelium with intestinal metaplasia in the esophagus? It is known that Barrett's esophagus is caused by chronic exposure of the esophagus to gastroesophageal reflux. Chang *et al.*<sup>[21]</sup> showed that squamous epithelium exposed to all-trans retinoic acid (ATRA) drove a sequence of events, beginning with the removal of surface squamous epithelia, which allowed for the esophageal sub-mucosal glands to access the luminal surface. The evidence implicating miRNAs in transdifferentiation/metaplasia is limited. However, a study by Tsonis *et al.*<sup>[22]</sup> implicated the let-7 miRNA family in regulating dedifferentiation (a critical event in transdifferentiation) in the lens and inner ear hair cell regeneration. In alveolar epithelial cells, miR-375 has been shown to regulate transdifferentiation *via* the Wnt/ $\beta$ -catenin pathway<sup>[23]</sup>. Finally, the miR-200 family and miR-205 have been implicated in driving epithelial to mesenchymal transdifferentiation, a crucial event in tumor metastasis<sup>[24]</sup>. These studies provide further evidence that fluctuations in miRNA expression play crucial roles in directing transdifferentiation.

ATRA interacts with retinoic acid receptors and retinoid X receptors (RXR) to mediate the suspected transdifferentiation response observed in the study by Chang *et al.*<sup>[21]</sup>. Importantly, lithocholic acid (LCA), a component of gastroesophageal refluxate, has been shown to interact with human RXR- $\beta$ <sup>[25]</sup>, providing evidence that ATRA exposure is relevant to the conditions

experienced in patients with chronic gastroesophageal reflux. Interestingly, RXR- $\beta$  expression is up-regulated in Barrett's esophagus compared with tissue from non-refluxing patients, and it also correlates with RXR- $\alpha$  expression<sup>[26]</sup>. This provides evidence that retinoid-induced differentiation is suppressed in normal squamous epithelia due to receptor down-regulation. In theory, following chronic exposure of the esophagus to refluxate, there could be a change in gene expression facilitated by differential expression of miRNAs targeting RXR receptor mRNA. Consequential up-regulation of RXR receptor translation would follow, allowing for increased binding of LCA to the RXR- $\beta$  receptor.

Among the retinoic acid targets are the *CDX1/2* genes, transcription factors shown to play a major role in driving intestinal gene expression required for developing a Barrett's esophagus phenotype<sup>[4,27,28]</sup>. Therefore, the initiation of RXR expression could be a crucial step in the early development of Barrett's esophagus.

There are examples of miRNA interaction with transcription factors forming negative feedback loops to regulate gene expression. One example includes the miR-200 family and miR-205 targeting of *ZEB1* and *ZEB2* transcription factors in epithelial-mesenchymal transition. The miR-200 family and miR-205 have been shown to directly repress *ZEB1* and *ZEB2* while these transcription factors regulate the transcription of the miR-200 family<sup>[29]</sup>. A miRNA regulatory loop, similar to that described by Bracken *et al.*<sup>[29]</sup>, could be directing the expression of transcription factors such as *CDX1/2* genes that are critical to the development of Barrett's esophagus.

Chronic gastroesophageal reflux is often associated with esophagitis. This condition provides the earliest disease state available for analysis prior to the development of Barrett's esophagus. Our laboratory has shown that some miRNAs are differentially regulated in response to chronic gastroesophageal reflux (unpublished results). miRNAs shown to be differentially regulated during exposure to acid or specific bile components may be involved in directing early molecular events required for the development of Barrett's esophagus. It is possible that miRNA de-regulation observed in patients with chronic reflux and esophagitis may allow RXR expression and initiate the early events required for Barrett's esophagus to develop.

## MAINTAINING A SPECIALIZED COLUMNAR EPITHELIUM

Whether miRNAs directly alter the gene expression profile in esophageal cells, thereby directing the development of a columnar epithelium with intestinal metaplasia, is unclear. Although the initial development of Barrett's esophagus may occur *via* transdifferentiation, it must be maintained. The sustained presence of the columnar epithelium suggests a continual gene expression profile is established in progenitor cells. It is possible that aberrant

miRNA expression acts to establish the required gene expression that allows progenitor cells to differentiate to a columnar phenotype. The role of miRNAs in driving differentiation is well established. An excellent example of this is miR-203, a miRNA down-regulated in Barrett's esophagus<sup>[30,31]</sup>.

### miR-203 and squamous epithelia

miR-203 has been implicated in driving terminal differentiation in skin epithelial cells<sup>[32]</sup>. The luminal surface of the esophagus is continually replaced by cells that migrate from the basal layer<sup>[33]</sup>. Once these basal cells become suprabasal, proliferative capacity is lost and terminal differentiation is initiated<sup>[32]</sup>. Suprabasal cells in the esophagus undergo terminal squamous differentiation to maintain a stratified squamous epithelium<sup>[33]</sup>. In skin epithelia, miR-203 has been shown to target and repress the transcription factor p63<sup>[32,34]</sup>. p63 plays a crucial role in maintaining stem cells in stratified squamous epithelium<sup>[32,35]</sup>. miR-203-directed repression of p63 acts to repress a cell's proliferative capacity and induce cell cycle exit<sup>[32,34]</sup>. It is therefore likely that induction of miR-203 expression is a crucial checkpoint required for terminal squamous differentiation. Whether miR-203 expression is linked with replenishment of the esophageal epithelium is unclear, although miR-203 expression is lost in Barrett's esophagus, and therefore it is likely that the miR-203-directed mechanism of epithelial replacement is lost in this tissue.

## miRNAs AND BARRETT'S ESOPHAGUS: CLINICAL INSIGHTS

Increasing evidence supports the application of knowledge about miRNAs to clinical settings. Recent publications have shown that miRNA expression patterns can be used as prognostic and pathogenic markers of numerous disease states, and can predict response to therapeutic strategies and outcomes from clinical procedures<sup>[36-40]</sup>. One clinically relevant example in carcinogenesis involves miR-21. miR-21 is reported to be up-regulated in a number of different solid tumors, and increased expression correlates with poor prognosis<sup>[38]</sup>. In the context of Barrett's esophagus, miR-196a expression has been shown to correlate with different disease states in the Barrett's metaplasia-dysplasia-carcinoma sequence<sup>[39]</sup>. Currently, Barrett's esophagus is the principle identifiable precursor to esophageal adenocarcinoma and, therefore, miR-196a expression may provide a valuable tool in early cancer detection. Standard clinical management of Barrett's esophagus involves continual endoscopic surveillance. Surveillance efficacy has been the topic of much scrutiny and a less invasive, more cost-effective patient management strategy is highly sought after<sup>[41,42]</sup>. Such an alternative management strategy could be one in which miRNA expression profiling may play a major role.

## miRNAs AND CANCER DEVELOPMENT

Aberrant miRNA expression has been linked with the development and progression of almost all cancers studied to date. The influence of miRNAs in the regulation and control of crucial cellular processes, including signaling, proliferation, apoptosis, motility and angiogenesis, has implicated a number of miRNAs in cancer development and progression<sup>[43]</sup>. Functional studies of miRNAs in cancer development have identified miRNAs acting as both oncogenes (e.g. miR17-92 cluster) and tumor suppressors (let-7)<sup>[44-46]</sup>. Forced expression of the miR17-92 cluster in mice leads to the accelerated development of B-cell lymphoma<sup>[45]</sup>. Also, the miR17-92 cluster is over-expressed in lung cancer, and this is associated with an increase in cellular proliferation<sup>[45,47]</sup>. Let-7 acts as a tumor suppressor through negative regulation of Ras<sup>[44,46]</sup>. In lung cancer, decreased let-7 expression results in increased cellular proliferation<sup>[46]</sup>. Also, increased let-7 expression *in vivo* has been shown to inhibit lung cancer cell xenograft growth in mice<sup>[48]</sup>.

### Tumor profiling and classification

Advances in the ability to profile different aspects of tumorigenesis have led to the rapid identification of differentially expressed miRNAs in cancer<sup>[49]</sup>. Different miRNA expression profiles are observed for normal and tumorigenic tissues<sup>[49]</sup>, where miRNA expression is generally down-regulated in tumor compared with normal samples<sup>[15]</sup>. Studies of miRNA expression in cancer have identified miRNA tumor profiles which can be used to classify different tumor subtypes<sup>[10,15]</sup>. Studies have also constructed miRNA expression profiles that can identify tumor origin<sup>[15]</sup>. This is particularly useful in the classification of poorly differentiated tumors.

## CURRENT KNOWLEDGE OF miRNAs IN BARRETT'S ESOPHAGUS AND ESOPHAGEAL ADENOCARCINOMA

Recent reports describe altered miRNA expression in Barrett's esophagus and esophageal adenocarcinoma. Initial work performed by Feber *et al*<sup>[50]</sup> identified miRNA alterations in these conditions. This study<sup>[50]</sup> provided preliminary evidence for using miRNAs in the identification of patients at risk of esophageal adenocarcinoma development. Since then, several publications have described miRNA expression in Barrett's esophagus and esophageal adenocarcinoma<sup>[31,39,51,52]</sup>. In a further study, our laboratory performed miRNA microarray and q-PCR analysis of miRNA expression in squamous esophageal epithelia, normal gastric epithelia, Barrett's esophagus with intestinal metaplasia and esophageal adenocarcinoma<sup>[30]</sup>. Our analysis identified miRNAs differentially regulated in Barrett's esophagus development and its subsequent progression to esophageal adenocarcinoma. miRNAs with differential expression patterns included

miR-21, miR-143, miR-145, miR-194, miR-203, miR-205 and miR-215. With respect to Barrett's esophagus and esophageal adenocarcinoma, current literature regarding miRNA expression allows for speculation about the potential molecular consequences of the aberrant miRNA expression identified in our study.

### The oncomiR, miR-21

Our group's research has shown that miR-21 expression is up-regulated in Barrett's esophagus and esophageal adenocarcinoma, compared with squamous esophageal epithelia. This finding is in accord with other publications where miR-21 has been shown to be up-regulated in various solid tumors<sup>[38]</sup>. Elevated miR-21 has been implicated in many cellular processes required for neoplastic development and progression. Elevations in miR-21 have been shown to promote survival in myeloma cells<sup>[53]</sup>, confer apoptotic resistance in prostate cancer cells<sup>[54]</sup>, increase cell proliferation, migration and invasion in hepatocellular carcinoma cells<sup>[55]</sup>, and increase invasion and metastasis in colorectal cancer cells<sup>[56]</sup>. Also, reduced miR-21 expression has been shown to reduce proliferation in MCF7 breast cancer cells and tumor growth in a mouse xenograft model<sup>[57]</sup>, reduce anchorage-independent colony formation in hepatocellular carcinoma cells<sup>[58]</sup>, and reduce invasion, intravasation and metastatic capacity of colon cancer cells<sup>[56]</sup>. It is possible that the observed up-regulation of miR-21 in esophageal adenocarcinoma may either provide a selective advantage to cells within a metaplastic columnar epithelium, increasing the chance for neoplastic development, or confer on esophageal adenocarcinoma similar molecular traits to those reported in the literature.

### Dual roles for miR-194

miR-194 is up-regulated in Barrett's esophagus and esophageal adenocarcinoma<sup>[30]</sup>. miR-194 expression is regulated by HNF-1a, a transcription factor induced in Barrett's esophagus and esophageal adenocarcinoma<sup>[59]</sup>. A study by Hino *et al*<sup>[59]</sup> also showed that miR-194 expression is induced during intestinal epithelial cell differentiation. Furthermore, miR-194 expression is induced in metastatic pancreatic cell lines<sup>[60]</sup>. Taken together, these results could suggest that elevated miR-194 may be contributing to intestinal differentiation observed in Barrett's esophagus, and may also contribute to the molecular phenotype required for tumor metastasis.

### miRNAs as tumor suppressors

miR-143, miR-145 and miR-215 are down-regulated in esophageal adenocarcinoma<sup>[30]</sup>. Similar alterations are observed in other expression profiling studies. miR-143, 145 and 215 are all down-regulated in colonic adenocarcinoma<sup>[61,62]</sup>. miR-143 and 145 have been shown to be down-regulated in gastric cancer<sup>[63]</sup> and miR-145 has been shown to be down-regulated in lung cancer<sup>[64]</sup>.

More specific roles for miR-143, 145, and 215 in carcinogenesis have been elucidated. miR-143 has been



shown to target the *KRAS* oncogene, suppressing colorectal cancer cell growth *via* inhibition of *KRAS* translation<sup>[65]</sup>. Therefore, loss of miR-143 expression in esophageal adenocarcinoma could result in a loss of *KRAS* regulation contributing to neoplastic development. Also, miR-143 up-regulation in Jurkat T cells has been linked with the regulation of FAS-mediated apoptosis<sup>[66]</sup>. miR-145 has also been implicated in regulating apoptosis *via* a negative feedback loop involving TP53, and also *via* translational inhibition of RTKN in breast cancer cell lines<sup>[67,68]</sup>. Furthermore, miR-143 and miR-145 expression are induced by p53 in response to DNA damage<sup>[69]</sup>. Therefore, the loss of both miR-143 and miR-145 in the progression of Barrett's esophagus to esophageal adenocarcinoma may alter the cell's ability to direct the appropriate apoptotic responses.

miR-215 expression is induced by *p53*, and it acts in cooperation with miR-192 to regulate cell cycle events through their ability to induce cell cycle arrest<sup>[61,70]</sup>. Loss of miR-215 expression causes a reduction in the ability of cells to regulate proliferation, a key neoplastic attribute. It is therefore possible that miR-143, 145 and 215 may act as tumor suppressors, with loss of expression contributing to the development of esophageal adenocarcinoma.

## CONCLUSION

It is now clear that altered regulation of miRNA expression results in numerous cellular consequences. Functional studies of miRNAs, which drive different disease states including neoplastic progression, are increasing in number. This literature is matched by an increase in studies evaluating miRNA expression in the clinical setting as a possible prognostic and diagnostic marker, with miRNA analysis of blood samples providing exciting potential for new avenues in disease diagnosis<sup>[71]</sup>. Although miRNA research in Barrett's esophagus and esophageal adenocarcinoma is in its early stages, increasing evidence allows for speculation about the potential roles of miRNAs in the development of Barrett's esophagus and its progression to esophageal adenocarcinoma.

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## A focus on parietal cells as a renewing cell population

Sherif M Karam

Sherif M Karam, Department of Anatomy, Faculty of Medicine and Health Sciences, United Arab Emirates University, Al-Ain PO Box 17666, United Arab Emirates

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Correspondence to: Sherif M Karam, MD, PhD, Professor, Department of Anatomy, Faculty of Medicine and Health Sciences, United Arab Emirates University, Al-Ain PO Box 17666, United Arab Emirates. [skaram@uaeu.ac.ae](mailto:skaram@uaeu.ac.ae)

Telephone: +971-3-7137493 Fax: +971-3-7672033

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### Abstract

The fact that the acid-secreting parietal cells undergo continuous renewal has been ignored by many gastroenterologists and cell biologists. In the past, it was thought that these cells were static. However, by using <sup>3</sup>H-thymidine radioautography in combination with electron microscopy, it was possible to demonstrate that parietal cells belong to a continuously renewing epithelial cell lineage. In the gastric glands, stem cells anchored in the isthmus region are responsible for the production of parietal cells. The stem cells give rise to three main progenitors: prepit, preneck and preparietal cells. Parietal cells develop either directly from the non-cycling preparietal cells or less commonly *via* differentiation of the cycling prepit and preneck cell progenitors. The formation of a parietal cell is a sequential process which involves diminishment of glycocalyx, production of cytoplasmic tubulovesicles, an increase in number and length of microvilli, an increase in number and size of mitochondria, and finally, expansion and invagination of the apical membrane with the formation of an intracellular canaliculus system. Little is known about the genetic counterparts of these morphological events. However, the time dimension of parietal cell production and the consequences of its alteration on the biological features of the gastric gland are well

documented. The production of a new parietal cell takes about 2 d. However, mature parietal cells have a long lifespan during which they migrate bi-directionally while their functional activity for acid secretion gradually diminishes. Following an average lifespan of about 54 d, in mice, old parietal cells undergo degeneration and elimination. Various approaches for genetic alteration of the development of parietal cells have provided evidence in support of their role as governors of the stem/progenitor cell proliferation and differentiation programs. Revealing the dynamic features and the various roles of parietal cells would help in a better understanding of the biological features of the gastric glands and would hopefully help in providing a basis for the development of new strategies for prevention, early detection and/or therapy of various gastric disorders in which parietal cells are involved, such as atrophic gastritis, peptic ulcer disease and gastric cancer.

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**Peer reviewers:** Vittorio Ricci, MD, PhD, Department of Physiology, Human Physiology Section, University of Pavia Medical School, Via Forlanini 6, Pavia, 27100, Italy; Bronislaw L Slomiany, PhD, Professor, Research Center, C-875, UMDNJ-NJ Dental School, 110 Bergen Street, PO Box 1709, Newark, NJ 07103-2400, United States

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### INTRODUCTION

The gastric parietal cells have attracted great interest in scientists mainly due to their dramatic morphological and biochemical changes which occur during acid

secretion. Little attention is paid to the fact that these cells are genetically programmed to have a limited life span and that they undergo perpetual renewal. In this editorial the plan is to focus on the dynamism of the parietal cell population and the tools utilized over the years to study this phenomenon.

Leblond<sup>[1]</sup> generally defined renewing cell populations by two major criteria: (1) Frequent mitoses which are restricted to a definite subpopulation of cells; and (2) Cell loss which is required to balance cell production and to keep the steady state of the population. In addition, he described 5 successive stages in the life history of renewing cells. They are first present as stem cells which divide and produce new stem cells as well as uncommitted and/or committed progenitor cells which represent the second stage in the renewing cell life. The uncommitted progenitor cells exhibit dual lineage features and eventually become committed progenitor cells with features of one lineage. These progenitor cells are usually capable of undergoing equivalent mitosis and thus amplifying the population before entering the next stage. Transit cells represent the third stage during which cellular specification gradually occurs by synthesizing new gene products. This may be encountered morphologically by gradual changes in cell structure, immunocytochemically by expression of new proteins or sugar residues, and biochemically by changes in enzymatic activities, protein composition and messenger RNA expression. The fourth stage is that of the mature cells which have completed their differentiation and thus become actively functional. In the fifth stage of terminal cells there is a gradual deterioration and eventually death and elimination<sup>[1,2]</sup>.

Parietal cells belong to the oxyntic (gastric) epithelium which is composed of one layer of heterogeneous cell populations. These cell populations have a common source of origin, but remarkably vary in their morphological features and functional potentials. The gastric epithelium is organized to form many long tubular units (gastric or oxyntic glands). Based on the allocation of the different cell types, each gastric gland is divided into pit, isthmus, neck and base regions (Figure 1)<sup>[3,4]</sup>.

Studying the dynamism of parietal cells is important for several reasons: (1) The gastric epithelium is highly vulnerable and the turnover of its cells is one of the effective defense mechanisms against damaging agents (e.g. alcohol and drugs); (2) Analysis of the normal production and loss of parietal cells provides the basis for understanding the pathogenesis of several diseases that affect parietal cell mass (e.g. Zollinger-Ellison syndrome and chronic atrophic gastritis); (3) The common gastric pathogen, *Helicobacter pylori*, is believed to play an important role in the pathogenesis of gastritis and peptic ulcer disease and may have an effect on parietal cell dynamics; (4) Studying gastric adenocarcinoma and “parietal cell carcinoma”<sup>[5]</sup> requires better understanding of the normal process of parietal cell production and loss; and (5) Identifying the origin of parietal cells, their immature

forms and the factors controlling their differentiation may lead to the design of new drugs for the treatment of peptic ulcer disease, chronic atrophic gastritis and gastric tumors.

## METHODS USED TO STUDY PARIETAL CELL DYNAMISM

Over the years, four main methods have been used to study the dynamic features of gastric epithelial parietal cells.

### Search for mitotic figures

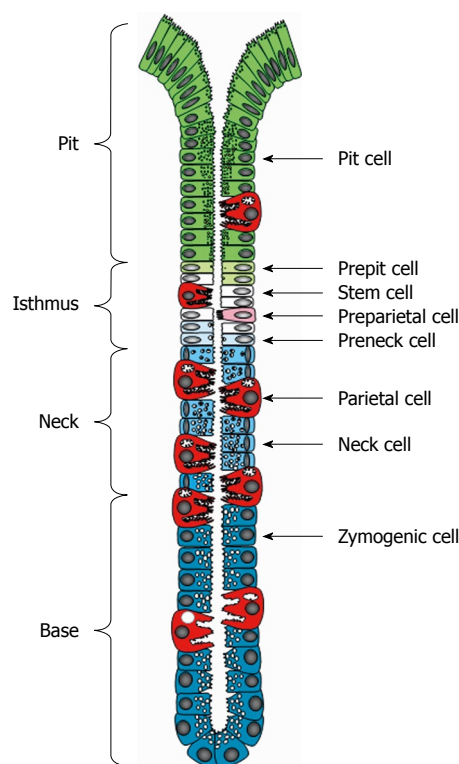
The microscopic search for mitotic figures was the method used by pioneers in this field who first discovered mitotic cells at the bottom of gastric pits<sup>[6]</sup>. Some of these mitotic figures were thought to be parietal cells<sup>[7]</sup>. The area of mitotic activity described by Bizzozzero<sup>[6]</sup> was later called the “isthmus” by Stevens *et al*<sup>[8]</sup>. Bizzozzero<sup>[6]</sup> correlated the presence of mitotic cells with the gradual increase in the secretion content of the pit lining cells from the bottom of the foveola to the free surface. He then concluded that the cells at the bottom of the pits were poorly differentiated, multiply by mitosis and migrate outward to the free surface while completing their differentiation. Deep in the mucosa, cells of the long tubular glands (including parietal cells) were thought to be stable permanent structures. Bizzozzero's conclusion was then confirmed by Benseley<sup>[9]</sup> who also observed degenerated cells from the pit within the gastric lumen. Benseley<sup>[9]</sup> then proposed that, upon reaching the free surface, pit cells were desquamated in response to damage by food. Later this food damaging idea was considered a rapid replacement of the surface epithelium following damage induced by dilute acid or alcohol. This replacement was generally considered as a regenerative process in response to damage.

A disadvantage of this mitosis search method is that no measurement of time is involved, and thus the rate at which cells are replaced cannot be calculated. The mitotic index is the parameter that can be estimated by this method. This index would only be valuable when relative measurements are needed in comparing various agents on the renewal of a cell population. However, in that case, it should be assumed that the agents have no effect on the duration of mitosis.

### Colchicine method

In the 1950s, the colchicine method of arresting and accumulating mitotic cells at metaphase was applied in the gastric epithelium by Stevens *et al*<sup>[8]</sup>. The site of mitotic activity, the isthmus region, became apparent. Moreover, by examining fasting and normally-fed rats sacrificed at different times of the day 4 h after colchicine injection, it was found that the mitotic activity and desquamation of cells were not dependent on ingested food. Thus, it was concluded that mitosis is not just a





**Figure 1** Diagrammatic representation of the gastric unit or gland. It is composed of 4 regions: pit, isthmus, neck and base and populated by a heterogeneous population of cell types. Pit, parietal and neck cells originate in the isthmus from stem (granule-free) cells and their immediate descendants (prepit, preparietal and preneck cells). Neck cells are not end cells and therefore, at the neck-base border, they transform into zymogenic cells.

response to damage, but is a sign of the normal constant “renewal” phenomenon. Moreover, Stevens *et al*<sup>[8]</sup> found that the mucous cells lining the surface and pit turnover very quickly and have a turnover time of about 3 d. Deep in the glandular epithelium, the turnover time of mucus-secreting neck cells was estimated to be about 1 wk.

### <sup>3</sup>H-thymidine radioautography

Mitosis occurs rapidly, so unless mitotic figures are frequent they may not be observed even with the colchicine method. A third method was thus developed which took advantage of the long duration of S-phase, the preparatory stage for mitosis. In mice, while the combined duration of metaphase and anaphase is about 0.4 h, the period of DNA synthesis (S-phase) extends over 7.3 h<sup>[10,11]</sup>, therefore the chances of detecting S-phase cells are high. If a radioactive DNA-precursor is injected into an animal, it will be incorporated into the DNA of dividing cells and will be retained throughout the entire life span of their daughter cells. Thus, by using radioautography not only would sites of mitotic activity be visualized, but also the post-mitotic differentiation and migration pathway of the daughter cells may be traced<sup>[12]</sup>. Based on these facts, the third method of radio-thymidine labeling of dividing cells was developed and used to study cell proliferation and migration in the gastric epithelium.

In 1960, Messier *et al*<sup>[13]</sup> demonstrated in radioautographs taken at different time intervals, evidence of the migration of mucus-secreting pit cells in rats. Labeled cells were initially present in the isthmus; within a few days they were found at the free surface. This observation was later confirmed in the mouse<sup>[14]</sup>, hamster<sup>[15]</sup>, and man<sup>[16,17]</sup>.

To analyze the dynamic parameters of a renewing cell population, <sup>3</sup>H-thymidine may be administered either by single injection, by multiple injections or by continuous infusion. It should be assumed that the population is in a steady state; i.e. cell production balances cell loss. Then, any of the three methods may be used to determine the rate at which the cells turn over (turnover rate) and the time taken for cells to be replaced (turnover time). The turnover rate may be defined as the fraction of the cell population which is replaced per unit time, whereas the turnover time is the interval taken for the replacement of the total number of cells in the population.

**Single injection method:** The use of the single injection method is based on the premise that some cells in S-phase will incorporate the label during the period between injection and sacrifice (30 min). These cells will eventually divide and yield all the cells in the population. With this single injection method, it is possible to determine the percentage of cells in the population which are synthesizing DNA and, thereby, preparing to divide. Animals, therefore, are sacrificed 30 min after a single injection of <sup>3</sup>H-thymidine, and the labeling index would be the ratio of the number of labeled cells to the total number of cells (labeled plus unlabeled cells). Because of diurnal variation, it is recommended that the average of labeling indices measured at different times throughout a 24-h period be used. The calculation of the number of new cells produced or the labeling index depends on the length of time each dividing cell spends in the S phase. Therefore, in order to calculate the turnover rate, the duration of S phase must be known. It may be obtained by plotting the percentages of labeled mitoses at different times (hours) following a single <sup>3</sup>H-thymidine injection. The distance between the ascending and descending portions of the resulting curve measured at the 50% labeling level provides the mean duration of the S phase in hours. In the mouse pyloric antrum, the S duration is 7.3 h<sup>[11]</sup>. The turnover rate is then calculated by dividing the labeling index by the S phase duration and is expressed as the percentage of cells formed per hour. The turnover time is the inverse of the turnover rate, i.e. 100/turnover rate = turnover time expressed in hours.

The single injection method has two disadvantages: the results depend on the time of day the experiment was carried out (diurnal variation), and the cells labeled after a single injection are not all necessarily committed to a single cell line.

**Multiple injection method:** The multiple injection

method is based on accumulating a labeled fraction of the cell population within a certain period of time, and then following the disappearance of these labeled cells at different time intervals after the last injection. Thus, the percentage of labeled cells would decrease steadily with time along a regression line. The rate of labeled cell loss is obtained from the decreasing slope of the line. Assuming a steady state, the rate of cell loss would be equal to the turnover rate.

The proliferative capacity of certain types of cells can be determined not only from the 30-min labeling experiment (as mentioned above) but also from the multiple injection experiment. This can be done by counting the number of silver grains over cell nuclei at different time intervals following the last injection. The percentages of cells with a given grain count are then plotted against grain count. Similar curves are prepared at the other time intervals. If the labeled cells have divided, the grains would be equally distributed to daughter cells and thus, a shift in the grain count curves with time toward a lower number of grains should occur.

A disadvantage of this method is the stress induced with the multiple injections over several days. Thus the following method is usually preferred.

**Continuous infusion method:** The continuous infusion method is based on the radio-labeling of newly produced cells in the population for different periods of infusion time. This technique minimizes the disadvantages of the single (diurnal variation) and the multiple (stress) injection methods. The percentage of labeled cells would increase steadily with time along a regression line. The rate of cell production, which under steady state is equal to the turnover rate, is the slope of the line. The “production rate” of a certain cell type can be estimated by simply multiplying the turnover rate by the number of these cells in the population. Assuming steady state, the “exit rate” of these cells would be equal to their production rate.

### Genetic manipulation

The use of genetic engineering technology for studying cell specification and differentiation has become a powerful tool for unraveling the role of the parietal cell population in maintaining homeostasis of the gastric glands. Promoters of parietal cell-specific genes are available to deliver biologically interesting foreign gene products exclusively to a parietal cell lineage throughout the lifespan of the organism<sup>[18]</sup>. These promoters can be used to design parietal cell-specific gain-of-function experiments as well as loss-of-function experiments. Thus, the mouse has become the model system for chimeric, transgenic, chimeric-transgenic, knock-out, and knock-in technology. These models will help answer many questions of parietal cell dynamics, including factors regulating their production and maintaining their organization along the pit-gland unit.

## SIZE OF THE PARIETAL CELL POPULATION

Parietal cells are scattered along the gastric glands and occupy much space due to their large size. Helander *et al*<sup>[19,20]</sup> estimated that in gastric mucosal tissue sections, the parietal cell number comprises 16%-21% of all epithelial cells, in rats<sup>[19]</sup> and 12% in humans<sup>[20]</sup>.

When some individual gastric glands of adult mice were followed in serial cross sections starting from the bottom of their base regions all the way up to opening of their pits, the parietal cell number was found to comprise about 13% of the total cell population, representing an average of 26 parietal cells out of the 194 epithelial cells present per gland<sup>[3]</sup>. An average of 4 parietal cells was found in the pit; 6 in the isthmus; 5 in the neck and 11 in the base region.

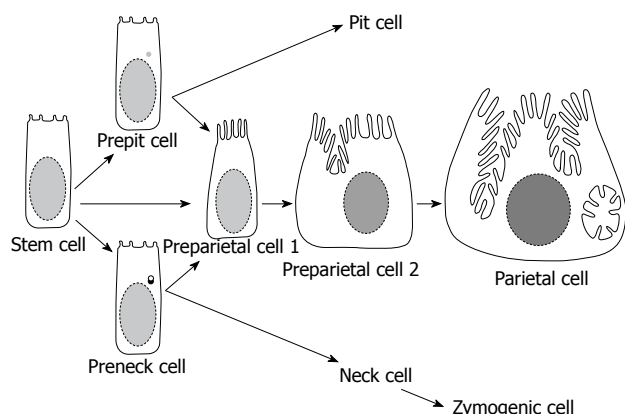
Preparietal cells are the least numerous cell type found in the gastric gland. They comprise about 0.3% of all epithelial cells, representing 0-3 cells per gland<sup>[3]</sup>.

## PARIETAL CELL PROGENITORS AND THEIR SPECIFICATION

Over the years, several theories have been raised regarding the source of parietal cells. The first idea was that parietal cells were able to divide<sup>[7]</sup> and to reproduce themselves. This idea was later refuted by studies using the colchicine arrest technique<sup>[8]</sup>, <sup>3</sup>H-thymidine radio-autography<sup>[17,21,22]</sup>, and the method of time-grain count curve<sup>[23]</sup>. Then, various investigators<sup>[24,25]</sup> re-supported the self renewal theory. It was Hunt *et al*<sup>[21]</sup> who first demonstrated radio-labeled parietal cells in paraffin sections of the rat stomach only 2 d after a pulse of <sup>3</sup>H-thymidine injection. No labeled parietal cells were found earlier than that. Hunt *et al*<sup>[21]</sup> suggested that parietal cells are not able to divide and that they originate from the mucus-secreting neck cells which were labeled at earlier time intervals. On the other hand, studies using electron microscopy<sup>[26]</sup> and in regenerating mouse gastric mucosa<sup>[27]</sup> have led to the proposal that parietal cells come from immature mucus-secreting pit cells.

In the 1960s, undifferentiated cells were identified in the oxyntic epithelium of the immature hamster<sup>[28]</sup> and bullfrog tadpole<sup>[29]</sup>. It was suggested that these cells are epithelial stem cells which are the source of parietal cells. The idea of the gastric epithelial stem cell was supported by Hattori<sup>[30]</sup> but questioned and even denied by Tamura *et al*<sup>[25]</sup>. Kataoka<sup>[14]</sup> first reported the existence of these stem cells which could be the source of parietal cells in the mouse. Later Kataoka *et al*<sup>[31]</sup>, observed some parietal cells which contained a few mucous granules and proposed that they came from the mucus-secreting neck cells.

In a systematic electron microscopic study, all cell types lining the epithelial unit of the mouse stomach were defined and quantified in serial cross sections. The tissue sections included the whole thickness of the gastric units starting from the bottom of the base region next to the



**Figure 2** Diagram summarizing the origin and differentiation program of parietal cells. The stem cell either directly or indirectly (via prepit or preneck cells) give rise to the committed progenitors of parietal cells (preparietal cells). Preparietal cells evolve in 4 successive stages (2 are depicted here) to become mature parietal cells. Preparietal cell 1 is characterized by long microvilli and preparietal cell 2 by an incipient canaliculus which expands around the nucleus in the mature parietal cell.

muscularis mucosae, up to the orifice of the pit where mucus-secreting surface cells are found. In addition to the well recognized mature cell types of the gastric epithelium (pit, neck, parietal and zymogenic cells), four different poorly differentiated cell types were identified in a narrow region along the gastric gland (Figure 1)<sup>[3]</sup>. This is the isthmus region observed earlier by Stevens *et al*<sup>[8]</sup>, which could be delimited between the first pit cell inward and the first neck cell outward (Figure 1)<sup>[3]</sup>. While one of these isthmal cells did not show any sign of differentiation, each of the three others exhibited one sign of early commitment. Thus, the first cell type was named granule-free (stem) cell and the three others, prepit, preneck and preparietal cells, reflecting their early commitment to three different cell lineages<sup>[3]</sup>.

In 30-min <sup>3</sup>H-thymidine-labeling experiments, three of the isthmal cells incorporated the radio-marker and thus were proliferative<sup>[32]</sup>. The preparietal cells, however, were never seen in mitosis and did not incorporate the label early after injection of the isotope. Thus, it was concluded that preparietal cells cannot divide and may have come from other cells which are capable of dividing and differentiating. In the isthmus, granule-free cells are similar to many embryonic cells. These cells have significant free ribosomes and dispersed chromatin, and large nucleoli, but other organelles are fewer and smaller. In addition, these are the most proliferative cell type within the gastric epithelium. All these criteria made granule-free cells good candidates as the source, not only for parietal cell lineage, but also for all other cell lineages (Figures 1 and 2).

The search for signs of cell lineage specification in the Golgi apparatus of granule-free (stem) cells revealed that they constitute three different subtypes<sup>[32]</sup>: (1) Cells with primitive Golgi having no sign of secretory activity and, hence, named “undifferentiated granule-free cells”; (2) Cells with trans-Golgi face including prosecretory

vesicles with homogeneous dense material similar to those in the pit cell lineage and, hence, considered to be “prepit cell precursors”; and (3) Cells with trans-Golgi face exhibiting prosecretory vesicles with irregular dense material in the center and light periphery similar to those of the neck cell lineage, hence, named “preneck cell precursors”.

Three different forms of preparietal cells have also been identified based on (1) the absence of secretory granules (as in granule-free cells) or (2) the presence of dense granules (as those of the pit cell lineage) or (3) the presence of cored-granules (as those of the neck cell lineage). The expression of pit- or neck-like mucous granules in a preparietal cell reflected the diversity in parietal cell origin and the phenotypic plasticity of the derivatives of granule-free cells. Thus, each subtype of granule-free cells provided a slightly different phenotype of preparietal cells which would then all pool into a single phenotype (parietal cells). Whether or not this phenotypic plasticity is unidirectional (from mucous to parietal), or bidirectional is not known. However, the occasional existence of a few mucous granules in mature parietal cells and the absolute absence of any parietal cell features (long microvilli or canaliculus, or H,K-ATPase expression) in mature mucous (pit or neck) cells suggest that it is unidirectional and only from prepit or preneck cell precursors to preparietal cells.

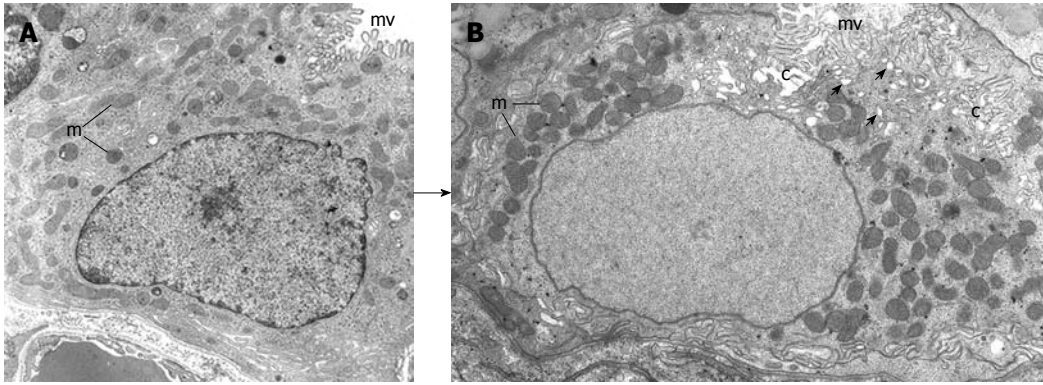
The origin of parietal cells was also demonstrated by radioautography. A pulse of <sup>3</sup>H-thymidine given to a group of mice first appeared in granule-free cells after 30 min. After 1 d, label started to appear in preparietal cells. At least 2 d later, some labeled mature parietal cells appeared, reflecting the short time required for their differentiation<sup>[33]</sup>.

The occasional presence of bi-nucleated parietal cells might give the impression that parietal cell mitosis could occur. However, during the years of our studies we have never visualized a mitotic parietal or even a preparietal cell, or observed an immuno-labeled (using antibodies for the nuclear CC-3 phosphoprotein or the proliferating cell nuclear antigen or by using the bromodeoxyuridine method) or a <sup>3</sup>H-thymidine-labeled cell. Our interpretation for these bi-nucleated parietal cells is that their early ancestors (prepit cell precursors, preneck cell precursors or undifferentiated granule-free cells) underwent an incomplete mitosis i.e. during telophase, karyokinesis occurred without cytokinesis and then the cell differentiated and matured as a bi-nucleated parietal cell<sup>[33]</sup>.

## DIFFERENTIATION COMMITMENT OF PARIETAL CELLS

Once a precursor cell enters the pathway to become a parietal cell, the gradual acquisition of specific features for acid secretion means differentiation commitment. It was Arnold<sup>[28]</sup> who first reported the presence of immature parietal cells in the hamster embryo. Similarly,





**Figure 3** Electron micrographs depicting 2 stages of the differentiating preparietal cells as seen in the human stomach. Original magnification,  $\times 12000$ . A: Preparietal cell showing relatively long numerous microvilli (mv). The mitochondria (m) are relatively few and small; B: Preparietal cell showing an incipient canaliculus (c) and relatively numerous mitochondria (m) which appear larger than those in A. The apical cytoplasm shows few tubulovesicles (small arrows) and long numerous microvilli (mv). A is reproduced from Karam *et al*<sup>[4]</sup> with permission from Wiley-Blackwell/AlphaMed press.

differentiating oxyntic cells were identified in the metamorphosing bullfrog tadpole<sup>[29]</sup>. In the adult mouse stomach, such growing cells were also identified<sup>[31]</sup> and named “preparietal cells”<sup>[3]</sup>. These cells are also found in the human stomach<sup>[4]</sup>. They gradually lose features of their precursors (large nucleoli, many free ribosomes, and relatively few organelles) and simultaneously acquire features of acid-secreting cells (long apical microvilli, intracellular canaliculi, and large numerous mitochondria).

There are four successive stages in the maturation of preparietal cells (two of them are diagrammatically presented in Figure 2)<sup>[33]</sup>. The first stage was revealed only when preparietal cells were amplified in a transgenic mouse model<sup>[34]</sup>. This stage is characterized by an increased number of apical microvilli and diminishment of their glycocalyx. The second stage (similar to that described by Arnold<sup>[28]</sup> and Forte *et al*<sup>[29]</sup>) is characterized by elongation of apical microvilli to reach the same size and shape as those of mature parietal cells (Figure 3A)<sup>[4,33]</sup>. This stage is diagrammatically represented in Figure 2 as preparietal cell 1. In the third stage, gradual invagination of the apical cytoplasm leads to the formation of an intracellular incipient canaliculus on one side of the nucleus. In addition, a few tubules and vesicles appear in the apical cytoplasm as an early indication of the formation of the tubulovesicular system (preparietal cell 2 in Figures 2 and 3B)<sup>[4,33]</sup>. In the fourth stage, the canaliculus expands and appears at both sides of the nucleus. The four stages are characterized by abundant free ribosomes and a relatively large nucleolus. With maturation of the cell from the first to the fourth stage, there is a gradual increase in the cell size and the size and number of mitochondria are gradually increased (Figure 3)<sup>[4,33]</sup>.

## PARIETAL CELL MIGRATION AND FORMATION OF TWO PHYSIOLOGICALLY DISTINCT POPULATIONS

In the mouse, the central position of preparietal cells in

the isthmus and the distribution of mature parietal cells throughout the four unit regions is the first indication of the bi-directional migration of parietal cells. In man, rat and rabbit, parietal cells are also scattered in the four regions, but are fewer in the pit<sup>[4,35]</sup>.

Continuous <sup>3</sup>H-thymidine-infusion in mice for different time intervals varying from 1 up to 52 d shows that labeled parietal cells start to appear in the isthmus after 3 d of infusion. With time, labeled parietal cells increase in the isthmus and then gradually appear in the pit region and, with time, ascend the pit. Simultaneously, labeled parietal cells make their appearance in the neck region and descend, eventually crossing the neck-base border and continuing their migration toward the bottom of the gland. An estimation of the turnover rate of parietal cells in the four unit regions indicated that it was 2.84, 3.20, 1.86, and 0.53, respectively in the pit, isthmus, neck and base. Since the average number of parietal cells in each region is known, simple calculations revealed that, every month, 6 parietal cells are produced in one isthmus. Within the same period, 3 cells appear in the pit and 3 in the neck. Thus, every month the newly produced parietal cells in the isthmus equal the sum of those appearing in both the pit and neck (6 cells). This indicates that there is a steady state in the parietal cell population.

It is likely that parietal cells located in the pit are those which originated from prepit cell precursors which were already assigned to migrate upward. The same might apply to basal parietal cells which might have originated from preneck cell precursors and are committed for inward migration.

The bidirectional migration of parietal cells along the pit-gland axis is associated with a gradual loss in their functional activity. Thus, young parietal cells present in the isthmus and neck would be expected to be structurally and functionally different from the old cells found in the pit and base regions. Evidence for this comes from at least five groups of studies on the heterogeneity of parietal cells. (1) It was Lawn<sup>[36]</sup> who first noticed that



parietal cells in the neck region have narrow and less extensive canaliculi than basal ones. Helander<sup>[37]</sup> then reported that neck parietal cells have fewer tubulovesicles and more elaborate secretory canaliculi than base parietal cells. This was later confirmed by Jacobs *et al*<sup>[38]</sup> who, in a morphometric study, found that parietal cells in the neck region have higher canalicular volume and rER surface area than those located in the base region. Electron microscopic studies on the mouse stomach revealed that, while almost all isthmus and neck parietal cells are morphologically normal, 25% of those in the pit and those in the base show signs of degeneration<sup>[33]</sup>. Similar but less pronounced degeneration of old parietal cells was also found in the rabbit<sup>[35]</sup> and this was enhanced by the omeprazole-induced inhibition of acid secretion; (2) After a 24-h-fasting, Coulton *et al*<sup>[39]</sup> were able to actively stimulate neck parietal cells with food but base parietal cells remained inactive. They proposed that basal parietal cells are either switched off or have a function other than acid secretion; (3) In a histochemical study, Firth *et al*<sup>[40]</sup> found that the H,K-ATPase activity is prominent in neck parietal cells, but absent in the basal cells; (4) In gastric glands isolated from rabbits, measurements of the morphological responsiveness of parietal cells to acid inhibitors and secretagogues revealed that luminal parietal cells respond much more efficiently than basal parietal cells<sup>[41]</sup>; and (5) The detection of H,K-ATPase  $\beta$ -subunit mRNA using a digoxigenin-labeled cRNA probe revealed a differential pattern of expression. Parietal cells located in the isthmus-neck region have high level of mRNA, while the level was low in the senescent pit and base parietal cells<sup>[41]</sup>.

The senescence-related decrease in functional and synthetic activity of parietal cells could be a sign of an alteration either in the membrane receptors, or in an intracellular mediator of the cAMP activation pathway, or in the effector proteins (proton pumps) themselves.

## ROLE OF PARIETAL CELLS AS GOVERNORS OF PROLIFERATION AND DIFFERENTIATION OF STEM/PROGENITOR CELLS

Several genetic manipulation studies have revealed different lines of evidence confirming the important role that parietal cells play as governors of both progenitor/stem cell proliferation and their differentiation into other cell lineages in the oxyntic glands. (1) In the first approach, forced expression of Simian virus 40 large T antigen gene in preproparietal cells to induce their proliferation and thus block parietal cell production was also associated with a block of zymogenic cell differentiation and a progressive increase in the number of progenitor cells starting from embryonic day 18<sup>[34,42]</sup>. With age, the situation in these mice became more dramatic due to development of dysplastic changes leading to invasive gastric adenocarcinoma with distant

metastasis<sup>[43]</sup>; (2) Genetically engineered ablation of parietal cells using the attenuated Diphtheria toxin gene in transgenic mice was also associated with a block in terminal differentiation of zymogenic cells and a massive increase in the proliferation of the multipotent stem cells and committed progenitors<sup>[44]</sup>; (3) Genetic enhancement of parietal cell apoptosis leading to a reduction in their population is also associated with a blockade in zymogenic cell differentiation and glandular hyperplasia<sup>[45]</sup>; and (4) Deletion mutants of the potassium channel, KCNQ1 in parietal cells of both mice and rats showed altered zymogenic cell lineage differentiation and hyperproliferation of glandular progenitor cells<sup>[46]</sup>.

## PHYSIOLOGICAL VS INDUCED DEATH OF PARIETAL CELLS

In physiologically renewing cell populations, before the life of a cell ends, it enters a "terminal stage" where its functional activity deteriorates and it gradually degenerates until death<sup>[1]</sup>. An occasional degeneration of parietal cells has been reported in the mouse by Kataoka *et al*<sup>[31]</sup>. When parietal cells degenerate they either show signs of necrosis or apoptosis<sup>[33]</sup>. Dead parietal cells are immediately eliminated from the epithelium by different mechanisms. Necrotic cells are usually extruded into the gland lumen, whereas, apoptotic ones are phagocytosed by neighboring pit cells, zymogenic cells or connective tissue macrophages invading through a break in the basement membrane<sup>[33,35]</sup>.

In addition to their physiological loss, death of parietal cells has been induced by various compounds. (1) Omeprazole is known to bind to the catalytic  $\alpha$  subunit of H,K-ATPase and hence, inhibit acid secretion. This covalent binding was found to be associated with a reduction in the amount of H,K-ATPase due to induced degeneration of parietal cells in rabbits<sup>[35]</sup>; (2) H<sub>2</sub> receptor antagonists (ranitidine and cimetidine) which inhibit acid secretion have also been shown to enhance parietal cell degeneration and loss in mice<sup>[47]</sup>; and (3) The orally active, cell-permeant neutrophil elastase inhibitor DMP-777 induces a rapid degeneration and loss of parietal cells in rodents<sup>[48]</sup>. This induced loss of parietal cells is associated with enhanced progenitor cell proliferation and, hence, hyperplasia develops.

## CONCLUSION

Revealing the renewal concept of parietal cells and analyzing their dynamic features have improved our understanding of the biology and pathophysiology of the gastric glands. For example: (1) The fact that parietal cells are not static provides an explanation for their functional heterogeneity. Therefore, young parietal cells in the isthmus and neck regions are more active in acid secretion than old parietal cells which have migrated to the glandular base; (2) Maturation of parietal cells in the isthmus region seems to be necessary to maintain the normal

proliferation and differentiation program of gastric epithelial stem cells and their immediate descendents; (3) Migration of parietal cells to the base region seems to be a prerequisite for the transformation of mucous neck cells into pepsinogen-secreting zymogenic cells; and (4) Regeneration of parietal cells explains the recurrence of symptoms following therapy aimed at inhibiting acid secretion and explains the possibility of hyperplastic changes and carcinoids.

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## A systematic review of efficacy and tolerability of mebeverine in irritable bowel syndrome

Mahnaz Darvish-Damavandi, Shekoufeh Nikfar, Mohammad Abdollahi

Mahnaz Darvish-Damavandi, Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran 1417614411, Iran

Shekoufeh Nikfar, Department of Pharmacoeconomics and Pharmaceutical Administration, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran 1417614411, Iran; Food & Drug Laboratory Research Center, Deputy for Food & Drug Affairs, Ministry of Health & Medical Education, Tehran 1417614411, Iran

Mohammad Abdollahi, Faculty of Pharmacy, and Pharmaceutical Sciences Research Center, and Endocrinology & Metabolism Research Center, Tehran University of Medical Sciences, Tehran 1417614411, Iran

**Author contributions:** Darvish-Damavandi M searched the literature, read the papers, gathered the data and drafted the manuscript; Nikfar S reviewed the papers, conducted the meta-analysis and reviewed the manuscript; Abdollahi M conceived the study, supervised/reviewed the entire study and edited the manuscript.

**Correspondence to:** Mohammad Abdollahi, Professor, Faculty of Pharmacy, and Pharmaceutical Sciences Research Center, and Endocrinology & Metabolism Research Center, Tehran University of Medical Sciences, Tehran 1417614411, Iran. [mohammad@tums.ac.ir](mailto:mohammad@tums.ac.ir)

Telephone: +98-21-66959104 Fax: +98-21-66959104

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women and 203 (37%) men in all subtypes of IBS. The pooled relative risk (RR) for clinical improvement of mebeverine was 1.13 (95% CI: 0.59-2.16,  $P = 0.7056$ ) and 1.33 (95% CI: 0.92-1.93,  $P = 0.129$ ) for relief of abdominal pain. The efficacy of mebeverine 200 mg compared to mebeverine 135 mg indicated RRs of 1.12 (95% CI: 0.96-1.3,  $P = 0.168$ ) for clinical or global improvement and 1.08 (95% CI: 0.87-1.34,  $P = 0.463$ ) for relief of abdominal pain. Thus, mebeverine is mostly well tolerated with no significant adverse effects; however, its efficacy in global improvement of IBS is not statistically significant.

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**Key words:** Clinical trial; Meta-analysis; Mebeverine; Placebo; Irritable bowel syndrome; Systematic review

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### Abstract

We evaluated the efficacy and tolerability of mebeverine, a musculotropic antispasmodic agent, in irritable bowel syndrome (IBS) and compared its usual dosages by meta-analysis. Medical databases and all relevant literature were searched from 1965 to June 2009 for any placebo-controlled clinical trials of mebeverine, using search terms such as mebeverine, clinical trials, and IBS. Eight randomized trials met our criteria, including six trials that compared mebeverine with placebo and two that compared mebeverine tablets with capsules. These eight trials included 555 patients randomized to receive either mebeverine or placebo with 352 (63%)

### INTRODUCTION

Irritable bowel syndrome (IBS) is a complex and widely-encountered syndrome. It is a condition characterized by abdominal pain associated with disordered defecation in the absence of any demonstrable abnormality. Despite recent advances in the treatment of IBS<sup>[1-3]</sup> the exact pathophysiology of IBS is still incompletely understood<sup>[4]</sup>. Alteration in neurohumoral mechanisms and psychological factors, bacterial overgrowth, genetic factors, gut motility, visceral hypersensitivity, and immune system factors are currently believed to influence the



pathogenesis of IBS<sup>[4,5]</sup>. There are three IBS subgroups: those with constipation, those with diarrhea, and those with alternating constipation or diarrhea<sup>[6]</sup>. The treatment of IBS is targeted at the management of constipation, diarrhea, and abdominal pain and usually includes pharmacotherapy with alosetron and other 5-HT(3)-receptor antagonists<sup>[7]</sup>.

Mebeverine is an antispasmodic that has been successfully used in the management of IBS for many years. Mebeverine is a musculotropic agent that has antispasmodic activity and regulatory effects on the bowel function<sup>[8]</sup>. During oral administration at doses of 135-270 mg *tid*, it shows no typical anticholinergic side effects, such as dry mouth, blurred vision, and impaired micturition. The incidence of side effects caused by mebeverine has not been demonstrated to be higher than that of a placebo<sup>[9]</sup>. This agent is now sold in approximately 56 countries, and its efficacy and tolerability have been demonstrated in 10 controlled studies and in many open clinical trials<sup>[9-19]</sup>. Although several clinical trials on the utility of mebeverine in patients with IBS exist, no statistical meta-analysis has been done regarding its efficacy and safety. In the present work, we systematically reviewed all the available data to examine the dose level efficacy and tolerability of mebeverine in IBS by a meta-analysis technique.

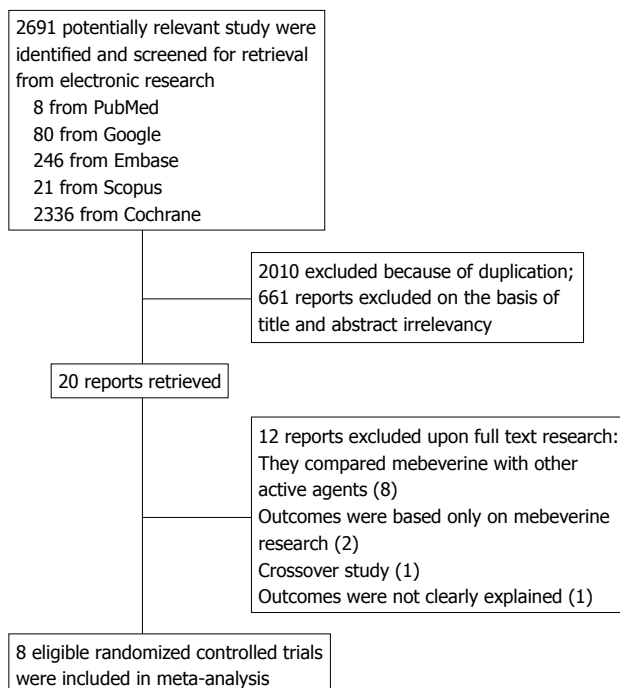
## DATA SOURCES AND META-ANALYSIS

PubMed, Embase, Scopus, Cochrane, and Google were searched from 1965 to June 2009 for clinical trials on the efficacy of mebeverine *vs* placebo. The search terms were mebeverine, clinical trial, and IBS. No language restriction was applied. The reference list from retrieved articles was also reviewed for additional applicable studies.

A total of 2691 results were examined and studies that were duplicates, case studies, and uncontrolled trials were eliminated. A high fiber diet or fiber supplementation with mebeverine was not considered a source of exclusion. Trials were disqualified if they compared mebeverine with other active agents, had not used a placebo, had used a combination of drugs, were crossover studies, and their outcomes did not relate to efficacy. Included studies used at least one clinical end point of "global assessment of symptoms by the patient or physician" or "abdominal pain and distention". The definition of global response varied widely among studies. Some trials recorded improvement *vs* no improvement, whereas others evaluated the subject's global assessment of relief. Responders in the included studies were patients who showed a global response according to the study's definition. In studies lacking a global response definition, patients who showed global improvement in symptoms were included. Two reviewers independently extracted data on patients' characteristics, therapeutic regimens, dosage, trial duration, and outcome measures. Disagreements, if any, were resolved by consensus. Among eight included studies, two compared mebeverine 135 mg with

**Table 1** Jadad quality score of randomized, controlled trials included in the meta-analysis

Study	Total score	Withdrawals and dropouts	Blinding	Randomization
Kruis <i>et al</i> <sup>[21]</sup> 1986	4	0	2	2
Connell <sup>[13]</sup> 1965	5	1	2	2
Tasman-Jones <sup>[22]</sup> 1973	4	0	2	2
Berthelot <i>et al</i> <sup>[11]</sup> 1981	4	0	2	2
Secco <i>et al</i> <sup>[19]</sup> 1983	4	0	2	2
Enck <i>et al</i> <sup>[23]</sup> 2005	5	1	2	2
Gilbody <i>et al</i> <sup>[24]</sup> 2000	4	1	2	1
Inauen <i>et al</i> <sup>[25]</sup> 1994	3	1	0	2



**Figure 1** Flow diagram of the study selection process.

mebeverine 200 mg, and the remaining studies compared mebeverine with placebo (Figure 1).

The methodological quality of included trials was assessed using the Jadad score, which judges the descriptions of randomization, blinding, and dropouts (withdrawals) in the trials<sup>[20]</sup> (Table 1). This is summarized as follow: (1) whether randomized or not (yes = 1 point, No = 0); (2) whether randomization was described appropriately or not (yes = 1 point, No = 0); (3) double blind (yes = 1 point, No = 0); (4) was the double blinding described appropriately (yes = 1 point, No = 0); and (5) whether withdrawals and dropouts described or not (yes = 1 point, No = 0). The quality scale ranges from 0 to 5 points with a low quality report of score 2 or less and a high quality report of score at least 3.

Data from selected studies were extracted in the form of 2 × 2 tables. All included studies were weighted and pooled. The data were analyzed using Statsdirect (2.7.7; 9/13/2009). Relative risk (RR) and 95% confidence intervals (95% CI) were calculated using the Mantel-

Table 2 Characteristics of studies comparing mebeverine and placebo included in meta-analysis

Study	Treatment duration (wk)	Dosage		IBS Subtype	Sex (F/M)	Mean age (yr)	
		Placebo	Mebeverine			Placebo	Mebeverine
Kruis <i>et al</i> <sup>[21]</sup> 1986	4-8-12-16	Placebo open branch <i>n</i> = 40	100 mg <i>qid</i> <i>n</i> = 40	All subtype	23/17	F = 43 M = 41	F = 43 M = 41
Connell <sup>[13]</sup> 1965	12	Wheat bran 15 g/d <i>n</i> = 40	400 mg/d <i>n</i> = 20	All subtype	25/15	40	40
Tasman-Jones <sup>[22]</sup> 1973	4	<i>n</i> = 12	400 mg/d <i>n</i> = 12	All subtype	14/10	43	43
Berthelot <i>et al</i> <sup>[11]</sup> 1981	8	<i>n</i> = 33	400 mg/d <i>n</i> = 36	All subtype	74/37	56	56
Secco <i>et al</i> <sup>[19]</sup> 1983	4	<i>n</i> = 15	400 mg/d <i>n</i> = 15	All subtype	15/15	45	45
Enck <i>et al</i> <sup>[23]</sup> 2005	16	Placebo <i>n</i> = 40 Dietary treatment <i>n</i> = 40	<i>n</i> = 40	All subtype		43	36

Table 3 Outcome results of studies comparing mebeverine with placebo included in meta-analysis

Study	Adverse effect		Relief of abdominal pain		Global or clinical improvement	
	Placebo	Mebeverine	Placebo	Mebeverine	Placebo	Mebeverine
Kruis <i>et al</i> <sup>[21]</sup> 1986	-	-	11/40	9/40	12/40	6/40
Connell <sup>[13]</sup> 1965	3/22	2/22	-	-	1/22	11/22
Tasman-Jones <sup>[22]</sup> 1973	-	-	7/24	15/24	7/24	15/24
Berthelot <i>et al</i> <sup>[11]</sup> 1981	-	-	-	-	24/33	31/36
Secco <i>et al</i> <sup>[19]</sup> 1983	-	-	9/15	12/15	-	-
Enck <i>et al</i> <sup>[23]</sup> 2005	-	-	-	-	16/40	8/40

Table 4 Characteristics of studies comparing two dosage forms of mebeverine included in meta-analysis

Study	Treatment duration (wk)	Dosage		IBS subtype	Sex (F/M)	Mean age (yr)	
		Meb 200 mg <i>bid</i>	Meb 135 mg <i>bid</i>			Meb 200 mg	Meb 135 mg
Gilbody <i>et al</i> <sup>[24]</sup> 2000	4-8	<i>n</i> = 92	<i>n</i> = 92	Abdominal pain predominant	142/42	34	32
Inauen <i>et al</i> <sup>[25]</sup> 1994	3	<i>n</i> = 24	<i>n</i> = 24	All subtype	36/12	43	37

Meb: Mebeverine.

Table 5 Outcome results of studies comparing two dosage forms of mebeverine included in meta-analysis

Study	Adverse effect		Outcomes of therapeutic efficacy		Relief of abdominal pain		Global or clinical improvement	
	Meb 200 mg	Meb 135 mg	Meb 200 mg	Meb 135 mg	Meb 200 mg	Meb 135 mg	Meb 200 mg	Meb 135 mg
Gilbody <i>et al</i> <sup>[24]</sup> 2000	66/107	63/106	74/92	69/92	65/92	64/92	64/92	59/92
Inauen <i>et al</i> <sup>[25]</sup> 1994	No serious adverse effect	No serious adverse effects			19/24	23/24	22/24	19/24

Haenszel and DerSimonian-Laird methods. The Cochran *Q* test was used to test heterogeneity. The event rate in the experimental (intervention) group against the event rate in the control group was calculated using L'Abbe plots as an aid to explore the heterogeneity of effect estimates. Funnel plot analysis was used as a publication bias indicator.

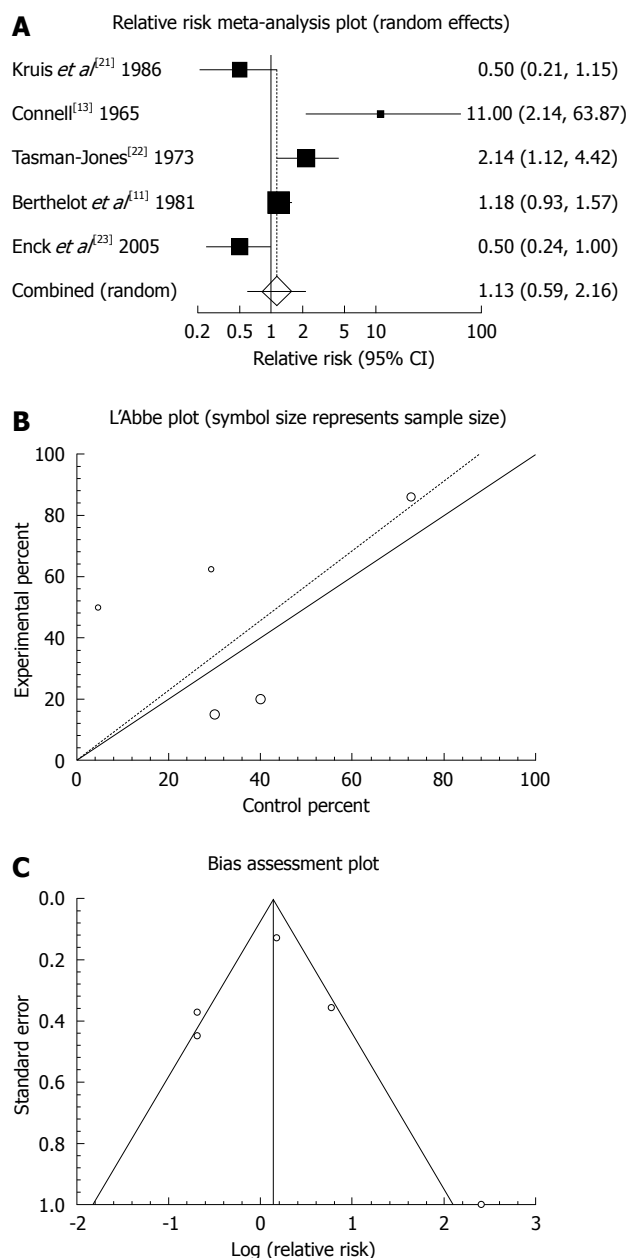
## RESULTS

The electronic searches yielded 2691 items: eight from PubMed, 80 from Google, 246 from Embase, 21 from Scopus, and 2336 from Cochrane. Of these, 20 were scrutinized in full text, eight were considered eligible and had a well-defined global response outcome and

were included in this analysis (Figure 1). The quality of the eligible studies was assessed by Jadad score. From eight studies, seven had Jadad scores  $\geq 4$ <sup>[11,13,19,21-24]</sup> and the other study had a Jadad score of 3<sup>[25]</sup> (Table 1). These eight trials included 555 patients randomized to receive either mebeverine or placebo. 352 (63%) were women and 203 (37%) were men. All subtypes of IBS were represented. Abdominal pain was prevalent in only one study<sup>[24]</sup>. Patient's characteristics, type, and dosage of mebeverine and placebo, duration of treatment, and outcomes (clinical improvement and the relief of abdominal pain) for each study are shown in Tables 2-5.

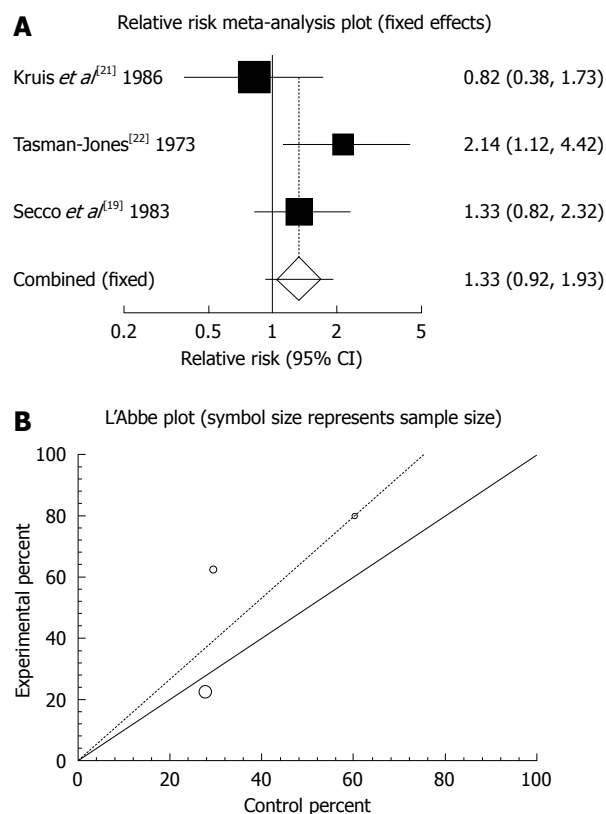
### Efficacy of mebeverine compared to placebo

The summary RR for global or clinical improvement in



**Figure 2** Individual and pooled relative risk (A), heterogeneity indicators (B), and publication bias indicators (C) for the outcome of "global or clinical improvement" in the studies comparing mebeverine vs placebo therapy.

five trials including<sup>[11,13,21-23]</sup> was 1.13 with a 95% CI of 0.59-2.16 and a non-significant RR ( $P = 0.7056$ , Figure 2A). The Cochrane  $Q$  test for heterogeneity indicated that the studies were heterogeneous ( $P = 0.0022$ , Figure 2B) and could not be combined, thus the random effects for individuals and summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for clinical response among mebeverine *vs* placebo therapy was 0.217719 (95% CI: -5.538784 to 5.974221,  $P = 0.9118$ ), and Kendall's test on standardized effect *vs* variance indicated  $\tau_{au} = 0.2$ ,  $P = 0.8167$  (Figure 2C). Summary RR for relief of abdominal pain in three trials<sup>[19,21,22]</sup> was 1.33 with a 95% CI of 0.92-1.93, a non-significant RR ( $P = 0.129$ , Figure 3A). The Cochrane  $Q$  test for heterogeneity



**Figure 3** Individual and pooled relative risk (A) and heterogeneity indicators (B) for the outcome of "relief of abdominal pain" in the studies comparing mebeverine vs placebo therapy.

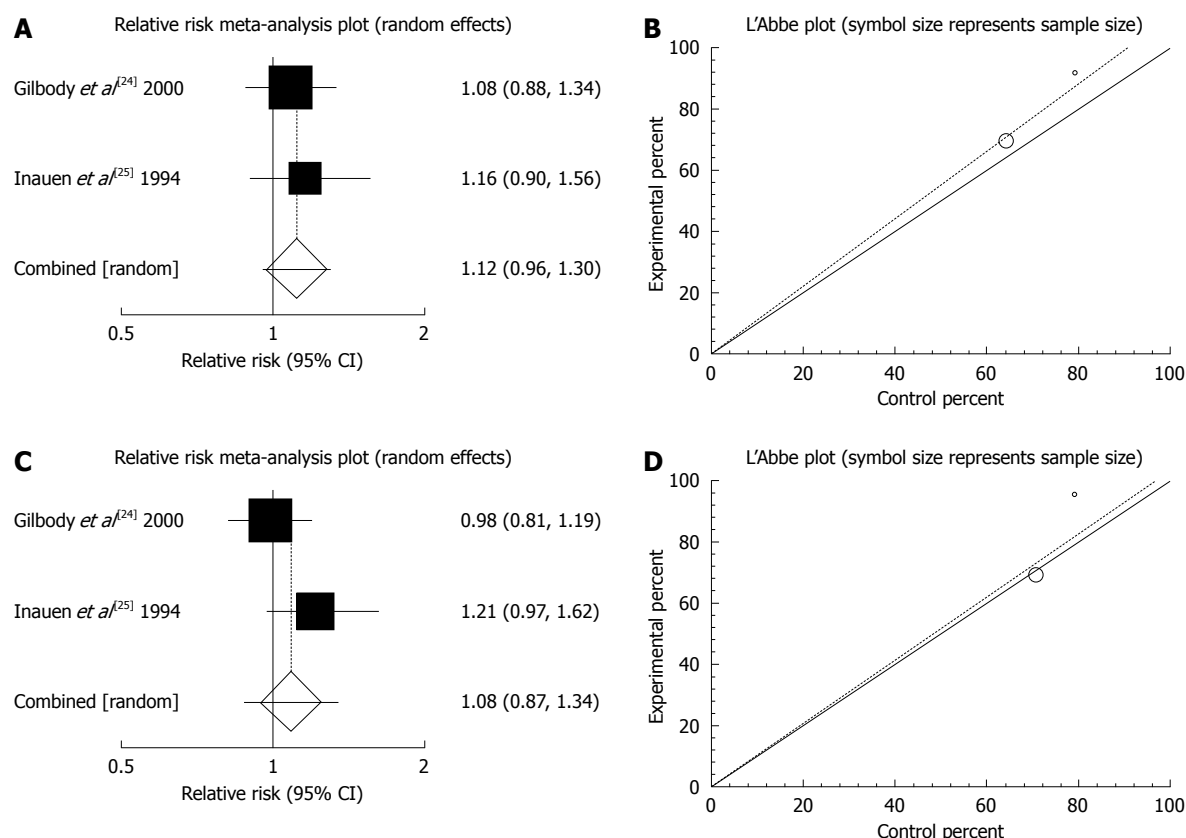
indicated that the studies were homogenous ( $P = 0.1871$ , Figure 3B) and could be combined, thus fixed effects for individuals and summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for clinical response among mebeverine *vs* placebo therapy could not be calculated because of too few strata.

### Tolerability of mebeverine compared to placebo

Adverse effects were rare or unknown in four of the six studies where mebeverine was compared to placebo. In two studies, 24% (15/62) of the mebeverine group and 22.5% (14/62) of the placebo group reported adverse effects<sup>[13,23]</sup>.

### Efficacy of mebeverine 200 mg compared to mebeverine 135 mg

The summary RR for global or clinical improvement in two trials<sup>[24,25]</sup> was 1.12 with a 95% CI of 0.96-1.3 and a non-significant RR ( $P = 0.168$ , Figure 4A). The Cochrane  $Q$  test for heterogeneity indicated that the studies were homogenous ( $P = 0.6654$ , Figure 4B) and could be combined, but because of few included studies, the random effects for individuals and summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for clinical response among mebeverine *vs* placebo therapy could not be calculated because of too few strata. Summary RR for relief of abdominal pain in two trials<sup>[24,25]</sup> was 1.08 with a 95% CI



**Figure 4** Individual and pooled relative risk and heterogeneity indicators for the outcome of “global or clinical improvement (A, B)” and “relief of abdominal pain (C, D)” in the studies considering mebeverine 200 mg compared to mebeverine 135 mg.

of 0.87-1.34, a non-significant RR ( $P = 0.463$ , Figure 4C). The Cochran  $Q$  test for heterogeneity indicated that the studies were homogenous ( $P = 0.1398$ , Figure 4D) and could be combined, but because of few included studies, the random effects for individuals and summary of RR were applied. Regression of normalized effect *vs* precision for all included studies for clinical response among mebeverine *vs* placebo therapy could not be calculated because of too few strata.

### Tolerability of mebeverine 200 mg compared to mebeverine 135 mg

Of the two studies comparing mebeverine 200 mg to mebeverine 135 mg, only one of them reported adverse effects in about 61.5% (66/107) of the mebeverine 135 mg group and 59.5% (63/106) of the mebeverine 200 mg group<sup>[24]</sup>. The other study found no serious adverse effects in the trial<sup>[25]</sup>.

## DISCUSSION

The results of this meta-analysis demonstrate that the clinical improvement and relief of abdominal pain observed for mebeverine is not statistically significant compared to placebo.

It is well tolerated without any significant adverse effects. The meta-analysis also showed that mebeverine 200 mg is as effective as mebeverine 135 mg in the

clinical improvement and relief of abdominal pain. The results also indicated no significant adverse effects for mebeverine 200 mg.

Although placebo effects in IBS clinical trials that measure a global outcome, are highly variable<sup>[26]</sup>, the last meta-analysis on myorelaxants indicated that compounds like mebeverine are superior to placebo for global improvement of IBS and reducing pain. This drug showed significant efficacy on global assessment despite a high placebo effect (38% global improvement). The efficacy was also significant and in the same range for pain relief<sup>[9]</sup>. The present results are also consistent with a previous report that indicated low incidence of side effects<sup>[11]</sup>.

Another systematic review on the safety and tolerability of antispasmodics in the treatment of IBS also confirmed the low incidence of adverse effects associated with mebeverine incidence (0.1-0.6 events per patient-year of exposure) and the investigators provided a favorable judgment regarding tolerance of mebeverine in dosages of both 600 mg and 400 mg<sup>[27,28]</sup>. Among other active agents for the treatment of IBS, probiotics can be used only as supplements of standard therapy. In addition, low doses of antidepressants induce clinical response and reduce abdominal pain score in patients with IBS<sup>[1,2]</sup>. Selective serotonin reuptake inhibitors (SSRIs) are often better tolerated than tricyclic antidepressants and have anxiety reducing benefits with a potential value



in IBS<sup>[7,29]</sup>. Despite this, results of a recent meta-analysis showed that SSRIs overall are not significantly better than placebo for the relief of individual IBS symptoms<sup>[2]</sup>. Recent trials have demonstrated that alosetron, a 5-HT<sub>3</sub> receptor antagonist, is effective in the treatment of IBS in non-constipated female *vs* placebo and *vs* mebeverine<sup>[7,30]</sup>. However, mebeverine could still be useful, particularly in treating males and constipated female with IBS, and it could diminish stool frequency or improve global feeling in diarrhea predominant IBS patients<sup>[9,31]</sup>. Thus it can be concluded that mebeverine is more effective than placebo in the management of diarrhea- or constipation-predominant IBS, without significant adverse effects.

Moreover, mebeverine 200 mg *bid* was shown to be therapeutically equivalent to mebeverine 135 mg *tid* in treatment of abdominal pain in IBS without a higher incidence of adverse effects. Studies also confirmed that both formulations of mebeverine were regarded as effective in more than 80% of cases. Tolerability was also excellent, with only few adverse effects and compliance close to 100% for most of patients. Of course, reducing the number of daily doses from three to two is an advantage of the mebeverine SR capsule in terms of patients' compliance<sup>[24,25,32]</sup>.

Fortunately, all the included studies in the present meta-analysis were well randomized, had acceptable Jadad scores, and included all subtypes of IBS (diarrhea predominant, constipation predominant, pain predominant and alternating). Some general limitations are unavoidable in meta-analyses, such as dissimilarities among patient characteristics (age, sex, lifestyle, and compliance), different duration of treatment, and different IBS subtypes; however, in this meta-analysis the high homogeneity of the included trials helped us to reach convincing conclusions. Of course, it would have been better to individualize patients based on IBS subtype and sex and evaluate outcomes for each subtype and gender, but it was not always applicable in the present study. Indeed, there is a need for more controlled, randomized trials considering the above-mentioned limitations.

## CONCLUSION

Although the effects of mebeverine on clinical improvement and relief of abdominal pain are not statistically significant, it could be considered clinically effective until more studies are added to this meta-analysis to increase the power of the conclusions. Comparing doses, the mebeverine capsule (200 mg *bid*) is effective and well tolerated without significant adverse effects and, in terms of compliance, it could be considered as an appropriate form of dosage.

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## Hepatitis D: Scenario in the Asia-Pacific region

Zaigham Abbas, Wasim Jafri, Sajjad Raza

Zaigham Abbas, Department of Hepatogastroenterology, Sindh Institute of Urology and Transplantation, Karachi 74200, Sindh, Pakistan

Wasim Jafri, Department of Medicine, The Aga Khan University Hospital, Karachi 74800, Pakistan

Sajjad Raza, Dow Medical College, Karachi 74200, Pakistan

**Author contributions:** Abbas Z conceived the topic; all authors did an independent search which was matched and divided different segments of the Asia-Pacific region for review; Abbas Z wrote the final draft; Jafri W and Raza S reviewed the final draft.

**Correspondence to:** Zaigham Abbas, FCPS, FRCP, Department of Hepatogastroenterology, Sindh Institute of Urology and Transplantation, Karachi 74200, Sindh, Pakistan. [drzabbas@gmail.com](mailto:drzabbas@gmail.com)  
Telephone: +92-21-35857172 Fax: +92-21-99215469

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### Abstract

Hepatitis D virus (HDV) infection is present worldwide and affects all age groups. Around 18 million people are estimated to be infected with HDV. An important trend in HDV infection is global decline. HDV prevalence has decreased significantly in Europe since the 1970s and 1980s when it was first reported. The Asia-Pacific region now seems to be where HDV is a major health concern. There is a lack of available data from most of the countries from this region; hence, the true status of HDV cannot be determined. In South Asia, most of the countries have conditions that are favorable for the spread of hepatitis B and other related infections. Countries like Pakistan and Iran have shown an increase in HDV prevalence over a period of time. Other countries and region like China, Turkey, Australia, Japan, India and Taiwan, some of which had very high HDV prevalence in the past, have shown a decline in the incidence, but high prevalence persists in some. Intravenous drug abusers, homosexual men and women, prostitutes, and people on hemodialysis are the groups with very high HDV prevalence.

### INTRODUCTION

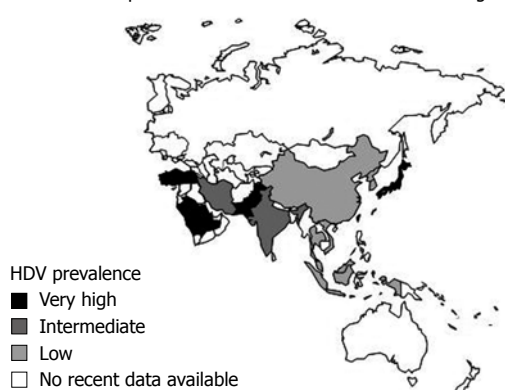
Hepatitis D virus (HDV) infection is present worldwide and affects all age groups. However, it does not have uniform distribution and its general pattern is parallel to that of hepatitis B virus (HBV). Its prevalence is highest in some parts of Africa, South America, Romania, Russia and the Mediterranean region included Southern Italy<sup>[1]</sup>. It is also noteworthy that approximately 5% of the global HBV carriers are co-infected with HDV. Out of approximately 350 million carriers of HBV worldwide, 18 million people are infected with HDV<sup>[2]</sup>.

Hepatitis D prevalence has declined significantly in Europe since the 1970s and 1980s when it was first reported. The new foci of infection now seem to be in the developing countries. The Asia-Pacific region seems to be where HDV exists with high prevalence rates in some of the countries<sup>[3]</sup>.

### HDV

HDV is also known as hepatitis delta virus, and is a defective RNA virus that requires HBV for its virion assembly and penetration into hepatocytes<sup>[4]</sup>. There are three genotypes of HDV, identified on the basis of analysis of HDV genomes from various parts of the world<sup>[5]</sup>. The most prevalent worldwide is genotype I, which is related to a broad spectrum of pathogenicity.

Hepatitis Delta-Scenario in the Asia-Pacific region


**Figure 1** Hepatitis D prevalence in the Asia-Pacific region.

The United States, Middle East and Europe are the places where genotype I is predominant, with some geographically based subtypes<sup>[6,7]</sup>. Genotype II is predominant in the Far East<sup>[5]</sup>. Genotype III is associated with severe forms of hepatitis and is predominant in Northern South America<sup>[8]</sup>.

## GLOBAL TREND OF DECLINE

An important trend in worldwide HDV infection is a global decline in the prevalence of hepatitis D infection, which is true for both acute and chronic forms of the disease<sup>[9]</sup>. This decreasing trend is the result of global HBV vaccination, increasing awareness, improved prevention strategies and socioeconomic conditions<sup>[10]</sup>. Italy, which was considered a traditionally prevalent area for HDV and where the virus was first reported, has shown a steady decline in the prevalence of this infection from 23% in 1987 to 14% in 1992 and 8.3% in 1997<sup>[11-13]</sup>. Figure 1 summarizes the prevalence of hepatitis D in the Asia-Pacific region, incorporating the recent data.

## HDV IN PAKISTAN

In South Asia, most of the countries offer conditions that are favorable for the spread of hepatitis B and other related infections. These conditions mainly include lack of defensive strategies and approaches for these infections, extreme and persistent poverty, densely populated areas, and deteriorating public health and educational infrastructure. According to a study conducted from January 1994 to April 2001, the prevalence of HDV infection in Pakistani hepatitis B surface antigen (HBsAg)-positive individuals was 16.6%. A large belt with high prevalence exists in the middle of the country, which comprises rural areas<sup>[10]</sup>. The predominant genotype of HDV is genotype I and that of HBV is genotype D<sup>[5]</sup>.

A recently conducted study shows a very high HDV prevalence of 58.6% in HBsAg-positive patients who visited liver clinics in Karachi and Jacobabad. The study showed a higher prevalence compared to that in a previous study by Mumtaz *et al*<sup>[10]</sup>. The prevalence

in these patients coming from districts of Sindh and Balochistan Provinces was 67% in Jacobabad, 65.4% in Jafferabad, 60% in Naseerabad, 69.2% in Kashmore, 65.2% in Quetta and 36.6% in Karachi<sup>[14]</sup>.

The high prevalence could be due to the frequent use of therapeutic injections and drips, contaminated needles, surgical and dental equipment, reusing traditional razors by barbers, use of injectable drugs, and sexual transmission<sup>[10]</sup>. There is higher seroprevalence of HDV in younger male subjects who are positive for HBsAg.

## HDV IN INDIA

In India, the trend is much different from that in Pakistan and HDV infection does not seem to be very common. It is suggested that the infection is switching towards low prevalence in this country<sup>[15]</sup>. A number of studies have been done in India to estimate the prevalence of HDV infection but there is lack of a national survey.

In Northern India, the prevalence of hepatitis D in HBsAg-positive individuals from New Delhi was reported to be 8.1% in 1996<sup>[16]</sup> and 10.6% in 2005<sup>[15]</sup>, which was lower than in Chandigarh in Northern India, where the infection was reported endemic in 1995 and showed a prevalence of 14.2%<sup>[17]</sup>. In Central India, a study in Indore showed higher prevalence of 5.7% in patients with chronic liver disease, 1.9% in those with acute viral hepatitis, 15% in those with hepatic failure, and 2.3% in those with chronic renal failure<sup>[18]</sup>. In Kolkata, the prevalence was found to be 3.3% in 1998<sup>[19]</sup>.

In Mumbai, according to a study done in 1992, the HDV prevalence was 37.46% in HBsAg-positive patients. There was a higher HDV prevalence of 63% in patients with fulminant hepatitis<sup>[20]</sup>. However, another study from this city showed a prevalence of 16% in patients with acute viral hepatitis, 17% in asymptomatic HBsAg carriers, and 19% in patients with chronic liver disease. Among the high-risk population, HDV prevalence was 20% in chronic renal failure patients, 29% in medical professionals, and 38% in recipients of multiple transfusions<sup>[21]</sup>. Delta infection in Ludhiana was reported to be 33% in children with a high prevalence of HDV<sup>[22]</sup>. Another study from Ludhiana in the same year showed a prevalence of 10% in HBsAg-positive patients<sup>[23]</sup>. In Southern India, low HDV prevalence in patients undergoing hemodialysis was reported in a study published in 1991<sup>[24]</sup>. However, there is high prevalence of HDV in the resident tribes of Nicobar and Andaman islands<sup>[25]</sup>.

## HDV IN IRAN

HDV is a major public health issue in Iran<sup>[26]</sup>. Studies from different areas of the country show varied prevalence rates. Although overall HBV, and therefore, HDV prevalence might have decreased in Iran, studies have indicated an increase in HDV occurrence in HBsAg-positive patients. Anti-HDV prevalence in asymptomatic HBV carriers from Southern Iran was 14% in 1989<sup>[27]</sup>. A recently published study reported a prevalence of 9.7%



in chronic HBV patients from Shiraz, which represented a decrease in prevalence from 1989<sup>[28]</sup>. In Midwest Iran, a low prevalence of 2.4% in 1989 was reported in HBsAg carriers<sup>[26]</sup>. A study conducted during 1986-1988 has estimated the prevalence of hepatitis D in various high-risk groups. The prevalence of anti-delta was found to be 2.5% (3/120) in asymptomatic chronic carriers of HBsAg, 33.33% (2/6) in hemophilic patients, 44.5% (16/36) in HBsAg-positive hemodialysis patients. Five out of eight patients with hepatocellular carcinoma (HCC) were also positive for anti-HDV<sup>[29]</sup>. In 2000, 1.3% of blood donors positive for HBsAg and 25.2% of HBsAg-positive hemodialysis patients were found to be anti-HDV positive<sup>[30]</sup>.

In Tehran, HDV prevalence was found to be 5.7% in chronic HBV patients in 2004<sup>[31]</sup>. In Golestan Province, Northeastern Iran, a recent study has shown an anti-HDV prevalence of 5.8% in HBsAg carriers<sup>[32]</sup>. Another study also has shown an HDV prevalence of 5.8% (8/139) in this part of Iran. These studies suggest an increase in HDV prevalence over the past decade<sup>[33]</sup>.

In Babol, Northern Iran, HDV prevalence was 2% in chronic hepatitis B patients in 2002<sup>[34]</sup>. However in Kerman, Southern Iran, HDV prevalence was found to be 20.7% in chronic hepatitis B patients<sup>[35]</sup>. In Tabriz, Northwestern Iran in 2000, HDV prevalence was observed to be 6.15% in chronic HBV patients<sup>[36]</sup>. A recently published study (2008) has shown a high HDV prevalence of 31.57% in HIV/HBV co-infected individuals in Kermanshah, Western Iran<sup>[37]</sup>.

## HDV IN AFGHANISTAN

Not enough data are available from Afghanistan to estimate recent prevalence of HDV. A study was conducted between 1976 and 1984 in which sera were collected from 362 persons in various epidemiological groups with chronic and acute HBV infection. Some sera were also collected from drug addicts and hemophiliacs with antibodies for hepatitis B. It was seen that hepatitis D was common in drug addicts, hemophiliacs, hemodialysis patients and prisoners. However, HDV was uncommon in homosexual men, people with sporadic hepatitis B, and in people from other endemic areas for hepatitis B. HDV prevalence was 18% in patients with chronic liver disease, 2% in asymptomatic carriers of hepatitis B, and 5% in acute hepatitis B patients. No infection was observed in institutionalized mentally retarded persons and health-care workers. During the 9 years of the study, no change was seen in HDV frequency<sup>[38]</sup>.

## HDV IN MAINLAND CHINA

In mainland China, HDV infection is not very prevalent but it does exist. The results of a study done in Sichuan Province in 1987 have suggested a low prevalence of 0.8% in HBsAg-positive patients, although the prevalence rate of HBV was high<sup>[39]</sup>. In 1989, the prevalence of anti-

HDV was 4.3% in Tibet, Inner Mongolia, and Xinjiang, whereas no HDV infection was observed in Henan, Fujian, Liaoning, Heilongjiang, Guangxi, Sichuan, Beijing and Shanghai<sup>[40]</sup>. In 1990, 2346 liver samples were tested from 17 different areas of China for the intrahepatic hepatitis D antigen (HDag). The observed detection rate was 9.47%<sup>[41]</sup>.

In the area of Guangzhou, the prevalence of HDag in 1990 was 13.3% in adults and 13.6% in children, which showed an insignificant difference in prevalence between the two age groups<sup>[42]</sup>. In the area of Shijiazhuang, the prevalence of HDV infection was observed to be 12.92% in 1990 and the study suggested Shijiazhuang as a high-HDV-prevalence area<sup>[43]</sup>.

In the province of Henan, a study conducted between 1991 and 1993 showed that 3.5% of the HBsAg-positive blood samples were anti-HDV positive. The investigators did not observe any significant difference of HDV prevalence with sex. However, noteworthy differences were observed in different age groups, particularly in those aged > 60 years<sup>[44]</sup>. In Shandong province, 7.72% of the HBsAg-positive patients were found to be anti-HDV positive in 1998. The prevalence rates were 13.15% in patients with hepatitis B and only 3.16% in HBsAg carriers<sup>[45]</sup>. A 2006 study conducted in Wuhan City has shown a low prevalence of HDV infection of 2.22% in intravenous drug users. However, the HBV and HCV prevalence rates were much higher in this population<sup>[46]</sup>.

## HDV IN HONG KONG, CHINA

In Hong Kong, hepatitis D has high prevalence rates among intravenous drug abusers (IVDAs). These findings were reported by a study that tested the sera of a large number of patients with acute or chronic HBV infection for anti-HDV between January 1988 and December 1990. HDV was detected in 13 out of 14 IVDAs, which corresponds to a prevalence of 93% in this group, whereas the prevalence was only 0.15% in 664 non-IVDAs<sup>[47]</sup>.

## HDV IN TAIWAN, CHINA

Taiwan is considered to be endemic for hepatitis B, but as a result of effective immunization, HBV prevalence has decreased markedly. Hepatitis D prevalence in Taiwan was very high in the 1990s and before, but the prevalence has decreased greatly since then and new cases of HDV infection are now encountered rarely<sup>[48]</sup>.

In 2003, the HDV prevalence in HBsAg carriers was 15.3% (56/366)<sup>[49]</sup>. A study published in 1997 identified 77 patients with acute HDV superinfection among 527 consecutive HBsAg carriers over the past 12 years. From June 1983 to May 1995, the prevalence decreased considerably by each 3-year period (23.7, 15.5, 13.1 and 4.2%, respectively). This change in the endemicity might have occurred due to the effective preventive measures taken against sexually transmitted diseases (STDs) and promiscuity and encouragement to use disposable

needles<sup>[50]</sup>. In 1990, a high HDV superinfection prevalence of 24.7% (21/85) was observed in HBsAg carriers from Southern Taiwan<sup>[51]</sup>.

Prostitutes are considered a high-risk group for hepatitis B and D. In 1993, the HDV prevalence in adult licensed prostitutes was 55%, 36% in adult unlicensed prostitutes, and 16% in teenage unlicensed prostitutes. Important factors identified for this high HDV prevalence were use of unsterilized needles for tattooing and ear piercing, and frequent sexual contact with multiple partners, which resulted in genital ulcers<sup>[52]</sup>. From 1986 to 1989, a two fold increase in HDV prevalence was observed in prostitutes. However there was no change in the HDV prevalence in IVDAs and the general population<sup>[53]</sup>. In 1992, the HDV prevalence in HBsAg carriers was 9.6% in STD patients, 33.1% in prostitutes, 2.2% in blood donors from the general population, and 68.1% in drug abusers<sup>[53]</sup>.

In 2002, HDV prevalence in IVDAs was 39%. From 1986 to 1997, a decreasing rate of 4.7% every year was observed in this high-risk group<sup>[54]</sup>. In 1990, 91% (119/131) of the HbsAg-positive IVDAs were found to be positive for anti-HDV, which showed an extremely high HDV prevalence in this high-risk group<sup>[55]</sup>. In 1989, 78.5% of the HbsAg-positive parenteral drug abusers tested positive for anti-HDV in Southern Taiwan<sup>[56]</sup>. In 1988, a study showed a high HDV prevalence of 85% in 151 IVDAs/HBsAg carriers, and in 1986, the results of a study showed that HDAG was detected in 78.9%<sup>[19]</sup> of 115 IVDAs/HBsAg carriers<sup>[57,58]</sup>.

In 1988, a study showed an HDV prevalence of 10.3% (3/29) in children with acute hepatitis B, and 1.4% (1/68) in children with chronic HBV. No asymptomatic HBsAg carrier child tested positive for HDV<sup>[59]</sup>.

## HDV IN JAPAN

In Japan, certain areas are highly HDV prevalent, especially Miyako Islands in Okinawa<sup>[60]</sup>. A study published in 1990 has assessed the prevalence of HDV infection in Japan at different time periods. Hepatitis D was first observed in 1979-1983 and the prevalence was 16% in acute hepatitis B, 6.8% in HBV carriers, and 26% in chronic liver disease. Anti-HDV was later rarely observed. The findings of this study suggest limited sporadic HDV infection in Japan<sup>[61]</sup>. In 1993, a study compared the HDV prevalence rates between area of high HBV incidence, i.e. Kamigoto Islands and an area of average HBV carriage rate, i.e. Oita City. The HDV prevalence in Kamigoto Islands was 8.3%, while that in Oita City was 0%. The study pointed towards the possible risk of outbreak of this infection in HBV prevalent areas in the future<sup>[62]</sup>.

As compared to other parts of the country, Okinawa Islands, especially Miyako Islands, which are located in the southernmost part of the country, are considered to be endemic for the virus, where HDV genotype IIb is prevalent<sup>[60]</sup>. In 1995, the HDV prevalence in Miyako Islands was 23.5%<sup>[63]</sup>. In 1998, the HDV prevalence in

Miyako Islands was 21.1%. It has been suggested that the rate of HDV prevalence is likely to increase with increasing age or underlying disease severity<sup>[64]</sup>. However, in 2000, the HDV prevalence further decreased to 8.5% among the 4728 inhabitants of the islands<sup>[65]</sup>.

In Irabu Islands, Okinawa the HDV prevalence among HBsAg-positive patients was 23.6% in 1997, which indicated another high-prevalence area in Okinawa. The incidence rates were different in the seven districts of Irabu Islands, and ranged from 0% to 63.3%<sup>[66]</sup>.

## HDV IN KOREA

In Korea, HDV prevalence was estimated to be 0.85% in 1985. In 2003, a study was conducted in which 194 HBsAg-positive Korean patients were tested for anti-HDV, out of which, seven (3.6%) tested positive. Six of these patients had HCC and one had cholangiocarcinoma. Therefore, HDV was mainly associated with patients with HCC with a prevalence rate of approximately 4%, which has not changed greatly during the past 20 years<sup>[67]</sup>.

## HDV IN INDONESIA

In Indonesia, only a few studies are available on the prevalence of HDV, thus, this infection does not seem to be a major problem in this area unlike some other Asian countries. A study published in 1988 assessed the prevalence of hepatitis D in pregnant women in Bandung, a densely populated area of Indonesia. Out of the 926 pregnant women included in the study, only 2.8% (26) were carriers of HBsAg, among which, none tested positive for delta antibodies in spite of the fact that in this Indonesian population, HBsAg was frequent<sup>[68]</sup>. However, in 2003, the prevalence was found to be < 0.5% in the HBsAg carriers of Surabaya, which is also very low<sup>[69]</sup>.

## HDV IN MALAYSIA

In Malaysia, hepatitis D was first described in 1986 in some population groups. The HDV prevalence was found to be 12.5% in cases of acute hepatitis B, 6.7% in homosexual individuals, and 17.8% in drug abusers who were positive for HBsAg<sup>[70]</sup>. In 1989, the HDV prevalence was found to be 4.9%<sup>[71]</sup>. In 1996, 0.9% of the 923 jaundiced patients were found to be positive for anti-HDV<sup>[72]</sup>. In 1985, surveillance results for the detection of anti-HDV in IVDAs showed an absence of anti-HDV. However, in 1986, a prevalence of 17.8% was observed in the same group. The prevalence increased to 20% in 1989 and in 1994, 34% of the drug addicts tested positive for anti-HDV<sup>[73]</sup>.

## HDV IN THAILAND

In Thailand, hepatitis D is prevalent among IVDAs, a

high-risk group for viral hepatitis. A study conducted in 1988 tested 84 HBsAg-positive IVDAs, of which, 65.48% showed anti-HDV positivity. The HDV prevalence in 20 chronic hepatitis patients was 11.11% and 8.33% in 12 cirrhotic patients. No anti-delta antibodies were detected in 46 asymptomatic carriers<sup>[74]</sup>. A study in 2002 tested 55 HBsAg-positive sera of IVDAs, among which, 12 (21.8%) tested positive for anti-HDV. Among these anti-HDV-positive sera, eight (66%) tested positive for HDV RNA, and all had genotype I virus<sup>[75]</sup>.

Another study in 2002 tested 269 sera from the Hmong people of Northern Thailand for the seroprevalence of viral hepatitis. Despite the high seroprevalence of 76.0% for hepatitis B, the seropositivity of HDV was only 0.7%<sup>[76]</sup>.

## HDV IN PHILIPPINES

A study was conducted in 1990 to estimate the prevalence of HDV. Of the 64 patients with acute viral hepatitis, 1.6% tested positive for HDV. HBV was present in 40.6% of the acute viral hepatitis patients<sup>[77]</sup>.

## HDV IN VIETNAM

Vietnam is considered to be highly endemic for hepatitis B, which is one of the most important public health concerns. In order to estimate the prevalence of HBV and HDV in this area, a cross-sectional seroprevalence study was conducted in Thai Binh Province in 2007. Nineteen percent of the samples were HBsAg-positive, out of which, 1.3% were positive for HDV<sup>[78]</sup>. Thus, HDV does not seem to be prevalent in this HBV-endemic area. The results of another study conducted in Ho Chi Minh City in 2003 has shown that, except for HDV, other hepatitis viruses are spreading among the patients with liver diseases<sup>[79]</sup>.

## HDV IN AUSTRALIA

A study was conducted in Melbourne to establish the epidemiology of hepatitis D over a period of 15 years. Three thousand nine hundred and eighty-six HBsAg-positive patients were tested for HDV markers between 1971 and 1985. Hepatitis D markers were detected mostly in IVDAs and among their close contacts, and the results suggested the introduction of HDV in this group in 1970. The delta infection prevalence in carriers with no chronic liver disease was the highest (19.2%) among the IVDAs. Out of the 23 carriers with recurrent acute hepatitis, all tested positive for HDV markers and 20.1% of the carriers with chronic liver disease tested positive for the HDV markers<sup>[80]</sup>.

Apart from IVDAs, homosexual men are another high-risk group for HBV and HDV. In a study from Sydney in 1989, 204 homosexual men with acute or chronic HBV were tested for markers of HDV (total antibody to delta and delta antigen). Eight tested positive

for total antibody to delta, whereas delta antigen was detected in only one of the tested individuals, which showed a total HDV prevalence of 4.4%. All individuals who tested positive for the markers were IVDAs<sup>[81]</sup>.

## PACIFIC ISLANDS

Kiribati is an island located in the central tropical Pacific Ocean. HDV prevalence in this island is said to be very high. A serological survey was undertaken between May 1985 and February 1986 in which blood samples collected from 13 different places of the Southeast Asia and Pacific region were tested for HIV, HBV and HDV. The maximum HDV prevalence was observed in Kiribati, which was 84% in HBsAg-positive patients<sup>[82]</sup>. Another study in 1989 reported a prevalence of 69% (90/130) in individuals infected with HBV<sup>[83]</sup>.

Nauru, an isolated Pacific Island is considered hyperendemic for HDV. High HDV prevalence was reported here in 1986, in a study in which 2645 HBsAg-positive patients from several places in the Western Pacific were tested for HDV<sup>[84]</sup>. According to the results of a national epidemiological survey, published in 1989, in which 88% of the population was screened for HBV and HDV, 69.1% were reported positive for HBV markers, whereas HDV superinfection was observed in 22.7% of hepatitis B carriers<sup>[85]</sup>.

## HDV IN SAUDI ARABIA

HDV prevalence in Saudi Arabia was 8% (3/36) in HBsAg carriers in 1986<sup>[86]</sup>. The prevalence in the Riyadh area in the same year was 22.2% in patients with chronic hepatitis B, 7.9% in those with acute hepatitis B, and 6.7% in HBsAg carriers. In Najran, the prevalence was 9.6% in HBsAg carriers, and 5.3% in Al-Hafouf. In the areas of Khaiber and Jaizan, no anti-delta was found in the tested HBsAg carriers<sup>[87]</sup>. In 1987, a high HDV prevalence of 32% was detected in HBsAg-positive Saudi patients with liver disease, while the prevalence was 13% in patients with other illnesses. In healthy individuals, the prevalence was found to be 5.4%<sup>[88]</sup>. In 1988, the prevalence was 8% in HBsAg carriers in Gizan, an area considered hyperendemic for HBV<sup>[89]</sup>. A prevalence rate of 9.7% was detected in HBsAg-positive pregnant women in 1988. However, follow-up of 17 infants showed no indication of perinatal transmission<sup>[90]</sup>. In 1991, the prevalence in HBsAg-positive patients was found to be 17.6%<sup>[91]</sup>. In 1998, a study conducted in Jeddah showed an HDV prevalence of 13.6%. The HDV prevalence in IVDAs/carriers of HBsAg was 14.7% while there was 0.0% prevalence in non-IVDAs positive for HbsAg<sup>[92]</sup>.

In 2004, the HDV prevalence among HBsAg-positive healthy donors was 3.3%, while that in clinic- and hospital-based HBsAg patients was 8.6%. HDV infection is expected to decrease in Saudi Arabia with decreasing HBV prevalence due to global vaccination<sup>[93]</sup>.



## HDV IN OMAN

In Oman, according to a study conducted in 1991, the HDV prevalence was 7.7% (1/13) in HBsAg-positive dialysis patients and 22.2% (2/9) in HBsAg-positive renal transplant patients who have been previously transfused<sup>[94]</sup>. In 1994, HbsAg was detected in 11% of patients with kidney transplants and 12.7% of patients on dialysis. Anti-HDV was detected only in one HBsAg-positive patient on dialysis and two HbsAg carriers with renal transplants, which shows a low HDV prevalence among these patients<sup>[95]</sup>.

## HDV IN LEBANON

Hepatitis D was first detected in Lebanon in 1987 when a study reported 57% HDV prevalence in patients with chronic active hepatitis<sup>[96]</sup>. In 2007, the results of a study in which 258 HBsAg-positive patients from 10 health centers were included showed 1.2% (3/258) HDV prevalence, which shows a decrease in prevalence since 1987<sup>[97]</sup>.

## HDV IN TURKEY

In Turkey, like many other countries, a decline in HDV infection has been observed, but it is still a significant public health concern<sup>[98]</sup>. A recently published meta-analysis of HDV seropositivity has shown the decline in HDV infection but also points to the significant health concern in low socioeconomic parts of the country<sup>[99]</sup>. HDV prevalence is much higher in the southeast of the country, being 27% in chronic hepatitis B patients and 46% in hepatitis-B-induced cirrhosis patients. This is in comparison to the HDV prevalence in the West of the country of 5% in chronic hepatitis B patients and 20% in hepatitis-B-induced cirrhosis patients. The analysis also compared prevalence of HDV before and after 1995. It was observed that HDV prevalence in chronic hepatitis B patients decreased from 38% to 27% in Southeast Turkey and from 29% to 12% in Central Turkey. HDV prevalence in cirrhosis patients has decreased from 66% to 46% in Southeast and from 38% to 20% in West Turkey<sup>[99]</sup>.

## CONCLUSION

The prevalence of hepatitis D shows a decreasing trend due to preventive measures like vaccination against hepatitis B and awareness campaigns with regard to risk factors for the transmission of hepatitis B and D. Although preventive measures against hepatitis B including vaccination have decreased the prevalence of hepatitis D, there is no effective way of preventing HDV infection in HBV carriers in endemic areas. This can only be achieved by educating such individuals to prevent further exposures to risk factors. In spite of the global trend of decline, significant and persistent transmission is present in some countries.

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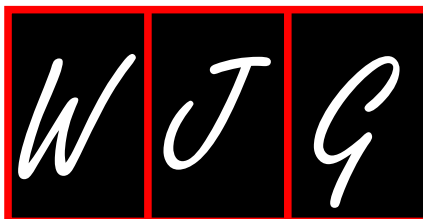


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## Effects of perinatal protein deprivation and recovery on esophageal myenteric plexus

Flavio M Greggio, Ricardo BV Fontes, Laura B Maifrino, Patricia Castelucci, Romeu Rodrigues de Souza, Edson A Liberti

Flavio M Greggio, Ricardo BV Fontes, Laura B Maifrino, Patricia Castelucci, Romeu Rodrigues de Souza, Edson A Liberti, Autonomic Nervous System Laboratory (VQM), Department of Anatomy, Instituto de Ciencias Biomedicas da Universidade de Sao Paulo, 2415 Av Prof Lineu Prestes, 05508-900, São Paulo, Brazil

**Author contributions:** Greggio FM and de Souza RR performed and evaluated the NADPH reactions; Castelucci P performed and evaluated the immunohistochemistry; Maifrino LB and Fontes RBV analyzed qualitative data concerning the NADH and AChE reactions; Liberti EA designed the research and evaluated the transmission electron microscopy.

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**Correspondence to:** Ricardo BV Fontes, MD, Autonomic Nervous System Laboratory (VQM), Department of Anatomy, Instituto de Ciencias Biomedicas da Universidade de Sao Paulo, 2415 Av Prof Lineu Prestes, 05508-900, São Paulo, Brazil. [rbvfontes@yahoo.com.br](mailto:rbvfontes@yahoo.com.br)

Telephone: +55-11-30917226 Fax: +55-11-30917366

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### Abstract

**AIM:** To evaluate effects of pre- and postnatal protein deprivation and postnatal recovery on the myenteric plexus of the rat esophagus.

**METHODS:** Three groups of young Wistar rats (aged 42 d) were studied: normal-fed (N42), protein-deprived (D42), and protein-recovered (R42). The myenteric neurons of their esophagi were evaluated by histochemical reactions for nicotinamide adenine dinucleotide (NADH), nitroergic neurons (NADPH)-diaphorase and acetylcholinesterase (AChE), immunohistochemical reaction for vasoactive intestinal polypeptide (VIP), and ultrastructural analysis by transmission electron microscopy.

**RESULTS:** The cytoplasm of large and medium neu-

rons from the N42 and R42 groups were intensely reactive for NADH. Only a few large neurons from the D42 group exhibited this aspect. NADPH detected in the D42 group exhibited low reactivity. The AChE reactivity was diffuse in neurons from the D42 and R42 groups. The density of large and small varicosities detected by immunohistochemical staining of VIP was low in ganglia from the D42 group. In many neurons from the D42 group, the double membrane of the nuclear envelope and the perinuclear cisterna were not detectable. NADH and NADPH histochemistry revealed no group differences in the profile of nerve cell perikarya (ranging from 200 to 400  $\mu\text{m}^2$ ).

**CONCLUSION:** Protein deprivation causes a delay in neuronal maturation but postnatal recovery can almost completely restore the normal morphology of myenteric neurons.

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**Key words:** Esophagus; Enteric nervous system; Myenteric plexus; Proteins; Light microscopy; Transmission electron microscopy

**Peer reviewer:** Dr. Claudia Zwingmann, PhD, Professor, Department of Medicine, University of Montreal, Centre de Recherche, 264 Rene-Levesque Est, Montreal, QC, H2X 1P1, Canada

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### INTRODUCTION

Several studies have been published that deal with the



effects of several forms of malnutrition on the myenteric plexus (MP) of the gastrointestinal tract. From the amassed data, it is obvious that different regions of the gut are differently affected in their morphology, both quantitatively and qualitatively<sup>[1-5]</sup>. However, information whether these changes are reversible following re-feeding are scarce.

The effects of re-feeding on both the peripheral and central nervous system (CNS) have been investigated. The optic nerve of rats undergoes a reduction in the number of myelinated fibers when subjected to malnourishment; that effect is reversed when adequate protein intake is restored early<sup>[6]</sup>. Similar results have been verified in the tibial and ulnar nerves of monkeys<sup>[7]</sup>. In the CNS, however, neurons lost in the hippocampal formation of rats or alteration of the dendritic orientation of pyramidal cells in the neocortex of rats are never recovered with a normal protein diet<sup>[8,9]</sup>.

That behavior, however, should not be expected from all other neurons. Cell size and MP appearance in colonic neurons return to baseline after restoration of a normal protein-content diet, as shown in our previous work, in sharp contrast to CNS neurons<sup>[1]</sup>. The estimated number of myenteric neurons of the small intestine in protein-recovered animals of the same age does not differ from that of normally nourished rats<sup>[4]</sup>. The reaction to undernourishment would thus be more correctly described as a developmental delay from the morphological standpoint, but not fully recovered in functional terms.

Several ultrastructural changes have also been described after protein deprivation. Recently, we have demonstrated drastic alterations in the esophageal neurons of 21-d-old weanling rats submitted to pre- and post-natal protein deprivation, such as altered disposition of both nuclear chromatin and ribosomes in the granular reticulum, as well as a poorly developed granular component of the nucleolus. These data are further compatible with a delay in the development of the myenteric neurons<sup>[3]</sup>. However, information on the structural and ultrastructural characteristics of the MP of the esophagus specifically remains unknown as to whether these modifications are reversible after adequate protein rehabilitation in the early postnatal period. Therefore, in the present work, we evaluated some of the histochemical, immunohistochemical, and ultrastructural features of the myenteric neurons of the esophagi of protein-deprived and protein-recovered young rats (42 d old).

## MATERIALS AND METHODS

### Experimental animals

This study was conducted according to current legislation on animal experiments of the Instituto de Ciencias Biomedicas da Universidade de Sao Paulo (ICB-USP). Young adult male and female Wistar rats (200-240 g body weight) were mated during a period of 7-10 d. After conception, the females were placed in individual cages and maintained under standard conditions at 21°C with a 12-h light/dark cycle. During pregnancy, the nourished mothers (N) received an AIN-93G<sup>[10]</sup> normal protein diet and the malnourished (protein-deprived)

mothers (D) received the AIN-93G low-protein diet (5% casein) (Rhoister, Sao Paulo, Brazil). Both groups were supplied with water *ad libitum*.

Following birth, the dams and pups of both groups continued to receive their respective assigned diets. At the end of the weanling period (21 d) N and D male pups were separated from the dams and maintained on their respective diets until day 42. They were studied as N42 and D42 groups, respectively. Exactly half of the malnourished pups from the first phase (up to 21 d) received the AIN-93G normal protein diet from day 22 to 42 and formed the protein-recovery group, R42.

The animals from all groups (N42, D42 and R42) were weighed, euthanized with hypnol (Fontoveter, Sao Paulo, Brazil) and had their esophagi entirely removed. After measurement of the surface area using a planimeter<sup>[11]</sup> (OTT, Germany), the esophagi were processed using the following techniques.

### Histochemical nicotinamide adenine dinucleotide (NADH)-diaphorase reaction

NADH-diaphorase histochemistry was employed as our “pan-neuronal” marker for the MP; the controversy about the ideal neuronal marker has not been satisfactorily settled and shall not be addressed here<sup>[11]</sup>. Five animals from each group were used for NADH-diaphorase reaction histochemistry<sup>[12,13]</sup>. After being washed in Krebs solution (120 mmol/L NaCl, 5 mmol/L KCl, 25 mmol/L NaHCO<sub>3</sub>, 1.2 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 1.2 mmol/L MgSO<sub>4</sub>, 2.5 mmol/L CaCl<sub>2</sub>, and 11.5 mmol/L glucose), the oral portion of each esophagus was ligated with cotton threads and filled with Krebs solution using a syringe needle introduced in the lumen. When the walls of the viscera were slightly distended, the needle was withdrawn and a ligature was performed in the aboral section. After incubation in Krebs solution at room temperature for 15-30 min, the specimens were transferred to a permeabilizing agent (0.3% Triton-X in Krebs solution) for 90 s and then washed in three 10-min cycles with Krebs solution.

The esophagi were incubated for 60 min at 20°C in 20 mL of a solution that contained 0.5 mg/mL nitro blue tetrazolium (Sigma, St Louis, MO, USA) in 5 mL distilled water, 5 mL 0.1 mol/L sodium phosphate buffer (pH 7.3), 10 mL distilled water and 0.5 mg/mL β-nicotinamide adenine dinucleotide (reduced form). The reaction was stopped by fixation in 4% buffered formaldehyde (24 h). Subsequently, the esophagi from all groups (N42, D42 and R42) were opened longitudinally and three circular fragments, 2 mm<sup>2</sup> each, were obtained from the oral (*Or*), medium (*Me*) and aboral (*Ab*) parts using a puncture device. The mucosa and submucosa from the fragments were then removed, washed in distilled water, arranged as whole-mount preparations in glycerol on a microscope slide, and sealed with Entellan (Merck KGaA, Darmstadt, Germany).

### Histochemical nitroergic neurons (NADPH)-diaphorase reaction

The histochemical NADPH-diaphorase reaction<sup>[14,15]</sup> was

performed in five esophagi from each animal group (N42, D42 and R42). The specimens were washed in Krebs solution and filled with 4% paraformaldehyde in phosphate buffer pH 7.4. After ligation, the oral and aboral parts from each esophagus were immersed in the same fixative solution for 2 h at 4°C, washed in phosphate-buffered 0.9% NaCl for 2.5 h, and incubated in medium that contained 1 mg/mL  $\beta$ -NADPH (reduced form; Sigma) in phosphate buffer for 35 min. Thereafter the *Or*, *Me* and *Ab* circular fragments were obtained from each esophagus and prepared as whole-mount preparations as mentioned above.

### Demonstration of acetylcholinesterase (AChE) activity

In an attempt to characterize further the cytoarchitecture of the MP, indirect detection of cholinergic neurons was attempted by demonstration of AChE activity<sup>[3]</sup>. Three esophagi from each group (N42, D42 and R42) were subjected to the direct coloring method to demonstrate AChE<sup>[16-18]</sup>. The specimens were filled with 4% paraformaldehyde in phosphate buffer (pH 7.4), ligated at their oral and aboral limits, and immersed in the same fixative solution for 2 h at 4°C. After this period, the esophagi were opened and the *Or*, *Me* and *Ab* circular fragments incubated overnight at 4°C in solution A - hyaluronidase (Hialozime; Apsen, Brazil), Krebs solution and tetraiso-propylpyrophosphoramidate (Sigma). The fragments were then washed in Krebs solution and further incubated overnight at 4°C in a solution that contained 50% of solution A plus 0.17 mol/L acetylthiocholine iodide, 0.1 mol/L phosphate buffer (pH 7.1), 100 mmol/L sodium citrate, 30 mmol/L cupric sulfate, 5 mmol/L potassium ferricyanide and 0.3% Triton X-100. The mucosal and submucosal layers were removed and the fragments were dehydrated in an increasing alcohol series (70%-100%), immersed in benzene for 20 min, and mounted on microscope slides with Entellan.

### Immunohistochemistry

Vasoactive intestinal peptide (VIP) staining<sup>[19]</sup> was performed on three fresh segments of esophagus (approximate area: 3 mm<sup>2</sup>). Segments were removed from three animals each of the N42, D42 and R42 groups and placed in PBS (0.15 mol/L NaCl in 0.01 mol/L sodium phosphate buffer, pH 7.2) that contained nicardipine (10<sup>-6</sup> mol/L; Sigma) to inhibit tissue contraction. The specimens were then pinned out tautly, mucosa side down, onto a balsawood board and fixed overnight at 4°C in a paraformaldehyde solution (0.2 mol/L, pH 7.3). The specimens were then cleared with three 10-min cycles in 100% dimethylsulfoxide, followed by three 10-min washes in PBS. All samples were stored at 4°C in PBS that contained sodium azide (0.1%). After removing the mucosa, submucosa, and circular muscular layers, the whole-mount preparations were pre-incubated in 10% normal horse serum in PBS that contained 1% Triton X-100 for 30 min at room temperature to reduce nonspecific binding and to permeabilize the tissue. Rabbit anti-VIP antibody was then used at a concentration of 1:2000. This antibody was diluted in antibody diluent (1.8% NaCl in 0.01 mol/L sodium phos-

phate buffer that contained 0.1 sodium azide). Following incubation in the primary antibody for two nights in a humid chamber at 4°C, the specimens were washed three times in PBS (10-min cycles) and then incubated for 1 h at room temperature with a fluorescence-labeled secondary antibody (Donkey anti-rabbit IgG Alexa 488 at 1:500; Molecular Probes, Eugene, OR, USA). After washing in PBS, the specimens were mounted in 0.5 mol/L sodium carbonate-buffered glycerol (pH 8.6). The specimens were finally examined for fluorescence on a Leica Microscope equipped with the appropriate filter for Alexa 488 fluorescence (set to 00 for Alexa 488). The images were captured using an Image Proplus System.

### Transmission electron microscopy (TEM)

Following systemic perfusion with a fixative solution (3% glutaraldehyde in Millonig's buffer (pH 7.2-7.4) with 0.25% tannic acid)<sup>[20]</sup>, esophagi of three animals from each group (N42, D42 and R42) were removed. Two fragments (approximately 2 mm in length) from the *Or*, *Me* and *Ab* sections were obtained from each specimen and incubated for 6 h in the same fixative solution. Specimens were subjected to post-fixation treatment in a 2% osmium tetroxide solution and embedded in adhesion medium. Ultra-thin sections were stained with uranyl acetate/lead citrate<sup>[21]</sup> and examined with a JEOL JEM-1010 (Jeol, Tokyo, Japan) electron microscope.

### Nerve cell perikarya

A semiautomatic device (Axioskop 40, AxioCam HRC, Axiovision software 4.5; Zeiss, Germany) was utilized for morphometric neuronal examination. The cross-sectional (profile) areas of 20 neuronal perikarya from each *Or*, *Me* and *Ab* circular fragment stained with NADH and NADPH were determined in each specimen at 400 × magnification.

### Statistical analysis

mean  $\pm$  SE were calculated and compared by analysis of variance (ANOVA), Student's *t* test, and Duncan's test for multiple comparisons as appropriate, with the level of significance set at  $P < 0.05$ <sup>[22]</sup>.

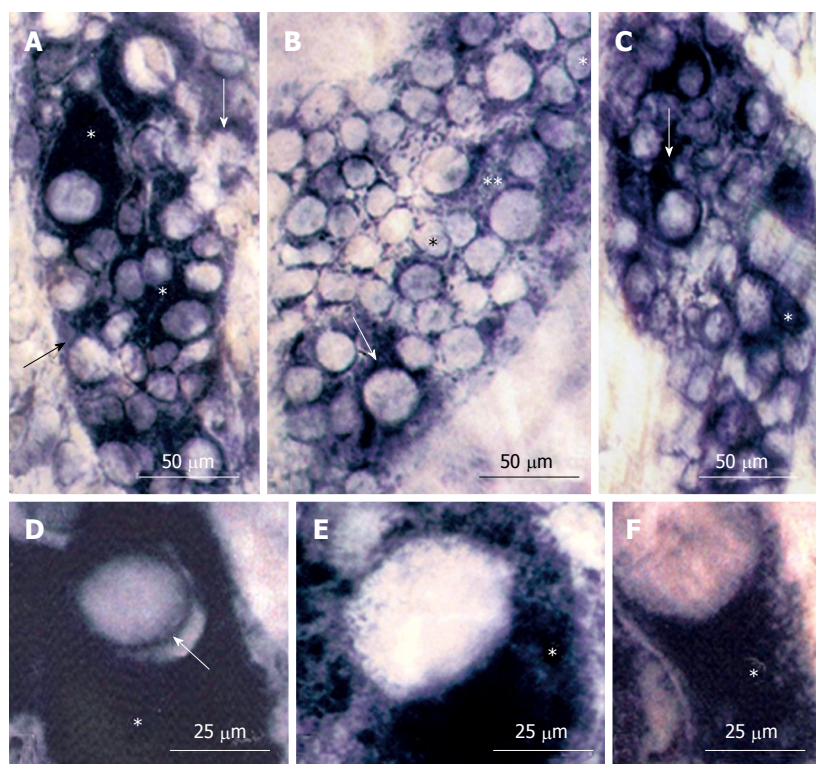
## RESULTS

### Histochemistry (NADH, NADPH and AChE)

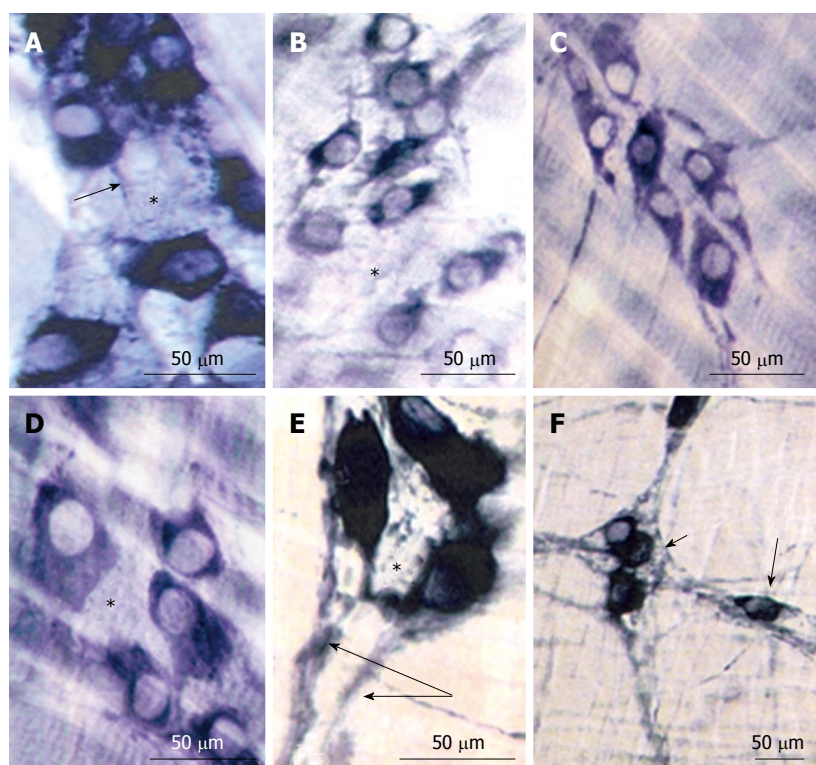
No significant differences were detected among the *Or*, *Me* and *Ab* esophageal segments in all groups. The NADH-diaphorase-stained whole-mount preparations of esophagi from all groups (N42, D42, R42) had myenteric neurons of varying size and reactivity (Figure 1). Most of the large and medium neurons in animals from the N42 and R42 groups had intense staining of the cytoplasm; an aspect detected in only a few large neurons of the D42 group (Figure 1A-C). Under high magnification, the dark and homogeneous aspects of the cytoplasm from N42 neurons were partially observed in R42 neurons. In contrast, the neurons from the D42 group had cytoplasm with granular aspects (Figure 1D-F).

The reactivity for NADPH-diaphorase was detected





**Figure 1 NADH-diaphorase reaction.** A: N42 group. Myenteric neurons of large and medium sizes with intensely reactive cytoplasm (\*) and neurons of diverse size weakly reactive (arrows); B: D42 group. Nuclei of small neurons (\*). The cytoplasm of large neurons had low (\*\*) or diffuse (arrow) reactivity; C: R42 group. Note large neurons with intense (arrow) and diffuse (\*) cytoplasmic reactivity; D-F: Large neurons, respectively from N42 (D), D42 (E) and R42 (F) groups. The well-delineated nucleus (arrow) from N42 was not clearly detected in malnourished and protein-recovered animals. Compare the patterns of reactivity of the cytoplasm (\*).



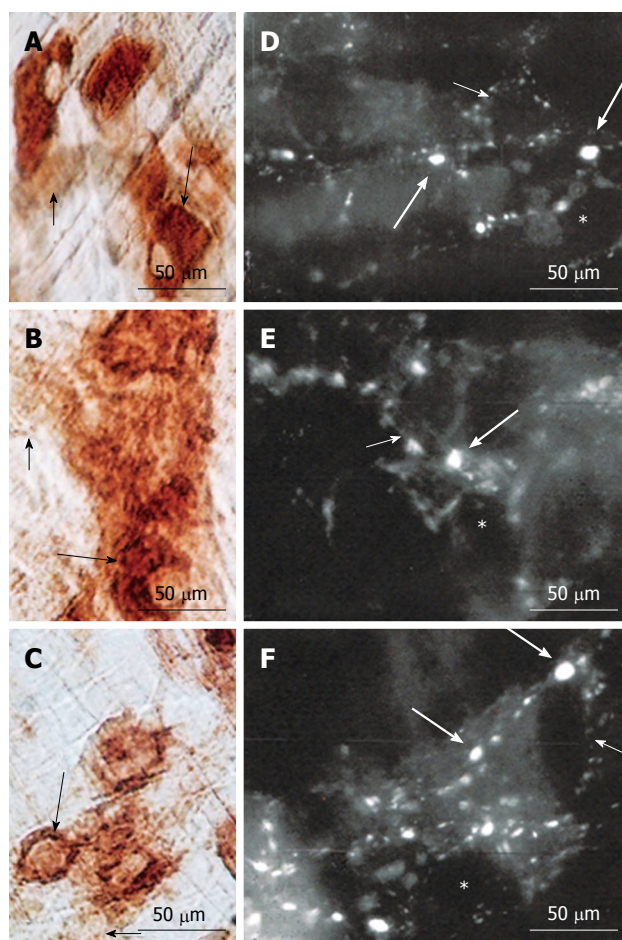
**Figure 2 NADPH-diaphorase reaction.** A and D: N42 group. Note that most of the myenteric neurons were intensely reactive and spaces inside the ganglia (\*) are surrounded by thin neuronal branches (arrow); B and E: D42 group. Neurons with diverse intensities of reaction, spaces inside the ganglia (\*) and thick neuronal meshes (double-arrow) were evident; C and F: R42 group. Neuronal meshes surrounded the ganglion (small arrow). Some neurons were detected inside the meshes (large arrow).

in both neurons and meshes from the MP. The space inside the myenteric ganglia of all groups (N42, D42, R42) was occupied by non-nitroergic neurons (Figure 2). The cytoplasm from most of the neurons from the N42 group exhibited intense NADPH-diaphorase staining with only a few showing weaker staining (Figure 2A and D). The variability of intensity distinguished the cytoplasm of the myenteric neurons from the D42 and R42

groups, however, more neurons from the R42 group exhibited darker staining (Figure 2B and C). The intra- and interganglionic meshes were detected in all groups (Figure 2A, E and F).

The myenteric ganglia from all groups contained neurons either strongly or weakly reactive for AChE. Qualitatively, the reactivity was diffuse in the neurons from the D42 and R42 groups (Figure 3A, C and E).





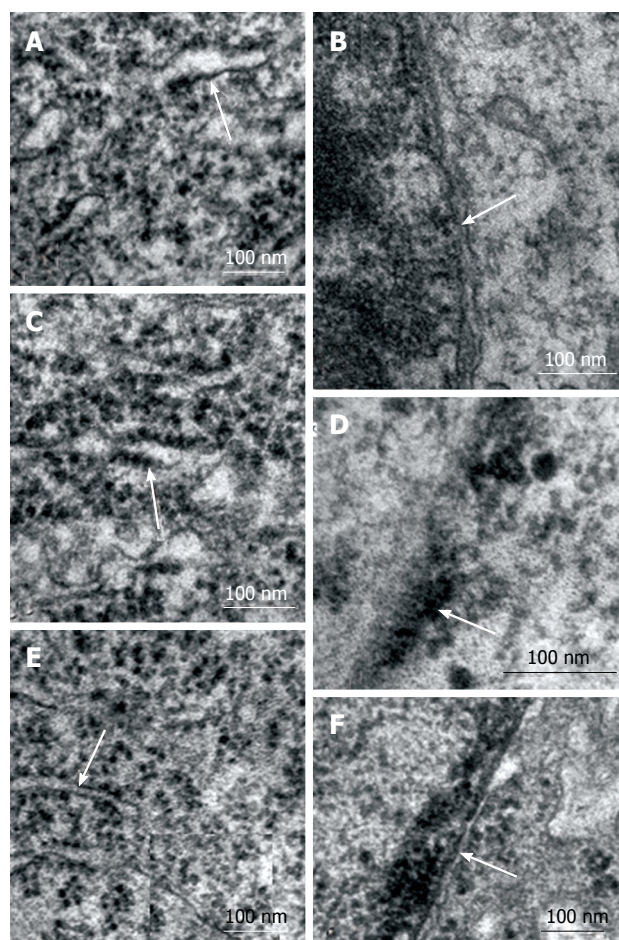
**Figure 3** AChE reactive (A, C and E) and VIP immunoreactive (B, D and F) myenteric neurons. Intensely (large arrows) and weakly (small arrows) reactive neurons were observed in N42 (A), D42 (C) and R42 (E) groups. Large and small varicosities (respectively, large and small arrows) were present around the neurons (\*) in the N42 (B), D42 (D) and R42 (F) groups. Apparently, the varicosities were more abundant in the N42 and R42 groups.

### Immunohistochemistry

The immunohistochemical reaction for VIP revealed the presence of large and small varicosities around the shadows of the neuron perikarya. The low density of these structures in ganglia of D42 animals was evident. No apparent differences were observed between the N42 and R42 groups concerning these structures (Figure 3B, D and F).

### TEM

Specific organelles of the myenteric neurons were selected for comparative analysis using TEM. The granular reticulum from all groups exhibited ribosomes aligned on the outer surface of the regularly arranged membrane of the cisternae (Figure 4A, C and E). The nuclear double membrane and the perinuclear cisterna were well defined in the myenteric neurons from the N42 and R42 groups and the electron-dense nuclear chromatin was detected on the inner membrane of the nuclear envelope (Figure 4B and F). In several neurons of the D42 group, the double membrane of the nuclear envelope and the perinuclear cistern were not distinguishable (Figure 4D). The euchro-



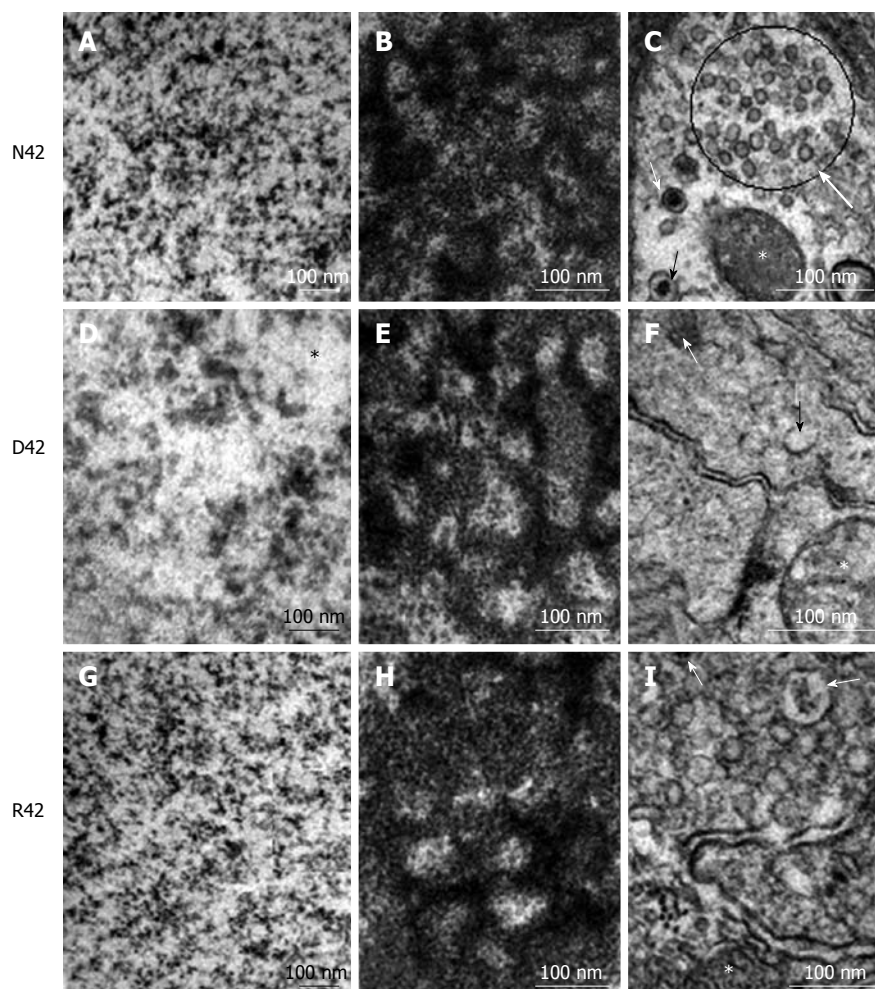
**Figure 4** Electron micrographs of myenteric neurons from N42 (A and B), D42 (C and D), and R42 (E and F) groups. A: In all groups, the granular reticulum showed that the ribosomes were aligned on the outer surface of the regularly arranged membrane (arrows). A well-defined nuclear double membrane and perinuclear cisterna were observed in neurons from N42 (B) and R42 (F) rats. Note that these structures were not delineated in the neurons of malnourished animals (D).

matin was homogeneously dispersed in the nucleus of myenteric neurons from all groups (Figure 5A, D and G). No group-specific differences in the granular and fibrillar parts of the nucleoli were detected (Figure 5B, E and H). Agranular, large, and small granular vesicles were observed in the neurons from all groups. However, large granular vesicles were only delineated in the N42 group. In the D42 and R42 groups, many of the neurons had ill-defined membranes and showed a weak electron-dense content (Figure 5C, F and I).

### Nerve cell perikarya

In all groups, most of the NADH-stained neurons had perikarya that ranged in cross-sectional area from 200 to 400  $\mu\text{m}^2$  (percentage of neurons in that range: 62%, 78% and 59%, respectively) with the following respective mean  $\pm$  SD: 375  $\pm$  169, 323  $\pm$  134 and 393  $\pm$  133  $\mu\text{m}^2$ . The perikarya of nitrergic neurons (NADPH+) also ranged from 200 to 400  $\mu\text{m}^2$  for the N42, D42 and R42 groups (66%, 67%, 62%, respectively), with the following





**Figure 5** Electron micrographs of myenteric neurons. A, D and G: Nuclear chromatin (\*) homogeneously distributed; B, E and H: Granular and fibrillar parts of the nucleolus were similarly arranged; C, F and I: Many small vesicles (circle) were detected in neurons from all groups. Compare the intensity of large granular vesicles among the groups (arrows) (\*) mitochondria.

respective mean  $\pm$  SD:  $376 \pm 118$ ,  $366 \pm 132$  and  $382 \pm 117 \mu\text{m}^2$ . No statistically significant differences were detected among any of the groups with respect to the neuronal area profiles for the NADH and NADPH reactions.

## DISCUSSION

In our previous work, we examined the effects of malnutrition on the MP of diverse regions of the gut in 21-d-old weanling rats subjected to pre- and postnatal protein deprivation using diverse time-honored histochemical methods<sup>[1-3,23-25]</sup>. We observed a decrease of approximately 15% in the cross-sectional areas of neurons in the colon of malnourished rats relative to normal-fed controls<sup>[1]</sup>. Average perikaryon area of myenteric neurons in the small intestine of malnourished animals was approximately half that observed for the normally fed controls. Moreover, most of the neurons that were reactive to NADH-diaphorase from malnourished animals showed weaker cytoplasmic staining<sup>[2]</sup>. In the esophagus, perikaryon area of myenteric neurons did not differ significantly between normally fed and protein-deprived animals. Nevertheless, the reactivity for AChE was clearly reduced in neurons from protein-deprived animals<sup>[3]</sup>.

At a later stage of MP development (42 d old), we

observed less NADH-diaphorase staining in large intestinal neurons from protein-deprived rats than in the controls, although the average size of the nerve cell perikaryon area was not significantly different from that of normally fed animals<sup>[1]</sup>. In the small intestine of rats of the same age, there was no detectable difference in the average perikaryon area of myenteric neurons between normally fed and protein-deprived animals. However, AChE histochemical activity and choline acetyltransferase (ChAT) immunoreactivity showed that most ganglionic neurons from the protein-deprived animals were unstained or moderately stained<sup>[4]</sup>. Similar aspects were detected in the MP from the duodenum of 90-d-old rats given protein and vitamin B complex. The effects did not alter the mean perikaryon area but did considerably reduce the reactivity of the myenteric neurons for NADPH-diaphorase (nitroergic neurons)<sup>[5]</sup>.

The absence of neuron cell profile reduction in protein-deficient animals (D42) shown in the present study is in line with previously described data using the esophagus of 21-d-old protein-deficient animals<sup>[3]</sup>. This observation also fits with data from other viscera of the digestive tract, such as the small intestine, where the neuron cell profiles were similar in 42-d-old nourished, malnourished and protein-recovered rats<sup>[4]</sup>. However, the myenteric neurons from the large intestine of protein-deprived 42-d-old rats

had a smaller mean perikaryon area than that normally found in fed and protein-recovered animals<sup>[4]</sup>. This finding corroborates data from 3-mo-old rats subjected to inadequate nutrition over a long time period. A decrease in the perikaryon area of myenteric neurons has been verified in the duodenum and ascending colon of these animals<sup>[26,27]</sup>. Mean area of neuronal perikarya does not change with age<sup>[28,29]</sup>. However, exposure of the myenteric neurons to other factors, such as dietary sodium intake and chagasic infection, effectively causes an irreversible hypertrophy of the neuron perikaryon area<sup>[30,31]</sup>. Although myenteric neurons may react differently depending on the extrinsic (i.e. dietary) and/or intrinsic (i.e. aging) factors they are exposed to, early protein recovery can reestablish their mean perikaryon area<sup>[1,4]</sup>. This has also been recently confirmed in the CNS (personal communication from Dr. Liberti EA).

Most of the reactive neurons in the D42 and R42 groups exhibited a diffuse pattern of AChE staining with a predominance of weakly reactive cells in the D42 group. Similar findings were verified in myenteric neurons from the colon of a chagasic mouse<sup>[32]</sup> and this fits with our previous observations from esophagi of 21-d-old malnourished animals. In that study, we only observed a few myenteric neurons that showed intense AChE staining in protein-deprived animals<sup>[3]</sup>. In the present study, although the myenteric neurons from the N42 and R42 animals had similar mean perikaryon areas assessed with AChE staining, the pattern of reactivity of neurons from the R42 group resembled that of the D42 group. This result is not in accordance with observations from the small intestine. In the small intestine, we have detected the same pattern of reactivity for nourished and protein-recovered animals when the myenteric neurons are immunohistochemically stained for ChAT<sup>[4]</sup>.

We observed similar patterns of VIP immunoreactivity in R42 and N42 cells. In addition, the pattern of NADH and NADPH reactivity of myenteric neurons was similar to that of AChE. However, the homogeneity of the reaction for NADH detected in the cytoplasm of most neurons from the N42 group was not totally recovered in neurons of the R42 group. Moreover, although the R42 group exhibited many intensely staining neurons for NADPH, their presence was far less frequent than that for the N42 group. The ultrastructural aspects of the large granular vesicles support this assertion. Although some neurons from the R42 group exhibited electron-dense areas, most of them clearly had less dense content. In consideration of the current study, it is possible that different enteric neurotransmitter systems respond differently to the protein-recovery regimen.

The deleterious effect of protein deficiency on myenteric neurons of the esophagus has not been previously considered. Our data from protein-deprived (D42) and protein-recovered (R42) animals suggest that protein deficiency retards neuronal maturation in a manner that is to some extent, but far from completely recovered with 20 d of protein reinstatement.

## COMMENTS

### Background

Malnutrition affects the myenteric plexus (MP) of the gastrointestinal tract in different manners according to location. Definite changes in neuronal population, morphometry and morphology have been described.

### Research frontiers

It is not known whether morphometrical and morphological parameters return to normal after adequate refeeding, that is, whether modifications induced by malnutrition are permanent.

### Innovations and breakthroughs

The present study shows that postnatal refeeding of perinatally malnourished rats restores almost all of the normal characteristics of the neurons of the MP of the esophagus.

### Applications

The present study suggests that adequate nutrition can restore almost all of the changes induced by malnutrition on MP neurons. Changes in malnourished individuals should thus be reverted with adequate nutrition with hopefully significant clinical impact.

### Peer review

The manuscript by Dr. Greggio and co-workers describes the evaluation of myenteric neurons from esophagi of young rats. They investigated normal, protein-deprived and protein-recovered groups of rats. This is an extension to studies previously done by this group and others, and an important aspect previously not done is the investigation of whether the structural and ultrastructural characteristics of the MP of the esophagus are reversible. The manuscript is in general very clearly written and the data are convincing.

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## Significance and relationship between Cripto-1 and p-STAT3 expression in gastric cancer and precancerous lesions

Jian-Guo Zhang, Jing Zhao, Yan Xin

Jian-Guo Zhang, Jing Zhao, Yan Xin, The Fourth Laboratory of Cancer Institute, Department of Tumor Pathology of General Surgery Institute, the First Affiliated Hospital of China Medical University, Shenyang 110001, Liaoning Province, China

**Author contributions:** Zhang JG performed the whole experiment, statistical analysis and wrote the manuscript; Xin Y designed the study, provided financial support, vital experimental equipments for this work and was involved in revising the manuscript; Zhao J participated in the experiment.

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**Correspondence to:** Yan Xin, Professor, The Fourth Laboratory of Cancer Institute, Department of Tumor Pathology of General Surgery Institute, the First Affiliated Hospital of China Medical University, Shenyang 110001, Liaoning Province, China. [yxin@mail.cmu.edu.cn](mailto:yxin@mail.cmu.edu.cn)

Telephone: +86-24-83282351 Fax: +86-24-83282375

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### Abstract

**AIM:** To explore the relationship between Cripto-1 (CR-1) and tyrosine phosphorylation STAT3 (p-STAT3) expressions in gastric cancer (GC) and gastric carcinogenesis and metastasis.

**METHODS:** The PV9000 immunohistochemical method was used to detect the expression of CR-1 and p-STAT3 in 178 cases of GC, 95 matched normal gastric mucosa, 40 chronic atrophic gastritis (CAG), 48 intestinal metaplasia (IM) and 25 dysplasia (DYS).

**RESULTS:** The positive rates of CR-1 and p-STAT3 expression were significantly higher in CAG (65.0% and 60.0%), in IM (83.3% and 77.1%), DYS (80.0% and 68%) and GC (71.3% and 60.1%) than in normal gastric mucosa (43.2% and 41.1%,  $P < 0.05$ ), respectively. The expressions of CR-1 and p-STAT3 (78.3% and 66.7%) were significantly higher in GC with lymph

node metastasis than in those without metastasis (53.1% and 42.9%,  $P < 0.05$ ). CR-1 expression was also related to histological and Lauren's types of GC ( $P < 0.001$ ). Furthermore, there was positive relationship between CR-1 and p-STAT3 expressions in GC ( $r_k = 0.189$ ,  $P = 0.002$ ).

**CONCLUSION:** The up-regulation of CR-1 and p-STAT3 may play important roles in gastric carcinogenesis and lymph node metastasis. CR-1 and p-STAT3 expression in GC was positively correlated, and the relevant molecular mechanism requires further investigations.

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**Key words:** Cripto-1; Phosphorylation STAT3; Gastric cancer; Precancerous lesions; Immunohistochemistry

**Peer reviewers:** Masanori Hatakeyama, MD, PhD, Professor, Department of Microbiology, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan; Vittorio Ricci, MD, PhD, Department of Physiology, Human Physiology Section, University of Pavia Medical School, Via Forlanini 6, Pavia 27100, Italy; Nathalie Perreault, PhD, Associate Professor, Department of Anatomy and Cell Biology, University of Sherbrooke, 3001 12th Avenue North, Sherbrooke, J1H5N4, Canada

Zhang JG, Zhao J, Xin Y. Significance and relationship between Cripto-1 and p-STAT3 expression in gastric cancer and precancerous lesions. *World J Gastroenterol* 2010; 16(5): 571-577 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i5/571.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i5.571>

### INTRODUCTION

Human Cripto-1 (CR-1), the founder member of the epidermal growth factor-Cripto-FRL1-Cryptic family, plays an important role during early embryonic development and cellular transformation<sup>[1]</sup>. CR-1 is also an oncogenic growth factor involved in tumorigenesis through influencing cell proliferation, survival, migration



and invasion<sup>[2-4]</sup>. CR-1 is absent or in low levels in normal tissues but over-expressed in most malignant tumors, such as breast, colon, stomach, pancreas, lung, cervix and ovary cancers, which is being assessed as a tumor-specific target for immunotherapy<sup>[11,5]</sup>. *STAT3*, a well recognized oncogene, is an important member of the STAT family that are normally inactive within the cytoplasm and become activated by tyrosine phosphorylation in response to cytokines and growth factors<sup>[6]</sup>. Its signal pathway is closely associated with the proliferation, differentiation and apoptosis, and constant activation of *STAT3* can promote cell proliferation and carcinogenesis<sup>[7,8]</sup>. Given the importance of CR-1 over-expression and *STAT3* activation in carcinogenesis, we investigated the expression and clinicopathological significance of CR-1 and phosphorylation *STAT3* (p-*STAT3*) in gastric cancer (GC), and also explored the relationship between abnormal expressions of the two oncoproteins in GC.

## MATERIALS AND METHODS

### Clinicopathological data and tissue microarray construction

Surgically resected GC specimens were collected from the First Affiliated Hospital of China Medical University, including 178 cases of GC, 95 matched normal gastric mucosa (obtained at > 5 cm apart from the edge of primary tumor focus), 40 chronic atrophic gastritis (CAG), 48 intestinal metaplasia (IM), and 25 dysplasia (DYS). There were 121 males and 57 females. The mean age of patients was 61 years. According to Borrmann's classification, gross types of primary tumors were classified as follows: 4 cases of Borrmann I, 22 cases of Borrmann II, 140 cases of Borrmann III, and 12 cases of Borrmann IV. In the light of the WHO's histological classification of GC, 178 cases were classified as follows: 2 papillary adenocarcinoma, 9 well and 65 moderately and 74 poorly differentiated adenocarcinoma, 3 undifferentiated adenocarcinoma, 19 mucinous adenocarcinoma and 6 signet ring cell carcinoma. Samples were fixed in 10% formalin, embedded in paraffin, cut into 4  $\mu$ m thick sections and constructed in blocks for tissue microarray. All the samples were evaluated by two experienced pathologists for diagnosis. None of the patients had received chemotherapy or radiation therapy preoperatively.

### Immunohistochemistry

Expression of CR-1 and p-*STAT3* in GC, precancerous lesions and normal gastric mucosa were detected using an IHC method. The PV-9000 kit was purchased from Beijing Zhongshan Golden Bridge Biotechnology Company. Mouse monoclonal antibody against human CR-1 was from the R&D systems (working dilution 1:80). Rabbit monoclonal antibody against human p-*STAT3* was from the Signalway Antibody (working dilution 1:25). All procedures were implemented according to the manufacturer's instructions. For negative controls, sections

were treated with 0.01 mol/L phosphate-buffered saline instead of primary antibodies.

### Immunohistochemical staining evaluation

Specific immunoreactivity of CR-1 protein was located in the cytoplasm, while p-*STAT3* protein was located in the nucleus and cytoplasm. Two hundred cells from two selected representative fields of each section were counted by two independent observers for the determination of their immunostaining intensity. Staining intensity (A) was classified as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). The percentage of positive cells (B) examined in 200 cells were divided into 0 (< 5%), 1 (5%-25%), 2 (26%-50%), 3 (51%-75%) and 4 (> 75%). According to the product of A and B, the IHC result was classified as 0, negative (-); 1-4, weakly positive (+); 5-8, moderately positive (++) and 9-12, strongly positive (+++).

### Statistical analysis

Statistical analysis was performed using SPSS 11.5 Package, and  $\chi^2$  test, Fisher's exact test and Kendall's *tau-b* test were used to differentiate the rates of different groups and test the correlation between the two factors.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Expression of CR-1 in normal gastric mucosa, CAG, IM, DYS and GC

The immunoreactivity of CR-1 protein was located diffusely in the cytoplasm. The positive rates of CR-1 presence in CAG (65.0%, 26/40), IM (83.3%, 40/48), DYS (80.0%, 5/25) and GC (71.3%, 127/178, Figure 1A) were significantly higher than that in normal gastric mucosa (43.2%, 41/95, Figure 1B), respectively,  $P < 0.05$ ; there was no statistically significant difference in CR-1 expression between DYS and GC ( $P = 0.246$ ), but the positive rate of CR-1 in IM was significantly higher than that in GC (Table 1).

### Expression of p-STAT3 in normal gastric mucosa, CAG, IM, DYS and GC

The immunoreactivity of p-*STAT3* protein was located in the nucleus and cytoplasm. The positive rates of p-*STAT3* expression in CAG (60%, 24/40), IM (77.1%, 37/48), DYS (68.0%, 17/25) and GC (60.1%, 107/178, Figure 1C) were significantly higher than that in normal gastric mucosa (41.1%, 35/95, Figure 1D), respectively,  $P < 0.05$ . There was no significant difference in p-*STAT3* expression among IM, DYS and GC ( $P = 0.087$ ,  $P = 0.103$ , Table 2).

### Correlation between CR-1, p-STAT3 expression and clinicopathological features of GC

Tables 3 and 4 showed the correlation of immunohistochemical (IHC) expression of CR-1 and p-*STAT3* with clinicopathological parameters. Statistical analysis showed



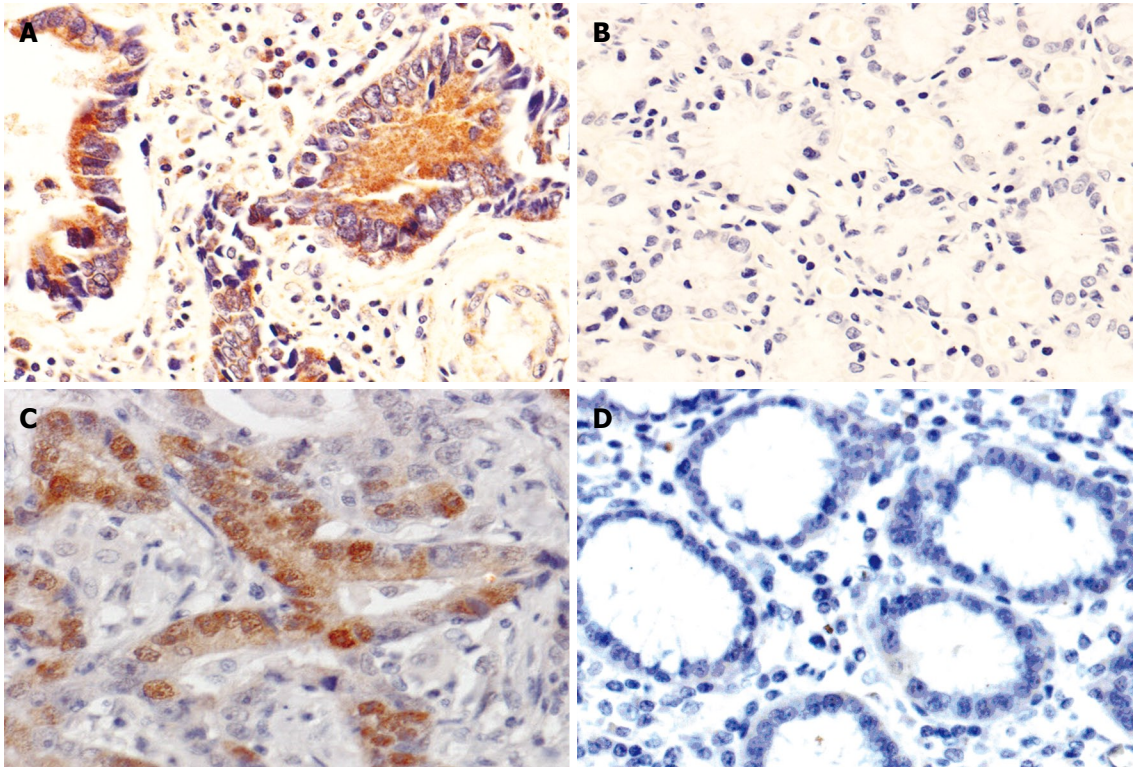


Figure 1 Expression of Cripto-1 (CR-1) and phosphorylation STAT3 (p-STAT3) in gastric cancer (GC) (A, C) and normal gastric mucosa (B, D). IHC PV9000  $\times$  400.

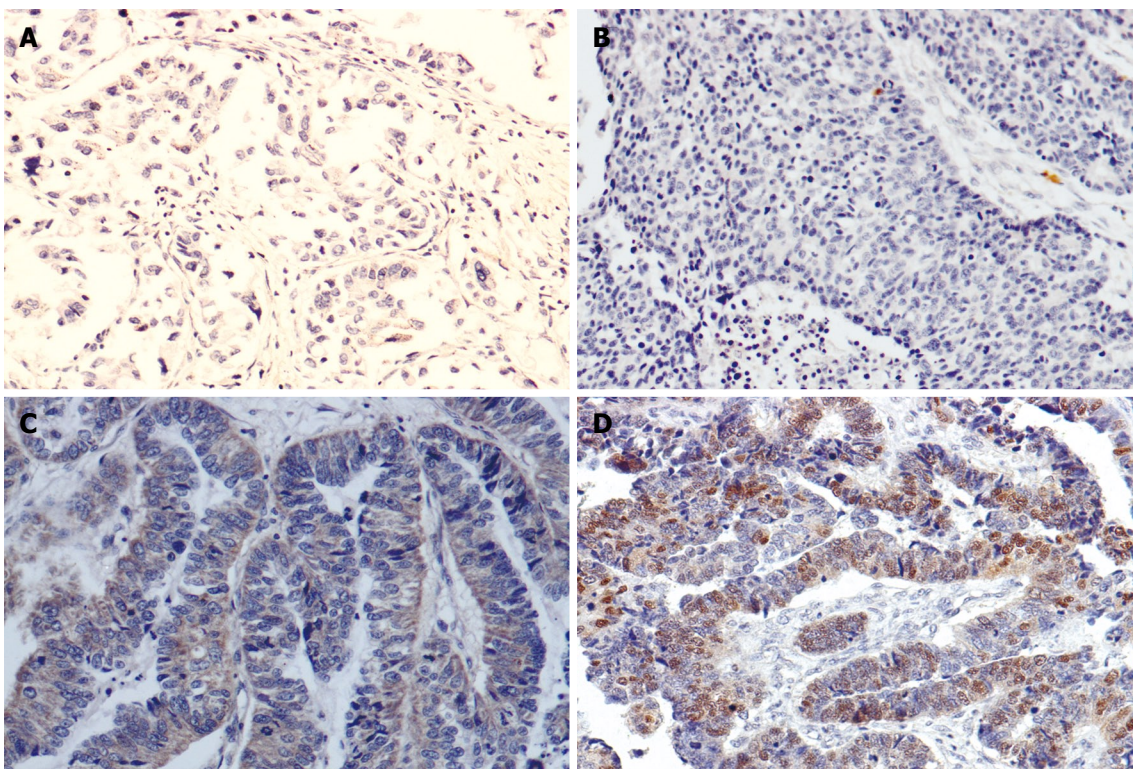


Figure 2 Expression of CR-1 and p-STAT3 in GC without lymph node metastasis (A,B) and with lymph node metastasis (C,D). IHC PV9000  $\times$  200.

the expression of CR-1 was related to histological differentiation ( $P < 0.001$ ), Lauren's types ( $P < 0.001$ ) and lymph node metastasis ( $P = 0.006$ ) (Figure 2A and C), but not related to the age and gender of patients, or Bor-

rmann's classification of GC. There was no relation between p-STAT3 expression and gender, age, Borrmann's classification, histological types or Lauren's types, but p-STAT3 expression was significantly higher in tumors

Table 1 Expression of CR-1 protein in normal gastric mucosa, chronic atrophic gastritis, intestinal metaplasia, dysplasia and GC

Groups	n	CR-1 expression				+ + + + (%)	$\chi^2$	P
		-	+	++	+++			
Normal mucosa	95	54	23	11	7	43.2	8.394/25.813	< 0.039 <sup>a</sup> / <sub>&lt; 0.001<sup>b</sup></sub>
CAG	40	14	11	12	3	65.0	4.214/4.192	0.239 <sup>c</sup> /0.242 <sup>d</sup>
IM	48	8	18	19	3	83.3	9.169	0.027 <sup>e</sup>
DYS	25	5	13	6	1	80.0	13.051/4.135	0.005 <sup>f</sup> /0.246 <sup>g</sup>
GC	178	51	63	38	26	71.3	21.236/3.347	< 0.001 <sup>h</sup> /0.341 <sup>i</sup>

<sup>a</sup>Normal mucosa *vs* CAG; <sup>b</sup>Normal mucosa *vs* IM; <sup>c</sup>CAG *vs* IM; <sup>d</sup>CAG *vs* DYS; <sup>e</sup>IM *vs* GC; <sup>f</sup>Normal mucosa *vs* DYS; <sup>g</sup>DYS *vs* GC; <sup>h</sup>Normal mucosa *vs* GC; <sup>i</sup>CAG *vs* GC. CR-1: Cripto-1; GC: Gastric cancer; CAG: Chronic atrophic gastritis; IM: Intestinal metaplasia; DYS: Dysplasia.

Table 2 Expression of p-STAT3 protein in normal gastric mucosa, chronic atrophic gastritis, intestinal metaplasia, dysplasia and gastric carcinoma

Groups	n	p-STAT3 expression				+ + + + (%)	$\chi^2$	P
		-	+	++	+++			
Normal mucosa	95	56	25	11	3	41.1	18.405	0.012 <sup>a,b</sup> / <sub>&lt; 0.001<sup>c</sup></sub>
CAG	40	16	22	2	0	60.0		0.113 <sup>a,d</sup> /0.003 <sup>a,e</sup>
IM	48	11	28	6	3	77.1	6.604	0.087 <sup>f</sup>
DYS	25	8	7	8	2	68.0	6.259	0.022 <sup>a,g</sup> /0.103 <sup>h</sup>
GC	178	71	69	23	15	60.1	10.322/7.220	0.016 <sup>i</sup> /0.065 <sup>j</sup>

<sup>a</sup>Fisher's exact test; <sup>b</sup>Normal mucosa *vs* CAG; <sup>c</sup>Normal mucosa *vs* IM; <sup>d</sup>CAG *vs* IM; <sup>e</sup>CAG *vs* DYS; <sup>f</sup>IM *vs* GC; <sup>g</sup>Normal mucosa *vs* DYS; <sup>h</sup>DYS *vs* GC; <sup>i</sup>Normal mucosa *vs* GC; <sup>j</sup>CAG *vs* GC.

with lymph node metastasis (66.7%) than in those without metastasis (42.9%),  $P < 0.05$  (Figure 2B and D).

### Correlation between expressions of CR-1 and p-STAT3 in GC

The positive relationship was observed between CR-1 and p-STAT3 expression in GC ( $r_s = 0.189$ ,  $P = 0.002$ ) (Table 5).

## DISCUSSION

Human CR-1, also known as teratocarcinoma-derived growth factor-1 (TDGF-1), was originally isolated and cloned from a human NTera-2 teratocarcinoma cDNA library and was classified among the epidermal growth factor (EGF) family of peptides due to amino acid sequence similarities within the EGF-like domain<sup>[9]</sup>. CR-1 maps to human chromosome 3p21.3 and encodes a 188-amino acid glycosylphosphatidylinositol-linked glycoprotein. CR-1 protein contains a signal sequence, a characteristic EGF-like domain, a second cysteine-rich region motif (CFC domain), and a hydrophobic COOH-terminus<sup>[1]</sup>. Several findings suggested that CR-1 can specifically bind to Glypican-1, a membrane-associated heparan sulfate proteoglycan, and activate the tyrosine kinase c-Src, triggering the mitogen-activated protein kinase and Akt signaling pathways and then promote cell proliferation, survival, migration, and invasion<sup>[4,10]</sup>. In addition, by forming complex with activin and growth factor  $\beta$  (TGF- $\beta$ ) ligand and the receptor, CR-1 inhibited activin and TGF- $\beta$ -signaling, while activin and TGF- $\beta$  are potent inhibitors

of cell growth in various target tissues<sup>[11,12]</sup>. Disruption of their signaling was associated with carcinogenesis. CR-1 may be a potential target for therapy in human malignancies<sup>[5]</sup>. In fact, anti-CR-1 antisense oligonucleotides or neutralizing blocking anti-CR-1 monoclonal antibodies have been shown to strongly inhibit the *in vitro* and *in vivo* growth of human breast, colon, ovarian, testicular, and leukemia carcinoma cells<sup>[5,13,14]</sup>.

Bianco *et al.*<sup>[15]</sup> determined CR-1 plasma levels using a sandwich-type ELISA in 21 healthy volunteers, 54 patients with breast cancer, 33 patients with colon carcinoma, and 21 patients with benign breast lesions. Very low levels of CR-1 were detected in the plasma of healthy volunteers ( $0.32 \pm 0.19$  ng/mL). A significant increase in the levels of plasma CR-1 was found in patients with colon carcinoma ( $4.68 \pm 3.5$  ng/mL) and in patients with breast carcinoma ( $2.97 \pm 1.48$  ng/mL,  $P < 0.001$ ), indicating that measurement of plasma CR-1 level can help in early detection of malignancies. Similar observations were made in IHC analysis and real-time reverse transcription-PCR detection. Zhong *et al.*<sup>[16]</sup> evaluated CR-1 expression in 118 cases of GC, and found that the positive rate of CR-1 was associated with lymph node metastasis, liver metastasis and late TNM stage ( $P < 0.05$ ). Our IHC investigation showed that the expression of CR-1 in CAG, IM, DYS and GC was significantly higher than that in normal gastric mucosa, suggesting the over-expression of CR-1 may contribute to malignant transformation of the gastric mucosa. We speculated that CR-1 may play an important role as a tumorigenic factor and early gastric tumorigenic molecule during gastric carcinogenesis. In



Table 3 Correlation between CR-1 expression and clinicopathological features of GC

Groups	n	Cripto-1 expression				+ → + + + (%)	$\chi^2$	P
		-	+	++	+++			
Gender							4.052	0.269
Female	57	11	24	14	8	80.7		
Male	121	40	39	24	18	66.9		
Age (yr)							2.920	0.417
≤ 61	90	22	33	23	12	75.6		
> 61	88	29	30	15	14	67.0		
Borrmann type								0.195 <sup>1</sup>
Bor I type	4	0	3	0	1	100		
Bor II type	22	6	9	7	0	72.7		
Bor III type	140	41	48	27	24	70.7		
Bor IV type	12	4	3	4	1	66.7		
WHO's histological types								< 0.001 <sup>1</sup>
Papillary. ade.	2	0	1	1	0	100		
Well-diff. ade.	9	3	2	2	2	66.7	17.872	< 0.001 <sup>2</sup>
Moderately-diff. ade.	65	8	28	11	18	87.7		
Poorly-diff. ade.	74	27	27	16	4	63.5		
Undiff. ade.	3	0	1	2	0	100		
Mucinous ade.	19	11	3	3	2	42.1		
SRC	6	2	1	3	0	66.7		
Lauren's types							28.523	< 0.001
Intestinal type	74	11	29	14	20	68.4	19.566	< 0.001 <sup>3</sup>
Diffused type	85	29	31	21	4	65.9		
Mixed type	19	11	3	3	2	42.1		
Lymph node metastasis							12.394	0.006
No	49	23	14	9	3	53.1		
Yes	129	28	49	29	23	78.3		

<sup>1</sup>Fisher's exact test; <sup>2</sup>Well-Moderately-diff. *vs* Poorly-diff. ade; <sup>3</sup>Intestinal type *vs* Diffused type. ade.: Adenocarcinomas; diff.: Differentiated; SRC: Signet ring cell carcinoma.

Table 4 Correlation between p-STAT3 expression and clinicopathological features of GC

Groups	n	P-STAT3 expression				+ → + + + (%)	$\chi^2$	P
		-	+	++	+++			
Gender							3.091	0.391
Female	57	18	27	7	5	68.4		
Male	121	53	42	16	10	56.2		
Age (yr)							2.177	0.544
≤ 61	90	37	35	13	5	58.9		
> 61	88	34	34	10	10	61.4		
Borrmann type								0.909 <sup>1</sup>
Bor I type	4	1	2	0	1	75.0		
Bor II type	22	11	8	2	1	61.2		
Bor III type	140	55	53	20	12	60.7		
Bor IV type	12	4	6	1	1	66.7		
WHO's histological types								0.100 <sup>1</sup>
Papillary. ade.	2	0	0	0	2	100		
Well-diff. ade.	9	4	4	0	1	55.6		
Moderately-diff. ade.	65	23	23	10	9	64.5		
Poorly-diff. ade.	74	35	29	7	3	52.7		
Undiff. ade.	3	1	2	0	0	66.7		
Mucinous ade.	19	7	8	4	0	63.2		
SRC	6	1	3	2	0	83.3		
Lauren's types							11.417	0.076
Intestinal type	74	27	26	9	12	63.5		
Diffused type	85	37	35	10	3	56.5		
Mixed type	19	7	8	4	0	63.2		
Lymph node metastasis							9.632	0.021
No	49	28	16	3	2	42.9		
Yes	129	43	53	20	13	66.7		

<sup>1</sup>Fisher's exact test.



**Table 5** Relationship between CR-1 and p-STAT3 expression in GC

Cripto-1 expression	p-STAT3 expression				Total
	-	+	++	+++	
-	24	21	6	0	51
+	28	22	9	4	63
++	13	17	3	5	38
+++	6	9	5	6	26
Total	71	69	23	15	178

$r_s = 0.189$ ,  $P = 0.002$ .

IM, the positive rates of CR-1 was significantly higher than in normal gastric mucosa ( $P < 0.05$ ), and in GC of the intestinal type was significantly higher than that in the diffused type ( $P < 0.001$ ), indicating that CR-1 may be involved in the occurrence of intestinal type of GC. The expression of CR-1 in the well-to-moderately differentiated cancer group was higher than in the poorly differentiated cancer group ( $P < 0.001$ ), which showed that CR-1 expression was correlated with differentiation of GC. Moreover, our results showed a positive correlation between CR-1 expression and lymph node metastasis, in GC with lymph node metastasis, the positive rates of CR-1 was significantly higher than that without metastasis ( $P < 0.05$ ), which was consistent with the reported findings<sup>[16]</sup>, indicating that CR-1 may participate in the development and lymph node metastasis of GC. CR-1 can act as a prognostic indicator for GC patients.

STAT3 maps to human chromosome 2q13-14.1 and encodes 750-795 amino acids protein. STAT3 protein contains six basic domains: an N-terminal conserved sequence, a helix domain, a DNA binding domain, a joining region, a Src homology 2 domain (SH2) and a transcription activation domain. The SH2 domain, being most conserved region in STAT3, acts as the cardinal part in STAT3 function by participating in tyrosin phosphorylation. STAT3 is mainly located in the cytoplasm when the cells are in resting state. Upon induction (activation) of tyrosine phosphorylation by a wide variety of cytokines or growth factors, STAT3 is activated and forms p-STAT3. p-STAT3 dimerizes and translocates to the nucleus, further regulating transcription of target genes<sup>[17,18]</sup>. Many reports have shown that p-STAT3 is abnormally activated in most malignant tumors, including squamous cell carcinoma of the head and neck, breast and GCs<sup>[7,19,20]</sup>. Lee *et al.*<sup>[20]</sup> detected p-STAT3 expression in 307 cases of GC, correlative analyses between p-STAT3 and clinical parameters demonstrated a positive correlation with prognosis of patients with GC. In this study, we found that the expression of p-STAT3 in CAG, IM, DYS and GC was significantly higher than that in normal gastric mucosa ( $P < 0.05$ ), but there was no significant difference in p-STAT3 expression between DYS and GC, suggesting that DYS of gastric mucosa with p-STAT3 abnormal activation has a canceration tendency, and p-STAT3 may be involved in the early events of gastric carcinogenesis. There was no relation between p-STAT3 expression and gender,

age, Borrmann's types, histological types or Lauren's types, but p-STAT3 expression was significantly higher in GC with lymph node metastasis than without metastasis ( $P < 0.05$ ), which suggested that the level of p-STAT3 expression may indicate the potential for lymph node metastasis and facilitate the prognostic judgment of GC.

CR-1 can induce activation of the cytoplasmic tyrosine kinase c-Src through specifically binding to Glypican-1<sup>[21]</sup>, and also can indirectly interact with epidermal growth factor receptor (EGFR) to promote cell survival and proliferation<sup>[22]</sup>; STAT3 can be activated by both EGFR and c-Src<sup>[23-25]</sup>. Based on the previous findings mentioned above and the results in our study that there is a significant association between CR-1 expression and p-STAT3 activation in GC ( $r_s = 0.189$ ,  $P = 0.002$ ), we presume that STAT3 may be the downstream molecule of CR-1, and over-expressed CR-1 protein participated in occurrence and development of GC through activating STAT3 signaling pathway. The relevant molecular mechanism requires further investigations.

In conclusion, CR-1 and p-STAT3 may take part in the occurrence, development and metastasis of GC, which can provide theoretical foundation for early diagnosis and judging the prognosis of GC. We discovered that the CR-1 expression in GC was positively related to p-STAT3 expression. These findings provide new insights into understanding the molecular mechanism involved in gastric carcinogenesis and progression, and may lead to the development of new approaches for early detection and effective therapy. However, whether CR-1 and p-STAT3 collaborate to contribute to gastric carcinogenesis and development need further studies.

## COMMENTS

### Background

Gastric cancer (GC) is one of the malignant diseases with highest incidence and mortality rates and it is of great significance to investigate the mechanisms behind the occurrence and development of GC. The over-expression of Cripto-1 (CR-1) and phosphorylation STAT3 (p-STAT3) has been shown to play an important role in the tumorigenesis and cancer progression. Therefore, the exploration into the correlation and significance of the expression of CR-1 and p-STAT3 in GC and precancerous lesions may sheds a new light on early detection and therapy of GC.

### Research frontiers

CR-1 and p-STAT3 have been shown to exert oncogenic effects in various human neoplasms. In this study, the authors investigated the expression of CR-1 and p-STAT3 proteins in atrophic gastritis, normal mucosa, intestinal metaplasia (IM) and GC to explore its correlation with gastric carcinogenesis and metastasis. They found that over-expression of CR-1 and p-STAT3 may take part in early carcinogenesis and metastasis of GC.

### Innovations and breakthroughs

CR-1 and STAT3 are over-expressed in a wide range of human cancers such as gastric, colorectal and breast carcinomas, and may play a key role in cancer pathogenesis. Over-expression of CR-1 and STAT3 increases cancer cell proliferation, migration, invasion and angiogenesis. This is the first study to report that the expression of CR-1 and p-STAT3 in GC was positively correlated, and speculate that there might be a functional relationship between CR-1 and STAT3, where STAT3 might be a the downstream molecule of CR-1.

### Applications

The positive rates of CR-1 and p-STAT3 expression were significantly higher in chronic atrophic gastritis, IM, dysplasia and GC than that in normal gastric mucosa. And the expression rates of CR-1 and p-STAT3 protein was also

significantly higher in GC with lymph node metastasis when compared with those without metastasis. Detection of CR-1 and p-STAT3 protein expressions might be helpful in early detection and prognosis judgment of GC patients. In addition, CR-1 and p-STAT3 may serve as a potential therapeutic target for GC.

### Terminology

Human CR-1, also known as teratocarcinoma-derived growth factor-1 (TDGF-1), is a member of the epidermal growth factor-Cripto-FRL1-Cryptic family including mouse CR-1, cryptic *Xenopus* FRL-1, zebrafish one-eyed pinhead and chick Cripto. CR-1 functions as an oncogene involved in *in vitro* cellular transformation and enhancement of cancer cell proliferation, migration, invasion and angiogenesis. STAT3 belongs to the STAT family of signal transducers and activators of transcription, which play a critical role in mediating cellular responses to various stimuli, mainly from those of growth factors and cytokines.

### Peer review

In this study, Zhang *et al* investigated the expression of CR-1 and p-STAT3 proteins in normal mucosa, IM and GC to explore its correlation with gastric carcinogenesis and metastasis. In general, this is an interesting study with new precision on known markers for GC progression.

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## Understanding of chemoprophylaxis and concordance in inflammatory bowel disease

Adam Low, Melanie Love, Robert Walt, Katherine Kane, Bertus Eksteen, Jason Goh

Adam Low, Melanie Love, Robert Walt, Katherine Kane, Jason Goh, Department of Gastroenterology, Queen Elizabeth Hospital, University Hospital Birmingham NHS Foundation Trust, Edgbaston, Birmingham, B15 6HT, United Kingdom  
Bertus Eksteen, Department of Liver Medicine, Queen Elizabeth Hospital, University Hospital Birmingham NHS Foundation Trust, Edgbaston, Birmingham, B15 6HT, United Kingdom

**Author contributions:** Low A was responsible for co-ordinating patient recruitment and data collection, analysing the data and drafting the manuscript; Love M, Walt R and Eksteen B were responsible for patient recruitment and editing the manuscript; Kane K assisted with questionnaire design and patient recruitment; Goh J conceived the idea for the study and assisted with manuscript preparation and drafting; all authors read and approved the final manuscript.

**Correspondence to:** Dr. Jason Goh, Department of Gastroenterology, Queen Elizabeth Hospital, University Hospital Birmingham NHS Foundation Trust, Edgbaston, Birmingham, B15 6HT, United Kingdom. [jason.goh@uhb.nhs.uk](mailto:jason.goh@uhb.nhs.uk)  
Telephone: +44-121-6271627 Fax: +44-121-6277827

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### Abstract

**AIM:** To assess patients' understanding for the reasons for taking 5-aminosalicylic acid or ursodeoxycholic acid as chemoprophylaxis against colorectal carcinoma associated with inflammatory bowel disease (IBD).

**METHODS:** A questionnaire-based study using a 5 point opinion scale was performed. One hundred and ninety-two patients with colitis only and 74 patients with primary sclerosing cholangitis and IBD were invited to take part.

**RESULTS:** Overall response rate was 58%. Sixty-four percent of patients claimed full concordance with chemoprophylaxis for maintenance of remission. Eighty-four percent of patients considered daily concordance

during remission to be very important. Seventy-five percent stated they understood the reasons for taking the drugs. However, only 50% of the patients were aware of any link of their condition to bowel cancer. Seventy-nine percent of patients felt their concordance and understanding would be improved if they were informed of the chemoprophylactic potential of the medication.

**CONCLUSION:** Despite good self-reported concordance, half of the patients were unaware of an association between colitis and bowel cancer. Explaining the potential chemoprophylactic benefits may enhance patients' overall concordance to 5-aminosalicylic acid and ursodeoxycholic acid and help maintain remission.

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**Key words:** Inflammatory bowel disease; Primary sclerosing cholangitis; Colorectal cancer; 5-aminosalicylic acid; Concordance

**Peer reviewer:** Kiron M Das, MD, PhD, FACP, FRCP, Chief of Gastroenterology and Hepatology, Professor of Medicine, Director of Crohn's and Colitis Center of NJ, 1 Robert Wood Johnson Place, MEB Rm. 478, New Brunswick, NJ 08903, United States

Low A, Love M, Walt R, Kane K, Eksteen B, Goh J. Understanding of chemoprophylaxis and concordance in inflammatory bowel disease. *World J Gastroenterol* 2010; 16(5): 578-582 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i5/578.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i5.578>

### INTRODUCTION

The risk of colorectal carcinoma (CRC) associated with inflammatory bowel disease (IBD) has long been established<sup>[1]</sup>, increasing with the duration, extent and severity



of inflammation. Primary sclerosing cholangitis (PSC) is known to be associated with colitis and an increased risk of CRC<sup>[2,3]</sup>. Over 60% of PSC patients have associated IBD, with associated increased risk of CRC as well as hepatocellular carcinoma. Five percent of patients with ulcerative colitis will go on to develop liver dysfunction, primarily cholestasis, of which 40% will develop PSC<sup>[4]</sup>. The risk of colorectal cancer in colitis patients has been estimated to be as high as 30% over 35 years in some populations<sup>[1]</sup>. There are similar rates of mucosal cell metaplasia and dysplasia in ulcerative colitis and Crohn's colitis.

5-aminosalicylic acids (5-ASAs), for example mesalazine, are the most commonly prescribed anti-inflammatory drugs used in the management of IBD<sup>[5,6]</sup>. The evidence for their chemoprophylactic use in IBD is accumulating, including case control studies<sup>[7-10]</sup>, prospective cohort studies<sup>[11,12]</sup>, and a large UK based epidemiological study<sup>[5]</sup>. The use of ursodeoxycholic acid (UDCA) as a chemopreventative agent in PSC with ulcerative colitis is now accepted practice<sup>[13]</sup>. Chemoprophylaxis with these drugs is now part of core recommendations in the treatment of colitis published in guidelines, for example guidance issued by the British society of Gastroenterology<sup>[14]</sup>. The use of 5-ASA compounds for chemoprophylaxis in ulcerative colitis patients was considered to be evidence level 2 in recent guidance published by ECCO, and UDCA for PSC patients was considered to have an evidence level 1b<sup>[15]</sup>.

Surveillance techniques for the detection of CRC and metaplastic cell changes are invasive endoscopic procedures. A systematic review of the available evidence concluded that while cancer was detected at an earlier stage through surveillance colonoscopy screening of colitis patients, there was no clear evidence of prolonged survival<sup>[16]</sup>. There was indirect evidence for screening programmes cost effectiveness, but the acceptability of colonoscopy to all patients remains an important prohibitive factor. It remains to be seen if novel techniques such as chromoendoscopy, confocal microscopy and emerging molecular markers can directly influence survival.

Chemoprophylaxis with 5-ASAs and UDCA as a means of reducing the risk of colorectal cancer to IBD and PSC/IBD patients is becoming an increasingly attractive concept. Current literature on drug concordance in IBD has been largely focussed on 5-ASA and factors influencing non-adherence<sup>[17-25]</sup>. Important factors identified include male gender, young age at diagnosis, occupation and depression<sup>[17,18]</sup>. The preparation and treatment regime for 5-ASA have also been highlighted as contributing to non-concordance and therefore poorer outcome<sup>[18-20]</sup>. All studies emphasize the importance of the physician-patient relationship and utilizing management strategies to reduce non-adherence. The concerns over 5-ASA preparations and dosing regimes are being addressed through the novel delivery of mesalazine as a once daily preparation<sup>[26,27]</sup>. Despite this and advancing knowledge of chemoprophylaxis, relatively little is known about patients' understanding of the risk of developing colitis-associated CRC and the role of 5-ASA

in reducing these risks. There is no doubt that patient education is important in not only establishing a good patient-physician working relationship, but also improving outcome through improved concordance with treatment regimes. More energy and strategies, such as the employment of specialist IBD nurse practitioners, are being used to educate patients about their conditions from the time of first diagnosis.

The aim of our study was to assess patients' understanding of the risk of developing colitis-associated CRC and their understanding of the role of 5-ASA/UDCA as part of their treatment, as well as identifying means of improving overall concordance with chemoprophylaxis, through a qualitative questionnaire-based survey and patient feedback.

## MATERIALS AND METHODS

A simple patient questionnaire comprising of seven questions using a validated five-point opinion scale, was designed. The questionnaires were altered appropriately for the PSC/IBD patients giving two similar short questionnaires. Patients were also provided with clear written instruction on how to complete questions using a five-point opinion scale and contact details if they had any further queries. Finally, they were given the opportunity to provide feedback on proposed methods we could use to improve overall concordance as well as their own opinions on the matter.

The questions assessed disease activity, the importance patients assigned to daily concordance when both symptomatic and in remission, how often they forgot to take their medication, whether they understood the reasons for being on the medication, how well their doctor had explained the reasons for taking 5-ASA/UDCA and whether they were aware that 5-ASA/UDCA may help reduce the risk of developing CRC. PSC/IBD patients were also asked whether the UDCA was to treat their liver, bowel, or both to further assess how fully they understood reasons for taking UDCA daily. The patients' opinions on ways of improving concordance were also sought. They were asked to rate how much the following proposals could improve concordance: a once-daily preparation, a clear explanation of the reasons for taking the medication and how it works from a health care professional, and being provided with evidence that 5-ASA/UDCA can reduce risk of CRC.

One hundred and ninety two patients with IBD (both Crohn's disease and ulcerative colitis patients) receiving 5-ASAs were identified from our University Hospital Birmingham IBD database and a further 74 patients with PSC and associated IBD from our Liver Medicine department records were also included. The patients in the IBD group were in clinical remission at the time of the study. The PSC/IBD group were a post-liver transplant cohort with their colon intact.

Patients were given the questionnaires to complete on a voluntary basis when they attended the out-patient department clinics. All responses were anonymised for



results analysis. Patients who were not given the opportunity to complete a questionnaire in the clinic were posted questionnaires with a stamped, addressed return envelope.

The questionnaire was approved by the hospitals Clinical governance board. Responses were processed and analysed by two of the authors using a standardised database.

## RESULTS

Response rates were 59% (114/192) and 56% (42/74) for the IBD only and the IBD/PSC cohorts, respectively. Of the 42 patients in the IBD/PSC group who responded, 5 patients were excluded from further analysis as they were not taking UDCA (1 had never been prescribed it and 4 had stopped it due to side effects), making the total for subsequent analysis 37.

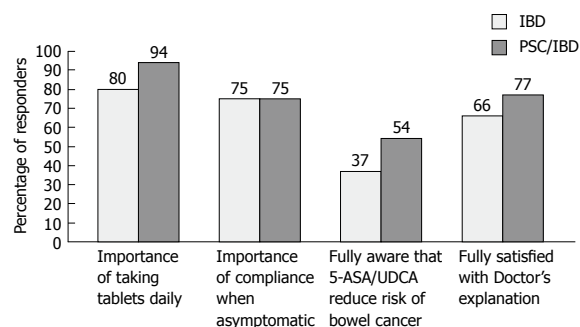
Sixty-four percent (98/151) of the total responders stated that they were fully concordant with 5-ASA/UDCA (60% IBD *vs* 78% IBD/PSC). Sixty-two percent of patients had had a flare up of symptoms in the past year, and of these, 67% reported full concordance with their medications, suggesting that disease activity may improve overall concordance.

Eighty-four percent (126/151) of patients considered daily concordance to be very important and 75% (114/151) maintained concordance to be very important while in remission, stating that they fully understood the reasons for being prescribed the medications. Sixty-seven percent (101/151) of patients felt they had been given a full explanation by their doctor for 5-ASA/UDCA maintenance treatment (66% IBD *vs* 70% IBD/PSC). Twenty percent of patients had only received a partial explanation while 13% (12% IBD *vs* 16% IBD/PSC) felt they had received no explanation from either their doctor or any health professional. A bar chart comparing the responses of the IBD cohort to the PSC/IBD cohort is shown in Figure 1.

Despite 75% of patients claiming full understanding of the reasons for 5-ASA/UDCA maintenance treatment, 50% of the total cohort reported that they were completely unaware of any link between their condition and CRC (54% IBD *vs* 37% IBD/PSC). Of the IBD/PSC cohort, only 55% of patients were aware that UDCA is protective for both bowel and liver, suggesting the patients may not have been fully informed, despite 75% believing they fully understood the reasons for taking UDCA.

Of the three methods proposed to improve overall concordance in the future, 92% of patients agreed that a once daily preparation of 5-ASA would improve their overall concordance, 87% felt that a clear and full explanation from a health professional would be beneficial, and 94% felt that it would be helpful if they were given evidence that 5-ASA would help to reduce the risks of developing colitis-associated CRC.

Methods of improving patient concordance with 5-ASA/UDCA are proposed in Table 1. Table 2 contains samples of feedback patients gave of ways that their concordance to chemoprophylactic medications could be



**Figure 1** A comparison of the IBD vs PSC/IBD cohorts. IBD: Inflammatory bowel disease; PSC: Primary sclerosing cholangitis; 5-ASA: 5-aminosalicylic acid; UDCA: Ursodeoxycholic acid.

**Table 1** The percentage of patients who agreed with three proposed methods of improving concordance with chemoprophylaxis

Proposed method to improve concordance	Agree (%)	Disagree (%)
Given evidence 5-ASA/UDCA reduces CRC risk	94	6
Once a day preparation	92	8
Clear explanation from Health Professional	87	13

5-ASA: 5-aminosalicylic acid; UDCA: Ursodeoxycholic acid; CRC: Colorectal carcinoma.

**Table 2** Some comments written by patients, including possible reasons for poor concordance and suggestions for improving concordance

Difficult to remember when feeling well. Reminders would be useful
Unaware of reasons for taking the medication
Need reassurance regarding side effects
Full explanation, counselling and education on condition
Newsletters and workshops on developments in research and treatment
Re-enforcement about why on treatment when it has been started and stopped in the past
Unfortunately you cannot teach common sense if they do not stick to the prescribed dose there is not a lot you can do
Smaller, easier to take tablets

improved. Some salient examples include “Re-enforcement about why I’m on a treatment when it has been started and stopped in the past would be useful” and “newsletters and workshops on developments in research and treatment...”.

## DISCUSSION

Patients had a high self-reported level of concordance with their medications. Our study made no attempt to verify this through biochemical testing<sup>[22]</sup>. A study using urinary analysis identified 6 (12.7%) out of 47 patients who self reported full concordance tested negative for mesalamine or its metabolite<sup>[23,24]</sup>. We did not verify patients’ concordance against the frequency of pharmacy prescription collection. These results suggest that overall concordance in our patient group may be lower than claimed.

It is clear from patients' responses that their understanding of the reasons for being on maintenance treatment and chemoprophylaxis is incomplete and that not all patients are fully informed about the risk of colitis-associated CRC. Patients need to have this information given to them by health professionals and reinforced by other resources. This information would also help IBD/PSC patients to better understand the need to continue their medication even when their condition is quiescent.

While the risk of CRC in IBD and PSC has been fully appreciated by health care professionals for decades, it is evident from our study that this knowledge is not being clearly communicated to patients. Potential obstacles may include time pressure in clinics, the physicians' reluctance to discuss cancer potential with an asymptomatic patient, or patients not retaining this information. From our unpublished data, many patients undergoing regular colonoscopy surveillance for IBD are not fully aware of the cancer association and the potential need for colectomy in the event of dysplasia detection.

Patient education has been demonstrated to improve clinical outcome in the management of diabetes mellitus<sup>[28]</sup> and can easily be applied to IBD. The role of nurse practitioners in gastroenterology, in particular IBD, is evolving. An exceedingly useful resource, they work alongside gastroenterologists in the clinics and on the wards to educate patients on their condition and treatment<sup>[29]</sup>. Additional resources for patient education include patient information leaflets in a language that is easy to interpret, support groups and relevant websites.

Reassurance with regards to the safety, efficacy and side effect profile of these medications has also been identified by our patients as an important factor to improve concordance. This observation is in keeping with the study by Loftus *et al.*<sup>[25]</sup>, who also identified a lower pill burden and less frequent dosing as important. Ninety-two percent of patients in our study believed that a once daily preparation would improve concordance. Several once daily 5-ASA preparations are now available<sup>[26,27,30]</sup>.

Optimising surveillance strategy using risk stratification and novel techniques coupled with advances in molecular markers may transform the way we manage this at-risk group. The emerging link between inflammation and cancer presents a very strong case for optimal disease control and chemoprophylaxis with 5-ASA and UDCA.

While the definitive randomised control study proving the benefit of chemoprophylaxis may remain elusive for the near future, the accumulating *in vitro*, *in vivo* and observational evidence are hard to ignore. To translate this into clinical benefit, it is important that physicians communicate these clinical rationales clearly to patients with IBD to improve the likelihood of them taking these medications even when they feel well.

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## COMMENTS

### Background

Risk of colorectal neoplastic changes increase with length and duration of inflammation, making inflammatory bowel disease (IBD) and primary sclerosing cholangitis (PSC) patients particularly at risk of colorectal carcinoma. Management of these patients now includes chemoprophylaxis with 5-aminosalicylic acid and ursodeoxycholic acid during disease quiescence as well as symptomatic flare ups. These therapies are now endorsed in guidance on disease management such as that issued by the British Society of Gastroenterology and European Crohn's and Colitis Organisation, and is therefore accepted clinical practice, yet little is known of patient's understanding of why they are taking these therapies, their understanding of the risks of colorectal carcinoma associated with their condition and how their concordance to chemoprophylaxis may be improved.

### Research frontiers

Research is focussing on improved surveillance techniques and screening in this high risk group to try to ensure early detection of neoplastic changes. However, interventions have little impact on overall patient survival and acceptability of invasive screening procedures such as colonoscopy remains in doubt. Therefore, management focuses increasingly on chemoprophylaxis in the meantime.

### Applications

Those prescribing chemoprophylaxis to IBD/PSC patients should take the time to explain to patients the reason for starting these therapies, even during times of disease quiescence, especially the fact that inflammation is associated with a higher chance of subsequent lower gastrointestinal carcinoma and that it is therefore important to minimise the risks with chemoprophylaxis. This information should not just be given at initial consultation, but should be regularly re-enforced to improve overall concordance with chemoprophylaxis. Specialist nurses have a key role in this educational aspect of disease management and patient education should be utilised for optimal results.

### Peer review

This paper is acceptable for publication with some revision.

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## Crypt abscess-associated microbiota in inflammatory bowel disease and acute self-limited colitis

Harry Sokol, Nadia Vasquez, Nadia Hoyeau-Idrissi, Philippe Seksik, Laurent Beaugerie, Anne Lavergne-Slove, Philippe Pochart, Philippe Marteau

Harry Sokol, Philippe Seksik, Laurent Beaugerie, Gastroenterology and Nutrition Department, Saint Antoine Hospital, Pierre et Marie Curie-Paris6 University, AP-HP, 184 rue du Faubourg Saint-Antoine, 75571, Paris, France

Nadia Vasquez, Philippe Pochart, Philippe Marteau, Laboratoire de Biologie EA 3199, CNAM, 2 rue Conte, 75003, Paris, France

Nadia Hoyeau-Idrissi, Pathology Department, Saint Antoine Hospital, Pierre et Marie Curie-Paris6 University, AP-HP, 184 rue du Faubourg Saint-Antoine, 75571, Paris, France

Anne Lavergne-Slove, Pathology Department, Lariboisière Hospital & Paris 7 University, 2 rue Ambroise-Paré, 75475, Paris, France

Philippe Marteau, Medicosurgical Department of Digestive Diseases, Lariboisière Hospital, AP-HP & Paris 7 University, 2 rue Ambroise-Paré, 75475, Paris, France

**Author contributions:** Sokol H, Marteau P and Lavergne-Slove A designed the research; Sokol H and Vasquez N performed the research; Sokol H, Vasquez N, Hoyeau-Idrissi N, Seksik P, Beaugerie L, Lavergne-Slove A, Pochart P and Marteau P analyzed the data; Sokol H and Marteau P wrote the paper.

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**Correspondence to:** Harry Sokol, MD, PhD, Gastroenterology and Nutrition Department, Saint Antoine Hospital, Pierre et Marie Curie-Paris6 University, AP-HP, 184 rue du Faubourg Saint-Antoine, 75571, Paris, France. [sokol\\_harry@yahoo.fr](mailto:sokol_harry@yahoo.fr)  
 Telephone: +33-1-49283162 Fax: +33-1-49283188

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### Abstract

**AIM:** To evaluate whether crypt abscesses from inflammatory bowel disease (IBD) patients contain bacteria and to establish their nature.

**METHODS:** We studied 17 ulcerative colitis patients, 11 Crohn's disease patients, 7 patients with acute self-limited colitis (ASLC) and normal colonic biopsies from 5 subjects who underwent colonoscopy for colon cancer

screening. A fluorescent *in situ* hybridization technique was applied to colonic biopsies to assess the microbiota composition of the crypts and crypt abscesses.

**RESULTS:** Crypts colonized by bacteria were observed in 42.9% and 3.6% of ASLC and IBD patients, respectively ( $P = 0.019$ ). Crypt abscesses colonized by bacteria were observed in 28.6% and 0.0% of ASLC and IBD patients, respectively ( $P = 0.035$ ).

**CONCLUSION:** These results do not support the hypothesis that crypt abscesses in IBD are the result of localized dysbiosis arising from persistence of living bacteria colonizing the crypts.

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**Key words:** Inflammatory bowel diseases; Crohn's disease; Ulcerative colitis; Crypt abscess; Microbiota

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### INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are the 2 most common types of inflammatory bowel disease



(IBD). Although their pathophysiology is still unknown, the gut microbiota is considered to play a crucial role<sup>[1]</sup>. The microbiota close to the mucosa differs from the luminal microbiota<sup>[2]</sup>. We and others described a luminal and mucosal dysbiosis in IBD<sup>[3-8]</sup> including a high proportion of unusual bacteria<sup>[6,8]</sup> and a restricted microbial biodiversity<sup>[7,9]</sup>. Nevertheless, no difference in the dominant microbiota was observed between inflamed and non-inflamed mucosa in CD and UC<sup>[5,10]</sup>.

It would be of great importance to establish the early events leading to IBD onset or to perpetuation of inflammation. Crypt abscesses are early lesions observed in IBD, particularly in UC<sup>[11]</sup>, consisting of dilated crypts containing polymorphonuclear cells. They can also occur in acute self limited colitis (ASLC)<sup>[12]</sup>, collagenous and lymphocytic colitis<sup>[13]</sup>, diverticula-associated colitis<sup>[14]</sup> and diversion colitis<sup>[15]</sup>. Defensins are antimicrobial peptides secreted in intestinal and colonic crypts and recent studies pointed out a defensin secretion defect in IBD patients<sup>[16-20]</sup>. The aim of this study was to evaluate if crypt abscesses in UC, CD and ASLC patients contained bacteria and, if so, to establish their nature i.e. investigate localized dysbiosis in this specific ecosystem.

## MATERIALS AND METHODS

### Patients

We studied 35 patients with acute colitis and crypt abscesses at histological examination of colon biopsy: 17 UC patients, 11 CD patients and 7 patients with ASLC (bacteria involved: *Shigella sonnei*, *Campylobacter sp.* and no identified pathogenic bacteria in the 5 other cases). We also analyzed normal colonic biopsies from 5 subjects who underwent colonoscopy for colon cancer screening. The characteristics of the patients are described in Table 1. Rectocolonic biopsies containing crypt abscesses were analyzed.

### Tissues, histological examination and fluorescent in situ hybridization (FISH)

Histological examination and assessment of the bacterial composition of the crypts and crypt abscess microbiota were performed as previously described using FISH with one general probe (Eubacteria) and 6 group-specific probes, [*Bacteroides-Prevotella*,  $\gamma$  Proteobacteria, *Bifidobacterium*, *Clostridium coccoides*, *Faecalibacterium prausnitzii* (*F. prausnitzii*) and *Lactobacillus-Enterococcus*]<sup>[21]</sup>.

**Histological examination:** Colonic biopsy sections were deparaffined in xylene and successively rehydrated for 3 min in 100%, 96%, and 70% ethanol. They were then stained with hematoxylin and eosin for morphological assessment.

**FISH:** Prior to FISH, sections were deparaffinized, rehydrated, and postfixed in 4% paraformaldehyde for 5 min. Fixation was stopped in phosphate-buffered saline (PBS) 3 × and slides were washed twice for 1 min in

Table 1 Characteristics of the patients

	UC	CD	ASLC
<i>n</i>	17	11	8
Male (%)	47.1	54.5	50.0
Mean age ( $\pm$ SE, yr)	42.0 $\pm$ 3.3	44.0 $\pm$ 3.9	45.6 $\pm$ 10.5
Mean disease duration ( $\pm$ SE, mo)	75 $\pm$ 23	50 $\pm$ 27	NA
Montreal classification (%)			
E1	0	NA	NA
E2	70.6	NA	NA
E3	29.4	NA	NA
L1	NA	0	NA
L2	NA	63.6	NA
L3	NA	36.4	NA
Analyzed segment (%)			
Rectum	64.7	36.4	71.4
Left colon	35.3	45.4	14.3
Right colon	0.0	18.2	14.3
Treatment (%)			
5-aminosalicylic acid	35.3	18.2	0.0
Corticosteroids	35.3	27.3	0.0
Azathioprine	17.6	18.2	0.0
Infliximab	0.0	18.2	0.0

UC: Ulcerative colitis; CD: Crohn's disease; ASLC: Acute self-limited colitis; NA: Not available.

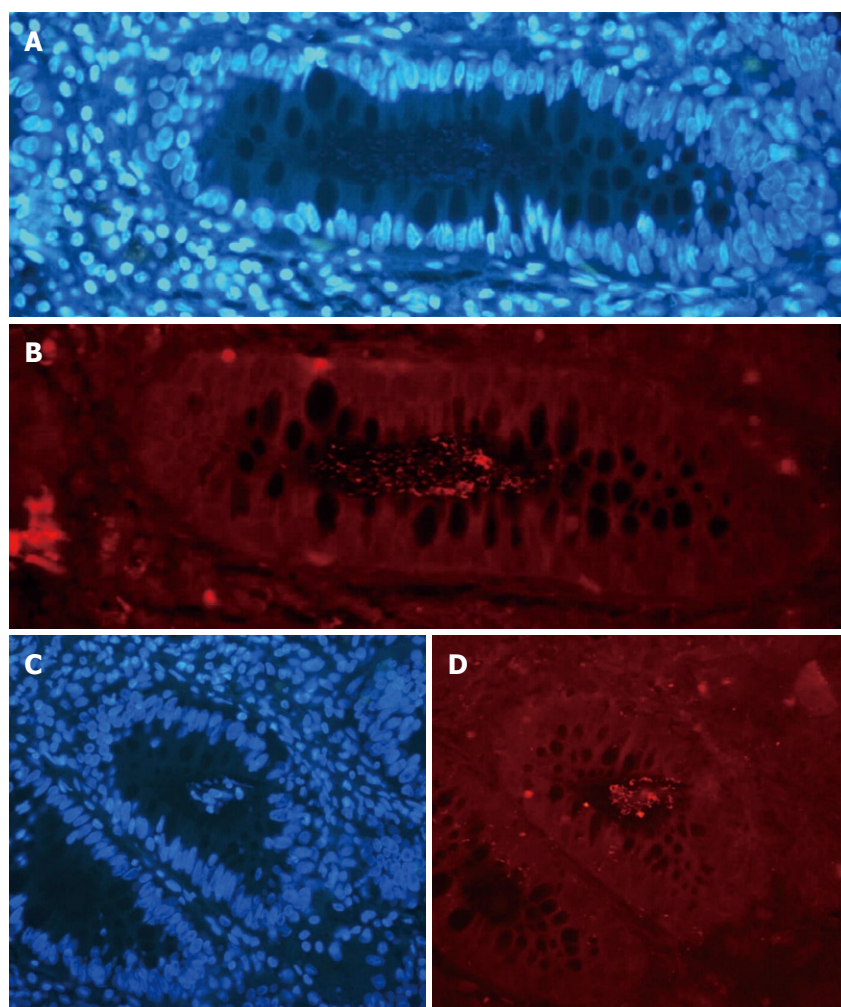
PBS 1 ×. Tissue sections were incubated 10 min at room temperature with Tris-EDTA buffer containing 10 mg/mL of lysozyme and then washed using the hybridization solution (0.9 mol/L NaCl, 20 mmol/L Tris HCl, pH 8, 0.01% SDS, 30% formamide). Fixed tissue sections were then hybridized with the previous hybridization solution containing 4.5 ng/ $\mu$ L of one of the 5'-end-Cy3-labeled 16S rRNA targeted oligonucleotide probes. Hybridizations were performed at 35°C overnight in a microscope slide incubator and stringent washings were carried out at 37°C (2 × 15 min) in a buffer containing 65 mmol/L NaCl, 20 mmol/L Tris HCl, pH 8.0, 5 mmol/L EDTA, and 0.01% SDS to remove nonspecific binding. The sections were mounted with Vectashield [mounting medium with 4',6'-diamidino-2-phenylindole (DAPI), Vector Laboratories, Burlingame, CA, USA]. DNA was stained with DAPI to visualize all cells.

### Detection of crypt abscess-associated-bacteria

Bacteria were visualized with an epifluorescence microscope Leica DMRB using Cy3- and DAPI-specific filters at 100 ×, 400 ×, and 1000 × magnification and images were captured with Leica DFC 300 FX camera and FW 4000 software (Leica microsystemes SAS, Rueil-Malmaison, France). The entire mucosal surface (crypts and crypts abscess) of each colonic biopsy section was examined for the presence of bacteria. Pure cultured bacteria belonging to each group were hybridized as positive and negative controls for the FISH procedure.

### Statistical analysis

We first performed a "patient analysis" considering that a patient had colonized crypts or crypt abscesses if at least one of his crypts or crypt abscesses was colonized



**Figure 1** Colonized crypt (A, B) and colonized crypt abscess (C, D). A, C: 4',6'-diamidino-2-phenylindole (DAPI); B, D: Hybridization with the Eubacteria probe.

by bacteria. As colonic biopsies harbored a different number of crypts or crypt abscesses, we also normalized the data by calculating in each patient group the ratio: total number of colonized crypts or crypt abscesses/total number of crypts or crypt abscesses. The  $\chi^2$  test was performed for comparison of qualitative variables.

## RESULTS

The FISH technique allowed detection of crypts and crypt abscesses colonized by bacteria as shown in Figure 1. Crypts colonized by bacteria (general probe targeting Eubacteria) were observed in 42.9% and 3.6% of ASLC and IBD patients (only UC patients), respectively ( $P = 0.019$ , Figure 2). No colonized crypt was observed in biopsies from CD patients. Crypt abscesses colonized by bacteria were observed in 2 patients with ASLC (28.6% of total ASLC patients) and in no biopsy from the 28 IBD patients ( $P = 0.035$ , Figure 2). Neither colonized crypts nor crypt abscesses were observed in colonic biopsies from control patients.

### Global and normalized analysis

We observed 2317, 2499 and 2405 crypts in UC, CD and ASLC groups, respectively. None were colonized by bacteria (general probe for Eubacteria) in CD patients

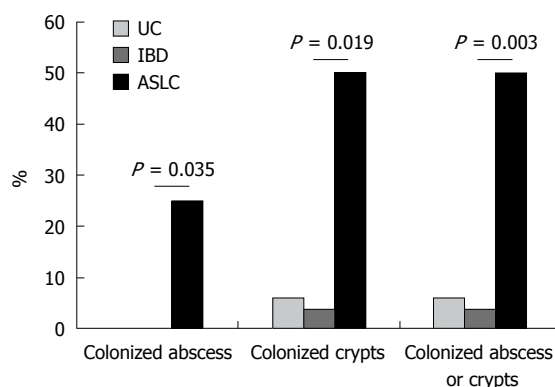
whereas 2 (0.09%) and 46 (1.91%) were colonized in UC and ASLC patients, respectively (Figure 3). We observed 121, 76 and 100 crypt abscesses in UC, CD and ASLC patients, respectively. Among these, 6 were colonized by bacteria in ASLC patients (6.0%) and none in UC and CD patients (Figure 3).

### Group/species-specific probe analysis

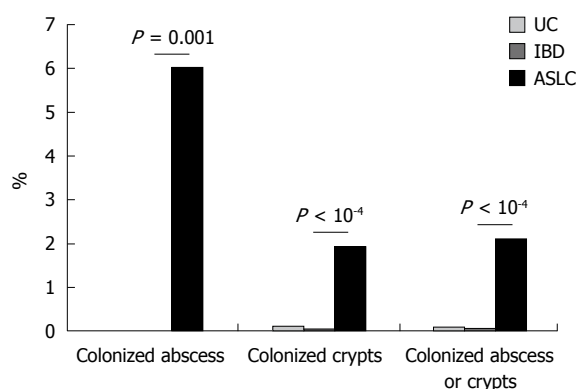
In order to determine what kind of bacteria was present in colonized crypts and crypt abscesses, we performed FISH analysis using 6 group- or species-specific probes on the sample previously identified to harbor colonized crypts or crypt abscesses. No fluorescent signal was detected in any of the crypt abscesses analyzed with the 6 specific probes. No bacteria-colonizing crypts were recognized by the *F. prausnitzii* or the *Lactobacillus-Enterococcus* probes. The 2 colonized crypts in UC patients contained *Enterobacteria* (detected by the *gamma Proteobacteria* probe) whereas 39.1%, 8.7%, 4.3% and 2.2% of the colonized crypts in ASLC patients contained bacteria from the *Bacteroides* (*Bacteroides-Prevotella* probe), the *Clostridium coccoides*, the *Enterobacteria* and the *Bifidobacteria* groups, respectively.

## DISCUSSION

This study shows for the first time that crypt abscesses



**Figure 2** Proportion of patients with at least one colonized crypt abscess, one colonized crypt or at least one crypt abscess or one crypt colonized by bacteria. UC: Ulcerative colitis; IBD: Inflammatory bowel disease; ASLC: Acute self-limited colitis.



**Figure 3** Proportion of crypt abscesses and crypts harboring bacterial colonization.

from IBD patients do not contain bacteria (aseptic abscess); colonic crypts of these patients are less often colonized than those from ASLC patients. These results do not support the hypothesis of the role of localized dysbiosis in the pathogenesis of crypt abscesses.

Dysbiosis is involved in the perpetuation of inflammation in IBD but it is presently not known whether it occurs early in the disease process and whether a localized dysbiosis in a specific ecological niche could trigger early inflammatory events. No previous study searched for bacteria inside crypt abscess in IBD or in other conditions. Swidsinski *et al.*<sup>[22]</sup> also looked at colonic crypts and found bacteria inside the crypts more frequently in IBD patients than in control subjects without inflammation or infection. In the current study, we did not find any colonized crypts in healthy subjects, and colonized crypts in IBD patients were infrequent. The differences between these 2 studies could arise from the population studied but also from the method used for the fixation of the colon biopsy. Indeed Swidsinski *et al.*<sup>[22]</sup> used a nonaqueous Carnoy solution which focuses at preserving the mucus layer on the surface epithelium while we used 4% buffered formalin. Nevertheless, in our hands, nonaqueous Carnoy solution and formalin gave similar results (unpublished data).

One can hypothesize that the results may be different

in a cohort of newly diagnosed patients without any antiinflammatory or immunomodulatory therapy. It is not possible to address this question. Nevertheless, the fact that 53.6% of the IBD patients in our cohort received no treatment or only 5-aminosalicylic acid, and that in 36% the IBD diagnosis was performed in the previous year suggested that the results would be similar in naive patients.

In ASLC, pathogenic bacteria induce an acute and transient breakdown of the intestinal barrier. On the other hand, many pathogenic bacteria involved in ASLC developed a strategy to escape the host immune system<sup>[23,24]</sup>. Alteration of the intestinal barrier could lead to deep penetration of gut microbiota bacteria (and also pathogenic bacteria) into the crypts where they could proliferate. In the current study, bacteria found in crypts from ASLC patients belonged to various phylogenetic bacterial groups of the normal gut microbiota, suggesting a non-specific intestinal barrier breakdown in this pathology, allowing the penetration of any bacteria in the crypts.

Our results do not support the hypothesis that crypt abscesses in IBD are the result of persistent localized dysbiosis with a focused reaction against high numbers of living bacteria specifically colonizing the crypts. On the other hand, the absence of entire bacteria in crypt abscesses does not rule out the implication of bacteria in their onset as one may hypothesize that bacteria could stimulate the genesis of crypt abscesses, with recruitment of polymorphonuclear leukocytes which would then destroy them but also contribute to chronic epithelial lesions. The dysbiosis in IBD seems to affect the surface of the whole mucosa (diffuse mucosal dysbiosis) and more studies should be performed to understand its specificity and the ways to influence it.

## COMMENTS

### Background

Ulcerative colitis (UC) and Crohn's disease (CD) are the 2 most common types of inflammatory bowel disease (IBD). Although their pathophysiology is still unknown, the gut microbiota is considered to play a crucial role. The microbiota close to the mucosa differs from the luminal microbiota. We and others described a luminal and mucosal dysbiosis in IBD. Nevertheless, no difference in the dominant microbiota was observed between inflamed and non-inflamed mucosa in CD and UC.

### Research frontiers

It would be of great importance to establish the early events leading to IBD onset or to perpetuation of inflammation. Crypt abscesses are early lesions observed in IBD, particularly in UC, but they can also occur in acute self limited colitis. The aim of this study was to search for a localized dysbiosis in crypt abscesses from UC, CD and acute self-limited colitis patients.

### Innovations and breakthroughs

The results do not support the hypothesis that crypt abscesses in IBD are the result of localized dysbiosis arising from persistence of living bacteria colonizing the crypts.

### Terminology

Dysbiosis: Breakdown in the balance between putative species of "protective" vs "harmful" intestinal bacteria.

### Peer review

This is an interesting and original study, which attempts to characterize dysbiosis associated with cryptitis and crypt abscesses in patients with early IBD. The results suggest that these lesions are in fact sterile.



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## Large endoscopic mucosal resection for colorectal tumors exceeding 4 cm

Philippe Ah Soune, Charles Ménard, Ezzedine Salah, Ariadne Desjeux, Jean-Charles Grimaud, Marc Barthet

Philippe Ah Soune, Charles Ménard, Ezzedine Salah, Ariadne Desjeux, Jean-Charles Grimaud, Marc Barthet, Department of Gastroenterology, Hôpital Nord, Chemin des Bourrelly, 13915 Marseille cedex 20, France

Author contributions: Ah Soune P wrote the paper; Ménard C collected the data; Salah E and Desjeux A participated in writing the introduction and discussion; Grimaud JC revised the paper; Barthet M was the leading author and was involved in preparing the whole manuscript.

Correspondence to: Marc Barthet, MD, Professor, Department of Gastroenterology, Hôpital Nord, Chemin des Bourrelly, 13915 Marseille cedex 20, France. [marc.barthet@ap-hm.fr](mailto:marc.barthet@ap-hm.fr)

Telephone: +33-491-968737 Fax: +33-491-961311

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### Abstract

**AIM:** To evaluate the feasibility and the outcome of endoscopic mucosal resection (EMR) for large colorectal tumors exceeding 4 cm (LCRT) undergoing piecemeal resection.

**METHODS:** From January 2005 to April 2008, 146 digestive tumors larger than 2 cm were removed with the EMR technique in our department. Of these, 34 tumors were larger than 4 cm and piecemeal resection was carried out on 26 colorectal tumors. The mean age of the patients was 71 years. The mean follow-up duration was 12 mo.

**RESULTS:** LCRTs were located in the rectum, left colon, transverse colon and right colon in 58%, 15%, 4% and 23% of cases, respectively. All were sessile tumors larger than 4 cm with a mean size of 4.9 cm (4-10 cm). According to the Paris classification, 34% of the tumors were type I s, 58% type II a, 4% type II b and 4% type II c. Pathological examination showed tubulovillous adenoma in 31%, tubulo-villous adenoma in 27%, villous adenoma in 42%, high-grade dysplasia in

38%, *in situ* carcinoma in 19% of the cases and mucosal carcinoma (m2) in 8% of the cases. The two cases (7.7%) of procedural bleeding that occurred were managed endoscopically and one small perforation was treated with clips. During follow-up, recurrence of the tumor occurred in three patients (12%), three of whom received endoscopic treatment.

**CONCLUSION:** EMR for tumors larger than 4 cm is a safe and effective procedure that could compete with endoscopic submucosal dissection, despite providing incomplete histological assessment.

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**Key words:** Endoscopic mucosal resection; Perforation; Colorectal carcinoma; Large polyps

**Peer reviewers:** Chris JJ Mulder, Professor, Department of Gastroenterology, VU University Medical Center, PO Box 7057, 1007 MB Amsterdam, The Netherlands; Walter E Longo, Professor, Department of Surgery, Yale University School of Medicine, 205 Cedar Street, New Haven, CT 06510, United States

Ah Soune P, Ménard C, Salah E, Desjeux A, Grimaud JC, Barthet M. Large endoscopic mucosal resection for colorectal tumors exceeding 4 cm. *World J Gastroenterol* 2010; 16(5): 588-595 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i5/588.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i5.588>

### INTRODUCTION

Endoscopic mucosal resection (EMR) allows complete and curative removal of the affected mucosa by excising through the middle or the deeper part of the submucosa following isotonic saline injection<sup>[1-3]</sup>. Thanks to a very simple technique, the endoscopic resection of superficial colorectal adenomas and tumors has been made

possible in selected patients with little or no ganglion risk. The therapeutic management of polyps larger than 3 cm, however, often relies on surgery. The frequency of degeneration is indeed increased, and piecemeal resection is carried out in 76% of cases<sup>[4]</sup>. Laparoscopic surgery has reduced the length of hospitalization, but a substantial morbidity rate of 20% and a mortality rate of 1% persist<sup>[5]</sup>. Endoscopic submucosal dissection (ESD) is a technique currently undergoing evaluation. This technique offers the advantage of a monobloc resection, enabling the analysis of the lateral margins and leading to a reduction of the risk of recurrence. However, this technique's learning curve is steep, and the morbidity associated with it is higher than that of mucosectomy<sup>[6]</sup>.

This study aimed to evaluate the feasibility and the outcome of endoscopic piecemeal mucosal resection of sessile polyps of the colorectum exceeding 4 cm in size.

## MATERIALS AND METHODS

### Patients

Between 2005 and 2007, our department carried out 146 mucosal resections larger than 2 cm throughout the gastrointestinal tract. Of these, 34 involved parietal tumors larger than 4 cm, and 26 tumors were colorectal. These large colorectal tumors exceeding 4 cm were treated by piecemeal resection in 25 of the 26 patients. Among these, 44% were female and the mean age was 71 years (46-89 years).

### Methods

The lesions were first identified by visual inspection (Figure 1A). Computed virtual chromoendoscopy with Fujinon Intelligent Color Enhancement<sup>®</sup> was used for estimating surface extension. The size of the lesions was measured and compared using open biopsy forceps. Deep tumor extension was estimated using three parameters. Polyps were classified according to the Paris morphological classification<sup>[7]</sup>. All lesions were analyzed according to Kudo's pit pattern classification<sup>[8]</sup>. Finally, endosonography was performed on all rectal lesions. Only lesions classified as T1N0 were treated endoscopically.

Certain lesions were excluded from the study and treated by surgery if they met one or more of the following criteria: (1) Type 0-III, Paris classification; (2) Type V, Kudo's classification; (3) Endosonographic lesions > T1 or N+; and (4) Absence of lifting after submucosal injection (negative lifting sign).

A video colonoscope type Fuji 450<sup>®</sup> was used for total colonoscopy, carried out under general anesthesia. Patients received endocarditis prophylaxis according to the recommendations of the *Société Française d'Endoscopie Digestive*<sup>[9]</sup>. Mucosal resection was performed using the conventional method<sup>[10]</sup>. In order to create a detachment of the pathological mucosa, a solution containing physiological serum (0.9%) and epinephrine (dilution of 1:10 000) was injected into the submucosa by means of a 25 gauge needle inserted into the operating channel

(Figure 1B). A diathermic snare was tightened around the elevated lesion, grasping also adjacent healthy mucosal tissue (Figure 1C). Two snare types were used: large snare of 6 cm × 3 cm (Jumbo Wilson Cook<sup>®</sup>, USA) and needle snare of 5.5 cm × 2.5 cm (Wilson Cook<sup>®</sup>, USA). Removal was performed with pure cutting current in the caecum and with endocut current in the remaining colon (Figure 1D). Considering that the injection could have masked the polyp's limits, the healthy peripheral mucosa was previously marked with a bistoury in order to ensure complete polyp removal. In case of doubt as to the normality of the resection margins, a complementary treatment was performed by applying a coagulation current to the mucosectomy margins, either *via* the tip of the snare or *via* argon plasma, at a flow rate of 1 L/min and a power of 60 W (Erbe<sup>®</sup>, Tuebingen, Germany). One-time resection was attempted in all patients (Figure 1E). If the mucosectomy was considered incomplete due to the persistence of residual adenomatous tissue, a further mucosectomy was planned.

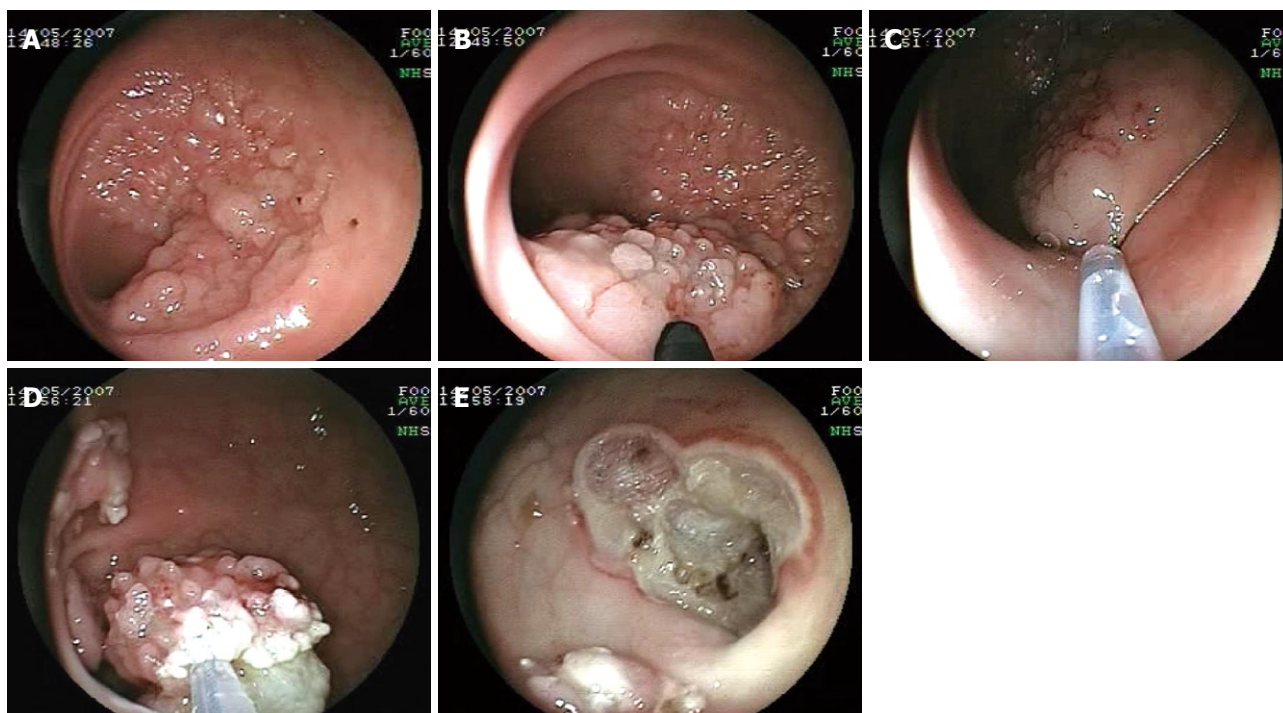
Each fragment of removed tissue was spread out and pinned on a 5 mm thick cork surface in order to avoid retraction of the polyp's base and permit improved histological assessment of the deep margins. Deep resection limits were marked with China ink and the tissue pieces were then fixed in 4% formol saline for 3 d. All fragments were routinely processed for paraffin embedding. Slices (5 µm) were stained with hematein-eosin-safran. Modified Vienna classification was used for the histological assessment according to the severity of dysplasia<sup>[11]</sup>. Lateral margins could not be evaluated due to the impossibility of displacing fragments in relation to each other. Histological assessment was carried out using the Japanese classification with deep submucosal invasion limited to 1000 µm<sup>[8]</sup>.

Following mucosal resection, colonoscopic surveillance included a first early endoscopic control examination between 3 and 6 mo, and further examinations at 1 and 3 years (Figure 2). Resection was considered complete if no residual adenomatous tissue was noted following completion of mucosectomy. Local recurrence was defined as the presence (on biopsy) of adenomatous tissue in areas of previously treated mucosa at endoscopic control between 3 and 6 mo after therapy. In the case of a recurrence of small-sized tumors, an additional mucosectomy was carried out. If a lesion could not be lifted due to fibrous tissue development in the submucosa, argon plasma coagulation (APC) following multiple biopsies was performed. When the biopsy revealed recurrent cancer, the patient underwent surgery.

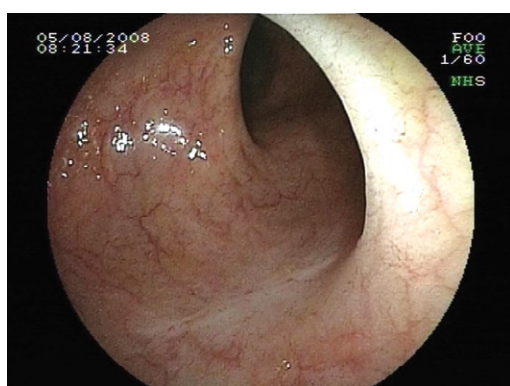
## RESULTS

### Lesion description and evaluation

The 26 sessile polyps were removed by piecemeal resection. The mean polyp size was 4.9 cm (4-10 cm). Lesion analysis according to the Paris classification revealed the presence of type 0-I s in 9 cases (34%), type



**Figure 1** Endoscopic piecemeal mucosal resection image. A: Large lateral spreading rectal tumor (adenoma with high grade dysplasia); B: Submucosal lifting of the tumor using saline with adrenaline 1/10 000; C: Capture of the lifted part of the tumor with a needle snare; D: Piecemeal resection of the rectal tumor; E: Final aspect at the end of the resection (procedure duration 70 min).



**Figure 2** One year follow-up: a scar is visible without any sign of recurrence.

0-II a in 15 cases (58%) and type 0-II b in one lesion (4%), with some mixed forms. The only lesion classified as type 0-II c (4%) showed low-grade dysplasia and two lesions classified as type 0-II a were intramucosal carcinomas. There was no ulcerated lesion of type 0-III. The most frequently identified pit pattern was pit pattern type IV in 24 cases (92%) followed by type III. The predominant location was the rectum in 15 cases (58%), and all lesions were classified as T1N0 (Table 1).

### Technique

A one-time resection was performed in 23 cases (88.5%). For two lesions located in the rectum (7.7%), three sessions were necessary, and for one lesion located at the right colonic angle (3.8%), two sessions were needed. The lesions' mean size was 6.7 cm (4-10 cm). Coagulation of

**Table 1** Lesion characteristics

Lesion description and evaluation	n (%)
Total number of lesions	26
Locations	
Rectum	15 (58)
Left colon	4 (15)
Transverse colon	1 (4)
Right colon	6 (23)
Size (mm)	
Mean	49
40-50	18 (65)
50-60	2 (8)
> 60	6 (27)
Paris classification	
0-I s	9 (34)
0-II a	15 (58)
0-II b	1 (4)
0-II c	1 (4)
Kudo's classification	
III (I+s)	2 (8)
IV	24 (92)
Anatomopathology	
Tubulous	8 (31)
Villous	11 (42)
Tubulovillous	7 (27)
Low-grade dysplasia	9 (35)
High-grade dysplasia	10 (38)
In situ carcinoma	5 (19)
Intramucosal carcinoma (type m2)	2 (8)
Submucosal-invading carcinoma	0

margins was performed as complementary treatment in 38.4% of cases. The mean duration of the intervention was 65 min (25-137 min).



Table 2 Lesion characteristics and treatment of recurrence

Case	Location	Size (cm)	Kudo's	Aspect	Histological type	Relapse interval time (mo)	Number of endoscopic treatments	Final treatment
1	Rectum	10	IV	0- I s	DHG	3	2	Argon plasma
2	Right colon	6	IV	0- II b	DBG	6	1	Argon plasma
3	Rectum	4	IV	0- II a + 0- II c	DBG	3	3	Surgery

### Pathology

All polyps were adenomatous. The most common histological type was villous adenoma in 42% (11 cases), followed by tubular adenoma in 31% (8 cases) and finally tubulovillous adenoma with a mixed villous and tubular architecture in 27% (7 cases). In total, 35% of lesions exhibited low-grade dysplasia (9 cases), 38% high-grade dysplasia (10 cases), 19% a carcinoma *in situ* (5 cases). Two polyps (8%) were reported as intramucosal carcinoma (m2), neither of which reached the submucosa. Contrary to the lateral margins (0%), deep margin analysis was possible on all samples. Resection was performed within a safe deep margin in 100% of cases.

### LEMR efficacy and follow-up

In total, 24 patients underwent endoscopic surveillance, while one patient refused to undergo control endoscopy. The mean duration of follow-up was 12 mo (3-37 mo). Three recurrences were detected (12.5%) (Table 2). All three patients had initially received a complementary treatment using APC. The median delay until recurrence diagnosis was 3 mo (3-9 mo). Two patients underwent endoscopic treatment and the remaining patient received surgical therapy. The first patient received an additional mucosectomy, followed by APC due to the absence of lifting after the injection. For the second patient, a single session of tissue destruction *via* coagulation was the immediate resort. These two endoscopically-treated recurrences were of the same histological type as the initial polyp. Following treatment of recurrences, a further endoscopic control was scheduled for 3 mo later. The third patient, who was followed-up for Crohn's disease which was surgically treated by ileorectal anastomosis, underwent rectal stump resection. After three successive endoscopic treatments, one using mucosectomy and the two others using APC, the low-grade dysplastic lesion detected at biopsy could not be eradicated. Histological examination of the surgically removed specimen revealed an infiltrating adenocarcinoma that was classified as pT2N0 with a colloid mucosal component of less than 50%. In total, endoscopic resection was effective in 96% of cases.

### Complications

Intra-operative bleeding occurred in two patients but in neither patient was there any need for blood transfusion or a drop in hemoglobin levels exceeding 2 g/dL. Late postoperative bleeding, at day 6, was observed in two patients (7.7%) but no case of severe bleeding as defined in the standards of practice of the American Society for

Gastrointestinal Endoscopy was noted<sup>[12]</sup>. Endoscopic injection therapy with epinephrine (diluted 1:10 000) was performed along with the deployment of hemostatic clips (Resolution® Boston Scientific, USA).

Perioperative perforation was diagnosed in one case (4%) upon visualisation of the peritoneal fat at the bottom of the resection zone. The abdominal radiography without preparation did not reveal a pneumoperitoneum because of the perforation's sub-peritoneal rectal location. The patient underwent conservative treatment with endoscopic closure of the perforation using clips, in addition to the administration of antibiotics and 48 h fasting.

Scar stenosis was observed in another patient who presented a 10 cm circumferential lesion within the upper rectum. The interval between the first colonoscopy and the diagnosis of the stenosis was 6 mo. The patient was asymptomatic and the endoscopic CRE balloon dilatation was able to traverse the stenosis.

Ten days after his examination, one patient experienced septic shock following *Staphylococcus epidermis* endocarditis. The patient had a bicuspid aortic valve, and underwent prosthetic valve replacement surgery. The microbiology did not support a bacterial translocation from the digestive tract.

## DISCUSSION

This study aimed to demonstrate the efficacy of piecemeal mucosectomy in the management of large non-polypoid colorectal lesions. The success rate was 96% despite a mean lesion size of 4.9 cm. These results are comparable to those reported in the literature, which show success rates of endoscopic treatment for large polyps (those exceeding 2 cm) ranging from 83% to 100% (Table 3). Kaltenbach *et al*<sup>[13]</sup> recently described a series of 125 mucosectomy-treated colorectal plane lesions whose mean size was 16.7 mm. Of the 62 patients followed up for a mean period of  $4.5 \pm 1.4$  years, none had developed colorectal cancer or metastasis, resulting in a success rate of 100%<sup>[13]</sup>.

The Japanese Society for Cancer of the Colon and Rectum's current criteria for curative endoscopic resection are: a submucosal invasion of less than 1000  $\mu$ m, moderate or well-differentiated lesion characteristics and the absence of vascular invasion<sup>[14,15]</sup>. Depending on the study reported, the presence of invasive lesions, which cross the muscular mucosa, varies from 0% to 44%, notably for sessile elevated tumors<sup>[1,16]</sup>. In our series, there was no submucosal invasion and only 2 cases (8%) with invasion of the mucosa (m2). This was



**Table 3** Resection of large sessile polyps *via* fragmentation: recurrence rates and efficacy

Author	Yr	n	Sessile lesions (%)	Size (cm)	Piecemeal (%)	Surveillance	Relapse (%)	Endoscopic efficacy (%)
Bedogni <i>et al</i> <sup>[48]</sup>	1986	66	75	≥ 3	100	3-85 mo	11	-
Walsh <i>et al</i> <sup>[49]</sup>	1992	65	100	≥ 3	100	2.8 mo	28	88
Binmoeller <i>et al</i> <sup>[50]</sup>	1996	170	73	3-6	100	> 6 mo	16	-
Kanamori <i>et al</i> <sup>[4]</sup>	1996	32	100	3-8.5	76	2.4 yr	0	100
Iishi <i>et al</i> <sup>[27]</sup>	2000	56	100	2-5	75	34 mo	39	83
Higaki <i>et al</i> <sup>[29]</sup>	2003	24	100	2-6	79	24 mo	22.2	91.3
Doniec <i>et al</i> <sup>[21]</sup>	2003	184	76	3-≥ 13	100	40 mo	3	-
Seitz <i>et al</i> <sup>[28]</sup>	2003	288	78	> 3	100	36 mo	17	-
Hurlstone <i>et al</i> <sup>[23]</sup>	2004	58	100	> 1.5	62	24 mo	17	96
Bories <i>et al</i> <sup>[51]</sup>	2006	50	100	1-> 5	55.8	17.3 mo	15	-
Katsinelos <i>et al</i> <sup>[52]</sup>	2006	59	100	≥ 2	61	> 1 yr	3.4	96.78
Arebi <i>et al</i> <sup>[31]</sup>	2007	161	100	2-> 8	100	9.2 mo	-	95.4

accounted for by the parietal extension assessment, which was based on a series of complementary examinations. On one hand, the Paris endoscopic classification allows the exclusion of ulcerated lesions<sup>[8]</sup>. On the other hand, magnified chromoendoscopy has been shown to permit a 97% diagnostic precision of deep submucosal invasion (69/71)<sup>[17]</sup>. However, another study found a specificity of only 50%<sup>[18]</sup>. Echoendoscopy with high-frequency mini probes was not used, as muscular mucosa is only visualized in 50% to 65% of cases<sup>[19,20]</sup>. Mini probes of 7.5 MHz were employed, permitting the detection of T1 cancers without separating mucosal and sub-mucosal lesions. The evaluation of extension depth is an essential step prior to resection in order to select the patients for whom endoscopic treatment will be curative with minimal lymphatic risk.

The late bleeding rate reported by Doniec *et al*<sup>[21]</sup> was only 2%. The higher late bleeding rate observed in our study (7.7%) can be explained by the 49 mm mean extension of the resection. Lesion size has been identified as an independent predictive factor of postpolypectomy delayed bleeding<sup>[22]</sup>. However, the bleeding complications of the current study and those aforementioned were effectively treated with the deployment of hemoclips and the injection of an adrenaline serum, without resorting to surgery. Perforation rates reported after the resection of polyps exceeding 2 cm have been shown to vary from 0% to 1.2%<sup>[23,24]</sup>. If the perforations are of small size (< 1 cm) and are properly diagnosed during endoscopy, they may be treated with endoscopic clip closure in 70% of cases<sup>[25]</sup>. Scar stenosis of the colon is a complication following endoscopic mucosectomy which has not yet been explained. Two factors appear to play a role: the initial lesion's extended circumferential shape, and its location in the upper rectum (where the diameter is smallest).

Local recurrence was detected in 13.4% of cases, despite APC of the area of the defect which has proved effective in preventing recurrences following piecemeal mucosal resection<sup>[26]</sup>. Previous studies have reported recurrence rates ranging from 0% to 39% for lesions larger than 2 cm<sup>[22,27]</sup>. In the largest series, that of Seitz *et al*<sup>[28]</sup>, 288 patients with large (> 3 cm) sessile and pediculated

polyps were treated using piecemeal resection, and the recurrence rate was 17% for a mean follow-up duration of 36 mo. The mean delay for diagnosis of recurrence was 5 mo. We followed the recommendations of Higaki concerning postpolypectomy surveillance<sup>[29]</sup>. Most recurrences occur within the first 6 mo. Our own experience and published data encourage close endoscopic control between 3 and 6 mo, and at 1 year, in cases of piecemeal resection. Patients who have undergone resection of a large dysplastic or cancerous polyp require tighter endoscopic control<sup>[30]</sup>. Several risk factors for recurrence have been identified; piecemeal resection, lesion size<sup>[31]</sup>, granulous appearance of the lesion, and a lesion location at the bottom of the rectum attaining the pectineal line<sup>[23,32]</sup>. Among these factors, endoscopic piecemeal procedure appears to play the most important role. In a series by Ishihara *et al*<sup>[33]</sup>, 78 esophageal squamous cell carcinomas of ≥ 2 cm were treated by endoscopy. The strongest predictor for recurrence was the number of resected fragments; 0/34 in patients with monobloc resection (0%), 4/24 in those with 2 and 4 fragments (15%) and 8/17 in those with more than 5 fragments (47%)<sup>[33]</sup>. In addition to the risk of local recurrence, piecemeal resection may prevent a good assessment of the lateral margins, with the consequence of incomplete interpretation and the risk of leaving small carcinomas around the main lesion. In a recent retrospective study by Kim *et al*<sup>[34]</sup>, 44 patients who had initially received imperfect EMR for colorectal cancers were subsequently treated by either EMR or ESD. Gross incomplete resection and deep margin positivity were found to be risk factors of residual cancer. No residual cancer cells were found after supplementary surgery in all cases with positive lateral resection margins. The authors proposed the hypothesis that the application of an electrocoagulation current can destroy residual cancer cells at the resection margins<sup>[34]</sup>. Hurlstone *et al*<sup>[35]</sup> have shown that recurrence rate could be decreased from 8.7% to 0.5% with the analysis of the pit patterns of the resection margins.

If the recurrence is of small size, removal by hot forceps is a therapeutic option. If the recurrence exceeds 5 mm, a further mucosectomy session may be carried out so as to obtain a complete histological analysis of

residual tumor tissue. In our series, such a thorough histological analysis was not possible in two out of three cases. The thermal lesion provoked by the coagulation current leads to a desmoplastic reaction in the submucosa, preventing another mucosal lifting (non lifting sign)<sup>[36]</sup>. Destruction of tumors by APC is an alternative therapeutic option. In a series of 68 recurrent colorectal adenomas following EMRP, APC treatment was effective in 90%<sup>[37]</sup>. We believe that in all cases without malignant recurrence, the best choice is to try to perform a new EMR, in order to assess the margins. However, the submucosal fibrotic scar due to thermal injury may prevent the lifting sign leading to a management by local destruction, mainly with APC treatment. Surgery remains essential in the presence of a carcinomatous recurrence whose extension depth cannot be properly assessed, and which therefore has a risk of metastatic spread<sup>[38]</sup>. In our series, one patient followed-up for Crohn's disease with ileorectal anastomosis underwent surgery. The operative specimen revealed an infiltrating cancer classified as pT2N0. A recent meta-analysis has shown that the positive predictive value of a plane low-grade dysplastic lesion, associated with chronic inflammatory bowel disease, correlated with the presence of colorectal cancer in 22% of cases<sup>[39]</sup>.

The technique of large EMR for rectal tumor might be challenged by two new techniques: ESD and transanal endoscopic microsurgery (TEMS). ESD permits single-fragment resection with safe margins in 70% of cases, as has been shown in a series of 200 colorectal lesions ranging in size from 20 to 150 mm<sup>[40]</sup>. There are only a few follow-up studies, but the risk of recurrence appears low. In a study by Fujishiro *et al*<sup>[41]</sup>, 35 rectal polyps were treated using the ESD method. Monobloc resection with safe margins was possible in 62.9% of cases, with a local recurrence rate of 2.8% for a mean follow-up duration of 36 mo. Initially developed for the treatment of superficial gastric tumors, the application of this technique in the colon exposes the patient to a higher risk of complications. For instance, ESD is associated with a higher risk of perforation, ranging from 5% to 14%, due to the thinness of the colonic wall<sup>[40,25]</sup>. The intra-operative bleeding risk is also significantly higher for ESD as compared to EMR (22.6% for ESD *vs* 7.6% for EMR,  $P < 0.01$ )<sup>[42]</sup>. The "inject and cut" mucosectomy has the advantage of being a simple technique that may be performed by the endoscopy practitioner with routinely-used equipment. In contrast, ESD practitioners require extensive training, operating initially on animals because the endoscopist's experience inversely correlates with the risk of perforation and monobloc resection rate<sup>[43,44]</sup>. The mean time required for the procedure is significantly higher and has been compared in a study by Oka *et al*<sup>[42]</sup>. The mean time for the resection of gastric lesions smaller than 1 cm was  $3.5 \pm 1.3$  min for EMR and  $58.5 \pm 28.7$  min for ESD ( $P < 0.01$ ). If the lesion is larger than 2 cm, the mean time required for the procedure is  $17.2 \pm 9.3$  min for EMR and  $123.8 \pm 101.4$  min for

ESD ( $P < 0.01$ )<sup>[42]</sup>. ESD is at present a non-standardized technique. It has the advantage of allowing monobloc resection of gastrointestinal tumors, which permits the assessment of lateral margins. However, the technique is difficult to learn, its complication rates are high and the time required for the procedure is long. To date, this technique cannot be proposed as a standard technique for the management of colorectal adenomas<sup>[44]</sup>.

For TEMS, major complications have been reported. In a series of 288 patients, 9% of patients experienced complications; severe digestive hemorrhages, perforations, and temporary anal incontinence. Non-surgical complications accounted for the low but relevant mortality rate of 0.3%. Duration of hospitalization was approximately 2 d<sup>[45]</sup>. Hurlstone *et al*<sup>[46]</sup> have evaluated EMR as an alternative to TEMS. In 62 patients, the success rate of endoscopic treatment was 98%, with a recurrence rate of 8%. Patients were discharged the same day as the procedure in 97% of cases<sup>[46]</sup>. Another major drawback of TEMS is economic, since it necessitates a financial investment of 50 000 Euros for the purchase of the equipment<sup>[47]</sup>.

In conclusion, endoscopic piecemeal resection is a safe and effective procedure. In spite of higher recurrence rates than with the ESD method, its technical simplicity, low complication rates, and lower costs are major advantages. Moreover, endoscopic management of recurrence has proven effective, with no risk of submucosal invasion in patients without chronic inflammatory colitis. A large lesion size ( $> 4$  cm) is not a limiting factor for an endoscopic approach, provided that the risk of submucosal invasion has been carefully evaluated. However, further studies are needed to specify the time intervals required for endoscopic surveillance and to develop new techniques that would allow the histological assessment of lateral margins.

## COMMENTS

### Background

Large colorectal adenomatous sessile polyps are neoplastic premalignant lesions, which carry a high risk of transformation into invasive cancer. The management of these lesions is usually surgical by segmental colonic resection. The treatment of these lesions by piecemeal mucosectomy represents a minimally-invasive procedure and the resection is effective for selected lesions.

### Research frontiers

Endoscopic mucosectomy is a technique approved for lesions less than 2 cm because it allows *en bloc* resection. The endoscopic resection of lesions more than 4 cm involves a systematic piecemeal resection, which could result in the absence of anatomopathologic analysis of the margins and may expose the patient to the risk of recurrence.

### Innovations and breakthroughs

This is an original study taking place in a single centre that describes the effectiveness and the feasibility of this technique as less risky and simpler than that of endoscopic submucosal dissection (ESD), for the management of polyps of large size (giant polyps). This study confirms that the size of the polyps should not be a contraindication for endoscopic treatment. However, the risk of deep parietal infiltration must be evaluated in a precise way because this controls the effectiveness of the treatment as well as the risk of metastatic dissemination to lymph nodes which could preclude the endoscopic resection. It is associated with a low risk of complications and recurrence.

## Applications

The results of this study show that mucosectomy for large colorectal polyps is effective whatever the size of the lesions if the estimation of lymph node infiltration is rigorously evaluated by the degree of infiltration into the layers of the wall of the colon. However, new studies should be realized in order to confirm these data and to determine the degree and regularity of follow-up monitoring.

## Terminology

Mucosectomy is an endoscopic technique of resection of a lesion that requires the separation of the submucosa using normal saline solution. ESD is a new method of resection, allowing the dissection of the lesion within the thickness of the submucosa or the interface between the submucosa and the muscularis propria. FICE: Technique of virtual chromoendoscopy by processing the image in a narrow spectral band.

## Peer review

Great paper, well written, appeals to our audience, favor acceptance.

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## Phlebotomy improves histology in chronic hepatitis C males with mild iron overload

Massimo Sartori, Silvano Andorno, Angelo Rossini, Renzo Boldorini, Cristina Bozzola, Stefania Carmagnola, Mario Del Piano, Emanuele Albano

Massimo Sartori, Silvano Andorno, Stefania Carmagnola, Mario Del Piano, Gastroenterology Unit, Azienda Ospedaliero-Universitaria Maggiore della Carità, Corso Mazzini, 18, 28100 Novara, Italy

Angelo Rossini, Hepatology Unit, A.O. Spedali Civili, 25100 Brescia, Italy

Renzo Boldorini, Cristina Bozzola, Emanuele Albano, Department of Medical Sciences University of East Piedmont "Amedeo Avogadro", 28100 Novara, Italy

**Author contributions:** Sartori M collected clinical data, and contributed to the study design and the writing of the manuscript; Andorno S performed statistical analysis; Rossini A collected clinical data and contributed to the study design; Carmagnola S collected clinical data; Boldorini R and Bozzola C performed histological analysis; Del Piano M contributed to the study design; Albano E supervised the work.

**Correspondence to:** Dr. Massimo Sartori, Gastroenterology Unit, Azienda Ospedaliero-Universitaria Maggiore della Carità, Corso Mazzini, 18, 28100 Novara, Italy. [sartori@fauser.edu](mailto:sartori@fauser.edu)

Telephone: +39-321-3733206 Fax: +39-321-3733345

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### Abstract

**AIM:** To investigate the usefulness of mild iron depletion and the factors predictive for histological improvement following phlebotomy in Caucasians with chronic hepatitis C (CHC).

**METHODS:** We investigated 28 CHC Caucasians with persistently elevated serum aminotransferase levels and non responders to, or unsuitable for, antiviral therapy who underwent mild iron depletion (ferritin  $\leq 70$  ng/mL) by long-term phlebotomy. Histological improvement, as defined by at least one point reduction in the staging score or, in case of unchanged stage, as at least two points reduction in the grading score (Knodell), was evaluated in two subsequent liver biopsies (before and at the end of phlebotomy,  $48 \pm 16$  mo apart).

**RESULTS:** Phlebotomy showed an excellent safety profile. Histological improvement occurred in 12/28 phlebotomized patients. Only males responded to phlebotomy. At univariate logistic analysis alcohol intake ( $P = 0.034$ ), high histological grading ( $P = 0.01$ ) and high hepatic iron concentration (HIC) ( $P = 0.04$ ) before treatment were associated with histological improvement. Multivariate logistic analysis showed that in males high HIC was the only predictor of histological improvement following phlebotomy (OR = 1.41, 95% CI: 1.03-1.94,  $P = 0.031$ ). Accordingly, 12 out of 17 (70%) patients with HIC  $\geq 20$   $\mu\text{mol/g}$  showed histological improvements at the second biopsy.

**CONCLUSION:** Male CHC Caucasian non-responders to antiviral therapy with low-grade iron overload can benefit from mild iron depletion by long-term phlebotomy.

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**Key words:** Hepatic iron; Hepatitis C; Oxidative stress; Aminotransferases

**Peer reviewers:** Chifumi Sato, Professor, Department of Analytical Health Science, Tokyo Medical and Dental University, Graduate School of Health Sciences, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan; Heitor Rosa, MD, PhD, Professor, Department of Gastroenterology and Hepatology, Federal University School of Medicine, Rua 126 n.21, Goiania - GO 74093-080, Brazil

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### INTRODUCTION

Hepatitis C virus (HCV) infection affects 130 million

people worldwide causing chronic hepatic inflammation that in about 20% of the patients evolves to liver cirrhosis and/or hepatocellular carcinoma<sup>[1]</sup>. The progression of chronic hepatitis C (CHC) is not linear and is greatly influenced by a variety of factors including male gender, age at the infection, obesity, co-infection with hepatitis B or HIV viruses, alcohol consumption and iron overload<sup>[2,3]</sup>. The diffusion of anti-HCV therapies based on the association of pegylated interferon and ribavirin will probably modify the natural history of the disease, as more than half of the treated patients achieve a sustained eradication of the virus<sup>[4,5]</sup>. Nonetheless, for those patients who do not respond to anti-viral therapy, have contraindications to the use of interferon or cannot afford the therapy costs there is an urgent need of other therapeutic options capable of preventing the progression of the disease.

Mild hepatic iron accumulation is frequent in CHC patients and is associated with higher aminotransferase levels and more severe fibrosis<sup>[6]</sup>. Moreover, experimental studies have shown that dietary supplementation with iron worsens hepatic damage in HCV-infected chimpanzees and promotes the onset of hepatocarcinomas in transgenic mice expressing the HCV core protein<sup>[7,8]</sup>.

Growing evidence indicates that HCV proteins stimulate the formation of reactive oxygen species within hepatocytes and oxidative stress associated with HCV infection is recognized as an important factor in the pathogenesis of hepatic damage in CHC<sup>[9]</sup>. In this context the capacity of iron to exacerbate oxidative damage suggests that iron accumulation might amplify oxidative stress-mediated events leading to both hepatocellular damage as well as to the pro-fibrogenic activation of hepatic stellate cells<sup>[10]</sup>.

In recent years several groups have reported on the efficacy of iron reduction therapies based on phlebotomy in combination or not with an iron-restricted diet in reducing aminotransferase levels in CHC patients non-responders to the interferon treatment<sup>[11-16]</sup>. However, in many of these studies the iron depletion (serum ferritin below 20 ng/mL) was rather severe<sup>[13-16]</sup>. We have previously reported that menstruating women experienced a milder CHC than men of the same age in relation to the lower hepatic iron concentration (HIC) due to blood loss<sup>[17]</sup>. As patient compliance to long-term iron depletion is an important factor in the success of this therapy we have investigated whether, and in which conditions, mild iron depletion (serum ferritin  $\leq$  70 ng/mL) might be effective in inducing histological improvement in a group of Caucasian patients with CHC not responding to anti-viral therapy or having contraindications to the use of interferon, who were histologically re-evaluated within 2-5 years from the first liver biopsy and the start of iron depletion therapy.

## MATERIALS AND METHODS

### Patient selection

In this study we investigated retrospectively 28 patients (21

men, 7 women; mean age  $58 \pm 7.6$  years) with CHC, who were either non responders ( $n = 13$ ) or had contraindications to antiviral therapy ( $n = 15$ ) who gave informed consent to be treated between January 2001 and December 2006 exclusively with iron reduction by phlebotomy in two Italian centres (Ospedale Maggiore della Carità in Novara and Spedali Civili in Brescia) and underwent liver biopsies before phlebotomy and 2 to 5 years after the achievement of iron depletion. Criteria of exclusion were alcohol intake  $> 80$  g/d, active drug addiction, HBs-Ag and/or HIV-Ab positivity, personal or familial history of haemochromatosis, haemoglobin  $< 13$  g/dL for men and  $< 11$  g/dL for women, interferon based therapy or immunosuppressive therapy during the last 6 mo, and refusal to undergo liver biopsies before and at the end of phlebotomy. The mean daily alcohol intake was evaluated by trained medical staff according to a standardized questionnaire, presented as part of a survey on life habits. Genetic tests for haemochromatosis mutations were not performed. However, HIC, iron index and the amount of iron removed during phlebotomy excluded phenotypic haemochromatosis.

All the patients underwent an initial period of bi-monthly or monthly phlebotomy of 250 mL of blood, until serum ferritin levels of 35 ng/mL or less were reached. Thereafter, maintenance phlebotomies were performed every 1 to 3 mo during a minimum 2 years period to maintain a serum ferritin level at 70 ng/mL or less. In the case of haemoglobin values  $< 11$  g/dL phlebotomy was postponed until haemoglobin levels exceeded the cut-off. For all patients histology was re-evaluated by a second liver biopsy after a period of  $48 \pm 16$  mo, as part of a study approved by a local ethical committee to evaluate on an individual basis whether iron depletion by phlebotomy was effective and worth being continued.

None of the patients were treated with antiviral drugs during the study. Eight patients were on oral treatment for chronic unrelated diseases (hypertension, diabetes, vascular diseases) and another diabetic patient was treated with insulin. All were allowed to continue with these treatments throughout the study period.

The demographic, clinical and laboratory characteristics of the patients included in the present study are summarized in Table 1. The study was planned according to the guide-lines of the local ethical committee in conformity the 1975 Declaration of Helsinki.

### Assessment of liver histology

Liver biopsies were performed using a modified Menghini procedure. Five micron-thick sections were stained with haematoxylin/eosin, Masson's trichrome and periodic acid-Schiff after diastase digestion as well as with the Gomori's method for reticulin and the Perls's method for iron staining. Only specimens with a minimum length of 15 mm and at least 6 portal tracts were considered adequate for histological assessment. Liver biopsy specimens were scored in a blind fashion (including the date - pre or post phlebotomy) by two expert pathologists (RB,

**Table 1** Epidemiological, clinical and laboratory characteristics at the time of their first liver biopsy of 28 patients with chronic hepatitis C included in the study

Variable <sup>1</sup>		Range	SD
Age (yr)	58	43-73	7.6
Gender (males/females)	21/7		
Body mass index (kg/m <sup>2</sup> )	24.5	19-38.4	3.9
HCV genotype (1/non-1)	17/11		
Drug addiction (yes/no)	2/26		
Previous transfusion (yes/no)	11/17		
Alcohol abuse (yes/no) <sup>2</sup>	3/25		
Alcohol intake (g/d)	15	0-60	18.35
Treatment before recruitment (interferon based therapy/no treatment)	13/15		
Aspartate aminotransferase (U/L, 0-40)	108	46-275	58
Alanine aminotransferase (U/L, 0-40)	130	52-318	57
$\gamma$ glutamyl transpeptidase (U/L, 0-50)	86	14-242	59
Hemoglobin (g/100 mL, 13.7-17)	14.7	11-18	1.6
Serum iron ( $\mu$ mol/L, 11-32)	24.3	73-240	6.9
Transferrin saturation (%; 20-50)	43	23-69	11
Serum ferritin (ng/mL, 5-365)	387	64-1051	238
Hepatic iron grade (Searle's score 0/1/2/3)	6/17/15/0	0-2	
Hepatic iron concentration ( $\mu$ mol/g dry tissue, < 25)	23.3	7.5-40.5	7
Histological grade (Knodel's score, median)	5	2-9	
Histological stage (Knodel's score 0/1/3/4)	0/14/9/5	1-4	
Advanced fibrosis or cirrhosis (Knodel's stage score 3-4) (yes/no)	14/14		
Steatosis grade (0/1/2/3) <sup>3</sup>	9/12/5/0	0-2	

<sup>1</sup>Values are means, counts or medians when indicated; <sup>2</sup>Yes:  $\geq 50$  g/d for men or 20 g/d for women; <sup>3</sup>Data were available for 26 out of 28 patients. Steatosis grade: 0, no steatosis; 1,  $\leq 33\%$  of hepatocytes; 2, 34%-66%; 3,  $> 66\%$ .

CB) unaware of the clinical data. In the case of discordant opinions, the two examiners analyzed the discrepancies to reach a consensus.

The original histology activity index proposed by Knodel<sup>[18]</sup> was used for grading inflammation/necrosis and for staging fibrosis. By this scoring system inflammation/necrosis score ranges from 0 to 18 (0-10 periportal  $\pm$  bridging necrosis, 0-4 intralobular degeneration and focal necrosis, 0-4 portal inflammation), while fibrosis stage includes only four stages: 0 (no fibrosis), 1 (fibrous portal expansion), 3 (bridging fibrosis), 4 (cirrhosis). Steatosis was scored semi-quantitatively as: 0, no steatosis, 1, steatosis  $\leq 33\%$  of hepatocytes, 2, steatosis in 34%-66% of hepatocytes, 3,  $> 66\%$  of hepatocytes. Intrahepatic iron deposition was evaluated in specimens stained by the Perls' method and evaluated using the Searle's semi quantitative score<sup>[19]</sup>. As in previous studies, patients were defined to have a histological improvement when they showed at least one point reduction in the staging score or, in the case of an unchanged staging score, at least a two point reduction in the grading score<sup>[20,21]</sup>.

### Measurement of hepatic iron content

HIC was measured by atomic absorption spectroscopy on

a portion of each liver biopsy, as previously reported<sup>[22]</sup>, and the values were expressed as  $\mu$ mol/g dry weight. Hepatic iron index was calculated to rule out phenotypic hemochromatosis.

### Statistical analysis

Stata Statistical Software (release 9.0 College Station, Stata Corporation, TX, USA) was used in all the statistical analyses. Each variable predictive or associated with presence of histological response was analysed with univariate logistic regression. The variables selected by the univariate analysis were entered into logistic regression models with the use of a forward stepwise elimination algorithm (terms with  $P > 0.05$  were eligible for removal). The paired Student's  $t$ -test was used to compare the continuous variables among groups and the Wilcoxon matched-pairs signed-rank test was used to compare discrete variables among groups.

## RESULTS

The long-term effects of mild iron depletion by phlebotomy were investigated in 28 CHC patients who were either non responders or had contraindications to antiviral therapy and received intensive phlebotomy followed by maintenance phlebotomy to maintain serum ferritin levels below 70 ng/mL. All the patients underwent a histological re-evaluation by a second biopsy within 2-5 years from the achievement of iron depletion. Two patients (a man aged 65 years and a woman aged 68 years who were treated with phlebotomy, respectively for 31 and 29 mo) were excluded from the study, because the second liver biopsy specimens did not meet the established criteria - minimum length and number of portal tracts - and, therefore, were not considered adequate. Both showed a decrease of HIC and aminotransferase levels  $> 50\%$ .

The patients showed excellent compliance to phlebotomy. In particular, none of the patients reported increased fatigue, reduced exercise capacity, cheilosis, koilonychia or other side effects related to mild iron depletion.

The changes in clinical and laboratory values before and at the end of therapy are shown in Table 2. As expected hemoglobin, transferrin saturation, serum iron, serum ferritin, hepatic iron grade and the HIC were significantly decreased in phlebotomized patients (Table 2). At the time of the second biopsy aspartate aminotransferase (AST) ( $P < 0.0001$ ), alanine aminotransferase (ALT) ( $P = 0.001$ ) and  $\gamma$  glutamyltranspeptidase (GGT) ( $P = 0.004$ ) levels were significantly lowered after phlebotomy (Table 2). Overall, ALT levels decreased in all 28 phlebotomized patients, while AST and GGT levels were lowered, in respectively, 27/28 (96%) and 22/28 (78%) of the patients (Table 3). Moreover, 7 out of the 28 phlebotomized patients (25%) achieved persistently normal aminotransferase levels during the iron-depleting treatment. No statistical correlation was found between the decreases of HIC and those of ALT levels ( $r = 0.22$ ,

**Table 2** Clinical and laboratory changes between the two liver biopsies of 28 patients with chronic hepatitis C included in the study

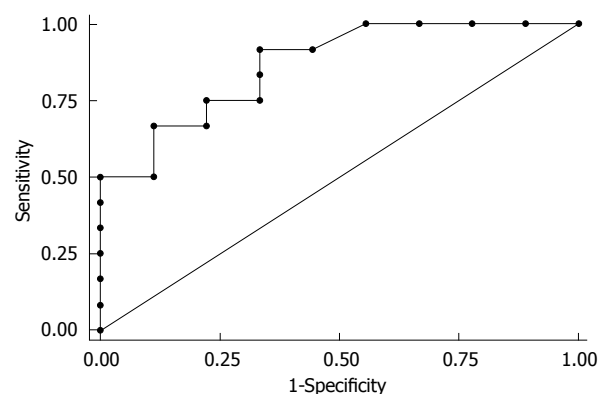
Variable	First biopsy <sup>1</sup>	Second biopsy <sup>1</sup>	P <sup>2</sup>
Body mass index (kg/m <sup>2</sup> )	24.5 ± 3.9	24.5 ± 3.8	0.93
Alcohol intake (g/d)	15 ± 18	7.5 ± 9.7	0.034
HCV-RNA (× 10 <sup>6</sup> copies/mL) <sup>3</sup>	4.54 ± 3.6	3.96 ± 3.5	0.11
Aspartate aminotransferase (U/L, 0-40)	108 ± 58	64 ± 49	0.0001
Alanine aminotransferase (U/L, 0-40)	130 ± 57	70 ± 43	< 0.0001
γ glutamyl transpeptidase (U/L, 0-50)	86 ± 59	56 ± 56	0.004
Hemoglobin (g/100 mL, 13.7-17)	14.7 ± 1.6	14 ± 1.4	0.0001
Serum iron (μmol/L, 11-32)	24.3 ± 6.9	16.4 ± 6	< 0.0001
Transferrin saturation (% , 20-50)	43 ± 11	24.5 ± 10	< 0.0001
Serum ferritin (ng/mL, 5-365)	387 ± 238	46 ± 51	< 0.0001
Hepatic iron grade (Searle's score 0/1/2/3)	6/17/5/0	27/1/0/0	< 0.0001
Hepatic iron concentration (μmol/g dry tissue, < 25)	23.3 ± 7	8 ± 5.6	< 0.0001
Histological grade (Knodell's score, median)	5	4.5	0.43
Histological stage (Knodell's score 0/1/3/4)	0/14/9/5	0/13/9/6	0.61
Steatosis grade (0/1/2/3) <sup>4</sup>	9/12/5/0	11/11/3/1	0.41

<sup>1</sup>Values are mean ± SD, counts or medians when indicated; <sup>2</sup>Paired Student's *t*-test or Wilcoxon matched-pairs signed-ranks test; <sup>3</sup>Data were available for 20 out of 28 patients; <sup>4</sup>Data were available for 26 out of 28 patients. Steatosis grade: 0, no steatosis; 1, ≤ 33% of hepatocytes; 2, 34%-66%; 3, > 66%.

$P = 0.26$ ). Mean alcohol intake slightly decreased during phlebotomy ( $15 \pm 18$  g/d *vs*  $7.5 \pm 9.7$  g/d,  $P = 0.034$ ) as a possible consequence of counseling. Serum HCV RNA levels were determined before and at the end of phlebotomy in 20/28 patients and no significant difference was observed ( $4.5 \pm 3.6 \times 10^6$  copies/mL *vs*  $4 \pm 3.5 \times 10^6$  copies/mL,  $P = 0.11$ ).

Overall, the scores for the necroinflammatory grading and the fibrosis staging were not modified by iron depletion (Table 2). However, by evaluating the histological improvement, as defined by at least one point reduction in the individual staging score or, in the case of unchanged staging, as at least two points reduction in the grading score, we observed that after phlebotomy 5 patients showed a reduction in both the grading and the staging scores and 7 had a two or more points reduction in the grading score with unchanged staging score (Table 3). Therefore, the frequency of histological improvements in the subjects undergoing mild iron depletion was 43% (12 out of 28).

Histological improvement was not significantly associated with the reduction of alcohol intake (reduction > 50%,  $P = 0.11$ ), or body mass index ( $P = 0.12$ ), nor was predicted by the magnitude of the reduction of the aminotransferase levels that occurred during iron depletion (reduction > 50%,  $P = 0.4$ ) (Table 3). However, the pa-



**Figure 1** Receiver operating characteristics (ROC) curve relative to the capacity of hepatic iron concentration (HIC) values to discriminate histological improvement in males with chronic hepatitis C patients undergoing long-term iron depletion by phlebotomy. HIC ≥ 20 μmol/g was the best cut-off value to discriminate CHC patients with histological improvement. Area under the curve = 0.866 (95% CI: 0.71-1.00).

tients with histological improvement had HIC significantly higher than in those without ( $26.55 \pm 6.37$  *vs*  $20.81 \pm 6.47$ ,  $P = 0.028$ ) (Table 3).

To determine the factors responsible for the histological improvement induced by long-term mild iron depletion, univariate logistic analysis of the putative predictors was performed (Table 4). We observed that only the males responded to treatment. Moreover, alcohol intake ( $P = 0.034$ ), high histological grading ( $P = 0.013$ ) and high HIC before phlebotomy ( $P = 0.043$ ) were significantly associated with the histological improvement (Table 4). At multivariate logistic analysis, high HIC was the only independent predictor (OR = 1.41, 95% CI: 1.03-1.94,  $P = 0.031$ ) of the histological improvement (Table 4). Furthermore, receiver operating characteristics (ROC) curve showed that HIC ≥ 20 μmol/g was a suitable cut-off value (sensitivity 91.7%; specificity 66.7%) to discriminate CHC patients with histological improvement (Figure 1). Indeed, 12 out of 17 men (70%) with HIC ≥ 20 μmol/g dry tissue at the time of the first biopsy achieved histological improvement following the mild iron depletion.

## DISCUSSION

Iron accumulation is a common feature of liver biopsies from CHC patients and HCV infected patients with increased hepatic iron content show increased serum aminotransferase and liver fibrosis as compared to patients without iron accumulation<sup>[6]</sup>. Moreover, elevated iron indices have been associated with a poor response to interferon therapy<sup>[6]</sup>.

At present, the mechanisms responsible for hepatic iron accumulation during HCV infection are not fully understood. It has been proposed that necroinflammation sustained by the virus together with genetic factors that modify iron trafficking might contribute to the alterations in iron homeostasis<sup>[6,23,24]</sup>. Recent studies in transgenic mice expressing the HCV polyprotein have shown



**Table 3** Individual values of serum aminotransferase, daily alcohol intake, hepatic iron concentration, histological staging and grading scores before (columns a) and at the end of long-term phlebotomy (columns b) in patients with or without evidence of histological improvement

No.	Age (yr)	Gender	Alcohol		BMI		AST		ALT		HIC		Staging		Grading		HI
			a	b	a	b	a	b	a	b	a	b	a	b	a	b	
1	53	M	30	20	21.9	21.6	81	57	138	76	7.9	5.1	3	1	4	3	Yes
2	56	M	0	0	20.8	20.4	74	57	114	54	22.4	2.8	3	1	7	2	Yes
3	50	M	40	20	25.5	25.9	183	50	158	36	19.8	4.9	4	4	8	3	Yes
4	59	M	0	0	22.9	23.2	275	194	121	76	24.9	12.1	4	4	5	7	No
5	68	M	0	0	24.0	24.3	104	50	116	78	21.7	5.2	1	1	4	4	No
6	58	F	0	0	21.9	21.6	72	67	117	77	29.3	8.6	3	3	5	5	No
7	67	F	0	0	22.7	22.7	109	88	148	93	17.3	8.8	1	3	3	4	No
8	65	F	0	0	19.6	20.2	155	215	220	195	23.3	7.3	3	4	6	7	No
9	53	M	0	0	22.8	22.4	139	113	159	100	7.5	3.7	4	4	4	6	No
10	51	M	30	30	23.9	23.9	50	32	92	53	40.5	24.8	1	1	4	2	Yes
11	64	F	10	10	38.4	37.7	129	144	147	138	32.5	12.7	4	4	5	7	No
12	52	M	30	10	25.3	24.9	64	54	138	74	30.4	22.8	3	1	4	2	Yes
13	54	M	20	20	24.2	24.2	52	42	94	62	15.8	9.2	1	1	2	3	No
14	65	M	0	0	30.5	30.8	47	34	73	53	22.8	11.8	4	4	6	4	No
15	73	M	10	10	20.9	20.9	140	94	221	120	30.1	3.1	3	1	6	4	Yes
16	42	M	50	30	24.2	23.5	96	22	64	19	28.5	13.1	3	3	9	2	Yes
17	56	M	0	0	23.3	23.6	95	42	103	57	23.3	3.6	3	1	9	9	Yes
18	55	M	0	0	24.5	23.5	103	36	113	40	28.7	3.6	1	1	5	3	Yes
19	43	M	30	10	29.4	28.1	256	61	318	110	21.5	5.4	1	1	9	3	Yes
20	57	M	20	0	27.7	27.7	124	50	135	90	14.3	3.6	1	3	5	7	No
21	50	F	0	0	20.7	21.1	46	37	52	49	25.1	5.4	1	3	5	5	No
22	59	M	60	20	26.9	28.1	96	20	114	22	32.2	1.8	1	1	7	5	Yes
23	62	M	20	0	24.6	24.6	90	30	142	32	19.7	5.4	1	1	3	7	No
24	67	M	50	10	24.8	24.8	52	48	59	54	16.1	5.4	1	3	5	9	No
25	66	M	10	10	23.7	23.3	94	51	128	70	23.3	12.5	3	3	9	7	Yes
26	54	F	0	0	19.3	21.2	64	21	83	23	16.1	3.6	1	1	5	7	No
27	64	F	0	0	29.7	29.7	68	25	85	29	28.7	7.2	1	3	5	7	No
28	51	M	10	10	22.7	22.7	160	50	201	76	17.9	10.7	1	3	4	3	No

Alcohol: Alcohol intake (g/d); BMI: Body mass index; AST: Aspartate aminotransferase (U/L); ALT: Alanine aminotransferase (U/L); HIC: Hepatic iron concentration ( $\mu\text{mol/g}$  dry tissue); Staging: Knodell's score; Grading: Knodell's score; HI: Histological improvement defined by at least one point reduction in the histological staging score or, in case of unchanged stage, as at least two points reduction in the histological grading score.

that iron accumulation is associated with a low mRNA expression of hepcidin<sup>[25]</sup>, a 25 amino acid peptide synthesized in the liver that regulates iron efflux from the enterocytes as well as the recycling of the metal by the macrophages<sup>[26]</sup>. Low liver hepcidin mRNA levels have also been detected in patients with CHC<sup>[27]</sup>, suggesting the possibility that iron accumulation in CHC might be due to an inappropriate hepcidin response. According to the importance of iron as a worsening factor in the progression of CHC several studies have demonstrated that lowering of hepatic iron by phlebotomy alone or in combination with an iron-restricted diet reduces serum aminotransferase levels in CHC patients who failed a sustained virological response by interferon-based treatment<sup>[11-14]</sup>. Although phlebotomy does not interfere with HCV RNA levels<sup>[12]</sup>, the efficacy of long term iron reduction in preventing the evolution of CHC has been substantiated by a recent histological follow-up study<sup>[16]</sup>. In their report Yano *et al*<sup>[16]</sup> showed that a 5 years iron-reducing treatment significantly lowered necroinflammatory grading and prevented the increase in fibrosis staging when they compared two sequential liver biopsies from non-responder CHC patients to interferon receiving or not phlebotomy. In addition, Kato *et al*<sup>[28]</sup> have reported that phlebotomy in combination with an iron-restricted

diet significantly reduces the development of hepatocarcinomas associated with CHC. Although none of these studies reported side effects of iron lowering (serum ferritin below 20 ng/mL), the long term compliance to such a condition might be problematic in many patients. Therefore, in the present study, we investigated whether, and in which condition, mild iron depletion (serum ferritin below 70 ng/mL) might be effective in inducing histological improvement. Considering that iron depletion might be a simple and inexpensive treatment for those patients not responding to antiviral therapy or having contraindications to the use of interferon it would be important to verify whether similar results might be obtained by a milder iron depletion.

The present study shows that attaining serum ferritin levels  $\leq 70$  ng/mL is effective in decreasing aminotransferase release in Caucasian CHC patients and that 25% of the phlebotomized subjects achieve persistently normal aminotransferase levels during the iron-depleting treatment. Unlike Yano's report<sup>[16]</sup> we did not observe significant changes in the median values of the necroinflammatory score among patients receiving phlebotomy. This likely reflects the lower degree of iron reduction achieved in our patients. However, the different scoring system used for evaluating necroinflammation, as well

**Table 4** Univariate and multivariate logistic regression analysis of the putative predictors of the histological improvement in 28 patients with chronic hepatitis C treated with phlebotomy

	Odds ratio (95% CI)	P
Univariate analysis		
Age (yr)	0.90 (0.80-1.01)	0.085
Male gender	Females are all non responders	
Drug addict (yes/non) <sup>1</sup>		
Previous transfusion (yes/non)	1.19 (0.26-5.5)	0.82
Alcohol abuse (yes/non) <sup>2</sup>	3 (0.24-38)	0.39
Alcohol intake (g/d) <sup>3</sup>	1.06 (1.004-1.11)	0.034
Body mass index (kg/m <sup>2</sup> )	0.96 (0.78-1.17)	0.69
HCV Genotype (1 vs non-1)	0.84 (0.18-3.95)	0.82
Previous antiviral treatment (yes/non)	0.71 (0.16-2.23)	0.66
Days between the two liver biopsies	1 (0.99-1.001)	0.91
Aspartate aminotransferase (U/L)	1.001 (0.99-1.01)	0.79
Alanine aminotransferase (U/L)	1.006 (0.99-1.02)	0.37
$\gamma$ glutamyl transpeptidase (U/L)	1.002 (0.99-1.01)	0.71
Hemoglobin (g/100 mL)	1.32 (0.79-2.21)	0.28
Serum iron ( $\mu$ mol/L)	0.99 (0.98-1.02)	0.9
Transferrin saturation (%)	0.97 (0.90-1.04)	0.42
Serum ferritin (ng/mL)	1.004 (0.99-1.008)	0.06
Histological grade (Knodel's score) <sup>3</sup>	2.4 (1.2-4.95)	0.013
Histological stage (Knodel's score)	1.32 (0.71-2.45)	0.12
Steatosis grade (0/1/2/3) <sup>4</sup>	0.7 (0.23-2.1)	0.53
Hepatic iron concentration ( $\mu$ mol/g dry tissue) <sup>3</sup>	1.16 (1-1.34)	0.043
Hepatic iron grading (Searle's score)	2.64 (0.69-10.1)	0.15
Multivariate analysis on male patients		
Hepatic iron concentration ( $\mu$ mol/g dry tissue)	1.41 (1.03-1.94)	0.031

<sup>1</sup>Only two subjects were drug addicts, so the univariate analysis was not performed; <sup>2</sup>Yes:  $\geq 50$  g/d for men or 20 g/d for women; <sup>3</sup>These variables were selected to enter in the multivariate model; <sup>4</sup>Data were available for 26 out 28 patients. Steatosis grade: 0, no steatosis; 1,  $\leq 33\%$  of hepatocytes; 2, 34%-66%; 3,  $> 66\%$ .

as the bias caused by the worsening of the grading in our phlebotomized patients who do not respond to iron depletion, might also account for the discrepancy. Nonetheless by taking into account the individual responses to treatment we have observed that mild iron depletion causes histological improvement in male subjects. In these patients multivariate logistic analysis reveals that increased HIC is the only independent predictor of the histological response to mild iron depletion. By using ROC curve we also found that a HIC  $\geq 20$   $\mu$ mol/g can be used as cut-off to discriminate the CHC patients with high probability of histological improvement while undergoing mild long-term iron depletion.

So far the mechanisms responsible for the beneficial effects of phlebotomy in preventing the evolution of CHC have not been characterized in detail. Recent observations suggest that HCV proteins stimulate the formation of reactive oxygen species within the hepatocytes<sup>[9]</sup>. Thus, iron accumulation probably amplifies oxidative stress-mediated events, worsening both hepatocellular damage and fibrogenic processes. Indeed, mild iron accumulation in the liver of transgenic mice over-expressing HCV polyprotein enhances lipid peroxidation<sup>[8]</sup>. Sup-

porting this view, a recent paper by Fujita *et al.*<sup>[29]</sup> shows that the immunohistochemical detection of 8-hydroxy-2' deoxyguanosine (8-OHdG), a well recognized marker of oxidative stress, is strongly increased in liver biopsies from CHC patients. In these patients hepatic 8-OHdG levels are significantly associated with the hepatic iron content as well as with the necroinflammation indices. Interestingly, iron depletion by phlebotomy greatly reduces the liver content of 8-OHdG in patients with CHC<sup>[30]</sup>, suggesting that the efficacy of phlebotomy in preventing the progression of CHC might rely on the reduction of oxidative damage promoted by the combination of HCV infection and iron accumulation. In this context, our observation that a high HIC is an independent predictor of the histological response to mild iron depletion further points to the possible importance of iron-mediated oxidative damage in the progression of CHC.

In conclusion, the study shows that mild iron depletion might be effective in improving liver histology in Caucasian CHC males with low-grade iron overload non-responders or with contraindications to established antiviral treatments. Prospective randomized studies should further address the long term efficacy of mild iron depletion in CHC patients with signs of iron accumulation.

## COMMENTS

### Background

Iron reduction therapies based on phlebotomy reduce aminotransferase levels and can improve liver histology in patients with chronic hepatitis C (CHC).

### Research frontiers

Although the management of patients with CHC has been improved by antiviral therapies, there is still an urgent need for alternative therapeutic options capable of preventing the progression of the disease in those patients who do not respond to antiviral drugs, have contraindications to the use of interferon and/or ribavirin or cannot afford the cost of therapy. Therefore, it is important to know which patients can benefit from iron reduction therapies.

### Innovations and breakthroughs

The present study shows that male CHC Caucasian patients, with low-grade iron overload (hepatic iron concentration  $\geq 20$   $\mu$ mol/g), non-responders or with contraindications to antiviral therapy can particularly benefit from mild iron depletion by long-term phlebotomy.

### Applications

This retrospective study demonstrates that mild iron depletion is a safe and effective strategy for preventing the histological progression of the disease in males with CHC and low-grade iron overload. Further randomized prospective studies are warranted.

### Terminology

In this study mild iron depletion means the achievement of a ferritin level below 35 ng/mL after intensive phlebotomy and a ferritin level below 70 ng/mL during long-term maintenance phlebotomy.

### Peer review

This work is very interesting in demonstrating that serial phlebotomy still remains a current method of treatment despite its medieval origins. The results of this study are interesting to clinicians.

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## Metabolic investigations in patients with hepatitis B and C

Abdul Aziz Mastoi, Bikha Ram Devrajani, Syed Zulfiquar Ali Shah, Qasim Rohopoto, Sikander Ali Memon, Marya Baloch, Ghulam Ali Qureshi, Waqas Sami

Abdul Aziz Mastoi, Sikander Ali Memon, Marya Baloch, M.A.Kazi Institute of Chemistry, University of Sind, Jamshoro 75000, Pakistan

Bikha Ram Devrajani, Syed Zulfiquar Ali Shah, Qasim Rohopoto, Department of Medicine, Liaquat University of Medical and Health Sciences, Jamshoro 75000, Pakistan

Ghulam Ali Qureshi, Medical Research Centre, Liaquat University of Medical and Health Sciences, Jamshoro 75000, Pakistan

Waqas Sami, Department of Biochemistry, University of Health Sciences, Lahore 54600, Pakistan

**Author contributions:** Devrajani BR, Mastoi AA and Rohopoto Q designed the study, collected, analyzed and interpreted the data, drafted and revised the paper; Qureshi GA guided and assisted during the study period regarding the specific protocol; Memon SA, Sami W and Baloch M participated in collection of data; Shah SZA participated in drafting, data analysis and interpretation.

**Correspondence to:** Dr. Bikha Ram Devrajani, Department of Medicine, Liaquat University of Medical and Health Sciences, Jamshoro 75000, Pakistan. [devrajani62@msn.com](mailto:devrajani62@msn.com)

Telephone: +92-22-2787999 Fax: +92-22-2729655

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### Abstract

**AIM:** To investigate the similarities and dissimilarities in patients with hepatitis B and hepatitis C, clinically and metabolically.

**METHODS:** Fifty patients with hepatitis B virus and hepatitis C virus infection were included in this study, along with fifty healthy controls for comparison purposes. Intravenous blood (10 mL) samples from patients and healthy subjects were collected and made to clot before serum was separated and immediately levels of the enzymes, alkaline phosphatase (ALK), creatinine phosphokinase (CPK), lactate dehydrogenase (LDH), serum glutamate oxaloacetate transaminase (s-GOT) and serum glutamate pyruvate transaminase (s-GPT) were determined by a kit method. For total con-

tent of each metal the serum samples were analyzed using atomic absorption spectrophotometry. Levels of cholesterol, triglycerides, urea, creatinine and uric acid were determined using a kit method on Microlab 300.

**RESULTS:** Serum magnesium and copper levels remained unchanged, whereas the concentration of zinc decreased and iron increased significantly in both groups of patients. Total antioxidant activity was significantly decreased in both hepatitis B and C. Among the enzymes analyzed, ALK, s-GPT, LDH and s-GOT were all significantly increased in both patients with hepatitis B and C whereas CPK was significantly decreased in patients with hepatitis B and remained unchanged in patients with hepatitis C.

**CONCLUSION:** The information accumulated by this study will help provide a better understanding of involved metabolic processes in order to design appropriate therapeutic approaches for treating these patients, so they can recover and lead normal lives.

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**Key words:** Hepatitis B virus; Hepatitis C virus; Anti-oxidant activity; Metal content; Enzyme activity

**Peer reviewer:** Shashi Bala, PhD, Post doctoral Associate, Department of Medicine, UMass Medical School, LRB 270L, 364 Plantation street, Worcester, MA 01605, United States

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### INTRODUCTION

The liver is one of the most important organs in energy



metabolism. Most plasma apolipoproteins, endogenous lipids and lipoproteins are synthesized in the liver. This depends on the integrity of liver cellular function, which ensures homeostasis of lipid and lipoprotein metabolism. Hepatitis B virus (HBV) infection, a major world health problem, is hyper-endemic in South-East Asia and sub-Saharan Africa. Being a major cause of morbidity and mortality, prophylaxis using the highly efficacious hepatitis B vaccine is recommended for those at risk<sup>[1]</sup>. The hepatitis C virus (HCV) is a linear, single-stranded RNA virus of the *Flaviviridae* family that was identified in 1989 and is recognized as the major causal agent of non-A, non-B hepatitis<sup>[2]</sup>. HCV is one of the leading causes of chronic liver disease worldwide, affecting 3% of the world's population. Diagnosis and treatment of HCV-related autoimmune features has become a clinical challenge in HCV-infected patients, in whom chronic liver disease associated with severe autoimmune features may contribute to a very poor prognosis<sup>[3,4]</sup>.

Enzymes are biocatalysts and catalyze relevant bio-reactions. Metals such as magnesium (Mg), copper (Cu), zinc (Zn) and iron (Fe) are the cofactors of enzymes. Enzymes are released into the blood by injured tissue along with the metals and each affected tissue can be identified by evaluation of the variation in enzyme activity and metals in blood serum. Metals are present in bones, tissues and body fluids and are built into key ingredients in enzymes and hormones. They also assist in every aspect of life from production of hormones, vitamins and energy, digestion, neurotransmission and muscle contraction to regulation of pH, metabolism, cholesterol and blood sugar. Our physical wellbeing is more dependent upon the minerals we take into our system than upon calories or vitamins or carbohydrates and proteins.

In the present study, the estimation of serum enzyme activity of alkaline phosphatase (ALK), glutamate pyruvate transaminase, (SGPT or ALT), lactate dehydrogenase (LDH) and serum glutamic oxaloacetic transaminase (SGOT or AST) along with levels of metals (Cu, Fe, Mg, and Zn) are evaluated in patients with hepatitis B and C to study similarities and dissimilarities in these hepatitis groups, with the aim of defining biochemical mechanisms to help in selecting or designing chemotherapy suitable for these patients.

## MATERIALS AND METHODS

Third-generation micro-ELISA assays were used for detection of hepatitis B surface antigen, antibody to hepatitis B core and surface antibody, secretory form of hepatitis B envelop antigen (HBeAg), antibody to secretory form of HBeAg, and ELISA for antibody to HCV. Clinical and laboratory features are helpful, but liver biopsy is essential for definitive diagnosis. Once the diagnosis was confirmed, 50 patients in each group of HBV and HCV were included in this study. Fifty healthy controls were included for comparison purposes. The

**Table 1** Clinical data of patients with HBV and HCV (mean  $\pm$  SE)

	Controls	Hepatitis B	Hepatitis C
Age (yr)	40.5 $\pm$ 1.5	43.5 $\pm$ 1.8	42.8 $\pm$ 1.3
Sex (M/F)	35/15	33/17	36/14
Albumin (g/dL)	3.68 $\pm$ 0.083	3.20 $\pm$ 0.079 <sup>c</sup>	3.07 $\pm$ 0.090 <sup>c</sup>
Total protein (g/dL)	8.40 $\pm$ 1.23	6.84 $\pm$ 0.80 <sup>b</sup>	6.74 $\pm$ 0.82 <sup>c</sup>
Glucose (F) (mg/dL)	81.56 $\pm$ 1.47	77.94 $\pm$ 1.27 <sup>a</sup>	76.56 $\pm$ 1.43 <sup>a</sup>

<sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01, <sup>c</sup>*P* < 0.001 *vs* controls. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

**Table 2** Metabolic parameters measured in plasma of HBV/HCV patients and healthy controls (mean  $\pm$  SE, mg/dL)

	Controls	Hepatitis B	Hepatitis C
Urea	28.84 $\pm$ 0.89	24.28 $\pm$ 0.779 <sup>c</sup>	22.06 $\pm$ 0.832 <sup>c</sup>
Creatinine	0.73 $\pm$ 0.021	0.59 $\pm$ 0.015 <sup>a</sup>	0.57 $\pm$ 0.013 <sup>c</sup>
Uric acid	3.68 $\pm$ 0.069	5.13 $\pm$ 0.22 <sup>b</sup>	5.02 $\pm$ 0.169 <sup>c</sup>
Cholesterol	172.44 $\pm$ 3.03	127.56 $\pm$ 1.70 <sup>c</sup>	124.24 $\pm$ 1.77 <sup>c</sup>
HDL	30.42 $\pm$ 0.57	27.96 $\pm$ 0.36 <sup>b</sup>	27.44 $\pm$ 0.385 <sup>c</sup>
LDL	112.34 $\pm$ 2.98	97.88 $\pm$ 1.87 <sup>c</sup>	95.16 $\pm$ 1.81 <sup>c</sup>
Triglycerides	96.00 $\pm$ 4.33	95.30 $\pm$ 3.76 <sup>a</sup>	84.12 $\pm$ 1.51 <sup>b</sup>
Mg	19.76 $\pm$ 0.59	20.49 $\pm$ 0.70	20.94 $\pm$ 0.82
Fe	2.93 $\pm$ 0.14	3.14 $\pm$ 0.05 <sup>b</sup>	3.22 $\pm$ 0.07 <sup>b</sup>
Zn	4.16 $\pm$ 0.12	3.26 $\pm$ 0.11 <sup>c</sup>	3.38 $\pm$ 0.13 <sup>b</sup>
Cu	2.03 $\pm$ 0.05	2.08 $\pm$ 0.02 <sup>a</sup>	2.11 $\pm$ 0.03
Antioxidant activity	1.52 $\pm$ 0.05	0.37 $\pm$ 0.05 <sup>c</sup>	0.43 $\pm$ 0.05 <sup>c</sup>

<sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01, <sup>c</sup>*P* < 0.001 *vs* controls.

clinical data regarding patient and healthy control groups are shown in Table 1.

Intravenous blood (10 mL) samples from hepatitis B/C patients and healthy subjects were collected and made to clot before serum was separated by centrifuging at 5000 r/min for 20 min and immediately the enzymes ALK, creatinine phosphokinase (CPK), LDH, serum glutamate oxaloacetate transaminase (s-GOT) and serum glutamate pyruvate transaminase (s-GPT) were determined by a kit method. For total content of each metal the serum samples were analyzed using a Hitachi atomic absorption spectrophotometer (Tokyo, Japan). Cholesterol, triglycerides, urea, creatinine and uric acid were determined using a kit method on Microlab 300.

## Statistical analysis

All values are expressed as mean  $\pm$  SE. For comparison between the patients *vs* healthy controls, Student's *t* test, non-parametric Mann-Whitney test and SPSS 15 were used. A *P* value less than 0.05 was considered statistically significant.

## RESULTS

Table 2 shows plasma levels of urea, creatinine and uric acid in B and C hepatitis patients and healthy controls. Among the biochemical parameters associated with kidney function, the levels of urea and creatinine were significantly decreased in both HBV and HCV patients,

**Table 3** Enzyme activity in blood serum of patients with Hepatitis B/C and healthy controls

	Controls	Hepatitis B	Hepatitis C
ALK (IU/L)	115.64 ± 3.98	145.82 ± 8.47 <sup>b</sup>	120.34 ± 5.84
ALT or s-GPT (mU/mL)	22.34 ± 1.67	59.24 ± 3.28 <sup>c</sup>	89.60 ± 6.41 <sup>c</sup>
CPK (IU/L)	106.56 ± 6.52	86.10 ± 3.34 <sup>a</sup>	101.88 ± 3.99
LDH (U/L)	331.06 ± 9.13	353.72 ± 9.37 <sup>a</sup>	395.48 ± 11.16 <sup>c</sup>
AST or s-GOT (IU/L)	18.86 ± 0.71	40.88 ± 1.44 <sup>c</sup>	45.80 ± 1.95 <sup>c</sup>
ALT/AST ratio	1.13 ± 0.071	1.56 ± 0.083 <sup>c</sup>	1.94 ± 0.11 <sup>c</sup>

<sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01, <sup>c</sup>*P* < 0.001 *vs* controls.

however, uric acid was increased significantly in both patient groups. With regard to plasma cholesterol levels (total, HDL and LDL) and triglycerides in hepatitis groups (HBV and HCV) and control subjects; cholesterol level was decreased in both patient groups whereas triglyceride levels decreased significantly in HCV and remained unchanged in HBV group. Table 2 also shows the serum levels of Mg, Fe, Zn and Cu. There was no significant difference in Mg and Cu levels for both HBV and HCV groups. Serum levels of Fe increased significantly whereas Zn decreased significantly in both patient groups. Total antioxidant activity showed significant decreases in both groups of patients.

Table 3 shows activity of the enzymes ALK, ALT, AST, CPK and LDH. ALK was only increased significantly in HBV patients and remained unchanged in HCV patients. ALT increased significantly in both patient groups but markedly in HCV patients. CPK was decreased in HBV but remained unchanged in HCV patients. LDH increased significantly in both group of patients and markedly in HCV patients. AST increased in both groups of patients. The ratio between ALT and AST was increased significantly in both patient groups.

## DISCUSSION

Hepatitis C infection is associated with diabetes mellitus and insulin resistance and it is suggested that metabolic syndrome is common in patients with hepatitis C. Microalbuminuria is common in patients with diabetes and metabolic syndrome<sup>[5]</sup>.

A decade ago, various authors described the association of chronic HCV infection with a heterogeneous group of non-hepatic conditions, such as pulmonary fibrosis, cutaneous vasculitis, glomerulonephritis, Mooren's ulcer, porphyria cutanea tarda and lichen planus<sup>[6]</sup>. The drug regimens available for treating chronic HCV infection are monotherapy with interferon  $\alpha$  (IFN- $\alpha$ ) and combined therapy with IFN- $\alpha$  and ribavirin<sup>[7]</sup>. With progressive liver disease serum albumin levels fall, reflecting its decreased synthesis. We found a significant decrease in albumin levels in patients with both hepatitis B and C.

HCV viremia appears to be associated with lower serum cholesterol and triglyceride levels which implies that HCV itself might play a significant role in serum

lipid profiles of patients with chronic HCV infection<sup>[8]</sup>. In our study, low levels of cholesterol (total, HDL and LDL) were found in both groups of hepatitis patients whereas significant decrease in triglycerides was only observed in HCV patients. It is known that about 50% of insulin secreted by the pancreas is removed by first-pass extraction in the liver. Insulin promotes glycogen synthesis (glycogenesis) in the liver and inhibits its breakdown (glycogenolysis). It promotes protein, cholesterol, and triglyceride synthesis and stimulates formation of very-low-density lipoprotein cholesterol. The liver is the primary target organ for glucagon action, where it promotes glycogenolysis, gluconeogenesis, and ketogenesis<sup>[9]</sup>.

Among the metals, the central importance of Fe in the pathophysiology of disease is derived from the ease with which Fe is reversibly oxidized and reduced. This property, while essential for its metabolic functions, makes Fe potentially hazardous because of its ability to participate in the generation of powerful oxidant species such as the hydroxyl radical<sup>[8]</sup>. It is now well established that oxidants can cause the release of catalytic Fe<sup>[7]</sup>; thus, a vicious cycle is initiated that leads to the formation of more reactive oxygen species.

In our study, the serum level of Fe was elevated significantly in both HBV and HCV patients. These data on the relevance of Fe as a prognostic factor prompted us to ascertain whether HCV- and HBV-related liver damage is mediated by Fe accumulation. We have also observed decreased total oxidative activity in both patient groups and hence increases in Fe generate reactive oxygen species which may exceed the capacity of the antioxidant system and perpetuate oxidative stress to cells. Oxidative stress with the attendant low-grade inflammation is implicated in a number of pathological conditions, including aging, atherosclerosis, and diabetes<sup>[6]</sup>. In a study of patients with unexplained hepatic Fe overload, most were found to be insulin-resistant, which suggests a common etiologic link between hepatic Fe, hepatic dysfunction, and insulin resistance<sup>[10]</sup>. Insulin deficiency due to iron deposition in the interstitial pancreatic cells, with resultant excess collagen deposition and defective microcirculation<sup>[10]</sup> and insulin resistance<sup>[11]</sup>, are the likely mechanisms for type 2 diabetes. It has been shown that treatment with intravenous or oral chelation improves glucose tolerance in up to one-third of these patients, suggesting a causal role for Fe<sup>[12,13]</sup>. In one study it was shown that Fe overload may be responsible for insulin resistance, or *vice versa*<sup>[1]</sup>. Other metals such as Cu and Zn are essential trace elements for several metabolic processes. Regarding Zn, patients with chronic hepatic encephalopathy have been shown to have low serum Zn levels. Moreover, in a controlled study, significant improvement was seen in those patients on oral Zn supplementation<sup>[14]</sup>. In our patients with HBV and HCV, both groups showed significantly decreased Zn levels. Various studies have shown both Cu and Mg levels in serum remained unchanged in HBV and HCV patients, though content of Zn and selenium in plasma and erythrocytes were significantly lower in hepatitis C

and B patients<sup>[15,16]</sup>. Our results show a similar tendency in HBV and HCV patients.

Disturbances in the antioxidant system could play a role in the pathogenesis of chronic liver disease. During the course of chronic liver disease we may observe slight irregularities in iron status relating to both the serum and store pool of this element. The most significant disturbances are seen in patients with alcoholic cirrhosis of the liver<sup>[17]</sup>. These findings suggest that disturbances in antioxidant parameters in the blood of patients with chronic liver disease may be the cause of the peroxidative damage of cells. In both HBV and HCV, antioxidant activity is significantly decreased. The release of oxidative free radicals, deficiency in antioxidant enzymes and the expression of bcl-2 protein might play a role in the pathogenesis of viral hepatitis. The ability to measure bcl-2 protein in the serum could be useful as a prognostic marker in cancer patients<sup>[18]</sup>.

The availability of serum blood chemistry tests for screening both symptomatic and asymptomatic patients has resulted in a marked increase in the number of abnormal liver chemistry tests that must be interpreted by physicians. Usually the first step in the evaluation of a patient with elevated liver enzymes is to repeat the test to confirm the result. There is a hypothesis that enzymes conventionally associated with liver dysfunction, AST, ALT and  $\gamma$ -glutamyltransferase (GGT), may predict diabetes. However, the role of enzymes such as ALK, s-GPT, LDH, CPK and s-GOT or AST is controversial in many studies<sup>[15]</sup>. In our study, significantly increased levels of ALK, SGPT (or ALT), LDH and SGOT (or AST) were found, whereas, CPK activity decreased in both groups of hepatitis patients. A recent study showed that one-third of the hospitalized patients with liver cirrhosis are infected with HBV or HCV infection, with raised ALT/AST and ALK being more common with superadded viral infection<sup>[19]</sup>. Another study showed that there were also significant correlations between Fe status, as indicated by transferrin saturation or serum ferritin levels, and SGOT, SGPT, and GGT levels. Moreover, abnormal liver function, as represented by elevated levels of SGOT, SGPT, GGT and serum ALK, was observed more frequently in patients with Fe overload than in patients with a lower degree of Fe burden<sup>[16]</sup>.

AST is present in cytosolic and mitochondrial isoenzymes and is found in the liver, cardiac muscle, skeletal muscle, kidneys, brain, pancreas, lungs, leucocytes, and red cells<sup>[18]</sup>. ALT, a cytosolic enzyme, is found in its highest concentrations in the liver and is more specific to the liver<sup>[20,21]</sup>. The most common causes of elevated AST levels are chronic hepatitis B and C, autoimmune hepatitis, non-alcoholic steatohepatitis, hemochromatosis, Wilson's disease, celiac sprue, muscle damage and myocardial infarction<sup>[21,22]</sup>.

Among the liver enzymes, ALT is both sensitive and specific for liver disease of a hepatocellular injury type. Elevations in ALT levels should be interpreted as indicative of liver disease with only rare exceptions:

severe rhabdomyolysis or systemic myopathies. Our study showed that this enzyme increased significantly in both HCV and HBV patients. ALT to AST ratios greater than 1 are typically found in patients with viral hepatitis, drug-induced liver disease, autoimmune disorders, *etc.*, whereas ratios less than 1 are more often associated with alcohol-induced liver disease, ischemic forms of liver disease (passive congestion or under-perfusion), biliary tract obstruction and certain disorders that tend to result in a predominantly mitochondrial form of cell injury such as fatty liver of pregnancy, tetracycline toxicity, Reye's syndrome, *etc.* The use of this ratio can also be helpful when assessing the severity of liver disease, because once liver disease has progressed to cirrhosis (regardless of the underlying etiology) an elevated ALT to AST ratio often falls to values of 1 or less<sup>[23]</sup>. Indeed, in our study high ALT to AST ratios were observed in HCV and HBV patients.

From the results of our study, we conclude that parameters such as enzyme activity and the levels of metals such as Fe, Zn, Mg and Cu, along with total antioxidant activity, in patients with hepatitis B and C can be used to measure the status of the disease. We recommend further research in the area of antioxidant therapy.

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## COMMENTS

### Background

Patients with hepatitis B and C are at risk for poor nutritional status, poor response to bacterial and viral infections, stomach ulcers, kidney disorders, gallstones, liver cancer, and diabetes mellitus. All such events lead to life threatening complications and act as poor prognostic factors.

### Research frontiers

Disturbances in liver biochemistry, serum metal levels and dyslipidemia are usually observed in patients with chronic viral hepatitis. There has been no study to differentiate between the two hepatitis viruses in terms of metabolic derangement as compared with healthy subjects. This study is designed with the objective of investigating the similarities and dissimilarities in patients with hepatitis B and hepatitis C, clinically and metabolically.

### Innovations and breakthroughs

It was identified that serum magnesium (Mg) and copper (Cu) levels remained unchanged, whereas the concentration of zinc (Zn) decreased and iron (Fe) increased significantly in both groups of patients. Total antioxidant activity was significantly decreased in both hepatitis B and C. Among the enzymes analyzed, ALK, s-GPT, LDH and s-GOT were all significantly increased in both patients with hepatitis B and C, whereas CPK was significantly decreased in patients with hepatitis B and remained unchanged in patients with hepatitis C.

### Applications

By understanding the liver enzyme profile, serum biochemistry and metal levels in relation to hepatitis B and C virus, one can assess the exact health status of such patients and plan the specific and necessary parameters to control such life-threatening biochemical modifications. For the development of future therapeutic strategies, this study may need to be continued in a more advanced and extensive manner at different health care centers.

**Peer review**

This study shows the comparison of serum metal content of healthy individuals to HCV or HBV infected persons. As most of the parameters have already been known for HBV and HCV infected individuals, peer reviewer think it should be accepted as a brief report after the consolidation of the discussion part.

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## Hemodynamics in the immediate post-transplantation period in alcoholic and viral cirrhosis

Waleed K Al-Hamoudi, Saleh Alqahtani, Puneeta Tandon, Mang Ma, Samuel S Lee

Waleed K Al-Hamoudi, Gastroenterology Unit (59), College of Medicine, King Saud University, PO Box 2925, Riyadh 11461, Saudi Arabia

Saleh Alqahtani, Gastroenterology unit, King Faisal Specialist Hospital & Research Center, Jeddah 21499, Saudi Arabia

Puneeta Tandon, Mang Ma, Alberta Liver Transplantation Program, University of Alberta, Edmonton, Alberta, T6G 2X8, Canada

Samuel S Lee, Liver Unit, University of Calgary, Calgary, Alberta, T2N 4N1, Canada

Author contributions: Ma M designed the study; Al-Hamoudi WK wrote the manuscript; Alqahtani S, Tandon P and Al-Hamoudi WK conducted data collection; Al-Hamoudi WK, Ma M and Lee SS conducted data interpretation; all authors conducted manuscript revisions and final approval.

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Correspondence to: Dr. Waleed K Al-Hamoudi, Gastroenterology Unit (59), College of Medicine, King Saud University, PO Box 2925, Riyadh 11461,

Saudi Arabia. [wahamoudi@gmail.com](mailto:wahamoudi@gmail.com)

Telephone: +966-1-4671215 Fax: +966-1-4671217

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### Abstract

**AIM:** To study the hemodynamics in the immediate post transplant period and compare patients with alcoholic vs viral cirrhosis.

**METHODS:** Between 2000-2003, 38 patients were transplanted for alcoholic cirrhosis and 28 for postviral cirrhosis. Heart rate (HR), central venous pressure (CVP), mean arterial pressure (MAP), pulmonary capillary wedge pressure (PCWP), cardiac index (CI), systemic vascular resistance index (SVRI), pulmonary artery pressure (PAP), and pulmonary vascular resistance index (PVRI) were measured immediately and 24 h post transplantation.

**RESULTS:** Hyperdynamic circulation persisted at 24 h

following transplantation with an elevated CI of  $5.4 \pm 1.3$  L/(min  $\times$  m<sup>2</sup>) and  $4.9 \pm 1.0$  L/(min  $\times$  m<sup>2</sup>) in the viral and alcoholic groups, respectively, and was associated with a decreased SVRI. Within the first 24 h, there was a significant decrease in HR and increase in MAP; the extent of the change was similar in both groups. The CVP, PCWP, and SVRI increased, and CI decreased in the viral patients, but not the alcoholic patients. Alcoholics showed a lower PVRI ( $119 \pm 52$  dynes/(cm<sup>5</sup>  $\times$  m<sup>2</sup>) vs  $166 \pm 110$  dynes/(cm<sup>5</sup>  $\times$  m<sup>2</sup>),  $P < 0.05$ ) and PAP ( $20 \pm 7$  mmHg vs  $24 \pm 7$  mmHg,  $P < 0.05$ ) compared to the viral group at 24 h.

**CONCLUSION:** Hyperdynamic circulation persists in the immediate post-transplant period with a faster improvement in the viral group. Alcoholic patients have a more pronounced pulmonary vasodilatation.

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**Key words:** Hemodynamics; Cirrhosis; Alcohol; Viral; Allograft

**Peer reviewer:** Sheung-Tat Fan, Professor, Department of Surgery and Center for the Study of Liver Disease, The University of Hong Kong, Queen Mary Hospital, 102 Pokfulam Road, Hong Kong, China

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### INTRODUCTION

Patients with end stage liver disease manifest a hyperdynamic circulation characterized by a decrease in the systemic vascular resistance and arterial pressure, and

an increase in cardiac output<sup>[1-5]</sup>. Many patients with advanced cirrhosis also have pulmonary vascular abnormalities, ranging from hepatopulmonary syndrome to pulmonary hypertension. Although several studies have examined these aspects of the circulation in the medium- and long-term postoperative period after liver transplantation (weeks to months), the cardiovascular profile in the immediate postoperative period remains unclear.

To what extent these changes persist following liver transplantation is unclear, and whether patients with alcoholic cirrhosis who are also at risk of alcoholic cardiomyopathy manifest a different hemodynamic pattern compared to patients with post viral cirrhosis is yet to be determined.

Therefore we conducted a retrospective review of orthotopic liver transplantation (OLT) performed at the Alberta Liver Transplant program between 2000-2003 to assess the systemic and pulmonary hemodynamics within the first 24 h following liver transplantation in patients transplanted for both alcoholic and viral induced cirrhosis.

## MATERIALS AND METHODS

### Patients

Systemic and pulmonary hemodynamics for 66 patients (38 alcoholic, 28 viral) receiving a cadaveric liver transplantation for end stage liver disease between January 2000 and January 2003 were evaluated immediately following surgery and 24 h later. There were 26 male patients in the alcohol group and seven in the viral group. The average age was 51 years (range 35-57 years) and 49 years (range 41-74 years) in the two groups, respectively. All included patients were thoroughly evaluated for any coexisting cardiovascular or pulmonary disease as part of the pre-transplant workup.

Following liver transplantation, these patients were transferred to the intensive care unit at the University of Alberta hospital. Postoperative care included: (1) Frequent daily rounds conducted by the intensive care team, transplant hepatologist, transplant surgeons, and other consultants as required; (2) Endotracheal intubation and mechanical ventilation to maintain adequate oxygenation with frequent intra-arterial monitoring through a catheter inserted prior to surgery; intubation was discontinued as soon as possible following the transplantation; (3) Continuous monitoring of the heart rate (HR), blood pressure, and oxygenation, as well as a continuous electrocardiographic tracing; (4) Systemic and pulmonary hemodynamics, including cardiac index (CI), systemic vascular resistance index (SVRI), pulmonary capillary wedge pressure (PCWP), central venous pressure (CVP), mean arterial pressure (MAP), pulmonary artery pressure (PAP), and pulmonary vascular resistance index (PVRI) were measured every 8 h during the first 24 h through a pulmonary arterial (Swan Ganz) catheter inserted preoperatively; (5) Laboratory investigations including a complete cell count, electrolytes, kidney function, coagulation

Table 1 Pulmonary and systemic hemodynamics at 24 h

	Alcohol	Viral
MELD score	16.2 ± 6.6	16.5 ± 7.3
HR (beats/min)	88 ± 16	86 ± 13
MAP (mmHg)	94.0 ± 17.2	95.1 ± 11.4
PAP (mmHg)	20.3 ± 6.9	24.1 ± 7.5 <sup>a</sup>
PCWP (mmHg)	13.5 ± 6.5	15.2 ± 4.6
CVP (mmHg)	10.3 ± 4.7	13.0 ± 5.0
CI [L/(min × m <sup>2</sup> )]	4.8 ± 1.1	4.8 ± 0.9
SVRI [dynes/(cm <sup>5</sup> × m <sup>2</sup> )]	1391 ± 425	1398 ± 350
PVRI [dynes/(cm <sup>5</sup> × m <sup>2</sup> )]	119 ± 52	166 ± 110 <sup>a</sup>

<sup>a</sup>*P* < 0.05. MELD: Model for end-stage liver disease; HR: Heart rate; MAP: Mean arterial pressure; PAP: Pulmonary artery pressure; PCWP: Pulmonary capillary wedge pressure; CVP: Central venous pressure; CI: Cardiac index; SVRI: Systemic vascular resistance index; PVRI: Pulmonary vascular resistance index.

profile, liver enzymes, and albumin were obtained twice a day (or more if indicated) for the first few days; and (6) Fluid balance was charted every 8 h with measurements of total fluid input and output, including surgical drains. Patients who suffered a gastrointestinal bleed or who had a sudden unexplained drop in the hemoglobin (> 2 g/L) underwent the appropriate investigation and were not included in the study. Patients were also excluded from the study if they developed sepsis within the first 72 h following transplantation, required dialysis, had an adverse intraoperative or immediate post operative course requiring continuous pressure support, or were being transplanted for the second time. This study was approved by the Research Ethics Committee of the hospital.

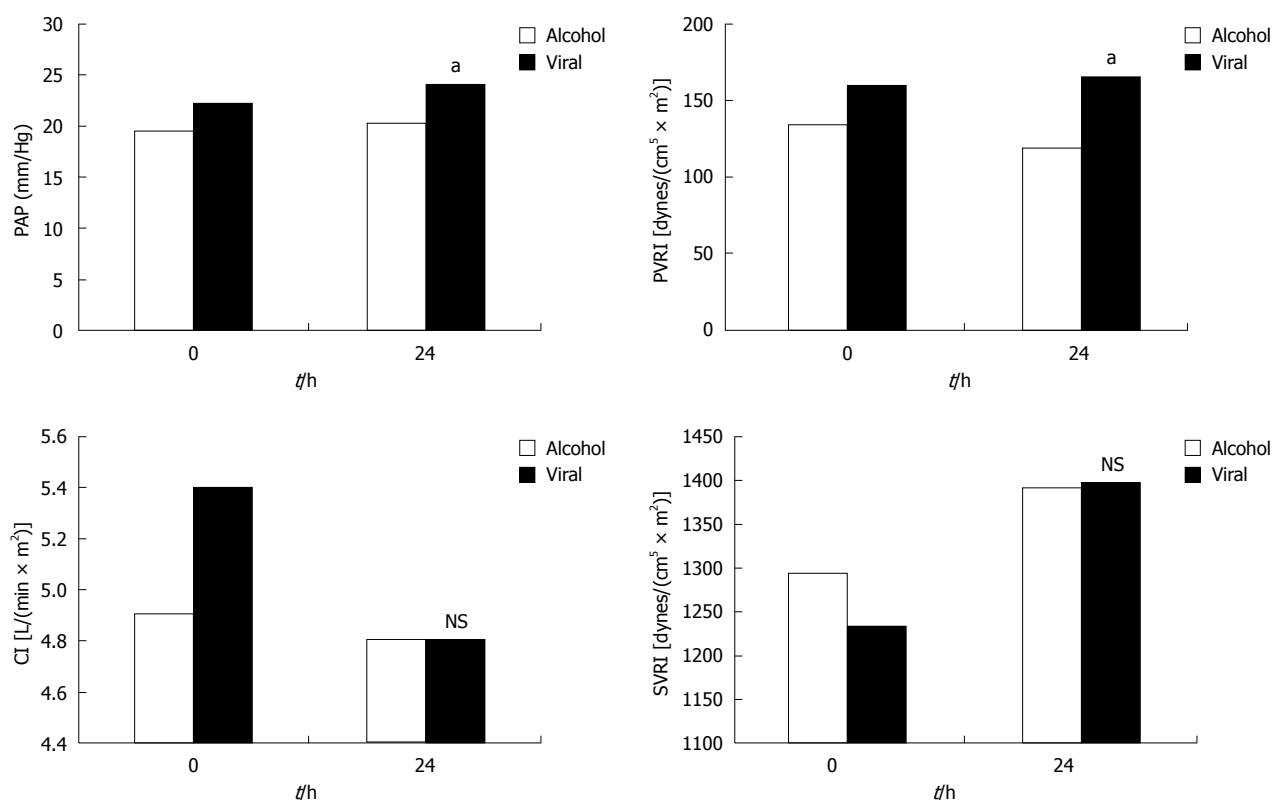
### Statistical analysis

Results were expressed as mean ± SD. Statistical analysis was done using STATA software (Version 8). Analysis was done using Student's *t*-test. A *P* value of less than 0.05 (two-sided test) was considered statistically significant. The correlations of disease severity graded by the model for end-stage liver disease (MELD) score and the various hemodynamic parameters were analyzed using Spearman's rank correlation coefficient.

## RESULTS

Hyperdynamic circulation persisted within the first 24 h with an elevated mean CI of 5.4 and 4.9 L/(min × m<sup>2</sup>), immediately following surgery in the viral and alcoholic group, respectively. The SVRI was also low at 1232 and 1294 dynes/(cm<sup>5</sup> × m<sup>2</sup>) in the two groups, respectively; however, these differences were not statistically significant.

Patients with alcoholic cirrhosis manifested a lower PVRI and a lower PAP, compared to the viral group at 24 h post transplant (*P* = 0.024 and 0.037, respectively) (Figure 1, Table 1). There were no significant differences in CVP, PCWP, HR, and MAP between the two groups at both time periods. The HR decreased significantly in association with an increase in the MAP over the first



**Figure 1** Hemodynamic changes within the first 24 h in both groups. \* $P < 0.05$ . NS: Not significant; PAP: Pulmonary artery pressures; PVRI: Pulmonary vascular resistance index; CI: Cardiac index; SVRI: Systemic vascular resistance index.

**Table 2** Hemodynamics immediately after, and 24 h post transplant

	Alcohol		Viral	
	Immediate	24 h post transplant	Immediate	24 h post transplant
HR (beats/min)	98 ± 19	88 ± 16 <sup>a</sup>	98 ± 14.5	86 ± 13 <sup>a</sup>
MAP (mmHg)	87 ± 12	94 ± 17 <sup>a</sup>	88.5 ± 14	95 ± 11 <sup>a</sup>
PAP (mmHg)	19.5 ± 5.6	20 ± 7.0	22 ± 7.0	24 ± 7.0
PCWP (mmHg)	11.5 ± 5	13.5 ± 6.5	12.5 ± 6	15 ± 4.6 <sup>a</sup>
CVP (mmHg)	9 ± 4.5	10 ± 4.5	10 ± 5.6	13 ± 5 <sup>a</sup>
CI [L/(min × m <sup>2</sup> )]	4.9 ± 1	4.8 ± 1	5.4 ± 1.3	4.8 ± 1 <sup>a</sup>
SVRI [dynes/(cm <sup>5</sup> × m <sup>2</sup> )]	1294 ± 390	1391 ± 425	1232 ± 411	1398 ± 349 <sup>a</sup>
PVRI [dynes/(cm <sup>5</sup> × m <sup>2</sup> )]	134 ± 78	119 ± 52	159 ± 88	166 ± 110

<sup>a</sup> $P < 0.05$ .

24 h in both groups. There was also an increase in the CVP, PCWP, SVRI, and a decrease in the CI in both groups over the first 24 h; these changes reached statistical significance only in the viral cirrhosis group (Table 2).

There were weak, insignificant correlations between the pre transplant MELD score and the CO, SVRI, PAP, PVRI with a Spearman's rank correlation coefficient of 0.0919, -0.1881, 0.0945, -0.0939, respectively.

## DISCUSSION

In this study, we showed that cirrhosis-associated hyper-

dynamic circulation persists in the immediate postoperative period, regardless of the etiology of the underlying liver disease. To our knowledge, this is the first study to compare hemodynamic changes in alcohol *vs* viral induced cirrhosis in the immediate postoperative period. Our results suggest that patients with viral-related cirrhosis have a rapid improvement in systemic hemodynamics, with a decrease in the cardiac output and an increase in the systemic vascular resistance; these changes were lacking in the alcohol group. We also showed that the increase in systemic vascular resistance was associated with a proportionate increase in CVP and PCWP unmasking the cardiac dysfunction of cirrhotic cardiomyopathy<sup>[3-5]</sup>. These results emphasize the additional effect of alcohol on the pathogenesis of the associated hyperdynamic circulation. Autonomic dysfunction has been clearly described in alcoholics<sup>[6,7]</sup>, alcoholic liver disease<sup>[8-10]</sup>, and to a lesser degree, in non-alcoholic chronic liver disease; the latter is thought to be related to the severity of the underlying liver disease<sup>[11,12]</sup>. Liu *et al*<sup>[13]</sup> demonstrated the role of vagal stimulation in the hyperdynamic circulation of cirrhotic rats, and by blocking this pathway the hyperdynamic circulation significantly decreased in the portal hypertensive rats. Lindgren *et al*<sup>[14]</sup> demonstrated that the autonomic, particularly vagal, nerve dysfunction of chronic liver disease is further exaggerated by alcohol abuse, suggesting that in alcoholic liver disease, sympathetic neuropathy adds to the parasympathetic dysfunction, which further contributes to the altered vascular

responsiveness in patients with alcoholic cirrhosis. It has also been suggested that alcohol consumption has a short and long term effect in reducing the vascular responses to endogenous vasoconstrictors<sup>[15,16]</sup>. Other factors that might contribute to the hyperdynamic circulation in both groups include vasoactive substances (such as nitric oxide and carbon monoxide), A-V shunts, increased blood volume, and central neural dysregulation<sup>[5,17-19]</sup>.

The consequences of these cardiovascular alterations can lead to significant cardiovascular derangements at the time of transplantation. OLT induces severe stresses on the cardiovascular system, during both intra- and postoperative periods<sup>[20,21]</sup>. Intraoperatively, the sudden reduction in the preload and the impaired cardiac contractility can result in significant reduction in cardiac output. Postoperatively, patients are at risk of hypovolemia from various factors, including hemorrhage, third space losses, and ongoing ascites formation. On the other hand, volume overload from aggressive fluid replacement can also stress the heart. Furthermore, the rapid improvement of systemic vasodilatation can result in a sudden increase in the afterload, adding extra stress to the heart. Metabolic derangements, in the form of acidosis, hypothermia, and electrolyte disturbance in the immediate postoperative period, can also impair the cardiac contractility and lead to hemodynamic instability and fluctuation. Hemodynamic depression caused by hypocalcemia-induced citrate intoxication from massive transfusion, or as a result of the reperfusion syndrome, has been described following OLT.

Cardiac complications following liver transplantation are common, involving 25%-70% of patients; fortunately most of these complications are mild and subclinical, with no significant impact on patient or graft survival<sup>[22-24]</sup>. Pulmonary edema following OLT is the most common cardiovascular complication, with at least 50% of these episodes developing within the first 24 h<sup>[21,25]</sup>. Careful fluid management and continuous cardiac monitoring in the immediate post transplant period is extremely important to avoid cardiac-related complications.

Studies examining hemodynamics after liver transplantation are limited and the results of those studies do not always agree with each other. Glauser<sup>[26]</sup> studied the systemic hemodynamics within the first 96 h following liver transplantation in 21 patients; his results suggested a progressive improvement towards normal within the study period. Navasa *et al.*<sup>[27]</sup> examined hemodynamics at 2 wk and 2 mo following liver transplantation, and suggested that most of the hemodynamic changes reverse. However, the limitations of these two studies are the small number of patients included (21 and 12, respectively), as well as the diverse indications for liver transplantation—thus only three and five patients were transplanted for alcoholic liver disease in the two studies, respectively. In contrast, other studies suggested persistence of hyperdynamic circulation for at least 6 mo<sup>[28-30]</sup>. In our study, we excluded factors that might affect the systemic circulation, such as anemia, sepsis, and renal failure. Patients

who were transplanted previously and were receiving their second or third transplant were also excluded; such surgeries disrupt local innervations and blood supply and might impact the vascular hemodynamics.

A low pulmonary vascular resistance has been described in patients with cirrhosis<sup>[31-34]</sup>. This also is thought to be related to endogenous vasodilators, as measured nitric oxide in exhaled air was higher in patients with cirrhosis compared to the general population<sup>[35]</sup>. Our data suggests that this pulmonary vasodilatory effect persists in the immediate post-transplant period. Furthermore, we showed that patients with alcoholic cirrhosis have a more profound decrease in the pulmonary vascular resistance associated with a significantly lower PAP; this could be related to a higher degree of intrapulmonary vascular shunting in patients with alcoholic cirrhosis and could, in part, be related to alcohol-induced depression in the vascular response to endogenous vasoconstrictors<sup>[36]</sup>.

In our study the MELD score did not significantly correlate with hemodynamic disturbance. We believe this is because the study cohort had a limited range of dispersion of MELD scores and hyperdynamic circulation, i.e. almost all patients had high MELD scores and also a significantly abnormal extent of hyperdynamic circulation. It is possible that inclusion of a larger range of less sick patients with lower MELD scores and less hyperdynamic circulation might have allowed a correlation to become more evident. However, such patients would not be considered for transplantation.

In conclusion, our data suggests that the systemic hyperdynamic circulation persists in the immediate post-transplant period. The pattern of changes in hemodynamics in the immediate post transplant period, as well as in the pulmonary circulation, differed in the two groups, suggesting an alcohol-related impact on the neurohumoral factors involved in the pathogenesis of hyperdynamic circulation of cirrhosis.

## COMMENTS

### Background

Cirrhosis is associated with hyperdynamic circulation and pulmonary vascular abnormalities; whether these abnormalities persist in the immediate post-transplantation, and differ according to the cause of cirrhosis remains unclear. The aim of this study was to compare the hemodynamics in alcoholic vs viral cirrhosis.

### Research frontiers

Cirrhosis is associated with significant cardiovascular abnormalities including hyperdynamic circulation, systemic vasodilatation, and cirrhotic cardiomyopathy. The immediate effect of liver transplantation on these cardiovascular changes has not been adequately studied. Prior studies on the effect of transplantation on the cardiovascular abnormalities were variable and not consistent.

### Innovations and breakthroughs

This is the first study to compare hemodynamics in the immediate post transplant period in patients with alcoholic and viral related cirrhosis. The results indicate that, although the hyperdynamic circulation persists in the immediate post-transplant period, systemic parameters improve faster in the viral group. Pulmonary hemodynamics differ significantly between alcoholic and postviral patients within the first 24 h, suggesting that alcoholics might have more pronounced pulmonary vasodilation than viral-cirrhotic patients.



## Applications

Following liver transplantation, careful patient monitoring and frequent assessment of the cardiac and fluid volume status is essential to avoid any cardiac-related adverse effects.

## Peer review

This is a retrospective study comparing the immediate hemodynamic status of patients with viral cirrhosis with that of those with alcoholic cirrhosis after liver transplantation. The authors found that the viral group has better recovery in hyperdynamic circulation after transplant than the alcoholic group, which has more pronounced pulmonary vasodilatation. This finding is interesting, but the clinical importance of this finding has to be further elaborated by the authors.

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## Effect of domperidone therapy on nocturnal dyspeptic symptoms of functional dyspepsia patients

Sheng-Liang Chen, Jie-Ru Ji, Ping Xu, Zhi-Jun Cao, Jian-Zhong Mo, Jing-Yuan Fang, Shu-Dong Xiao

Sheng-Liang Chen, Jie-Ru Ji, Ping Xu, Zhi-Jun Cao, Jian-Zhong Mo, Jing-Yuan Fang, Shu-Dong Xiao, Department of Gastroenterology, Shanghai Institute of Digestive Diseases, Renji Hospital, Shanghai Jiaotong University School of Medicine, 145 Middle Shandong Road, Shanghai 200001, China

Author contributions: Chen SL, Mo JZ, Fang JY and Xiao SD designed the research; Chen SL, Ji JR, Xu P and Cao ZJ performed the research; Chen SL analyzed the data and wrote the paper.

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Correspondence to: Dr. Sheng-Liang Chen, Department of Gastroenterology, Shanghai Institute of Digestive Diseases, Renji Hospital, Shanghai Jiaotong University School of Medicine, 145 Middle Shandong Road, Shanghai 200001, China. [chenslmd@yahoo.com.cn](mailto:chenslmd@yahoo.com.cn)

Telephone: +86-21-63200784 Fax: +86-21-63266027

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### Abstract

**AIM:** To investigate the incidence of nocturnal dyspeptic symptoms in patients with functional dyspepsia (FD) and whether prokinetic drugs can alleviate them.

**METHODS:** Eighty-five consecutive Chinese patients with FD were included in this study. One week after single-blinded placebo run-in treatment, baseline nocturnal intragastric pH, bile reflux and nocturnal dyspeptic symptoms of eligible patients, including epigastric pain or discomfort, abdominal distention and belching, were investigated with questionnaires. Patients exhibiting nocturnal dyspeptic symptoms were randomly and double-blindly assigned to domperidone group or placebo group. Nocturnal intragastric pH and percentage of duodenogastric bile reflux time were determined after treatment.

**RESULTS:** Of the 85 FD patients, 2 females without

nocturnal symptoms, who responded to placebo run-in treatment, were excluded from the study, 30 (36.1%) exhibited nocturnal dyspeptic symptoms with increased duodenogastric bile reflux time (intragastric bilirubin absorbance > 0.14) and mean gastric pH (confirming the existence of bile reflux) ( $P = 0.021, 0.023$ ) at night were included in the study. Of these 30 patients, 21 (70%) had overt nocturnal duodenogastric bile reflux, which was significantly higher than that of those without nocturnal symptoms ( $P = 0.026$ ). The 30 patients were allocated to domperidone group or placebo group ( $n = 15$ ). The nocturnal duodenogastric bile reflux and gastric pH were significantly decreased after domperidone treatment ( $P = 0.015, 0.021$ ). The severity score of nocturnal dyspeptic symptoms was also significantly decreased after domperidone treatment ( $P = 0.010, 0.015, 0.026$ ), which was positively correlated with the reduced nocturnal bile reflux or gastric pH ( $r = 0.736, 0.784, 0.753$  or  $r = 0.679, 0.715, 0.697$ ,  $P = 0.039, 0.036, 0.037$  or  $P = 0.043, 0.039, 0.040$ ).

**CONCLUSION:** A subgroup of Chinese FD patients show overt nocturnal dyspeptic symptoms, which may be correlated with the excessive nocturnal duodenogastric bile reflux. Domperidone therapy can alleviate these symptoms.

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**Key words:** Functional dyspepsia; Nocturnal dyspeptic symptoms; Duodenogastric bile reflux; Intragastric pH; Domperidone

**Peer reviewer:** Joachim Labenz, Associate Professor, Jung-Stilling Hospital, Wichernstr 40, Siegen 57074, Germany

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## INTRODUCTION

Functional dyspepsia (FD) is a common chronic disorder with non-specific upper abdominal symptoms such as persistent or relapsing pain or discomfort<sup>[1]</sup>. Its underlying pathophysiological mechanism has not been fully elucidated. Besides the role of excessive chemical (both acidic and alkaline) stimuli in the gastric lumen and/or hypersensitivity to these stimuli, impaired gastrointestinal (GI) motility is intensively involved in FD pathogenesis<sup>[2-4]</sup>. Delayed gastric emptying or intestinal peristalsis may influence GI secretion function which may in turn further affect GI motility, resulting in alterations in luminal chemical environment, abnormal duodenogastric bile reflux and FD symptoms<sup>[5]</sup>.

Although the optimal treatment of FD has not yet been established, empirical pharmacological interventions with prokinetic agents are effective in a subgroup of FD patients<sup>[6-8]</sup>. Domperidone, a peripheral dopamine (D<sub>2</sub>) receptor antagonist, acts as an antiemetic and prokinetic agent through its effects on the chemoreceptor trigger zone and motor function of the stomach and small intestine, thus promoting gastric emptying by augmenting gastric peristalsis and improving antroduodenal coordination<sup>[9,10]</sup>. Domperidone has been used in treatment of a variety of GI motility disorders, such as gastroparesis and gastroesophageal reflux disease, with an acceptable safety profile and a relatively good therapeutic efficacy for FD<sup>[7,10]</sup>.

In clinical practice, gastroenterological physicians frequently meet a subgroup of FD patients with obvious nocturnal dyspeptic symptoms such as epigastric pain or discomfort, abdominal distention or belching. Up to now, however, their prevalence and mechanism and whether prokinetic drugs can alleviate them are still unknown.

This study was aimed to evaluate the prevalence of nocturnal dyspeptic symptoms, the relation between such symptoms and nocturnal intragastric pH or duodenogastric bile reflux, and the effect of 2-wk oral administration of domperidone on these symptoms.

## MATERIALS AND METHODS

### Patients

Eighty-five consecutively patients, including 38 males at the age of 22-56 years who were diagnosed as FD according to Rome II criteria, were enrolled in this study. All patients were admitted because of dyspeptic symptoms and underwent routine biochemistry, bilimetry, upper GI endoscopy and abdominal ultrasonography. Inclusion criteria included the presence of dyspeptic symptoms for at least 3 mo in the past year, the absence of organic, systemic or metabolic disease, 2 or more dyspeptic symptoms present at least in 3 d per week such as epigastric pain or discomfort, abdominal distention and belching. Exclusion criteria were the presence of esophagitis, severe atrophic gastritis, erosive or ulcerative gastroduodenal lesions on endoscopy, heartburn as a predominant symptom, a history of peptic ulcer, major

abdominal surgery, or underlying psychiatric illness, and use of nonsteroidal anti-inflammatory drugs, steroids, or drugs affecting gastric motility and acid secretion during the last week.

### Study protocol

This study was conducted at Department of Gastroenterology, Shanghai Institute of Digestive Diseases, Renji Hospital, Shanghai Jiaotong University School of Medicine, according to the ethical principles in Declaration of Helsinki and the requirements of local laws and regulations. The study protocol was approved by the Ethics Committee of Renji Hospital. Written informed consent was obtained from each participant prior to the study.

Patients with a severity score of at least two individual symptoms decreased by 50% were excluded. One week after single-blinded placebo run-in treatment, baseline nocturnal intragastric pH, bile reflux and nocturnal dyspeptic symptoms of eligible patients, including epigastric pain or discomfort, abdominal distention and belching, were investigated. Those exhibiting nocturnal symptoms were randomly and double-blindly assigned to either domperidone group or placebo group. Patients in domperidone group received domperidone (10 mg *qid*, before meal and at bedtime) for 2 wk and those in placebo group received placebo. Nocturnal dyspeptic symptoms, intragastric pH and percentage of bile reflux time during nighttime (22:00 PM to 6:00 AM) were determined after treatment.

### Nocturnal intragastric pH monitoring and bilimetry

Following 2-h fasting, an initial manometric localization (CTD-Synectics, Sweden) of the lower esophageal sphincter (LES) was carried out. Nocturnal intragastric pH was then recorded using a pH sensitive microelectrode (Synectics Digtrapper MKIII, Medtronic Synectics, Sweden) connected to a portable Synectics medical Digtrapper III (CTD-Synectics). The electrode was inserted transnasally and positioned at about 8-10 cm below the manometrically determined LES. A two-point calibration of the probe was made before each recording, using standard buffers of pH 1 and pH 7. The Digtrapper data were downloaded onto a personal computer to calculate the nocturnal mean intragastric pH.

A fiber-optic spectrophotometer, Bilitec 2000 (Medtronic Synectics), was used to quantify duodenogastric bile reflux. The system consists of a probe (1.5-mm in diameter) that carries light signals into the stomach and back *via* a plastic fiber-optic bundle. Before each study, the probe was calibrated in water and located at 8-10 cm below the LES. An episode of duodenogastric bile reflux was defined as a rise of bilirubin absorbance above the cut-off level ( $> 0.14$  at 470 nm) lasting longer than 10 s<sup>[11]</sup>. The Digtrapper data were downloaded onto a personal computer to calculate the percentage of bile reflux time with the absorbance of bilirubin  $> 0.14$ .

### Evaluation of dyspeptic symptoms

Nocturnal dyspeptic symptoms were evaluated with a

**Table 1** Demographic and baseline clinical characteristics of FD patients with nocturnal dyspeptic symptoms *n* (%)

Characteristic	Placebo	Domperidone
Age (yr)		
mean $\pm$ SD	44.5 $\pm$ 8.6	43.9 $\pm$ 9.8
Range	25-56	22-50
Gender		
Male	6 (40)	7 (46.7)
Female	9 (60)	8 (53.3)
BMI (kg/m <sup>2</sup> )		
mean $\pm$ SD	21.3 $\pm$ 7.9	20.7 $\pm$ 8.1
Range	18.4-27.2	18.0-25.1
Current smokers	1 (4.2)	3 (5.9)
Alcohol use	5 (22.7)	6 (11.8)
Positive for <i>Helicobacter pylori</i>	0 (0)	0 (0)

FD: Functional dyspepsia; BMI: Body mass index.

**Table 2** Effect of domperidone on nocturnal duodenogastric bile reflux and gastric pH in FD patients with nocturnal dyspeptic symptoms (mean  $\pm$  SD)

Group	<i>n</i>	% of bile reflux time		Gastric pH	
		Baseline	After treatment	Baseline	After treatment
Placebo	15	3.4 $\pm$ 1.3	3.4 $\pm$ 1.5	4.9 $\pm$ 1.2	4.7 $\pm$ 1.1
Domperidone	15	3.5 $\pm$ 1.7	1.1 $\pm$ 0.6 <sup>a</sup>	5.2 $\pm$ 1.5	2.8 $\pm$ 0.9 <sup>a</sup>

<sup>a</sup>*P* < 0.05 vs baseline.

self-recorded questionnaire. Each patient was instructed on how to fill in the questionnaire. The symptom questionnaire consisted of 4 questions related to nocturnal upper gastrointestinal symptoms, including epigastric pain or discomfort, abdominal distention and belching. The intensity and frequency of each symptom were rated at 7 levels according to 3 grades with half steps between each rating: 0 = absent, 1 = mild, 2 = relevant and 3 = severe<sup>[12]</sup>. The severity of symptoms was scored as the product of intensity and frequency. The patients were asked to record the severity score of different nocturnal symptoms before and at end of the treatment.

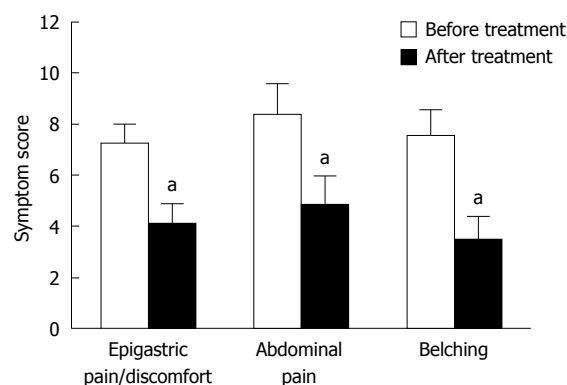
### Statistical analysis

All data were presented as mean  $\pm$  SD. Student's *t*-test and Wilcoxon rank sum test were used to compare the difference between means of before and after treatment. Incidence was compared using  $\chi^2$  test and correlation was assessed by Pearson correlation analysis. *P* < 0.05 was considered statistically significant.

## RESULTS

### Patients

Of the 85 patients, 2 females without nocturnal symptoms with a severity score of at least 2 individual symptoms decreased by 50% after placebo run-in treatment were excluded from the study, 30 (36.1%) including 13 males at the age of 22-56 years who exhibited overt noc-

**Figure 1** Severity scores of nocturnal dyspeptic symptoms in a subgroup of Chinese FD patients before and after domperidone (10 mg *qid*) treatment. Data are expressed as mean  $\pm$  SD, *n* = 15. FD: Functional dyspepsia. <sup>a</sup>*P* < 0.05 vs before treatment.

turnal dyspeptic symptoms were allocated to domperidone group or placebo group (*n* = 15). Demographic and baseline clinical characteristics did not differ between the two groups (Table 1).

### Nocturnal intragastric bilirubin and pH in FD patients with or without nocturnal dyspeptic symptoms

Of the 30 patients with nocturnal dyspeptic symptoms, 21 (70%) including 9 males had overt nocturnal duodenogastric bile reflux, which was higher than that of those without nocturnal symptoms (17.0%) including 2 males (*P* < 0.05). Moreover, the percentage of duodenogastric bile reflux time (intragastric bilirubin absorbance > 0.14) and mean gastric pH at night in the subgroup of patients with nocturnal symptoms was 5.9%  $\pm$  1.7% and 4.9%  $\pm$  1.9%, respectively, both were significantly higher than those in patients without nocturnal symptoms (bile reflux time = 2.4%  $\pm$  0.8%, mean gastric pH = 2.5  $\pm$  1.4, *P* < 0.05).

In domperidone group, 11 patients (73.3%) including 5 males had overt nocturnal duodenogastric bile reflux at baseline and mean nocturnal gastric pH > 4, which were significantly decreased after domperidone treatment (*P* < 0.05). In placebo group, 10 patients (66.7%) including 4 males had obvious nocturnal bile reflux and mean gastric pH > 4 at baseline, which were not markedly changed after placebo treatment (Table 2).

### Severity of nocturnal dyspeptic symptoms

The severity score of nocturnal dyspeptic symptoms such as epigastric pain or discomfort, abdominal distention and belching was significantly lower in FD patients after domperidone treatment than before domperidone treatment (4.1  $\pm$  0.8 vs 7.3  $\pm$  0.7, 4.9  $\pm$  1.1 vs 8.4  $\pm$  1.2 and 3.5  $\pm$  0.9 vs 7.6  $\pm$  1.0, *P* < 0.05, Figure 1). Improved nocturnal symptoms after domperidone treatment were positively correlated with reduced nocturnal duodenogastric bile reflux or intragastric pH in patients with marked duodenogastric bile reflux and mean nocturnal gastric pH > 4 at baseline (*r* = 0.736-0.784 or *r* = 0.679-0.715, *P* < 0.05) (Table 3).



**Table 3** Pearson R-values for improved nocturnal dyspeptic symptoms and reduced nocturnal bile reflux or gastric pH in domperidone-treated FD patients

	% of bile reflux time	Gastric pH
Epigastric pain/discomfort	0.736 <sup>a</sup>	0.679 <sup>a</sup>
Abdominal distention	0.784 <sup>a</sup>	0.715 <sup>a</sup>
Belching	0.753 <sup>a</sup>	0.697 <sup>a</sup>

<sup>a</sup>*P* < 0.05.

### Safety assessment

No patients stopped their medication or withdrew from the study due to side effects of domperidone during the study.

## DISCUSSION

The pathophysiological mechanism underlying FD is complex and its treatment remains a clinical challenge. Impaired GI motility seems to play a pivotal role in pathogenesis of FD. Gastric emptying is delayed in about 30% of FD patients<sup>[4,13]</sup>. It has been shown that domperidone and other prokinetic agents can markedly improve dyspeptic symptoms of FD patients<sup>[6-8]</sup>. However, the prevalence of nocturnal symptoms and the effect of domperidone on these symptoms have not been extensively elucidated.

Nocturnal intragastric pH and bilirubin level are hardly affected by food taking and other diurnal activities, thus relatively more stable and better reflecting the chemical environment in the gastric cavity. Increased gastric pH is thought to be an indicator of gastric bile reflux, which is related to impaired antroduodenal motility<sup>[14-16]</sup>. Therefore, the profile of gastric pH and bile reflux at night were selected as the main endpoints in the present study.

In this study, over 35% of FD patients exhibited nocturnal dyspeptic symptoms, and about 70% of the patients in this subgroup had a marked nocturnal duodenogastric bile reflux at baseline, manifested as prolonged time of absorbance of gastric bilirubin > 0.14 confirmed by alkalization of the acidic intragastric environment (mean pH > 4), suggesting that nocturnal dyspeptic symptoms of FD patients may be associated with abnormal nocturnal duodenogastric bile reflux. There is evidence that decreased antroduodenal motility is the main factor for increased duodenogastric reflux<sup>[15,16]</sup>. Our findings also indicate that nocturnal dyspeptic symptoms are related with impaired antroduodenal motility.

In our study, the severity score of nocturnal epigastric pain or discomfort, abdominal distention or belching, as well as nocturnal gastric pH and bile reflux in FD patients, were significantly lower after domperidone treatment than before domperidone treatment. Furthermore, alleviation of bile reflux was well correlated with the improved nocturnal symptoms, suggesting that the efficacy of domperidone therapy may be associated with

the inhibition of nocturnal duodenogastric bile reflux resulting from improved gastric peristalsis and antroduodenal coordination.

Since the placebo effect on FD can be high<sup>[17]</sup>, 1-wk single-blind placebo run-in treatment was used in patients before they received domperidone or placebo treatment in this study.

In conclusion, a subgroup of FD patients exhibit overt nocturnal dyspeptic symptoms, which may be related to an increased nocturnal duodenogastric bile reflux. Domperidone therapy can alleviate such symptoms by attenuating nocturnal duodenogastric bile reflux resulting from improved GI motility.

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## COMMENTS

### Background

The pathophysiological mechanism of functional dyspepsia (FD) is complex and its treatment remains a clinical challenge. There exists a subgroup of FD patients with nocturnal dyspeptic symptoms, such as epigastric pain or discomfort, abdominal distention or belching. Up to now, the prevalence and mechanism of FD and its effective drug therapy have not been established.

### Research frontiers

Impaired gastrointestinal motility plays a pivotal role in pathogenesis of FD, and empirical pharmacological interventions with prokinetic agents have been shown to be effective on FD. However, whether abnormal antroduodenal motility, which can lead to increased nocturnal duodenogastric bile reflux, contributes to nocturnal dyspepsia symptoms of FD patients and whether prokinetic drugs can alleviate these symptoms are still unknown.

### Innovations and breakthroughs

This is the first study to report that a subgroup of FD patients exhibit overt nocturnal dyspeptic symptoms, which may be related to excessive nocturnal duodenogastric bile reflux. Prokinetic therapy can alleviate these syndromes by attenuating nocturnal bile reflux, thus representing a promising treatment modality for nocturnal dyspeptic symptoms of FD patients.

### Applications

The incidence of nocturnal dyspeptic symptoms and the effect of prokinetic drugs on FD were studied in this study, which may provide valuable data for the treatment of nocturnal dyspeptic symptoms of FD patients.

### Peer review

A RCT in dyspeptic patients with nocturnal symptoms was described. The results are clear cut in the sense that many FD patients in China complain of nocturnal symptoms and that domperidone is a promising agent in treatment of these patients. The study also suggests that duodenogastric reflux during night is of a pathophysiological relevance.

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## Natural course of chronic hepatitis B is characterized by changing patterns of programmed death type-1 of CD8-positive T cells

Xue-Song Liang, Ying Zhou, Chen-Zhong Li, Mo-Bin Wan

Xue-Song Liang, Chen-Zhong Li, Mo-Bin Wan, Department of Infectious Diseases, Changhai Hospital, Second Military Medical University, Shanghai 200433, China

Ying Zhou, Department of Biochemistry and Molecular Biology, Second Military Medical University, Shanghai 200433, China

**Author contributions:** Liang XS and Zhou Y contributed equally to this work; Liang XS provided the vital reagents and analytical tools and wrote the manuscript; Zhou Y performed the majority of experiments; Li CZ took the charge of collecting all human materials; Wan MB designed the study and provided the financial support for this work.

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**Correspondence to:** Mo-Bin Wan, Professor, Department of Infectious Diseases, Changhai Hospital, Second Military Medical University, Shanghai 200433, China. [mobinwan@yahoo.com.cn](mailto:mobinwan@yahoo.com.cn)

Telephone: +86-21-81873504 Fax: +86-21-65518368

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### Abstract

**AIM:** To investigate if and how programmed death type-1 (PD-1) expression affects the natural course of hepatitis B virus (HBV) infection.

**METHODS:** Sixty-four patients in different natural stages of chronic HBV infection were enrolled in this study. PD-1 expression in total T cells was detected by flow cytometry. Levels of total CD8+ T cell responses and proliferation in relation to PD-1 expression levels were analyzed with intracellular staining and PD-1/PD-L1 blockage.

**RESULTS:** The PD-1 expression in T cells was dynamically changed during the natural course of chronic HBV infection, did not significantly increase in the immune tolerance phase, and returned to normal in the inactive

virus carrier stage. Blockage of the PD-1/PD-L1 pathway could not affect the T-cell response in the immune tolerance and inactive virus carrier stages of chronic HBV infection. However, it could significantly restore the T-cell response in the immune clearance stage of chronic HBV infection. Furthermore, the PD-1 expression level in T cells was associated with the alanine aminotransferase level during the immune clearance stage of chronic HBV infection.

**CONCLUSION:** The PD-1/PD-L1 pathway plays a different role in T-cell response during the natural course of chronic HBV infection.

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**Key words:** Programmed death type-1; Hepatitis B virus; Chronic hepatitis B; Natural stage; CD8+ T cell; Serum viral load; Programmed death ligand; T cell response

**Peer reviewer:** Emanuel K Manesis, MD, Professor of Medicine, Athens University School of Medicine, Liver Unit, Euroclinic, 19 Mavromateon Street, 104 34 Athens, Greece

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### INTRODUCTION

During chronic hepatitis B virus (HBV) infection, a dynamic balance between viral replication and host immune response is pivotal to the pathogenesis of liver

disease. In accordance with immune characteristics, chronic HBV infection can be clinically categorized into three periods, namely immune tolerance phase, immune clearance phase, and immune stable phase or inactive virus carrier phase<sup>[1,2]</sup>. It has been widely accepted that adaptive immune responses, particularly cellular immune responses, mediate the clearance of HBV<sup>[3-6]</sup>. Unfortunately, HBV-specific T-cell function is impaired in patients with chronic HBV infection characterized by low levels of antiviral cytokines, impaired cytotoxic T lymphocyte activity, and persistent viremia<sup>[7,8]</sup>. However, the mechanism underlying this T-cell malfunction in chronic HBV infection has not been completely understood<sup>[9]</sup>.

The immunologic receptor programmed death type-1 (PD-1), a 55 kDa transmembrane protein containing an immunologic receptor tyrosine-based inhibitory motif, was originally isolated from a T-cell line exhibiting a high sensitivity to apoptosis<sup>[10]</sup>. The PD-1/PD-L1 pathway has been well documented to play a negative role in the regulation of activation and proliferation of T-cells and production of cytokines<sup>[11-13]</sup>. There is evidence that the PD-1 pathway plays an important role in inhibiting the function of virus-specific CD8<sup>+</sup> T-cells in chronic viral infection involving human immunodeficiency virus (HIV)<sup>[14-16]</sup>, hepatitis C virus (HCV)<sup>[17,18]</sup>, and HBV<sup>[19]</sup>.

Although reports are available on the changes in expression levels of PD-1 and T-cell responses in patients with HBV infection<sup>[20]</sup>, the change pattern of PD-1 expression in the natural course of chronic HBV infection has not yet been presented. Understanding such changes in PD-1 expression and T-cell responses in the course of chronic HBV infection is crucial in the management of HBV carriers. For this reason, we analyzed the PD-1 expression in T cells and tested the role of the PD-1/PD-L1 pathway in the regulation of T-cell response during different stages of chronic HBV infection. Our results suggest that activated PD-1 signaling is closely related with T-cell malfunction in the immune clearance phase but not in the other two phases of chronic HBV infection.

## MATERIALS AND METHODS

### Subjects

Sixty-four patients with chronic HBV infection (53 males and 11 females), enrolled in this study, were positive for HBsAg and anti-HBc but negative for antibodies (Abs) to HCV, delta virus (HDV), HIV-1 and -2, and other symptoms of chronic liver damage. The patients were observed for more than 48 wk during which liver function and serum DNA level were tested once every 3 mo. Of the 64 patients, 9 were in the immune tolerance stage with the presence of HBeAg, high serum DNA level, normal serum alanine aminotransferase (ALT) and minimal or no evident inflammation on liver biopsy, 10 were in the inactive virus carrier stage and negative for HBeAg and positive for anti-HBe antibody with undetectable or low HBV DNA level, and 45 were in the immune

clearance phase with persistent elevated serum ALT level and positive serum HBV DNA. The morphology of liver was examined by ultrasonography or computerized tomography, which showed no radiologic or histological evidence of cirrhosis. None of the patients received antiviral therapy and/or immune regulate therapy before they were admitted (clinical information is listed in Table 1). Twelve healthy blood donors served as normal controls.

All the patients and normal controls were Chinese. Our study was approved by the local ethics committee, and all patients provided their written informed consent.

### Virology assessment

HBsAg, HBeAg, anti-HBs, anti-HBc, anti-HBe, and antibodies to HCV, HDV, HIV-1, and HIV-2 were detected by enzyme linked immunosorbent assay with commercially available kits (Sino-American Biotechnology Company, SABC). Serum HBV-DNA level was measured by fluorescent quantitative PCR with commercially available kits (PE/B/MJ/L, Shenzhen, China).

### Isolation of peripheral blood mononuclear cells

EDTA- and heparin-anticoagulated blood (5-7 mL) was collected from each patient and used either directly for fluorescence-activated cell sorting (FACS) or for peripheral blood mononuclear cell (PBMC) isolation. PBMC ( $2 \times 10^6$ - $6 \times 10^6$ ) were isolated by Ficoll-Hypaque density gradient centrifugation, washed twice in phosphate-buffered saline, and analyzed immediately.

### Flow cytometry

Cells were stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, and allophycocyanin (APC)-labeled monoclonal antibodies, respectively, according to their manufacturers' instructions. Flow cytometry was performed using FACSCalibur (Becton Dickinson, San Jose, CA). FACS data were analyzed using CellQuest software (Becton Dickinson Rutherford, NJ). The PE-labeled anti-PD-1 monoclonal antibodies were obtained from BD PharMingen (BD Biosciences, San Jose, CA). FITC-anti-CD4 and APC-anti-CD8 antibodies were purchased from eBioscience (San Diego, CA).

### PD-1/PD-L1 blockage

Fresh PBMC ( $1 \times 10^5$ - $5 \times 10^5$ ) isolated from patients with chronic HBV infection were incubated for 45 min at 37°C with anti-PD-L1 (10 g/mL) or isotype control antibody (IgG2b clone MPC 11; 10 µg/mL eBioscience, Boston, MA) or without anything, washed and co-incubated with the Dybeads CD3/CD28 T-cell expander ( $4 \times 10^7$ /mL, Invitrogen) for 3 d. On the third day of incubation, the cells were stained with surface antibodies, perforin, and granzyme B, respectively, for flow cytometry analysis.

### Intracellular perforin and granzyme B staining

*In vitro* expanded cells ( $0.2 \times 10^6$ - $0.3 \times 10^6$ ) were stained with anti-CD8 APC mAb (BD Biosciences) before they



Table 1 Demographic and clinical parameters of 4 groups

Subjects	Sex		Age (yr)	ALT (U/L)	eAg (+)	HBV DNA			
	Male	Female				ND	< 10 <sup>3</sup>	10 <sup>3</sup> -10 <sup>7</sup>	> 10 <sup>7</sup>
NC	8	4	23.5 ± 4.5	24.5 ± 9.7	-	12	-	-	-
Immune tolerance	7	2	26.0 ± 6.4	42.4 ± 28.9	9	0	0	2	7
Immune clearance	37	8	33.3 ± 8.8	298.0 ± 289.4	33	0	0	25	20
Inactive carrier	9	1	39.1 ± 12.1	23.8 ± 10.7	0	0	10	0	0

NC: Normal control; ND: Not done; ALT: Alanine aminotransferase; HBV: Hepatitis B virus.

were fixed and permeabilized (Cytofix-Cytoperm by BD Biosciences) for intracellular staining (ICS) with anti-perforin-FITC (BD Biosciences) or anti-GRZ-A-FITC (BD Biosciences) mAbs.

Proliferation of CD8 T cells

Freshly isolated peripheral lymphocytes were re-suspended at the concentration of 1 × 10<sup>6</sup> cells/mL in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (R10; Invitrogen) and stimulated with the Dybeads CD3/CD28 T cell expander (4 × 10<sup>7</sup> cells/mL, Invitrogen), with or without anti-PD-L1 Mab (10 g/mL) or isotype control antibody (IgG2b clone MPC 11; 10 µg/mL e-Bioscience, Boston, MA). On day 3, the cells re-suspended in 300 µL PBS were double-stained with anti-CD8-FITC and 7-amino-actinomycin D. Cellular data were acquired for analysis. The total number of cells in each well was calculated according to the following formula: Total number of cells = (number of live cells/number of beads) × 10<sup>5</sup>.

Statistical analysis

Wilcoxon matched paired test and Mann-Whitney test of SSPS 12.0 were used to assess the difference between different groups. Spearman correlation analysis of PD-1 expression and HBV viral titers or ALT level was performed. *P* < 0.05 was considered statistically significant. The test of significance was two sided.

RESULTS

PD-1 expression in CD8+ T cells during the natural course of chronic HBV infection

To determine the surface expression of PD-1 in total peripheral CD8+ T cells during the natural course of chronic HBV infection, the total number of peripheral T cells of 39 patients (9 in the immune tolerance phase, 10 in the inactive virus carrier phase, and 20 in the immune clearance phase) were analyzed by flow cytometry. The PD-1 expression levels in CD8+ T cells in the immune tolerance and inactive virus carrier stages of chronic HBV infection patients did not significantly differ from those of the normal controls. However, the PD-1 expression levels were significantly higher in the immune clearance stage of chronic HBV infection patients than in normal controls and in the other two natural stages of chronic HBV infection patients (Figure 1).

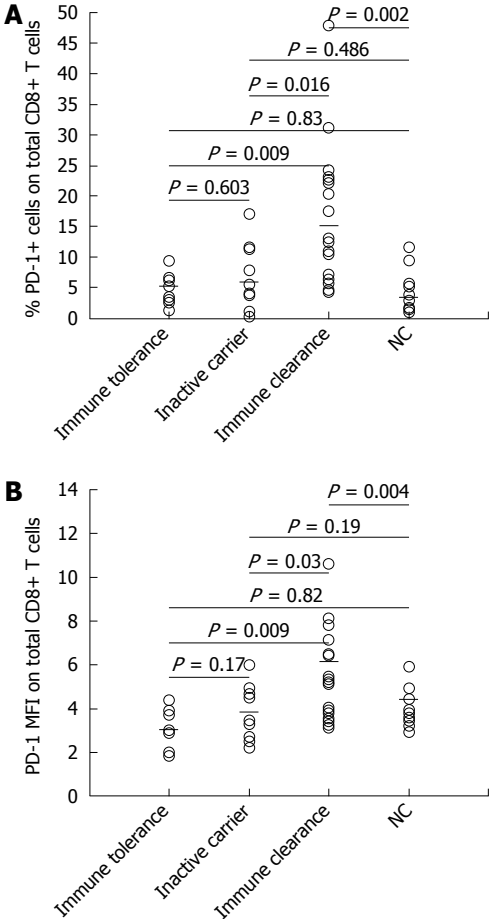
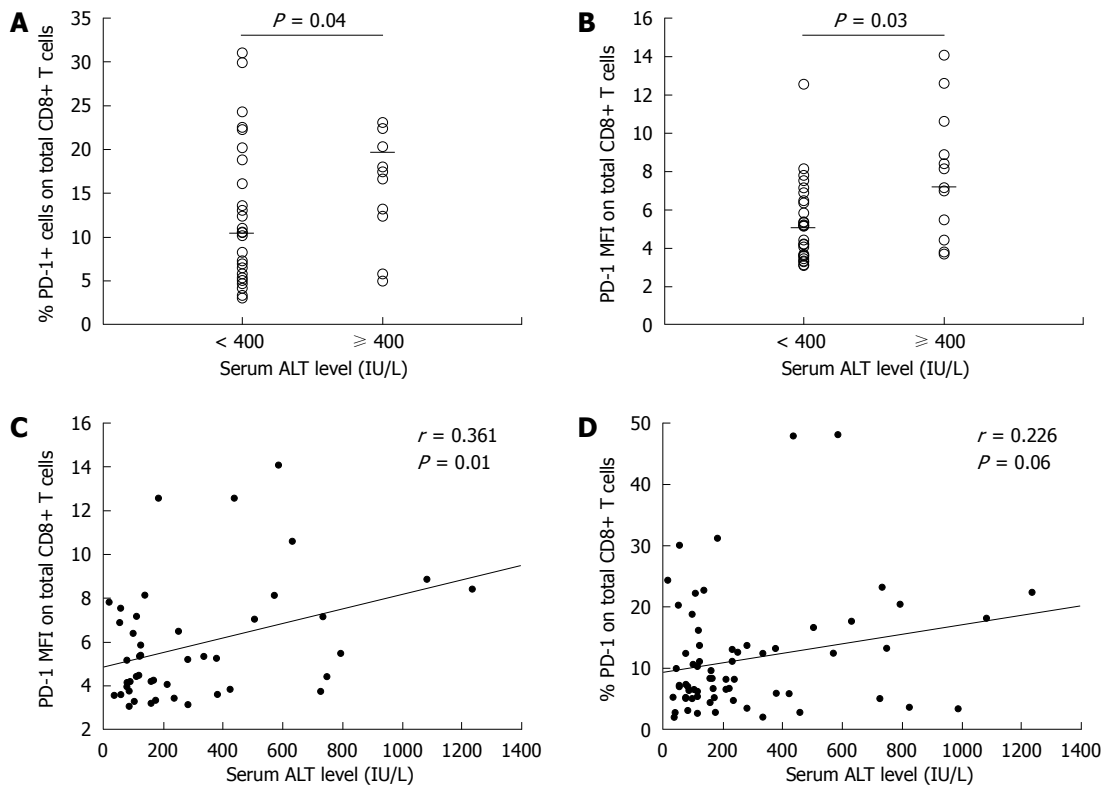


Figure 1 Programmed death type-1 (PD-1) expression in total peripheral CD8+ T cells in the natural stages of chronic hepatitis B virus (HBV) infection. A: The dot plots showing the percentage of PD-1+ CD8+ T cells in different natural stages of chronic HBV infection patients and normal controls and the horizontal bars indicating the median percentage of positive PD-1 in CD8+ T cells; B: The dot plots showing the PD-1 mean fluorescence intensity (MFI) expression in total CD8+ T cells, and the horizontal bars indicating the median PD-1 MFI expression level in CD8+ T cells. NC: Normal control.

PD-1 expression level was positively correlated with serum ALT level in the immune clearance stage of chronic HBV infection

The PD-1 expression level was significantly higher in the immune clearance stage of chronic HBV infection patients than in normal controls and in the other two natural stages of chronic HBV infection patients.

The patients were divided into two groups according to their ALT level. Based on the exacerbation or flare of



**Figure 2** PD-1 expression levels in the total T cells in the immune clearance stage of chronic HBV infection and liver inflammatory groups. A: The dot plots showing the percentage of positive PD-1 in CD8+ T cells in high and low liver inflammatory groups; B: The dot plots showing the PD-1 MFI expression level in the total CD8+ T cells in high and low liver inflammatory groups; C: Positive correlation between PD-1 MFI expression and alanine aminotransferase (ALT) levels in CD8+ T cells; D: Positive PD-1 percentage and ALT level in CD8+ T cells.

hepatitis B<sup>[21]</sup> and general indications of antiviral therapy for CHB with interferon<sup>[22]</sup>, we used 10 times the upper limit of normal ALT level ( $10 \times \text{ULT}$ , 400 IU/L) as the cut-off value in dividing the patients. Patients with their ALT level higher than 400 IU/L were defined as the high immune response group, while those with their ALT levels lower than 400 IU/L were defined as the low immune response group. The PD-1 expression level in CD8+ T cells was much higher in the high immune response group than in the low immune response group ( $P < 0.05$ , Figure 2A and B).

Further analysis showed that the PD-1 expression level was positively correlated with the ALT level in total CD8+ T cells ( $P < 0.05$ , Figure 2C and D).

#### **PD-1 expression level was not correlated with serum HBV DNA level in total T cells during the immune clearance phase of chronic HBV infection**

In this study, no significant correlation was found between the percentage and mean fluorescence intensity (MFI) of PD-1 expression and the serum viral load in total T cells. Similarly, no difference was observed in PD-1 expression in CD4+ T cells and CD8+ T cells between the HBe+ and HBe- groups.

#### **Relation between CD8+ T cell response and PD-1 expression level in the immune clearance stage of chronic HBV infection**

The relation between total CD8+ T cell response and

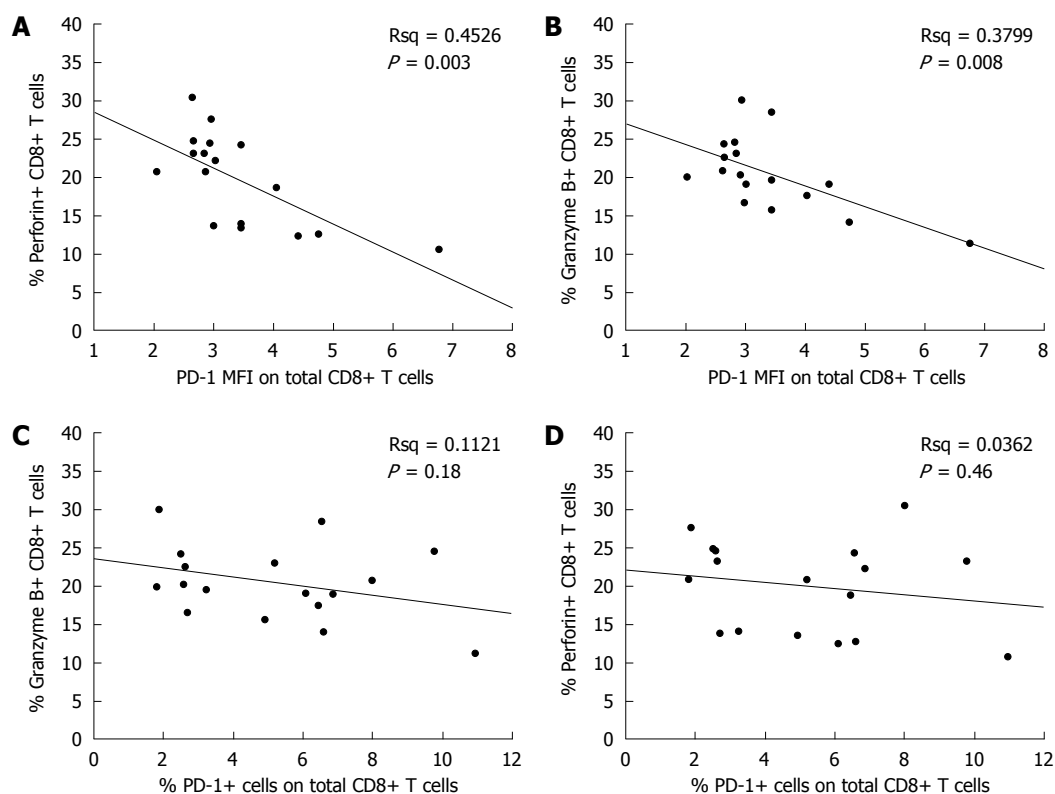
PD-1 expression level was analyzed with ICS, which showed that the CD8+ T cell response level was inversely correlated with PD-1 MFI (Figure 3A-D).

#### **Blocking PD-1/PD-L1 engagement in the total CD8+ T cells during the immune clearance stage of chronic HBV infection**

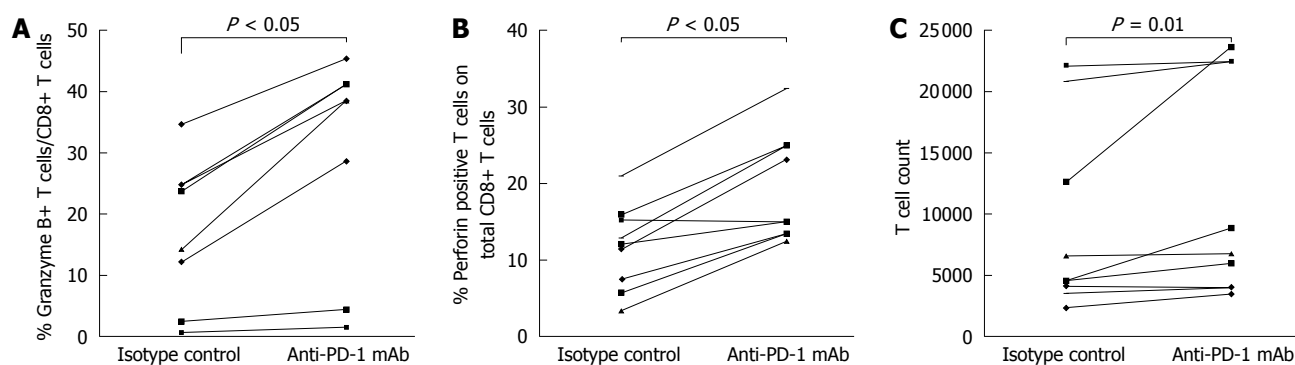
To investigate the role of PD-1 expression in CD8+ T cell responses in the natural stages of chronic HBV infection, expansion of CD8+ T cells and production of cytokines were detected 3 d after *in vitro* stimulation in the presence or absence of anti-PD-L1 antibody. The blockage of PD-1/PD-L1 significantly enhanced the expansion of T cells in the immune clearance stage of chronic HBV infection (Figure 4C), and increased the production of perforin and granzyme B by CD8+ T cells in the immune clearance, immune tolerance and inactive virus carrier stages of chronic HBV infection (Figure 4A and B).

## **DISCUSSION**

As a negative co-stimulatory receptor, PD-1 interacts with its ligands, PD-L1 and PD-L2, to attenuate T-cell responses and appears particularly important in regulating T-cell tolerance. In chronic infection with viruses such as HIV and HCV<sup>[18,23]</sup>, the PD-1 expression level remains high in virus-specific T cells. In our study, the PD-1 expression level in T cells was dynamically changed during the natural stage of chronic HBV



**Figure 3** Relation between PD-1 expression level in CD8+ T cells and CD8+ T-cell responses in the immune clearance stage of chronic HBV infection. A: Inverse relation between the percentage of perforin and PD-1 MFI in positive CD8+ T cells; B: Inverse relation between the percentage of granzyme B and PD-1 MFI in positive CD8+ T cells; C: No correlation between the percentages of granzyme B and PD-1 in positive CD8+ T cells; D: No significant correlation between the percentages of perforin and PD-1 in positive CD8+ T cells.



**Figure 4** Effect of anti-PD-L1 antibody on the function of CD8+ T cells in the immune clearance stage of chronic HBV infection. A, B: Significant restoration of CD8+ T cell response after blockage of PD-1/PD-L1; C: Restoration of T-cell proliferation after blockage of PD-1/PD-L1.

infection, indicating that it is up-regulated when the host antiviral immune is activated and down-regulated when the virus is cleared and the antiviral immune is inactivated.

Among the natural stages of chronic HBV infection, the immune clearance stage is the most critical phase for the host in eliminating the virus. In this study, the PD-1 expression level in T cells was significantly higher in the immune clearance stage than in the other two stages of chronic HBV infection and in normal controls. The PD-1 expression level in CD8+ T cells was related with serum ALT level but not with serum viral load, which is not consistent with the reported findings<sup>[14,18,24]</sup>. The

difference may be due to the different study groups and detection methods used. Furthermore, the PD-1 MFI in CD8+ T cells was inversely correlated with CD8+ T cell responses, and blockage of PD-1/PD-L1 interaction significantly restored the proliferation of T-cells and the secretion of antiviral cytokines in the immune clearance stage of chronic HBV infection, suggesting that the PD-1/PD-L1 pathway affects the progress of chronic HBV infection by regulating T-cell response in the immune clearance stage of chronic HBV infection.

CHB is characterized by loss of virus-specific CD8+ T cells and varying degrees of functional impairment of virus-specific T-cell responses. In patients with chronic

HBV infection, the PD-1 is highly expressed in virus specific CD8+ T cells, and the spectrum of anti-HBV immunity can be improved by blocking the PD-1<sup>[19]</sup>. In this study, the PD-1 expression level in CD8+ T cells was up-regulated, and blocking the PD-1/PD-L1 pathway increased the proliferation of T-cells and restored the response of CD8+ T cells in the immune clearance stage but not in the immune tolerance and inactive virus carrier stages of chronic HBV infection, suggesting that the PD-1 inhibitory pathway particularly acts on the immune clearance stage of chronic HBV infection, and that the PD-1 expression level and the role of the PD-1/PD-L1 pathway are dramatically changed with the antiviral immune change in the natural stage of chronic HBV infection.

In conclusion, PD-1 expression participates in modulating the host antiviral immunity throughout the natural course of chronic HBV infection. However, in this study, we only examined the total number of CD8+ T cells but did not calculate the number of HBV-specific CD8+ T cells as targets. Further study is needed on the virus-specific anti-viral immunity.

## COMMENTS

### Background

Adaptive immune response, particularly cellular immune response, mediates the clearance of hepatitis B virus (HBV). Unfortunately, HBV-specific T-cell function is severely impaired in chronic HBV infection patients, characterized by low levels of antiviral cytokines, impaired cytotoxic T lymphocyte activity and persistent viraemia. The mechanism underlying T-cell malfunction in chronic HBV infection has not yet been completely understood.

### Research frontiers

The programmed death type-1 (PD-1)/PD-L1 pathway plays a negative role in the regulation of activation and proliferation of T cells and production of cytokines. There is evidence that the PD-1 pathway plays an important role in inhibiting the function of virus-specific CD8+ T-cells in chronic viral infections involving human immunodeficiency virus, hepatitis C virus, and HBV. However, the change pattern of PD-1 expression in the natural course of chronic HBV infection has not been presented. The results of this study indicate that the PD-1/PD-L1 pathway plays a different role in T-cell response in the natural course of chronic HBV infection.

### Innovations and breakthroughs

Recent reports have highlighted the importance of the PD-1 pathway in T-cell response in different stages of chronic HBV infection. This study showed PD-1 expression participated in modulating host antiviral immunity throughout the natural course of chronic HBV infection.

### Applications

By discussing the changes in expression of PD-1 and T cell responses in the natural course of chronic HBV infection, this study may represent a future strategy for therapeutic intervention in treatment of patients with HBV infection.

### Terminology

PD-1: A 55 kDa transmembrane protein containing an immunologic receptor tyrosine-based inhibitory motif, which was originally isolated from a T-cell line exhibiting a high sensitivity to apoptosis. As a negative co-stimulatory receptor, PD-1 interacts with its ligands, PD-L1 and PD-L2, to attenuate T-cell responses and appears to be particularly important for regulating T-cell tolerance.

### Peer review

By employing well-designed immunological techniques, the authors found that the expression of PD-1 was significantly up-regulated in T-cells of patients in the immune clearance phase of chronic HBV infection and was closely correlated with increasing serum ALT levels but not with serum HBV DNA in the same phase, which may contribute to the elucidation of the pathophysiology underlying the different natural courses of chronic HBV infection.

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## Serum thymosin $\beta$ 4 levels in patients with hepatitis B virus-related liver failure

Tao Han, Ying Liu, Huan Liu, Zheng-Yan Zhu, Yan Li, Shi-Xiang Xiao, Zhen Guo, Zhen-Gang Zhao

Tao Han, Ying Liu, Huan Liu, Zheng-Yan Zhu, Yan Li, Shi-Xiang Xiao, Zhen Guo, Zhen-Gang Zhao, Department of Hepatology, Tianjin Institute of Hepatobiliary Disease, Tianjin Key Laboratory of Artificial Cells, Tianjin Third Central Hospital, Tianjin Medical University, 83 Jintang Road, Tianjin 300170, China

**Author contributions:** Han T designed the study and wrote the paper; Liu Y carried out the laboratory work and wrote the paper; Liu H assisted in the analysis of data; Zhu ZY assisted in the experiment; Li Y, Xiao SX, Guo Z and Zhao ZG collected the blood samples and clinical data.

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**Correspondence to:** Tao Han, MD, PhD, Professor, Department of Hepatology, Tianjin Institute of Hepatobiliary Disease, Tianjin Key Laboratory of Artificial Cells, Tianjin Third Central Hospital, Tianjin Medical University, 83 Jintang Road, Tianjin 300170, China. [hantaomd@126.com](mailto:hantaomd@126.com)

Telephone: +86-22-84112298 Fax: +86-22-84112208

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### Abstract

**AIM:** To investigate whether serum thymosin  $\beta$ 4 can provide diagnostic or prognostic information in liver failure patients caused by chronic hepatitis B virus (HBV) infection.

**METHODS:** Serum thymosin  $\beta$ 4 levels were measured in 30 patients with acute-on-chronic liver failure (ACLF), 31 patients with chronic liver failure (CLF), 30 patients with compensated liver cirrhosis (CR) and 32 patients with chronic hepatitis B and 30 healthy controls. Serum thymosin  $\beta$ 4 levels were measured by enzyme-linked immunosorbent assay and Child-Pugh and model for end-stage liver disease (MELD) scores were calculated for each patient on admission.

**RESULTS:** Compared with healthy controls, serum thymosin  $\beta$ 4 levels in ACLF, CLF, CR and chronic hepatitis B patients were significantly lower, 6.5047 (4.7879-10.5314)  $\mu$ g/mL vs 0.4632 (0.2759-0.8768)  $\mu$ g/mL, 0.6981 (0.5209-1.2008)  $\mu$ g/mL, 1.8053 (0.8110-2.3397)  $\mu$ g/mL, 3.7803 (1.8570-6.4722)  $\mu$ g/mL, respectively ( $P < 0.001$ ). The levels of thymosin  $\beta$ 4 in liver failure (ACLF or CLF) patients were markedly lower than that in CR ( $P < 0.001$ ), and a difference was also found between CLF and ACLF patients ( $P = 0.038$ ). In patients with chronic liver disease, there was a positive relationship between thymosin  $\beta$ 4 levels and albumin, choline esterase, and platelet ( $P < 0.001$ ), and negative relationship with alanine aminotransferase ( $P = 0.020$ ), aspartate aminotransferase, total bilirubin, international normalized ratio of prothrombin time, and Child-Pugh and MELD scores ( $P < 0.001$ ). Of the 61 liver failure patients, the thymosin  $\beta$ 4 levels of non-survivors were significantly lower than that of survivors ( $P = 0.007$ ). Receiver operating characteristics analysis identified a thymosin  $\beta$ 4 cutoff level of 0.5708  $\mu$ g/mL for predicting poor prognosis in all liver failure patients. The serial thymosin  $\beta$ 4 values were observed in 13 liver failure inpatients. Lower initial values were observed in the death. While greater improvement in thymosin  $\beta$ 4 value was found in those who recovered from the disease.

**CONCLUSION:** Serum thymosin  $\beta$ 4 can be used as an important potential predictor for liver failure caused by chronic HBV infection.

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**Key words:** Thymosin  $\beta$ 4; Liver failure; Serum; Hepatitis B virus; Biochemistry

**Peer reviewers:** Dr. Richard Parker, MB, ChB, MRCP (London), Department of Gastroenterology, University Hospital of North Staffordshire, Newcastle Road, Stoke on Trent, North Staffordshire, ST4 6QG, United Kingdom; Christopher O'Brien, MD, Professor of Clinical Medicine, Chief of Clinical Hepatology,

Center for Liver Diseases, Divisions of Liver and GI Transplantation, University of Miami School of Medicine, 1500 Northwest 12th Ave., Suite #1101, Miami, FL 33136, United States

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## INTRODUCTION

Chronic hepatitis B virus (HBV) infection remains one of the most challenging public health problems in Asia. Acute-on-chronic liver failure (ACLF) and chronic liver failure (CLF) are common serious conditions with a high mortality among chronic HBV infected patients. The prognosis is clearly related to the stage of the disease and early diagnosis has resulted in a significant reduction in mortality<sup>[1-4]</sup>. Unfortunately, a differential diagnosis from non-liver failure patient is sometimes very difficult to make since the current biomarkers used in clinical diagnosis are still lack of sensitivity and reliability<sup>[5,6]</sup>. Thus, it is very important to discover new biomarkers for liver failure diagnosis.

Thymosin  $\beta$ 4 is a 4.9-kDa polypeptide widely distributed in human tissues, which is considered as the major G-actin sequestering protein<sup>[7-9]</sup>. Thymosin  $\beta$ 4 has been shown to have multiple biological activities involved in a variety of physiologic and pathologic processes. For example, thymosin  $\beta$ 4 is known to promote wound healing, tumor metastasis and angiogenesis<sup>[10-12]</sup>. Recently thymosin  $\beta$ 4 has been found to stimulate cardiac cell migration and survival related to cardiac repair. It was also considered to play an important role in healing hypoxic injury in the heart by preventing apoptotic cell death of cardiomyocytes and reducing scarring<sup>[13]</sup>, meanwhile, hypoxia and oxidative stress also play a key role in the pathophysiology of liver failure. Recent findings suggested that thymosin  $\beta$ 4 could be beneficial for the treatment of chronic liver disease (CLD)<sup>[14]</sup>. These data have attracted more consideration of whether thymosin  $\beta$ 4 plays an important role in liver failure. However, the levels of thymosin  $\beta$ 4 in liver failure patients are still unknown.

This present study aims to determine serum thymosin  $\beta$ 4 levels in CLD patients with HBV infection, and to assess the potential usefulness of thymosin  $\beta$ 4 in diagnosis and prognosis prediction of HBV-related liver failure, including ACLF and CLF.

## MATERIALS AND METHODS

### Patients

The study included 61 patients with liver failure (44 males, 17 females), of whom 30 (23 males, 7 females) were diagnosed as having ACLF, the other 31 (21 males,

10 females) patients as having CLF. And 30 (20 males, 10 females) patients with Child-Pugh A cirrhosis, and 32 (26 males, 6 females) patients with chronic hepatitis B were also enrolled. All patients had chronic HBV infection. Those with concurrent hepatocellular carcinoma (HCC), splenectomy, fatty liver, and hepatitis C or alcohol-related liver diseases were excluded. ACLF was defined as acute deterioration of liver function within 4 wk in CLD, with severe jaundice [serum total bilirubin (TBIL)  $\geq 171 \mu\text{mol/L}$  or an increase of TBIL  $\geq 17.1 \mu\text{mol/L}$  per day] and coagulopathy [international normalized ratio of prothrombin time (INR)  $> 1.5$  or PTA  $< 40\%$ ]. CLF was defined as chronic decompensation of an end-stage liver disease, complicated with refractory ascites and/or encephalopathy and hepatorenal syndrome<sup>[3]</sup>. The diagnosis of liver cirrhosis (CR) was based on the clinical manifestation, physical examination, biochemical, endoscopic and ultrasound findings and/or liver biopsy, with features of liver fibrosis, portal hypertension and hypersplenism. All patients infected with HBV were recruited from January to July 2009 at Tianjin Third Central Hospital. Thirty healthy volunteers served as controls, including 20 males and 10 females, aged 41 (31-49) years [median (inter-quartile range, IQR)].

The study was approved by Tianjin Third Central Hospital Ethics Committee. Informed consent was obtained from either the patients or their immediate family members.

### Collection of clinical data

INR, platelets (PLT), serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), choline esterase (CHE), albumin (ALB), TBIL and renal function were tested. Child-Pugh and model for end-stage liver disease (MELD) scores were calculated on admission.

### Measurement of serum thymosin $\beta$ 4 levels

All the samples were collected in the morning when the patients were admitted to our hospital. Blood collected without anticoagulant was spun at  $2600 \times g$  for 15 min, and the serum obtained was immediately stored at  $-80^\circ\text{C}$  until thymosin  $\beta$ 4 measurement. Thymosin  $\beta$ 4 was measured with a newly developed commercial enzyme-linked immunosorbent assay (ELISA) (Immunodiagnostik AG, Bensheim, Germany). The test principle was based on a competition between antigen in the sample or standards and the antigen coated on the wells of microplate. A peroxidase-conjugated antibody was used for detection and quantification, and tetramethylbenzidine as a peroxidase substrate. The enzymatic reaction was terminated by acidic stop solution. The results were obtained by measuring the absorbance at 450 nm in an ELISA reader.

### Statistical analysis

Kruskal-Wallis and Mann-Whitney *U* tests were used. Correlations were analyzed by Spearman rank test. Results were expressed as median and IQR. Cutoff values for the identification of non-survivors with liver failure (ACLF

Table 1 Clinical characteristics of the patients [median (IQR)]

	ACLF	CLF	CR	Chronic hepatitis B	Healthy subjects
<i>n</i>	30	31	30	32	30
Age (yr)	54 (48-61)	50 (46-55)	50 (37-55)	39 (31-45)	41 (31-49)
Gender (M:F)	23:7	21:10	20:10	26:6	20:10
ALB (g/L)	30.9 (26.3-33.2)	25.7 (22.6-28.9)	42.2 (38.4-45.7)	46.7 (42.0-49.1)	39.3 (38.1-41.1)
ALT (IU/L)	174.0 (104.3-653.0)	38.0 (27.5-49.5)	35.5 (24.0-52.8)	57.5 (25.0-75.5)	30.5 (25.1-34.7)
AST (IU/L)	274.5 (74.0-521.0)	34.0 (21.0-71.0)	19.0 (9.3-32.3)	15.0 (10.0-20.5)	23.3 (19.2-31.3)
TBIL ( $\mu$ mol/L)	409.7 (208.1-474.7)	170.9 (98.2-215.2)	14.3 (12.5-18.1)	15.1 (12.7-20.5)	9.7 (8.75-13.2)
CHE	2699 (1760-3286)	1586 (1129-2035)	3526 (3287-4529)	4938 (4168-5465)	6784 (6553-7463)
INR	2.29 (1.94-2.70)	2.52 (2.27-2.85)	1.08 (1.01-1.13)	1.02 (1.01-1.04)	1.01 (0.95-1.02)
PLT	72.5 (44.5-104.0)	44.0 (30.5-79.0)	89.0 (78.0-99.8)	197.5 (178.0-225.0)	197.5 (187.0-222.3)
MELD	23.75 (19.81-29.55)	22.97 (19.16-27.98)	3.52 (2.39-5.23)	2.65 (1.72-4.44)	2.65 (2.05-3.70)
Child-Pugh score	11 (10-12)	12 (11-12)	6 (5-7)	5 (5-6)	5 (5-6)

ACLF: Acute-on-chronic liver failure; CLF: Chronic liver failure; CR: Cirrhosis; ALB: Albumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; CHE: Cholinesterase; INR: International normalized ratio of prothrombin time; PLT: Platelet; MELD: Model for end-stage liver disease.

Table 2 Correlation coefficients (*r*) and *P* values of thymosin  $\beta$ 4 values *vs* biochemical parameters in chronic liver disease patients

Characteristics	<i>r</i> value	<i>P</i> value
ALB	0.536	< 0.001
ALT	-0.210	0.020
AST	-0.553	< 0.001
TBIL	-0.581	< 0.001
CHE	0.656	< 0.001
INR	-0.605	< 0.001
PLT	0.541	< 0.001
Child-Pugh score	-0.629	< 0.001
MELD	-0.587	< 0.001

and CLF) and survivors were determined using the receiver operating characteristic (ROC) analysis. A *P* value of < 0.05 was considered to be statistically significant. All analyses were performed with SPSS 13.0 software.

## RESULTS

### Group comparison

The clinical characteristics of CLD patients in the study are listed in Table 1.

Thymosin  $\beta$ 4 levels in the groups of ACLF, CLF, CR, chronic hepatitis B (CHB) and healthy controls are shown in Figure 1. Patients in ACLF, CLF, CR and CHB groups had significantly lower median (IQR) thymosin  $\beta$ 4 levels than healthy controls, 0.4632 (0.2759-0.8768)  $\mu$ g/mL, 0.6981 (0.5209-1.2008)  $\mu$ g/mL, 1.8053 (0.8110-2.3397)  $\mu$ g/mL, 3.7803 (1.8570-6.4722)  $\mu$ g/mL *vs* 6.5047 (4.7879-10.5314)  $\mu$ g/mL, respectively (*P* < 0.001). The thymosin  $\beta$ 4 in ACLF and CLF patients was markedly reduced as compared with that in CR patients (*P* < 0.001). A difference was also found between ACLF and CLF (*P* = 0.038).

### Relationship between thymosin $\beta$ 4 and biochemical parameters in liver failure patients

Table 2 shows the relationship between thymosin  $\beta$ 4 values and biochemical parameters in CLD patients

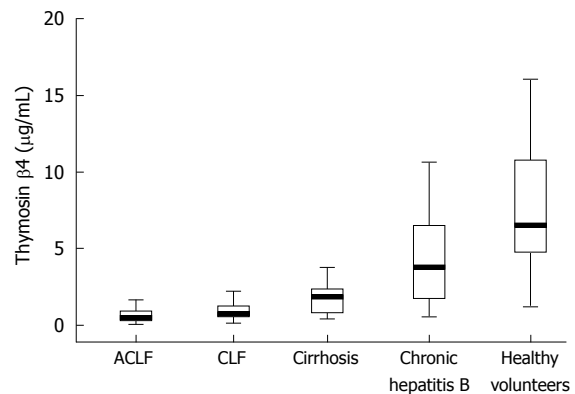


Figure 1 Comparison of thymosin  $\beta$ 4 levels ( $\mu$ g/mL) between ACLF, CLF, cirrhosis, chronic hepatitis B patients and healthy volunteers. ACLF: Acute-on-chronic liver failure; CLF: Chronic liver failure.

(including ACLF, CLF, CR and CHB). There was a positive relationship between thymosin  $\beta$ 4 levels and ALB, CHE, and PLT (*P* < 0.001). And in CLD patients, thymosin  $\beta$ 4 was negatively correlated with ALT (*r* = -0.210, *P* = 0.020), AST (*r* = -0.553, *P* < 0.001), TBIL (*r* = -0.581, *P* < 0.001), INR (*r* = -0.605, *P* < 0.001), Child-Pugh (*r* = -0.629, *P* < 0.001) and MELD scores (*r* = -0.587, *P* < 0.001).

### Analysis of liver failure group

Among the 61 liver failure patients, the survival rate was 63.93% (39 patients), while the non-survival rate was 36.07% (22 patients). The thymosin  $\beta$ 4 level of non-survivors was significantly lower than that of the survivors (*P* = 0.007) (Table 3). ROC analysis identified a thymosin  $\beta$ 4 cutoff value of 0.5708  $\mu$ g/mL [area under the ROC (AUROC) 0.710] with a sensitivity of 64.1% and a specificity of 68.2% for predicting poor prognosis in all liver failure patients (Figure 2A).

To further investigate the prediction value of thymosin  $\beta$ 4 for different liver failure status, the 61 patients were divided into two groups: CLF and ACLF. Table 3 shows the median (IQR) thymosin  $\beta$ 4 values of the two groups. In the ACLF group, ROC analysis identified a thymosin



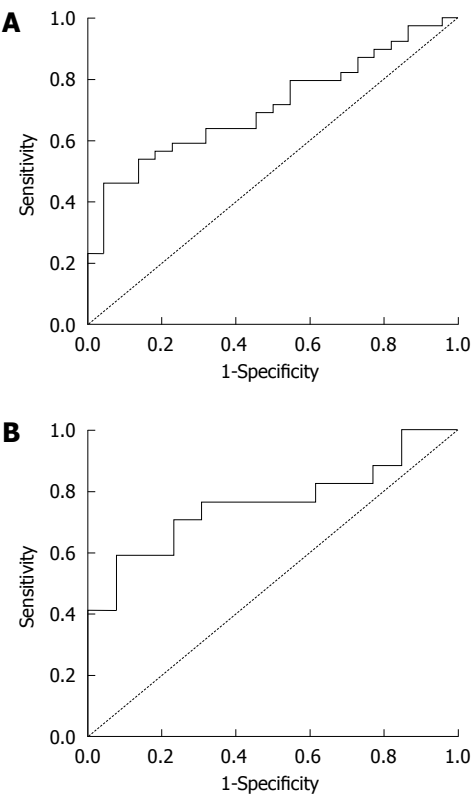


Figure 2 Receiver operating characteristics curve. A: Liver failure patients; B: ACLF patients.

Table 3 Comparison of thymosin $\beta$ 4 levels between survivors and non-survivors in liver failure group [median (IQR)]				
Group	n	Survivors	Non-survivors	P value
LF	61	0.8144 (0.3979-1.1510)	0.4699 (0.2771-0.6658)	0.007
ACLF	30	0.8144 (0.4042-1.0998)	0.3656 (0.2291-0.4458)	0.016
CLF	31	0.8329 (0.4306-1.4280)	0.6581 (0.5603-0.7713)	0.317

Table 4 Median (IQR) thymosin $\beta$ 4 values ( $\mu$ g/mL) in 13 liver failure patients					
Group	1 wk	2 wk	3 wk	4 wk	5 wk
Survivors	0.9402	1.2146	1.2762	1.2226	1.5486
Non-survivors	0.5458	0.6765	0.5056	0.3986	0.3488

$\beta$ 4 cutoff value of 0.3873  $\mu$ g/mL (AUROC 0.760) with a sensitivity of 76.5% and a specificity of 69.2 % for predicting poor prognosis (Figure 2B). In CLF group, lower thymosin  $\beta$ 4 levels were observed in non-survivors, but there was no statistically significant difference between the survivors and non-survivors of CLF group. Figure 3 shows the distribution of thymosin  $\beta$ 4 levels in the survivors and non-survivors with liver failure in the groups of ACLF and CLF.

**Dynamic changes of serum thymosin  $\beta$ 4 values in liver failure patients**

In this study, 13 liver failure patients were selected to

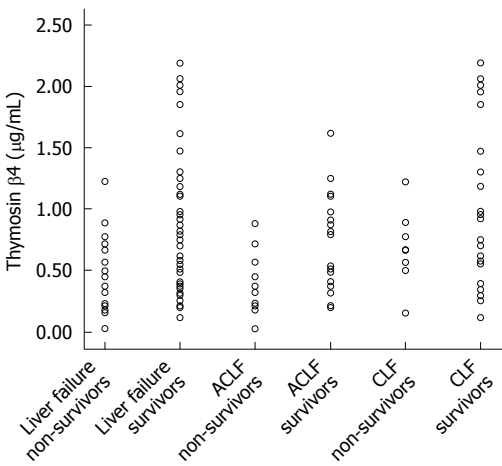


Figure 3 Thymosin  $\beta$ 4 levels in non-survivors and survivors of liver failure patients.

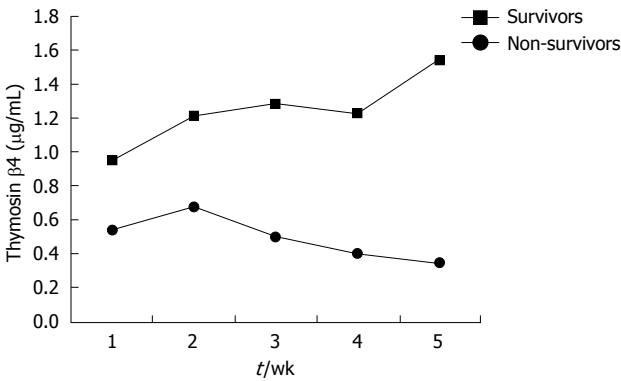


Figure 4 Median (inter-quartile range, IQR) thymosin  $\beta$ 4 values ( $\mu$ g/mL) observed in 13 liver failure patients.

observe the dynamic changes of their serum thymosin  $\beta$ 4 values. Among them, 6 patients survived and the other 7 patients died. Blood samples were taken from these patients every week during their hospitalization. The median thymosin  $\beta$ 4 concentrations of non-survivors were significantly lower than those of survivors ( $P < 0.001$ , Table 4). And greater improvement in thymosin  $\beta$ 4 values was observed in those who recovered than in the non-survivors (Figure 4). Figure 5 shows the serial thymosin  $\beta$ 4 changes in 6 typical liver failure patients of the 13 patients.

**DISCUSSION**

In this study, we found that serum thymosin  $\beta$ 4 levels were significantly lower in patients with chronic hepatitis B infection. The magnitude of reduction of thymosin  $\beta$ 4 was closely related to the severity of the hepatic injury. Serum thymosin  $\beta$ 4 concentrations were most significantly decreased in ACLF and CLF. Dynamic changes of serum thymosin  $\beta$ 4 values could reflect the recovery or death in some liver failure patients.

Liver failure occurs in the hepatocytes with extensive injury, which represents either a failure to regenerate after

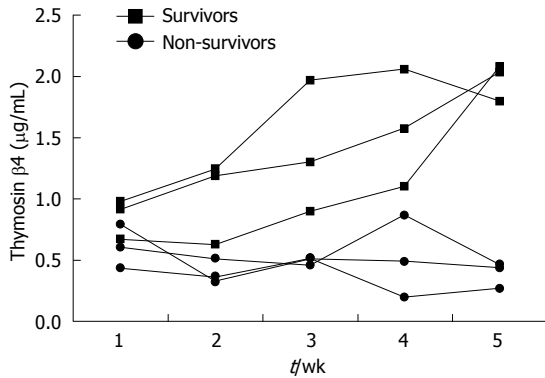


Figure 5 Serial thymosin  $\beta$ 4 concentrations in 6 patients with liver failure.

accelerated destruction of hepatocytes from necrosis or apoptosis<sup>[15]</sup>. As a result, surviving hepatocytes cannot maintain adequate metabolic functions. ALB and CHE are synthesized by hepatocyte, and their content can directly reflect the state of liver function. In this study, the levels of thymosin  $\beta$ 4 in CLD patients had a significantly positive correlation with ALB and CHE. In addition, a negative correlation between serum levels of thymosin  $\beta$ 4 and other parameters (INR, ALT, AST and TBIL) was found, which reflected the severity of acute hepatocellular injury in patients with liver failure. There was a strong correlation between composite scores of CLF (Child-Pugh and MELD scores) and thymosin  $\beta$ 4. Thymosin  $\beta$ 4 significantly decreased in ACLF and CLF patients. These results suggested that serum thymosin  $\beta$ 4 can be used as an important potential predictor of liver failure caused by chronic HBV infection.

Liver failure occurs when the extent of hepatocyte death exceeds the liver's regenerating capacity. Liver regeneration is considered to be suppressed in liver failure. Hepatocyte growth factor (HGF) is a promising therapeutic agent for the treatment of liver failure. HGF not only stimulates liver regeneration, but also acts as an antiapoptotic factor in experimental liver failure models<sup>[16]</sup>. Recent findings showed that thymosin  $\beta$ 4 upregulates the expression of HGF and downregulates the expression of PDGF- $\beta$  receptor in human hepatic stellate cells<sup>[14]</sup>. HGF could induce apoptosis of hepatic stellate cells and hepatocyte regeneration<sup>[17,18]</sup>. So it is conceived that thymosin  $\beta$ 4 protects the liver from injury. The study demonstrated that thymosin  $\beta$ 4 level was significantly lowered in liver failure patients, suggesting that thymosin  $\beta$ 4 might become a new therapeutic agent for liver failure caused by chronic HBV infection. Of course, more investigations will be needed to answer this question.

In addition, thymosin  $\beta$ 4 promotes wound healing and modulates inflammatory mediators in different tissue injury<sup>[19-21]</sup>. Thymosin  $\beta$ 4 reduces lethality and down-regulates inflammatory mediators in endotoxin-induced septic shock<sup>[22]</sup>. Liver failure is a systemic inflammatory reaction, which is characterized by a predominantly proinflammatory cytokine profile, causing the transition from stable clinical condition to severe deterioration in

liver function. So it is considered that the more severe the liver inflammatory reaction is, the lower level of thymosin  $\beta$ 4. In this study, thymosin  $\beta$ 4 significantly decreased in liver failure patients.

In this study, we also found that serum thymosin  $\beta$ 4 was an attractive parameter in the assessment of prognosis for liver failure patients. Thymosin  $\beta$ 4 levels were markedly lower in the non-survivors than the survivors in liver failure cohorts. ACLF group had the same result with the liver failure group. A trend toward higher thymosin  $\beta$ 4 levels was seen in survivors as compared with non-survivors of CLF patients. A possible explanation for the absence of a statistically significant difference could relate to heterogeneity in complications of CLF. In addition, the relatively small number of patients may not permit small differences to be detected. Dynamic changes of thymosin  $\beta$ 4 concentration may further help determine the prognosis of liver failure patients.

In conclusion, our study has demonstrated a clear relationship between reductions in serum thymosin  $\beta$ 4 level and severity of liver failure. Serum thymosin  $\beta$ 4 level could be used as an important potential marker for predicting the prognosis of HBV-related liver failure (ACLF and CLF) patients. Further investigations including comparison with Child-Pugh or MELD scores are needed to assess the thymosin  $\beta$ 4 value and explore the mechanism of lower thymosin  $\beta$ 4 levels in liver failure caused by chronic HBV infection. More studies in different etiologies and a larger number of subjects of liver failure should also be considered.

## COMMENTS

### Background

Chronic hepatitis B virus (HBV) infection remains one of the most challenging public health problems in Asian region. Acute-on-chronic liver failure (ACLF) and chronic liver failure (CLF) are common serious conditions with a high mortality among chronic HBV infected patients. The prognosis is clearly related to the stage of the disease and early diagnosis has resulted in a significant reduction in mortality. This study aims to discover a new biomarker for diagnosing liver failure caused by chronic HBV infection.

### Research frontiers

Thymosin  $\beta$ 4 is a 4.9-kDa polypeptide widely distributed in human tissues, which is considered as the major G-actin sequestering protein. Thymosin  $\beta$ 4 has been shown to have multiple biological activities involved in a variety of physiologic and pathologic processes. Recent findings suggested that thymosin  $\beta$ 4 could be beneficial for the treatment of chronic liver diseases (CLD). However, the levels of thymosin  $\beta$ 4 in liver failure patients are still unknown.

### Innovations and breakthroughs

In this study, the authors examined the serum thymosin  $\beta$ 4 level in the HBV-related CLD patients and investigated the relationship between thymosin  $\beta$ 4 values and biochemical parameters and observed the dynamic changes of serum thymosin  $\beta$ 4 in liver failure patients. The results suggested that a lower serum thymosin  $\beta$ 4 level could be a potential marker for predicting HBV-related liver failure (ACLF and CLF) prognosis.

### Applications

Serum thymosin  $\beta$ 4 level could become an important potential marker for liver failure diagnosis and is helpful in predicting the prognosis of patients with liver failure caused by chronic HBV infection.

### Terminology

ACLF was defined as acute deterioration of liver function within 4 wk in

CLD, with severe jaundice and coagulopathy. CLF was defined as chronic decompensation of an end-stage liver disease, complicated with refractory ascites and/or encephalopathy, and hepatorenal syndrome.

### Peer review

This study is an interesting piece of work investigating whether serum thymosin  $\beta$ 4 can provide diagnostic or prognostic information in liver failure patients caused by chronic HBV infection.

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## Clinical and endoscopic analysis of gastric Dieulafoy's lesion

Yi-Juan Ding, Liang Zhao, Jun Liu, He-Sheng Luo

Yi-Juan Ding, Liang Zhao, Jun Liu, He-Sheng Luo, Department of Gastroenterology, Renmin Hospital of Wuhan University, Jiefang Road 238, Wuhan 430060, Hubei Province, China  
 Author contributions: Ding YJ and Luo HS designed the research; Ding YJ, Zhao L and Liu J performed the research; Ding YJ and Zhao L analyzed the data and wrote the paper.  
 Correspondence to: Liang Zhao, PhD, Department of Gastroenterology, Renmin Hospital of Wuhan University, Jiefang Road 238, Wuhan 430060, Hubei Province, China. [airman-zhao@tom.com](mailto:airman-zhao@tom.com)  
 Telephone: +86-27-88041911-82135 Fax: +86-27-88041911  
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**Peer reviewer:** Mitsuhiro Fujishiro, MD, PhD, Associate Professor, Department of Endoscopy and Endoscopic Surgery, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan

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### Abstract

**AIM:** To investigate the incidence, location, clinical presentation, diagnosis and effectiveness of endoscopic treatment of gastric Dieulafoy's lesion (DL) in China.

**METHODS:** All patients who received emergency upper gastrointestinal (GI) endoscopy due to gastric DL from February 2000 to August 2008 at GI endoscopy center of Renmin Hospital of Wuhan University were included in this study. The clinical presentation, medical history, location and characteristics of DL methods and effectiveness of therapy of patients with DL were retrospectively analysed by chart reviews. Long-term follow-up data were collected at outpatient clinics or telephone interviews.

**RESULTS:** Fifteen patients were diagnosed with DL, which account for 1.04% of the source of bleeding in acute non-variceal upper GI bleeding. Common comorbidities were found in one patient with hypertension and diabetic mellitus. Hemoclip or combined therapy with hemoclip produced primary hemostasis in 92.8% (13/14) of patients.

**CONCLUSION:** DL is uncommon but life-threatening in China. Hemoclip proved to be safe and effective in controlling bleeding from DL.

### INTRODUCTION

Dieulafoy's lesion (DL), an abnormal arterial lesion in the digestive tract, was first described by Gallard in 1884, then designated by George Dieulafoy in 1898 and named for this French surgeon<sup>[1]</sup>. It refers to a submucosal "caliber-persistent" artery which protrudes through a minute 2-5 mm mucosal defect, which fails to diminish to the minute size of the mucosal capillary microvasculature and becomes elongated with a diameter 10 times that of the normal arteries at the same level<sup>[2,3]</sup>.

The most common and classic location of this lesion is in the proximal lesser curvature within 6 cm of the gastro-esophageal junction. However, uncommon occurrences in other sites including esophagus, duodenum, Billroth II anastomoses after gastrectomy, jejunum, colon, and rectum have also been described<sup>[3-5]</sup>. The erosion of "caliber-persistent" artery in DL may lead to massive and serious gastrointestinal (GI) bleeding, which accounts for 1%-5% of the origin of acute non-variceal upper GI bleeding<sup>[6-8]</sup>. Endoscopic diagnosis may be very difficult especially on the first episode due to the minute size of the lesion and the intermittent nature of the related bleeding. A wide variety of endoscopy techniques including injection of epinephrine (EPI) and sclerosing substances, heater and Argon probe coagulation, and mechanical



procedures including endoscopic band ligation (EBL) and hemoclippping have been applied in the treatment of the lesion and achieve satisfactory hemostasis with low recurrence and complication rates<sup>[5-11]</sup>. However, no single modality has been proven superior to the others. The choice depends on the endoscopist's preference and experience, as well as the clinical setting. Recently, there has been a trend suggesting that hemoclippping and EBL may be the first-line choice, while endoscopic ultrasound (EUS) guided angiotherapy appears to be promising<sup>[12,13]</sup>.

Although studies on the management and long-term outcome of GI DL have been widely documented, gastric DL and its clinical features in Chinese patients is poorly understood. In this retrospective study, we investigate the incidence, location, clinical presentation, diagnosis, and effectiveness of endoscopy treatment on gastrointestinal DL in Renmin Hospital of Wuhan University, with the aim of presenting experience on diagnosis and treatment of DL in China.

## MATERIALS AND METHODS

All patients who underwent emergency upper GI endoscopy due to acute non-variceal upper GI bleeding from February 2000 to August 2008 at GI endoscopy center of Renmin Hospital of Wuhan University were included in this study. Diagnosis of DL was established based on the following published criteria: (1) active arterial spurting or micropulsatile streaming from a minute mucosa defect; (2) visualization of a protruding vessel with or without active bleeding within a minute mucosal defect within normal surrounding mucosa; and (3) densely adherent clot with a narrow point of attachment to a minute mucosal defect or normal appearing mucosa<sup>[6,8,12]</sup>. Hemodynamic instability was defined as meeting one or more of the following criteria: (1) systolic blood pressure < 100 mmHg and pulse rate > 100 bpm; (2) orthostatic change in systolic blood pressure > 20 mmHg; and (3) decrease in hemoglobin of at least 2 g/dL in 24 h and need for blood transfusion before endoscopy.

Endoscopies were performed with Olympus video-endoscope after hemodynamic support treatment including intravenous fluids and blood transfusion if necessary. Endoscopic therapy was performed immediately after diagnosis with informed consent and the choice of endoscopy technique were decided by the endoscopist performing the procedure. Local injection was performed with an EPI solution 1:10000 in 4-quadrant fashion. Thermocoagulation was performed with a 2.3 mm diameter heat probe (HP) set at 25-30 J/pulse. Hemoclips (MD-850, Olympus) were applied with a rotatable clip-fixing device (HX-6UR-1) by using an endoscope with a 3.2 mm diameter accessory channel. Primary hemostasis was defined as no further bleeding from the site of DL for 5 min before withdrawal of the endoscope or disappearance of the vessel. Omeprazole 40 mg twice daily was given intravenously to all patients after initial endoscopy for 5-7 d and stopped if there was no further bleeding; feeding started 3-5 d after initial endoscopy

if no recurrent bleeding occurred. Recurrent bleeding was defined as hemodynamic instability due to bleeding symptoms or requirement of blood transfusions more than 5 units or decrease in hemoglobin more than 3 g/dL within 48 h associated with a fresh adherent clot or active bleeding from the same site of DL found in the initial endoscopy. Another endoscopy was performed 72 h after the initial hemostatic procedure or earlier if recurrent bleeding occurred in all patients. Angiography and gel-foam embolism were applied if it was difficult to identify the location of bleeding or achieve hemostasis after repeat endoscopy and surgery intervention was not selected by surgeon. The clinical presentation, medical history, location and characteristics of DL, methods and effectiveness of therapy of those patients were retrospectively analyzed by chart review. Long-term follow-up data were collected at outpatient clinics or by telephone interviews. This study was approved by the Ethics Committee of Renmin Hospital of Wuhan University and was in accordance with the Declaration of Helsinki.

## RESULTS

Fifteen out of 1433 patients with acute non-variceal upper GI bleeding were diagnosed as having DL from February 2000 to August 2008 at GI endoscopy center of Renmin Hospital of Wuhan University, Wuhan, China, which accounted for 1.04% of the sources of bleeding in acute non-variceal upper GI bleeding. The clinical presentation and endoscopy diagnosis of DL are listed in Table 1. Endoscopy treatment and outcome of DL are listed in Table 2. The median age was 36.1 years, ranging from 11-80 years. Most patients presented with melena and/or hematemesis while 1 female, an 80-year-old patient presented (patient No. 10) with only hypotension without external signs of bleeding. She had a previous history of hypertension and diabetic mellitus. No other common comorbidity causes for DL was found in other patients. No patients were receiving anticoagulant therapy, platelet aggregation inhibitors (PAI), or non-steroidal anti-inflammatory drugs medication.

Eleven patients presented with hemodynamic instability and needed blood transfusion before endoscopy. Active bleeding was detected during first endoscopy in 13 patients. A nonbleeding visible vessel was detected in 1 patient, and a minute mucosal defect below an adherent clot was detected in 1 patient. Three patients failed to establish diagnosis of DL during first endoscopy and needed more than one endoscopy to achieve conclusive diagnosis and exact location of DL. The most frequent location was the fundus (11/15, 73.3%), followed by the subcardinal area (2/15, 13.3%), and proximal corpus (2/15, 13.3%). For hemostasis, three endoscopic techniques were used: (1) only EPI in 1 case; (2) only hemoclippping in 11 cases; (3) EPI plus hemoclippping in 1 case; and (4) EPI, heat probe plus hemoclip in 1 case. Primary hemostasis was achieved in 14 patients. No endoscopic complications were found. Recurrent bleeding was detected in 3 patients (patient No. 8, 10 and 11) within 30 h. Angiography and gel-foam embolism were applied to identify location and hemostasis

Table 1 Clinical presentation and endoscopy diagnosis of DL

Patient No.	Sex/age (yr)	Clinical presentation	Hemodynamic instability	Location	Stigmata (forrest)	Diagnosis at initial endoscopy
1	M/40	Melena/hematemesis	+	Subcardinal area (remnant stomach)	I a	+
2	M/60	Melena/hematemesis	+	Subcardinal area	I a	+
3	M/36	Melena	-	Fundus	II a	+
4	F/15	Melena/hematemesis	+	Fundus	I b	+
5	M/11	Melena/hematemesis	+	Fundus	I a	+
6	M/20	Hematemesis	+	Proximal corpus	I a	+
7	F/54	Melena/hematemesis	+	Fundus	I a	+
8	M/23	Melena/hematemesis	+	Proximal corpus	I a	-
9	F/43	Melena	-	Fundus	I a	+
10	F/80	Hypotension	+	Fundus	II b	-
11	M/25	Melena/hematemesis	+	Fundus	I a	+
12	M/35	Melena/hematemesis	+	Fundus	I a	+
13	M/25	Melena/hematemesis	+	Fundus	I a	-
14	M/47	Melena/hematemesis	+	Fundus	I a	+
15	M/28	Melena/hematemesis	+	Fundus	I a	+

I a: Spurting bleeding; I b: Oozing bleeding; II a: Nonbleeding visible vessel; II b: Minute mucosal defect below an adherent clot. For patients who were not diagnosed with DL during initial endoscopy, the location and stigmata refer to the findings during repeated endoscopy. DL: Dieulafoy's lesion.

Table 2 Endoscopy treatment and outcome of DL

Patient No.	Endoscopy treatment	Primary hemostasis	Relapse within 30 h	Treatment for relapse	Hemostasis for relapse	Follow-up (mo)
1	EPI + hemoclip	+	-	-	-	22
2	EPI + HP + hemoclip	+	-	-	-	10
3	Hemoclip	+	-	-	-	17
4	EPI	+	-	-	-	33
5	Hemoclip	+	-	-	-	28
6	Hemoclip	+	-	-	-	39
7	Hemoclip	+	-	-	-	6
8	Hemoclip	+	+	Surgery	-	8
9	Hemoclip	+	-	-	-	44
10	-	-	+	2nd: angiography embolism; 3th: hemoclip	+	41
11	Hemoclip	+	+	Angiography embolism	-	-(death)
12	Hemoclip	+	-	-	-	17
13	Hemoclip	+	-	-	-	6
14	Hemoclip	+	-	-	-	35
15	Hemoclip	+	-	-	-	21

EPI: Epinephrine; HP: Heat probe.

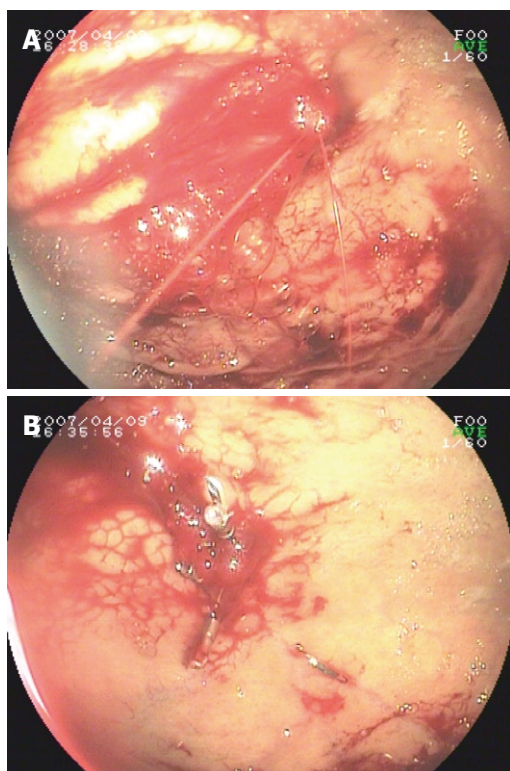
in patient No. 10 and No. 11 during the second bleeding. In patient No. 10, the source of bleeding was concluded to be located in the colon by angiography but colonoscopy failed to identify the source of bleeding in the colon. DL in the stomach was identified by gastroscopy during the third bleeding, while hemostasis was subsequently achieved by hemoclipping. Patient 11 died of recurrent bleeding and fever 1 d after embolization. For patient No. 8, a hemoclip was placed during the second endoscopy. Subsequently, surgical intervention was selected due to uncontrolled bleeding during the third episode. Multiple DL was identified at the same site during the initial endoscopy by emergency endoscopy during the operation. Hemostasis was achieved by oversewing at the location of DL. Typical bleeding of gastric DL and hemostasis after hemoclipping are shown in Figure 1A and B.

Follow-up was conducted in 14 patients, ranging from 6 to 44 mo after effective treatment. No recurrent bleeding was noted in all patients.

## DISCUSSION

In this study, we found that DL accounts for 1.04% (15/1433) of acute non-variceal upper GI bleeding according to an 8 years clinical investigation in a provincial general hospital in Middle China. This proportion, consistent with other previously published epidemiological series, indicates that DL appears not to be a common source of acute non-variceal upper GI bleeding in China<sup>[4,5,12,14,15]</sup>. On the other hand, 86.7% (13/15) patients with active bleeding and hemodynamic instability were diagnosed with DL, which suggests that DL should be fully recognized as a potentially severe cause of upper GI bleeding and a life-threatening GI emergency.

As some clinical and epidemiological data have previously suggested, DL usually affects elderly patients with significant comorbidities, including cardiovascular disease, respiratory disease, chronic renal failure, liver cirrhosis, neurological disease or medication, which influence blood



**Figure 1** Typical bleeding of gastric Dieulafoy's lesion (DL) in one male patient before (A) and after (B) hemoclippping.

coagulation<sup>[1,4,5,8,12,14,16]</sup>. It has been speculated that those systemic conditions may disturb normal angiogenesis, including the formation of aberrant caliber persistent vessels, thus increasing the incidence of DL. Our data detected only 2 patients > 60 years and 1 elderly patient with significant comorbidities including hypertension and diabetic mellitus. Having relatively few comorbidities in the present study may be attributed to the much younger mean ages of patients with DL, which seems not agree with those of previous studies. However, the actual incidence of DL in elderly patients may be underestimated in that (1) Elderly patients are more reluctant to undertake emergency endoscopy; (2) Concomitant systemic conditions in elderly patients may dramatically increase the risk of emergency endoscopy and compel clinicians to give up emergency endoscopy; and (3) Systemic conditions may obscure the manifestations of DL and decrease suspicion by clinicians. For example, melena or hematemesis was persistently absent in 1 elderly patient during 3 episodes of massive GI bleeding from DL in our study, which delayed the diagnosis of DL until repeat endoscopy was performed. Similar conditions may also occur in other elderly patients with DL, leading to failure of diagnosis.

Data on the distribution of DL in the GI tract showed that the majority of DL occurs in the stomach in the upper GI tract and is located in the region within 6 cm of the gastroesophageal junction<sup>[1,6,14,16,17]</sup>. In this study, the most frequent location of DL was also found at this classic site of DL. Therefore, this site should be highly suspicious and exhaustively explored during emergency

endoscopy in order to achieve an early diagnosis of DL. In our series, the first endoscopy failed to identify the location of DL in 3 patients. Failure to detect DL initially may be attributed to (1) intermittent bleeding and retracted vessels make DL invisible; (2) an excessive quantity of blood in stomach obscures the visual field; (3) alternative lesions give rise to GI bleeding already detected which prevents further detection of DL. In those special cases, repeat endoscopy is frequently necessary, and gastric lavage or position shifting may be needed for an accurate diagnosis.

Endoscopic therapy is currently advocated as a first-line choice for homeostasis of DL. A large variety of hemostasis techniques including injection of epinephrine or sclerosant, heater or Argon probe coagulation, elastic band ligation (EBL), hemoclip or a combination of those methods have been used for hemostasis of DL with permanent hemostasis achieved in more than 90% of patients<sup>[1,4-9,14-16,18-20]</sup>. Up to now, it has not been concluded that one single therapeutic modality is superior to another in well designed retrospective or randomized controlled trials<sup>[10,15,18]</sup>. The choice of technique should be individualized for each patient and depend on the type and location of DL, endoscopist's experience and competency, and the risk and complication of techniques. In the present study, hemoclip or combined therapy of EPI or HP followed by hemoclip achieved primary hemostasis in 92.8% (13/14) of patients. Recurrent bleeding occurred in only 1 patient with multiple DL during the second endoscopy. No recurrent bleeding occurred in long-term follow-up, demonstrating that the hemoclip is effective and safe for controlling bleeding from DL. According to our experience, hemoclippping has a special advantage in that (1) it is easier to perform technically than other techniques like EBL or sclerosis; (2) it causes less damage to the surrounding tissues, thus avoiding the possibility of necrosis or perforation caused by sclerosant injection or thermal coagulation; (3) it is effective for proximal gastric lesions with protruding vessels or active bleeding; and (4) it is an easy and safe method for controlling recurrent bleeding. Additionally, we found that use of EPI or HP before application of the hemoclip may achieve a better visual field in cases with profound bleeding, which will facilitate the application of the hemoclip.

Angiography and gel-foam embolism were applied to identify the location and hemostasis of DL in 2 patients with recurrent bleeding in our study. Bleeding from the colon as a false-positive result was concluded in 1 patient and death due to fever and profound bleeding immediately after gel-foam embolism occurred in another patient. Therefore, the accuracy and risk of this technique should be fully evaluated when choosing treatment for DL bleeding. Furthermore, bleeding from multiple DL was found in 1 patient during recurrent episodes of bleeding, which were controlled by surgical oversewing. Further explanation for this phenomenon is needed.

In conclusion, DL, as an uncommon cause, accounts for 1.04% of the instances of non-variceal upper GI bleeding in Middle China. Hemoclippping proved to be safe



and effective in controlling bleeding from DL. Further investigations are needed to accumulate more experience on endoscopic treatment for elderly patients with DL. Better options and approaches should be explored for identifying the location of DL which are difficult to define and refractory to routine endoscopy techniques.

## COMMENTS

### Background

Dieulafoy's lesion (DL) refers to submucosal "caliber-persistent" artery which fails to diminish to the minute size of the mucosal capillary microvasculature and becomes elongated with a diameter 10 times that of the normal arteries at the same level. The erosion of "caliber-persistent" arteries in DL may lead to massive and serious gastrointestinal (GI) bleeding, which accounts for 1%-5% of the origin of acute non-variceal upper GI bleeding. Endoscopy diagnosis may be very difficult especially on the first episode due to the minute size of the lesion and the intermittent nature of the related bleeding.

### Research frontiers

Endoscopy diagnosis may be very difficult especially on the first episode due to the minute size of the lesion and the intermittent nature of the related bleeding. A wide variety of endoscopy techniques including injection of epinephrine and sclerosing substances, heater and Argon probe coagulation, and mechanical procedures including endoscopic band ligation and hemoclippping have been applied in the treatment of the lesion and achieve satisfactory hemostasis with a low recurrence and complication rate. However, no single modality has been proven superior to the others.

### Innovations and breakthroughs

This study investigated the incidence, location, clinical presentation, and diagnosis of gastric Dieulafoy's lesion in a Chinese region and evaluated the options and effectiveness of endoscopic treatment for DL.

### Applications

This study indicated that DL is an uncommon cause of non-variceal upper GI bleeding in Middle China. Hemoclippping proved to be safe and effective in controlling bleeding from DL. Those results provide a background for further investigation for better options and approaches.

### Peer review

In the method section, the trends of selected endoscopic methods in the hospital should be described during the 8 years' study period, because almost all cases were treated by hemoclips.

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## Inhibition of allogeneic T-cell response by Kupffer cells expressing indoleamine 2,3-dioxygenase

Mao-Lin Yan, Yao-Dong Wang, Yi-Feng Tian, Zhi-De Lai, Lv-Nan Yan

Mao-Lin Yan, Yao-Dong Wang, Yi-Feng Tian, Zhi-De Lai, Lv-Nan Yan, Department of Hepatobiliary Surgery, Fujian Provincial Hospital, Fuzhou 350001, Fujian Province, China  
 Author contributions: Yan ML and Yan LN designed the study; Yan ML, Yan LN, Tian YF and Lai ZD performed the experiments and the data analysis; Yan ML wrote the manuscript; Wang YD and Yan LN revised the manuscript.  
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Correspondence to: Mao-Lin Yan, MD, Department of Hepatobiliary Surgery, Fujian Provincial Hospital, Fuzhou 350001, Fujian Province, China. [yanmaolin74@163.com](mailto:yanmaolin74@163.com)  
 Telephone: +86-591-87552554 Fax: +86-591-87552554  
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of 1-methyl-tryptophan and anti-FasL antibody. KC expressing IDO could induce allogeneic T-cell apoptosis.

**CONCLUSION:** In addition to Fas/FasL pathway, IDO may be another mechanism for KC to induce immune tolerance.

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**Key words:** Kupffer cell; FasL; Indoleamine 2,3-dioxygenase; T-cell proliferation

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### Abstract

**AIM:** To explore the possibility and mechanism of inhibiting allogeneic T-cell responses by Kupffer cells (KC) pretreated with interferon- $\gamma$  (IFN- $\gamma$ ) *in vitro*.

**METHODS:** The expressions of indoleamine 2,3-dioxygenase (IDO) mRNA and FasL mRNA in KC pretreated with IFN- $\gamma$  were studied with real-time polymerase chain reaction (PCR). The catabolism of tryptophan by IDO from KC was analyzed by high performance liquid chromatography. Allogeneic T-cell response was used to confirm the inhibition of KC *in vitro*. The proliferation of lymphocytes was detected using [ $^3$ H] thymidine incorporation. Cell cycle and lymphocyte apoptosis were evaluated by flow cytometric assay.

**RESULTS:** Real-time PCR revealed IDO mRNA and FasL mRNA expressions in KC pretreated with IFN- $\gamma$ , and IDO catabolic effect was confirmed by a decrease in tryptophan and increase in kynurenine concentration. KC expressing IDO and FasL in BABL/c mice acquired the ability to suppress the proliferation of T-cells from C57BL/6, which could be blocked by addition

### INTRODUCTION

The acceptance of the fetus during pregnancy by the mother is a successful model of tolerance against allogeneic tissues. The exact mechanism of immunosuppression in pregnancy will help develop novel therapeutic strategies in clinical transplantation. Indoleamine 2,3-dioxygenase (IDO) plays an important role in maintenance of maternal T-cell tolerance to fetal alloantigen, and IDO-specific inhibitor, 1-methyl-tryptophan (1-MT), can selectively reject allogeneic fetuses<sup>[1,2]</sup>, which was observed in liver allografts<sup>[3]</sup>. The mechanism of IDO immunosuppression may be the localized depletion of the essential amino acid tryptophan and formation of its metabolites<sup>[4-7]</sup>.

Kupffer cells (KC), act as effective antigen-presenting cells (APC) in the liver, can directly interact with passenger leukocytes and may play an important role in intrahepatic

immunoregulation. KC can regulate allogeneic T-cell response *in vitro* and *in vivo* by Fas/FasL pathway, but blocking anti-FasL antibody could not thoroughly suppress the effect of KC on T-cell proliferation<sup>[8]</sup>, and some other mechanisms may contribute to KC-dependent suppression. Many experiments<sup>[9,10]</sup> showed that APC, such as dendritic cells and monocyte-derived macrophages, could induce IDO expression by certain treatment and acquire the capacity to suppress T-cell proliferation.

In this study, we hypothesized that IDO expressed by KC was another mechanism of KC immunoregulation. To confirm this, we analyzed whether KC pretreated with interferon- $\gamma$  (IFN- $\gamma$ ) can be induced to express IDO and FasL, and the impact of KC expressing IDO and FasL on allogeneic T-cell response *in vitro*.

## MATERIALS AND METHODS

### Animals

Male BABL/c and C57BL/6 mice were purchased from the West China Animal Center and those aged 10-12 wk were used for the experiment.

### Lymphocyte and KC isolation

Spleens were harvested from male C57BL/6 mice and the lymphocyte (LC) was isolated as described previously<sup>[5]</sup>. KCs were isolated as described by Yan *et al.*<sup>[11]</sup>. This technique of cell isolation yielded 10-20 million KCs per liver on average, with a 92%-95% viability, as determined by trypan blue exclusion. KCs were determined by phagocytosed carbon bead and stained positively for nonspecific esterase.

### Relative quantitation for FasL mRNA and IDO mRNA expression

Total RNA of KC was extracted using TRIzol (Life Technologies, Rockville, USA) and subjected to reverse transcription polymerase reaction (RT-PCR) using the following primers: FasL 5'-GCACAGAAGGGAAGGAGTA-3' and 5'-CCAGGAGAATCGCAGTAGA-3'; IDO 5'-TATTGCTGTTCCCTACTG-3' and 5'-GGTCTTGACGCTCTACT-3'; GAPDH 5'-CCTCAAGATTGT-CAGCAAT-3' and 5'-CCATCCACAGTCTTCTGAGT-3'. And they yielded a fragment size of 163, 255 and 141 bp, respectively. Amplification was performed on an iCycler (Bio-Rad, Hercules, USA). For quantitative PCR, amplification was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, USA). Fluorescence was detected at 490 nm using the iCycler IQ real-time PCR (Bio-Rad, Hercules, USA). The Ct of negative samples was defined as 45. A comparative Ct method was used to quantitate IDO and FasL cDNA levels<sup>[12]</sup>. Melting curve analysis consisted of a denaturation step at 95°C for 1 min, lowered to 55°C for 30 s, and followed by 40 cycles of incubation, in which the temperature was raised to 95°C at a rate of 1°C/30 s per cycle with continuous reading of fluorescence. The size of amplification products was verified by agarose gel electrophoresis.

### Mixed leukocyte reaction

KC ( $2.0 \times 10^4$ ) from BABL/c was co-incubated with allogeneic spleen lymphocytes ( $4.0 \times 10^5$ ) from C57BL/6 in 0.2 mL RPMI1640 medium containing 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 mg/mL streptomycin. Positive controls consisted of KC pretreated with IFN- $\gamma$  (Cytolab Ltd., USA) for 24 h and allogeneic lymphocytes with anti-FasL antibody (MFL4; BD PharMingen, San Diego, CA) or 1-MT (Aldrich Chemical, Milwaukee, WI), or KC pretreated with IFN- $\gamma$  (200 U/mL) or without allogeneic lymphocytes, whereas negative controls included KC or lymphocytes only. These cells were cultured in a 37°C, 5% CO<sub>2</sub> incubator in 96-well flat bottom plates (COSTAR, Corning, NY, USA). After 5 d, [<sup>3</sup>H] thymidine (<sup>3</sup>H-TdR) (Inotech Biosystems, USA) was added and kept for 12 h and the number of counts per minute (cpm) was determined in a  $\beta$ -counter.

### High performance liquid chromatography for functional IDO expression

To verify functional IDO expression, tryptophan and kynurenine levels were measured in culture media. IDO activity was expressed as the concentration of kynurenine in the sample. KC was treated with IFN- $\gamma$  (200 U/mL) for 6 and 24 h in 200  $\mu$ L RPMI1640 containing 24.5  $\mu$ mol/L tryptophan. The supernatant was harvested and the quantity of kynurenine and tryptophan was determined by high performance liquid chromatography (HPLC) and UV detection (UV265 nm, Shimadzu, Japan). HPLC was performed according to Carlin *et al.*<sup>[13]</sup> with minor modifications.

Protein-free supernatant of 100  $\mu$ L was injected onto the column. Kynurenine and tryptophan concentrations in the supernatants were determined based on their peak heights relative to standard solutions obtained in the chromatograms. The standard solutions of kynurenine and tryptophan, purchased from Sigma, were prepared and kept fresh each day at concentrations ranging from 0 to 30  $\mu$ mol/L and submitted to precipitation with 30% trichloroacetic acid and incubation at 50°C for 30 min. Chromatography of the standards and the samples was performed under the same conditions.

### Flow cytometric analysis

KCs ( $1 \times 10^6$ ) were seeded and after an overnight incubation, they were pretreated with IFN- $\gamma$  (200 U/mL) for 24 h. T-cell alone was used as controls. KCs were cocultured with allogeneic T-cells (E/T = 1:1) for 24 h. T-cells were harvested and washed twice with 0.01 mol/L PBS and fixed with 70% ethanol for 2 h at 25°C. Finally, the cells were washed twice with PBS and stained with propidium iodide. The apoptosis of T-cells was analyzed by a flow cytometer (Beckman-Coulter, USA).

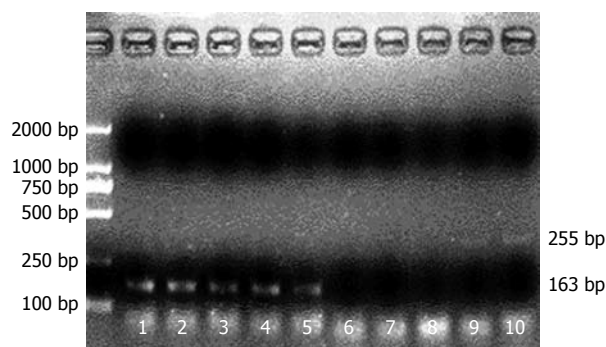
### Statistical analysis

The Chinese law on the Protection of Animals was followed. The data were presented as mean  $\pm$  SD. Parametric data was analyzed by Student's *t* test. *P* < 0.05 was considered statistically significant.

**Table 1** IDO mRNA expression of KC pretreated with IFN- $\gamma$  at different time points

Group	FasL Ct	IDO Ct	GAPDH Ct	FasL $\Delta$ Ct	IDO $\Delta$ Ct
0 h	45	45	19.23 $\pm$ 0.51	5.77 $\pm$ 0.51	25.77 $\pm$ 0.51
6 h	21.10 $\pm$ 0.52	25.67 $\pm$ 1.00	16.5 $\pm$ 0.61	4.60 $\pm$ 0.10 <sup>a</sup>	8.83 $\pm$ 0.95 <sup>a</sup>
24 h	21.07 $\pm$ 0.29	25.37 $\pm$ 0.23	16.57 $\pm$ 2.03	5.70 $\pm$ 0.10 <sup>a</sup>	9.47 $\pm$ 0.58 <sup>a</sup>
48 h		27.03 $\pm$ 0.21	17.1 $\pm$ 0.20		9.93 $\pm$ 0.15 <sup>a</sup>

<sup>a</sup> $P < 0.05$  vs KC group. IDO: Indoleamine 2,3-dioxygenase; KC: Kupffer cells; IFN- $\gamma$ : Interferon- $\gamma$ .



**Figure 1** Reverse transcription polymerase chain reaction (RT-PCR) shows indoleamine 2,3-dioxygenase (IDO) and FasL expression in Kupffer cells (KC) before and after activation with recombinant interferon- $\gamma$  (IFN- $\gamma$ ). RT-PCR showing FasL expression in KC before (lanes 1 and 2) and after activation for 6 h (lanes 3 and 4) or 24 h (lane 5) with IFN- $\gamma$ . RT-PCR showing IDO expression in KC before (lanes 6-8) and after activation for 6 h (lane 9) or 24 h (lane 10) with IFN- $\gamma$ .

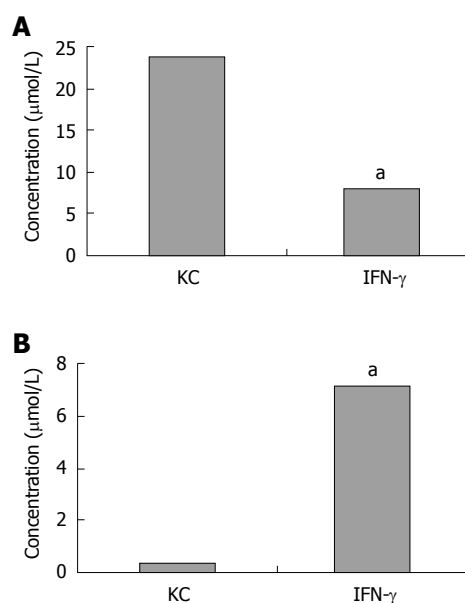
## RESULTS

### IFN- $\gamma$ induces the expression of IDO mRNA and FasL mRNA in KC

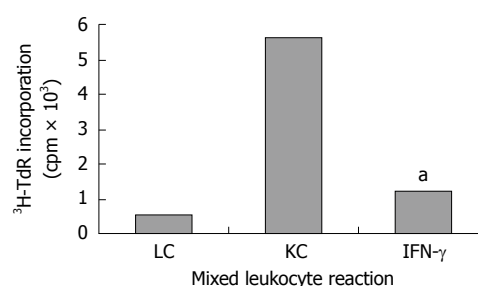
We measured KC FasL mRNA and IDO mRNA using real-time RT-PCR. Treatment of mouse KC with IFN- $\gamma$  resulted in the time-dependent induction of IDO and FasL. The results were expressed in terms of change in Ct values ( $\Delta$ Ct),  $\Delta$ Ct = average  $C_{t\text{specific gene}}$  - average  $C_{t\text{GAPDH}}$ . Because GAPDH is an abundant message, lower  $\Delta$ Ct values corresponded to a higher level of expression. Expression of FasL and IDO in KC pretreated with IFN- $\gamma$  was significantly decreased in comparison with KC without IFN- $\gamma$  pretreatment (Table 1). IDO and FasL expression was determined by RT-PCR in KCs before and after activation for 6 and 24 h with recombinant IFN- $\gamma$  (Figure 1).

### Tryptophan depletion of culture medium

To verify whether IDO induced by KC exerted its catabolic effect, tryptophan as well as kynurenine were measured by HPLC in KCs pretreated with or without IFN- $\gamma$  for 24 h. Tryptophan and kynurenine concentrations were determined using 1 mL of culture medium that was harvested from a 3.5 cm<sup>2</sup> culture dish. As expected, the kynurenine concentration significantly increased while tryptophan decreased. Tryptophan concentration in KC group and IFN- $\gamma$  group was 23.6  $\pm$  0.9  $\mu$ mol/L and 7.8  $\pm$  0.6  $\mu$ mol/L while kynurenine concentration was 0.3  $\pm$  0.08  $\mu$ mol/L and 7.1  $\pm$  1.4  $\mu$ mol/L, respectively (Figure 2).



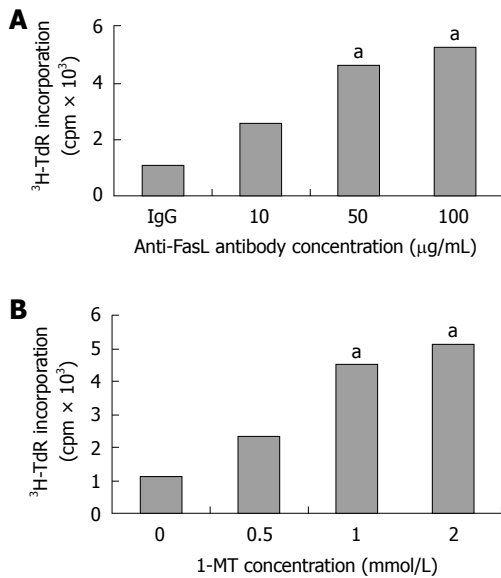
**Figure 2** Tryptophan (A) and kynurenine (B) concentration in cultures of KC. The kynurenine concentration significantly increased while tryptophan decreased in KC pretreated with IFN- $\gamma$  for 24 h. <sup>a</sup> $P < 0.05$  vs KC group.



**Figure 3** Reduction of allogeneic T-cell stimulation by IDO and FasL-expressing KC. Naive lymphocyte (LC), naive KC, KC pretreated with IFN- $\gamma$  were coincubated with allogeneic spleen T-cell for 5 d and cell proliferation measured by <sup>3</sup>H-TdR incorporation. <sup>a</sup> $P < 0.05$  vs KC group.

### Inhibition of allogeneic T-cell response by KCs expressing IDO and FasL

Whether KCs expressing IDO and FasL can inhibit the proliferation of allogeneic T-cells was confirmed in the experiment. KC pretreated with IFN- $\gamma$  for 24 h and naive KC were coincubated with allogeneic T-cells, respectively, the proliferation was determined by <sup>3</sup>H-TdR incorporation. KCs expressing IDO and FasL were significantly more effective than naive KC (Figure 3) in inhibiting the allogeneic T-cell proliferation ( $P < 0.05$ ).



**Figure 4** Anti-FasL antibody (A) or 1-methyl-tryptophan (1-MT) (B) enhances the proliferation of allogeneic T-cell stimulated with KC pretreated with IFN- $\gamma$ . <sup>a</sup>*P* < 0.05 vs control group.

#### 1-MT and anti-FasL antibody blocks the inhibitory effect of KC pretreated with IFN- $\gamma$

To confirm whether the inhibition of T-cell proliferation was due to the enzymatic activity of IDO, inhibitory tests were performed in the presence of 1-MT, anti-FasL antibody or IgG. The addition of 1-MT or anti-FasL antibody showed an augmented proliferative response in a dose-dependent manner as compared with the control group (Figure 4). These results indicate that the inhibitory effect of KC pretreated with IFN- $\gamma$  was induced by the IDO activity and Fas/FasL interaction.

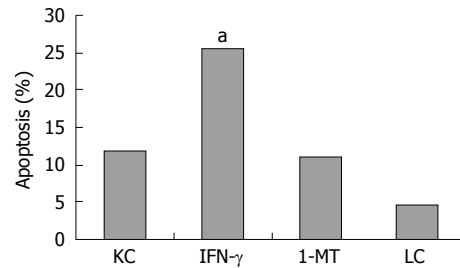
#### KCs induce allogeneic T-cell apoptosis

In order to clarify the underlying mechanism by which KC inhibits the proliferation of allogeneic T-cells, we confirmed that KC has the ability to induce apoptosis of allogeneic T-cells. The apoptosis rate of allogeneic T cells was 11.7%  $\pm$  1.2% in KC group, 25.53%  $\pm$  1.31% in IFN- $\gamma$  group, 10.9%  $\pm$  0.25% in 1-MT group and 4.53%  $\pm$  0.65% in LC group. Figure 5 shows that KC pretreated with IFN- $\gamma$  was more effective to induce T-cell apoptosis than naive KC, but the effect was abrogated by addition of 1-MT (1 mmol/L).

## DISCUSSION

KC pretreated with IFN- $\gamma$  can be induced to express IDO mRNA and FasL mRNA as shown in this study. KC expressing FasL and IDO can inhibit the proliferation of allogeneic T-cells and induce their apoptosis by increasing kynurenine and reducing tryptophan *in vitro*, which could be blocked by addition of 1-MT and anti-FasL antibody. These results are consistent with our hypothesis that IDO is another mechanism of KC immunoregulation.

The interaction between T-cell and KC has an important impact on antigen-specific immune response in



**Figure 5** KC induces apoptosis of allogeneic T-cell. The KC pretreated with IFN- $\gamma$  exhibit a significant allogeneic T-cell apoptosis. <sup>a</sup>*P* < 0.05 vs KC group.

the liver. The expansion of specifically activating T-cell requires the presence of KC, which holds a key position in regulation of hepatic immune response<sup>[14,15]</sup>. Apoptosis induced by Fas/FasL interactions have been proposed as a mechanism of immune privilege and peripheral tolerance<sup>[16]</sup>. Sun *et al*<sup>[8]</sup> identified that KC induced T-cell apoptosis and may be involved in the development of hepatic immune tolerance through Fas/FasL pathway. But the addition of anti-FasL antibody could not thoroughly block the inhibitory effect of KC on allogeneic T-cell proliferation. We propose that KC-induced T-cell apoptosis is dependent on the Fas/FasL pathway and IDO. IDO as well as FasL are known to be expressed in immune-privileged sites and believed to induce peripheral tolerance<sup>[11,2,17]</sup>. Our data showed that KC pretreated with IFN- $\gamma$  induced the expression of both FasL and IDO. The blockade of IDO activity by 1-MT would negate the induction of apoptosis *in vitro* by IFN- $\gamma$ -treated KC. This suggests that IDO induction is another mechanism by which IFN- $\gamma$  acts on KC to mediate apoptosis of T-cells.

Munn *et al*<sup>[7]</sup> confirmed that macrophages expressing IDO caused the lack of tryptophan in a cell-culture system. In the current series of experiments, the amount of tryptophan reduced by KC expressing IDO was not so dramatic as by macrophages. The phenomena were due to different activities of the IDO gene. In the presence of tryptophan, IDO-expressing KCs also suppressed the proliferation of allogeneic T-cells. So the concentration of tryptophan in culture may not be the only reason contributing to the effect. Some reports<sup>[4,6,18,19]</sup> have shown that tryptophan-derived catabolites, such as kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid and picolinic acid, also suppress the allogeneic T-cell responses *in vitro* and *in vivo*. The concentration of kynurenine in our study also increased after KCs were pretreated with IFN- $\gamma$ . Tryptophan metabolites may be another reason that IDO-expressing KCs inhibit the proliferation of allogeneic T-cells.

KCs expressing IDO and FasL may drive the differentiation of Th2/Th3. Th2 cells expressed higher levels of FAP-1 that inhibited Fas/FasL pathway and were not sensitive to FasL-induced apoptosis, but Fas/FasL pathway promoted the rapid death of Th1 cells and selective survival of Th2 cells<sup>[20]</sup>. It may be a mechanism for differential regulation of Th cells by KC. Furthermore, tryptophan metabolites, such as 3-hydroxykynurenine and quinolinic acid, can selectively induce apoptosis of



Th1 cells, but not Th2 cells<sup>[19]</sup>. It was associated with the activation of caspase 28 and cytochrome C released by mitochondrion without the participation of Fas/FasL interaction. KC pretreated with IFN- $\gamma$  may control the differentiation of Th cells by the two mechanisms.

In conclusion, our results showed that IDO expressed by KC is another mechanism associated with induction of allogeneic T-cell apoptosis in addition to Fas/FasL interaction.

## COMMENTS

### Background

The acceptance of the fetus during pregnancy by the mother is a successful model of tolerance against allogeneic tissue. Indoleamine 2,3-dioxygenase (IDO) plays an important role in maintenance of maternal T-cell tolerance to fetal alloantigens. The mechanism of IDO immunosuppression may be the localized depletion of tryptophan and formation of its metabolites. Kupffer cells (KC) plays an important role in intrahepatic immunoregulation and induction of tolerance by Fas/FasL pathway. But blocking the anti-FasL antibody could not thoroughly suppress the effect of KC on T-cell proliferation. IDO expressed by KC may be another mechanism of KC immunoregulation.

### Research frontiers

How to induce specific immune tolerance is a hot topic of research. Killing the transplantation antigen-activated T cells is an important way to induce specific immune tolerance. IDO plays an important role in inducing the apoptosis of antigen-activated T cells. The activation and apoptosis of T cell is the central link in the formation of immune tolerance, but the apoptosis requires the activation of antigen presenting cells in the liver mainly by KC. KC can regulate allogeneic T-cell response *in vitro* and *in vivo* by Fas/FasL pathway.

### Innovations and breakthroughs

The study found that KC pretreated with interferon- $\gamma$  (IFN- $\gamma$ ) could express IDO and FasL. KCs expressing IDO and FasL from BABL/c mice acquire the ability to suppress the proliferation of T-cells from C57BL/6, which could be blocked by 1-methyl-tryptophan and anti-FasL antibody. In addition to Fas/FasL pathway, IDO may be another mechanism in KC to induce immune tolerance.

### Applications

IDO may be another mechanism in KC to induce immune tolerance, indicating that KC may be the potential target for immune tolerance in liver transplantation.

### Terminology

IDO is a monomeric protein heme iron, which was found in rabbit intestinal tissues in 1967. IDO has been found widely expressed in the immune system, including the thymus, placenta, gastrointestinal mucosa, anterior chamber, epididymis and other immune tolerance or immunologically privileged sites, particularly in a number of inhibitory antigen-presenting cells. IDO is involved in the regulation of T-cell proliferation and apoptosis.

### Peer review

This is an interesting paper devoted to the role of IDO in KC in inhibiting allogeneic T-cell responses. In this study, the authors investigated whether KC pretreated with IFN- $\gamma$  can be induced to express IDO and FasL, and analyzed the impact of KC expressing IDO and FasL on allogeneic T-cell response *in vitro*. The study shows IDO is another mechanism of KC immune regulation. Overall, the study is interesting and significant, and the data are presented in a clear and logical manner.

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## DNA polymorphism and risk of esophageal squamous cell carcinoma in a population of North Xinjiang, China

Wen-Jing Ma, Guo-Dong Lv, Shu-Tao Zheng, Cong-Gai Huang, Qing Liu, Xing Wang, Ren-Yong Lin, Ilyar Sheyhidin, Xiao-Mei Lu

Wen-Jing Ma, Guo-Dong Lv, Shu-Tao Zheng, Cong-Gai Huang, Qing Liu, Xing Wang, Ren-Yong Lin, Xiao-Mei Lu, Medical Research Center, the First Affiliated Hospital, Xinjiang Medical University, Urumqi 830054, Xinjiang Uygur Autonomous Region, China

Ilyar Sheyhidin, Department of Thoracic Surgery, the First Affiliated Hospital, Xinjiang Medical University, Urumqi 830054, Xinjiang Uygur Autonomous Region, China

**Author contributions:** Ma WJ, Lv GD and Zheng ST performed the majority of experiments; Huang CG, Liu Q and Wang X provided vital reagents and analytical tools and were involved in editing the manuscript; Lin RY and Sheyhidin I coordinated in the collection of all the human material; Lu XM designed the study and wrote the manuscript in addition to providing financial support for this work.

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**Correspondence to:** Xiao-Mei Lu, Professor, Medical Research Center, the First Affiliated Hospital, Xinjiang Medical University, Urumqi 830054, Xinjiang Uygur Autonomous Region, China. [luxiaomei88@163.com](mailto:luxiaomei88@163.com)

Telephone: +86-991-4360051 Fax: +86-991-4360051

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### Abstract

**AIM:** To investigate the role of metabolic enzyme and DNA repair genes in susceptibility of esophageal squamous cell carcinoma (ESCC).

**METHODS:** A case-control study was designed with 454 samples from 128 ESCC patients and 326 gender, age and ethnicity-matched control subjects. Genotypes of 69 single nucleotide polymorphisms (SNPs) of metabolic enzyme (aldehyde dehydrogenase-2, *ALDH2*; alcohol dehydrogenase-1 B, *ADHB1*; *Cytochrome P450 2A6*, *CYP2A6*) and DNA repair capacity genes

(excision repair cross complementing group 1, *ERCC1*; *O*<sup>6</sup>-methylguanine DNA methyltransferase, *MGMT*; xeroderma pigmentosum group A, *XPA*; xeroderma pigmentosum group A, *XPD*) were determined by the Sequenom MassARRAY system, and results were analyzed using unconditional logistic regression adjusted for age, gender.

**RESULTS:** There was no association between the variation in the *ERCC1*, *XPA*, *ADHB1* genes and ESCC risk. Increased risk of ESCC was suggested in *ALDH2* for frequency of presence C allele of SNP [Rs886205: 1.626 (1.158-2.284)], *XPD* for C allele [Rs50872: 1.482 (1.058-2.074)], and *MGMT* for A allele [Rs11016897: 1.666 (1.245-2.228)]. Five variants of *MGMT* were associated with a protective effect on ESCC carcinogenesis, including C allele [Rs7069143: 0.698 (0.518-0.939)], C allele [Rs3793909: 0.653 (0.429-0.995)], A allele [Rs12771882: 0.719 (0.524-0.986)], C allele [Rs551491: 0.707 (0.529-0.945)], and A allele [Rs7071825: 0.618 (0.506-0.910)]. At the genotype level, increased risk of ESCC carcinogenesis was found in homozygous carriers of the *ALDH2* Rs886205 [CC vs TT, odds ratios (OR): 3.116, 95% CI: 1.179-8.234], *MGMT* Rs11016879 (AA vs GG, OR: 3.112, 95% CI: 1.565-6.181), Rs12771882 (AA vs GG, OR: 2.442, 95% CI: 1.204-4.595), and heterozygotes carriers of the *ALDH2* Rs886205 (CT vs TT, OR: 3.930, 95% CI: 1.470-10.504), *MGMT* Rs11016879 (AG vs GG, OR: 3.933, 95% CI: 2.216-6.982) and Rs7075748 (CT vs CC, OR: 1.949, 95% CI: 1.134-3.350), respectively. Three variants were associated with a protective effect on ESCC carcinogenesis, carriers of the *MGMT* Rs11016878 (AG vs AA, OR: 0.388, 95% CI: 0.180-0.836), Rs7069143 (CT vs CC, OR: 0.478, 95% CI: 0.303-0.754) and Rs7071825 (GG vs AA, OR: 0.493, 95% CI: 0.266-0.915). Increased risk of ESCC metastasis was indicated in *MGMT* for frequency of presence C allele [Rs7068306: 2.204 (1.244-3.906)], A allele [Rs10734088: 1.968 (1.111-3.484)] and C allele [Rs4751115: 2.178

(1.251-3.791)]. Two variants in frequency of presence C allele of *CYP2A6* [Rs8192720: 0.290 (0.099-0.855)] and A allele of *MGMT* [Rs2053139: 0.511 (0.289-0.903)] were associated with a protective effect on ESCC progression. Increased risk of ESCC metastasis was found in heterozygote carriers of the *MGMT* Rs7068306 (CG vs CC, OR: 4.706, 95% CI: 1.872-11.833).

**CONCLUSION:** Polymorphic variation in *ALDH2*, *XPD* and *MGMT* genes may be of importance for ESCC susceptibility. Polymorphic variation in *CYP2A6* and *MGMT* are associated with ESCC metastasis.

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**Key words:** Esophageal cancer; Metabolic enzyme gene; DNA repair gene; Carcinogenesis; Metastasis

**Peer reviewers:** En-Min Li, PhD, Professor, Department of Biochemistry and Molecular Biology, Medical College, Shantou University, 22nd Xinling Road, Shantou 515041, Guangdong Province, China; Dr. Thomas Wex, PhD, Clinic of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University Magdeburg, Leipziger Str. 44, 39120 Magdeburg, Germany

Ma WJ, Lv GD, Zheng ST, Huang CG, Liu Q, Wang X, Lin RY, Sheyhidin I, Lu XM. DNA polymorphism and risk of esophageal squamous cell carcinoma in a population of North Xinjiang, China. *World J Gastroenterol* 2010; 16(5): 641-647 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i5/641.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i5.641>

## INTRODUCTION

Esophageal cancer (EC) is one of the most common malignancies throughout the world, ranking the eighth in incidence and the sixth in mortality among tumors of all sites<sup>[1]</sup>. However, the incidence of EC varies extremely among different regions<sup>[1,2]</sup>. The high-risk areas for esophageal squamous cell carcinoma (ESCC) (with an incidence ranging from 15 to 150/100 000) included distinct areas of South America, and the so-called "Asian Esophageal Cancer Belt" from eastern Turkey, through Iraq, Iran and the southern former Soviet Union (Kazakhstan, Turkmenistan, Uzbekistan, Tajikistan) to Mongolia and Western/Northern China<sup>[3]</sup>. Residents in the Asian EC Belt have a 10-100 fold greater chance suffering from EC than those living in the neighboring areas. ESCC is the most frequent type of EC in Asia. More than half of all ESCC cases in the world occurred in China, in the southern parts of the Taihang mountains at the borders of Henan, Shanxi and Hebei Provinces (Lin xian/Linzhou and Anyang County in Henan, and Cixian in Hebei, which will be designated below as "Linxian area"), in northern Jiangsu (Huai'an county) and in northern Xinjiang (with age standardized rates of 90-150/100 000)<sup>[4,5]</sup>.

Epidemiological studies revealed that the incidence of ESCC is associated with environmental factors, such as tobacco smoking, alcohol consumption, exposure to Nitrosamines, and nutritional deficiencies<sup>[6-8]</sup>. However, since only a fraction of individuals exposed to these risk factors actually develop EC, the role played by genetic determinants in response to environmental exposures needs to be addressed.

Data have also identified chronic alcohol consumption as a significant risk factor for alimentary tract cancer<sup>[9]</sup>. Ethanol is almost totally broken down by oxidative metabolism *in vivo*. Individuals who accumulate acetaldehyde due to polymorphic differences in the genes encoding the enzymes responsible for acetaldehyde generation and detoxification have been thought to show an ethanol associated carcinogenesis.

Ethanol is first metabolized into acetaldehyde through several enzymatic and nonenzymatic mechanisms, the main enzymatic pathways being alcohol dehydrogenase, cytochrome P450 (*CYP*) and catalase. Most of the acetaldehydes generated during alcohol metabolism *in vivo* are promptly eliminated by aldehyde dehydrogenase-2 (*ALDH2*), and alcohol dehydrogenase-1B (*ADH1B*, previously called *ADH2*)<sup>[10,11]</sup>.

Cytochrome P450 is a Phase I enzyme responsible for activating most environmental pre-carcinogens, whereas glutathione S-transferases (*GSTs*) are Phase II enzymes capable of detoxifying the electrophile carcinogens that result from the action of *CYP* enzymes. *CYP2A6*, the main enzymes capable of activating nitrosamines and other carcinogens in humans, are expressed in esophageal mucosa of Brazilian patients, with a high degree of variation in expression<sup>[12]</sup>. The *CYP2A6* gene is located on chromosome 19, and 30 different alleles have been described<sup>[13]</sup>.

DNA repairing capacity (DRC) is an essential component in EC progression. Sufficient DNA repair activity ensures the stability and fidelity of the genome when exposed to carcinogens in the process of cell growth and differentiation<sup>[14]</sup>, whereas instability in the genome in cancer patients may indicate a possible involvement of defective DRC<sup>[15]</sup>. DNA repair processes generally consist of direct reversal (DR), base excision repair, nucleotide excision repair (NER), or mismatch repair pathways<sup>[16]</sup>. Each pathway is specific for the repair of one or more types of DNA damage.

Key proteins in the transcription-coupled NER pathway (involved in correcting UV-induced lesions, chemical adducts, and crosslinks) include xeroderma pigmentosum group A and D (*XPA* and *XPD*), and excision repair cross complementing group 1 (*ERCC1*)<sup>[17,18]</sup>.

*O*<sup>6</sup>-methylguanine DNA methyltransferase (*MGMT*) is a single protein responsible for the DR pathway. The major defense against alkylating mutations is from *MGMT*, a 207 amino acid DNA repair protein that transfers potentially carcinogenic *O*<sup>6</sup> alkylation adducts from the DNA to a cysteine residue of *MGMT*<sup>[19,20]</sup>. For each adduct removed, an *MGMT* molecule is inactivated, hence the capacity for each cell to repair DNA depends upon the total number of *MGMT* molecules in the cell.



When genes essential for a variety of DNA repair pathways display aberrant activities, this may influence DRC with consequent carcinogenesis and progression. Several studies suggested that reduced expression of certain DNA repair genes were associated with the risk of environment-related esophageal adenocarcinoma<sup>[21-23]</sup>. There are few studies that examined the roles of gene polymorphism, metabolic enzyme and several DNA repair genes in risk of ESCC.

In the present study, Sequenom MassARRAY system was utilized to determine the relationship between ESCC and seven genes (*ADHB1*, *ALDH2*, *ERCC1*, *MGMT*, *XPA*, *XPD*, and *CYP2A6*) involved in two different DNA damage and repair pathways. The relationship between the polymorphism of candidate DNA repair genes and ESCC was determined in peripheral blood mononuclear cells from 128 patients with newly diagnosed, untreated ESCC and 326 healthy controls from the northern Xinjiang.

## MATERIALS AND METHODS

### Study subjects

This study included 128 ESCC patients and 326 healthy controls. All cases were from the Northern Xinjiang, China. Patients were newly diagnosed with histologically confirmed primary ESCC and not treated with radiotherapy or chemotherapy previously from January 2006 to December 2008. Healthy control subjects were recruited from a cancer-screening program for early detection of EC in the same area and matched with ESCC patients on age ( $\pm 5$  years), gender, ethnicity and residence. The selection criteria included no individual history of cancer and digestive disease. After written consent of blood donation for research purposes was obtained from cases and control, each subject donated 5 mL peripheral blood collected in K<sub>2</sub>EDTA tubes and stored at -80°C until DNA was extracted.

### Single nucleotide polymorphism (SNP) selection

The International Haplotype Mapping ([www.hapmap.org](http://www.hapmap.org)) and Cancer SNP databases (<http://snp500cancer.nci.nih.gov/snp.cfm>) were used to select SNPs in seven genes. The selection criteria was that SNPs had an  $r^2$  of  $\geq 0.8$  for all SNPs with a minor allele frequency  $> 5\%$  in CHB. According to the above rules, 69 *Taq* SNPs were selected in seven genes, including 6 for *XPD*, 5 for *XPA*, 5 for *ERCC1*, 6 for *ALDH2*, 3 for *ADHB1*, 3 for *CYP2A6*, and 41 for *MGMT*.

### Interview

A trained interviewer administered a questionnaire to cases and control, gathering clinical and demographical information. The questions included demographic variables (age, gender, and ethnicity), and a comprehensive smoking and alcohol intake profile. Smoking habits, and alcohol status were all defined at 1 year prior to diagnosis for cases, or 1 year prior to interview for controls. Smoking of the patients was classified as never-smokers, ex-smokers (quit  $> 1$  year), and current smokers (smoking

currently or quit  $< 1$  year). Alcohol use was defined as patients who never consumed alcohol *vs* those who had consumed alcohol at any time in the past.

### Genotyping

Genomic DNA was extracted within 1 wk after sampling using proteinase K digestion followed by a salting out procedure according to the method published by Miller *et al*<sup>[24]</sup>. SNPs were genotyped using the Sequenom MassARRAY system. The i-PLEX assay was performed according to manufacturer's instructions ([www.sequenom.com](http://www.sequenom.com)) using 5 ng of genomic DNA.

SNP typing was conducted using the Sequenom<sup>TM</sup> iPLEX<sup>TM</sup> protocol. PCR reactions were performed in standard 384-well plates containing 10 ng genomic DNA, 0.5 U of *Taq* polymerase (HotStarTaq, Qiagen, CA), 500  $\mu$ mol of dNTPs and 100 nmol of both forward and reverse PCR primers. Thermocycling conditions within the ABI-9700 (Applied Biosystems, USA) consisted of an initial 15 min denaturation at 94°C, followed by 45 cycles of 20 s denaturing at 94°C, 30 s annealing at 56°C, and 60 s extension at 72°C. PCR products were purified by incubation at 37 using 0.15 U of Shrimp Alkaline Phosphatase for 30 min followed by a 5 min inactivation at 85°C. A primer extension reaction mixture including 0.1  $\mu$ L of a  $10 \times$  termination mix, 0.02  $\mu$ L DNA polymerase and 1000 nmol/L of the extension primers was used in both the initial (denaturation at 94°C for 30 s, followed by 5 annealing and extension cycles at 52 and 80°C, respectively) and secondary (40 cycles of 5 s denaturation at 94°C, 5 s annealing at 52°C and 5 s extension at 80°C) iPLEX reactions. A final extension for 3 min at 72°C was conducted prior to cooling at 20°C. Products were diluted and desalted with 15  $\mu$ L sterile water and 3  $\mu$ L of resin prior to spotting onto a SpectroChip for analysis in the Compact Mass Spectrometer, using Workstation software version TYPER 4.0 (Sequenom). Genotype accuracy was calculated for all SNPs tested at 99.95%.

For quality control, genotyping was repeated randomly in at least 5% of the samples, and two of the authors independently reviewed all results. Allele frequencies of controls were calculated using the formula (example genotypes AA, AB and BB): Allele B frequency = [number of genotypes AB + 2  $\times$  (number of genotypes BB)]/[2  $\times$  (number of genotypes AA + number of genotypes AB + number of genotypes BB)].

### Statistical analysis

All cases and controls were compared for age, gender and ethnicity to ensure frequency matching. Hardy-Weinberg equilibrium of allele distributions was tested in cases and controls, separately. We used unconditional multivariate logistic regression to assess the main effects of genetic polymorphisms on ESCC risk by estimating odds ratios (OR) and associated confidence intervals (CI). Genotypes were categorized into three groups when the allele frequencies were allowed (major allele homozygous, heterozygous and homozygous variants).



**Table 1** Characteristics of ESCC patients and controls *n* (%)

Variables	Controls ( <i>n</i> = 326)	Patients ( <i>n</i> = 128)	<i>P</i> value
Age (yr)			
Median (range)	61 (55-78)	63 (58-81)	0.950
Gender			
Female	160	58	0.470
Male	166	70	
Smoking status			
Non-smokers	112 (34.3)	26 (20.3)	0.007
Ex-smokers	152 (46.7)	66 (51.6)	
Current smokers	62 (19.0)	36 (28.1)	
Alcohol use			
Never	81 (24.9)	15 (11.7)	0.002
Ever	245 (75.1)	113 (88.3)	
Metastasis			
No		92 (71.9)	
Yes		36 (28.1)	
Ethnicity			
Kazakh	89 (27.3)	39 (30.5)	0.703
Uygur	87 (26.7)	30 (23.4)	
Han	150 (46.0)	59 (46.1)	

## RESULTS

### Subject characteristics

The frequency of 69 sequence variants was assessed in DNA samples from 128 ESCC cases and 326 controls from the population of North-Western China. The distributions of age and gender, smoking history, alcohol consumption history, and pathological grade among the study subjects are summarized in Table 1. There were no statistically significant differences among cases and controls in terms of mean age, gender and ethnicity, suggesting that the frequency matching was adequate.

As expected, the prevalence of smoking and alcohol was both significantly higher in ESCC cases than in the controls (Table 1).

There was a greater proportion of ex-smokers and current-smokers in the cases than in the controls ( $P = 0.007$ ). The percentage of ever alcohol users in the cases was higher than in the controls ( $P = 0.002$ ). There were 36 (28.1%) cases with metastasis and 92 (71.9%) cases without metastasis.

### Analysis of seven genes allele in ESCC carcinogenesis

The results in this study showed no associations between variations in the *ADH1B*, *ERCC1* and *XPA* genes and ESCC risk.

Possible allele and genotypes of investigated SNPs in relation to ESCC carcinogenesis are listed in Tables 2 and 3. Increased risk of ESCC was suggested in *XPD* for frequency of presence C allele of SNP [Rs50872: 1.482 (1.058-2.074)], in *ALDH2* for C allele of SNP [Rs886205: 1.626 (1.158-2.284)] and in *MGMT* for A allele of SNP [Rs11016897: 1.666 (1.245-2.228)]. Five variants of *MGMT* were associated with a protective effect on ESCC carcinogenesis: C allele [Rs7069143: 0.698 (0.518-0.939)], C allele [Rs3793909: 0.653 (0.429-0.995)], A allele [Rs12771882: 0.719 (0.524-0.986)], C allele [Rs551491: 0.707 (0.529-0.945)] and A allele [Rs7071825: 0.618 (0.506-0.910)].

### Analysis of seven genes genotype in ESCC carcinogenesis

At the genotype level, increased risk of ESCC was found in homozygous carriers of the *ALDH2* Rs886205 (CC *vs* TT, OR: 3.116, 95% CI: 1.179-8.234), *MGMT* Rs11016879 (AA *vs* GG, OR: 3.112, 95% CI: 1.565-6.181) and Rs12771882 (AA *vs* GG, OR: 2.442, 95% CI: 1.204-4.595). Increased risk of ESCC was also observed in heterozygotes carriers of the *ALDH2* Rs886205 (CT *vs* TT, OR: 3.930, 95% CI: 1.470-10.504), *MGMT* Rs11016879 (AG *vs* GG, OR: 3.933, 95% CI: 2.216-6.982) and Rs7075748 (CT *vs* CC, OR: 1.949, 95% CI: 1.134-3.350), respectively.

Three variants were associated with a protective effect on ESCC carcinogenesis: the carriers of the *MGMT* Rs11016878 (AG *vs* AA, OR: 0.388, 95% CI: 0.180-0.836), Rs7069143 (CT *vs* CC, OR: 0.478, 95% CI: 0.303-0.754) and Rs7071825 (GG *vs* AA, OR: 0.493, 95% CI: 0.266-0.915).

### Analysis of seven genes allele in ESCC progression

Tables 4 and 5 show the possible allele and genotypes of the investigated SNPs in relation to ESCC metastasis. Increased risk of ESCC metastasis was indicated in *MGMT* for frequency of presence C allele [Rs7068306: 2.204 (1.244-3.906)], A allele [Rs10734088: 1.968 (1.111-3.484)] and C allele [Rs4751115: 2.178 (1.251-3.791)].

Two variants were associated with a protective effect on ESCC carcinogenesis in frequency of presence C allele of *CYP2A6* [Rs8192720: 0.290 (0.099-0.855)] and A allele of *MGMT* [Rs2053139: 0.511 (0.289-0.903)].

### Analysis of seven genes genotype in ESCC progression

Increased risk of ESCC metastasis was found in heterozygote carriers of the *MGMT* Rs 7068306 (CG *vs* CC, OR: 4.706, 95% CI: 1.872-11.833).

## DISCUSSION

ESCC is one of the major health issues in China because of its high incidence and poor survival. Although the exact mechanism on EC is unclear, several possible mechanistic pathways have been proposed, including metabolic enzyme and DNA repair factors.

Alcohol consumption and tobacco smoking are established major risk factors for ESCC in Western populations<sup>[25,26]</sup>. However, studies in high-incidence regions are scarce and their results have been inconsistent<sup>[27-29]</sup>.

In the present study, we first conducted a case-control study to evaluate the associations between metabolic enzyme polymorphisms of *ADH1B*, *ALDH2* and *CYP2A6* and ESCC risk in population from northern Xinjiang, China. Consistent with previous studies<sup>[30-32]</sup>, the present study demonstrated that alcohol consumption and cigarette smoking are strongly associated with the incidence of ESCC. Moreover, individuals with *ALDH2* for frequency of presence C allele of SNP in Rs886205 and heterozygote carriers of the *ALDH2* in Rs886205 (CT *vs* TT) had significantly increased risk for ESCC carcinogenesis while individuals with frequency of

Table 2 Analysis of genes allele in ESCC carcinogenesis

Gene	Locus	Control allele frequency (n = 326)		Case allele frequency (n = 128)		OR (95% CI)	P value
		1	2	1	2		
ALDH2	Rs886205	448	204	200	56	1.626 (1.158-2.284)	0.005
XPD	Rs50872	522	130	187	69	1.482 (1.058-2.074)	0.022
MGMT	Rs7069143	356	296	162	94	0.698 (0.518-0.939)	0.017
MGMT	Rs3793909	535	117	224	32	0.653 (0.429-0.995)	0.046
MGMT	Rs12771882	165	487	82	174	0.719 (0.524-0.986)	0.040
MGMT	Rs551491	280	372	132	124	0.707 (0.529-0.945)	0.019
MGMT	Rs11016879	264	388	136	120	1.666 (1.245-2.228)	0.001
MGMT	Rs7071825	415	237	139	117	0.618 (0.506-0.910)	0.009

Table 3 Analysis of genes genotype in ESCC carcinogenesis n (%)

Gene/ genotype	Control (n = 326)	Cases (n = 128)	OR (95% CI)	P value
ALDH2				
Rs886205				
TT	40 (12.270)	5 (3.906)	1.0 (reference)	
CT	114 (34.969)	56 (43.750)	3.930 (1.470-10.504)	0.004
CC	172 (52.761)	67 (52.344)	3.116 (1.179-8.234)	0.017
MGMT				
Rs11016878				
AA	18 (5.522)	15 (11.719)	1.0 (reference)	
AG	130 (39.877)	42 (32.815)	0.388 (0.180-0.836)	0.013
GG	178 (54.601)	71 (55.466)	0.479 (0.229-1.002)	0.047
Rs11016879				
GG	118 (36.196)	17 (13.281)	1.0 (reference)	
AG	150 (46.012)	85 (66.406)	3.933 (2.216-6.982)	0.000
AA	58 (17.791)	26 (20.313)	3.112 (1.565-6.181)	0.001
Rs7069143				
CC	87 (26.687)	54 (42.188)	1.0 (reference)	
CT	182 (55.828)	54 (42.188)	0.478 (0.303-0.754)	0.001
TT	57 (17.485)	20 (15.625)	0.565 (0.306-1.043)	0.066
Rs12771882				
GG	181 (55.521)	63 (49.219)	1.0 (reference)	
AG	125 (38.344)	48 (37.500)	1.013 (0.711-1.712)	0.661
AA	20 (6.350)	17 (13.281)	2.442 (1.204-4.595)	0.011
Rs7075748				
CC	88 (26.994)	22 (17.188)	1.0 (reference)	
CT	156 (47.853)	76 (59.375)	1.949 (1.134-3.350)	0.015
TT	82 (25.153)	30 (23.438)	1.463 (0.782-2.740)	0.233
Rs7071825				
AA	46	24	1.0 (reference)	
AG	144	69	0.918 (0.519-1.625)	0.770
GG	136	35	0.493 (0.266-0.915)	0.024

presence C allele in *CYP2A6* of Rs8192720 were inversely associated with ESCC metastasis.

Genomic fidelity and genetic stability usually depend on the efficiency of DRC when an organism is exposed to environmental and endogenous carcinogens. Peltomäki<sup>[33]</sup> showed that aberrant DRC might be involved in the pathogenesis of some cancers.

Previous molecular epidemiological studies have found that the *XPD* polymorphism is associated with increased risks for head and neck cancers<sup>[34]</sup>. However, inconsistent findings were also reported, including inverse associations with esophageal adenocarcinoma<sup>[35]</sup>. Our results suggested that *XPD* for frequency of presence C allele of SNP in Rs50872 increased the risk of ESCC carcinogenesis, which is consistent with the reported findings<sup>[36,37]</sup>.

*MGMT* is a major gene in the pathway of DNA repair and frequently found to be silenced by CpG island hypermethylation in many cancers, such as gastric cancer<sup>[38]</sup> and esophageal adenocarcinoma<sup>[39]</sup>. However, the studies on the ESCC are still rare. Fang *et al.*<sup>[40]</sup> reported that 29% (5 of 17) of normal esophageal tissues, 50% (10 of 20) of basal cell hyperplasia, 67% (8 of 12) of dysplasia, and 72% (13 of 18) of ESCC samples obtained from Linzhou City of Henan Province in northern China had DNA hypermethylation in *MGMT* promoter region. Until now, no investigation has been carried out about the associations between *MGMT* polymorphism and ESCC in a population of high incidence region of Northern Xinjiang. Our findings displayed that *MGMT* for frequency of presence A allele of SNP in Rs11016897, carriers of *MGMT* Rs11016879 (AA *vs* GG, AG *vs* GG), Rs12771882 (AA *vs* GG), and Rs7075748 (CT *vs* CC) significantly increased the risk of ESCC carcinogenesis. Our results are in agreement with the previous studies on EC<sup>[41-43]</sup>.

Furthermore, increased risk of ESCC metastasis was indicated in *MGMT* for frequency of presence C allele in Rs7068306, A allele in Rs10734088 and C allele in Rs4751115, and carriers of *MGMT* Rs7068306 (CG *vs* CC).

In addition, variants of *MGMT* were associated with a protective effect on ESCC carcinogenesis, including C allele in Rs7069143, C allele in Rs3793909, A allele in Rs12771882, C allele in Rs551491, and A allele in Rs7071825, and the carriers of the *MGMT* in Rs11016878 (AG *vs* AA), Rs7069143 (CT *vs* CC) and Rs7071825 (GG *vs* AA). Frequency of C allele in Rs8192720 and A allele in Rs2053139 were associated with a protective effect on ESCC metastasis which was inconsistent with previous studies<sup>[44,45]</sup>.

Difference in study population may be one of the reasons leading to the different results. However, since this is only a retrospective study and our findings could not be stratified because of the relatively small number in the groups, further epidemiological studies with enlarged samples are worth doing to warrant the results.

A limitation of our study may be missing covariate data. There have been many studies of individual environmental risk factors for EC<sup>[46-48]</sup>. We did not collect information on dietary factors and occupational history and so we were unable to study gene-environmental interactions in the etiology of ESCC. Gene-smoking and gene-alcohol interactions cannot be accurately measured due to sample size considerations. However, an improved

Table 4 Analysis of genes allele in ESCC progression

Gene	Locus	Control allele frequency (n = 92)		Case allele frequency (n = 36)		OR (95% CI)	P value
		1	2	1	2		
CYP2A6	Rs8192720	153	31	68	4	0.290 (0.099-0.855)	0.018
MGMT	Rs7068306	137	47	41	31	2.204 (1.244-3.906)	0.006
MGMT	Rs10734088	135	49	42	30	1.968 (1.111-3.484)	0.019
MGMT	Rs4751115	112	72	30	42	2.178 (1.251-3.791)	0.005
MGMT	Rs2053139	93	91	48	24	0.511 (0.289-0.903)	0.020

Table 5 Analysis of genes genotype in ESCC progression n (%)

Gene/ genotype	Without metastasis (n = 92)	With metastasis (n = 36)	OR (95% CI)	P value
MGMT				
Rs7068306				
CC	60 (65.217)	12 (33.333)	1.0 (reference)	
CG	17 (18.478)	16 (44.445)	4.706 (1.872-11.833)	0.001
GG	15 (16.304)	8 (22.222)	2.667 (0.925-7.685)	0.064

understanding of the main separate effects of genes and environment will allow detailed gene-environmental interactions to be examined in larger-scale studies in the future. Our study population size is too small to avoid spurious results although they are suggestive, and further larger validation studies are certainly needed. The biological meaning and function of interesting SNPs need to be confirmed especially in advanced researches.

However, the strong points in our study are that both cases and healthy control subjects were recruited from the same area and matched on age, gender, ethnicity and residence, and all our control subjects were under Hardy-Weinberg equilibrium. Moreover, all our cases were pathologically confirmed, and followed by a strict quality control from genotyping.

To conclude, this study provides no evidence on a role of common variation of genes *ERCC1*, *XPA* and *ADHB1* in ESCC. However, our data indicated that variation in the *ALDH2*, *XPD* and *MGMT* genes may be related to the risk of ESCC carcinogenesis. The polymorphic variation in *CYP2A6* and *MGMT* are associated with ESCC metastasis.

## COMMENTS

### Background

Because of the high incidence and poor survival, esophageal cancer (EC) is one of the major health issues in Western/Northern, China. Although the exact mechanism is unclear, several possible mechanistic pathways have been proposed, including metabolic enzyme and DNA repair factors.

### Research frontiers

The relationship between gene polymorphism of metabolic enzyme, DNA repair and EC in Western/Northern China needs to be addressed. The present study indicated that metabolic enzyme and DNA repair factors were involved in the risk of esophageal squamous cell cancer (ESCC).

### Innovations and breakthroughs

The variation of *ALDH2*, *XPD* and *MGMT* genes may be of relevance to the risk of ESCC. The polymorphic variation of *CYP2A6* and *MGMT* were associated with ESCC metastasis.

### Applications

By understanding the gene polymorphism in *ALDH2*, *CYP2A6*, *MGMT* and

*XPD*, further researches may represent a future strategy for detecting the susceptibility to ESCC.

### Peer review

The paper deals with a very interesting subject. The authors analyzed genotypes of sixty-nine single nucleotide polymorphisms (SNPs) of metabolic enzyme and DNA repair capacity related genes in a moderate scale, and found some SNPs correlated with the risk of ESCC and tumor metastasis. These results are attractive and interesting. In general, the manuscript is written well and the data is presented clearly.

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## Rectal perforation from endometriosis in pregnancy: Case report and literature review

Adolfo Pisanu, Daniela Deplano, Stefano Angioni, Rossano Ambu, Alessandro Uccheddu

Adolfo Pisanu, Daniela Deplano, Alessandro Uccheddu, Department of Surgery, Clinica Chirurgica, University of Cagliari, Ospedale San Giovanni di Dio, Via Ospedale 46, 09124, Cagliari, Italy

Stefano Angioni, Department of Obstetrics and Gynecology, University of Cagliari, Ospedale San Giovanni di Dio, Via Ospedale 46, 09124, Cagliari, Italy

Rossano Ambu, Department of Pathology, University of Cagliari, Ospedale San Giovanni di Dio, Via Ospedale 46, 09124, Cagliari, Italy

**Author contributions:** Pisanu A was responsible for the study conception and design, manuscript writing, drafting the article and revising for intellectual content; Deplano D carried out data acquisition, and manuscript writing and revising for intellectual content; Angioni A and Ambu R revised the manuscript for intellectual content and performed supportive work; Uccheddu A revised the manuscript for important intellectual content.

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**Correspondence to:** Dr. Adolfo Pisanu, Department of Surgery, Clinica Chirurgica, University of Cagliari, Ospedale San Giovanni di Dio, Via Ospedale 46, 09124, Cagliari, Italy. [adolfo.pisanu@unica.it](mailto:adolfo.pisanu@unica.it)

Telephone: +39-70-6092420 Fax: +39-70-6092417

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consistent with decidualization of the rectal wall. Only 20 cases of intestinal perforation due to endometriosis have been reported in the literature. This report is believed to be the first case of spontaneous rectal perforation from endometriosis in pregnancy, and it shows the potential occurrence of serious and unexpected complications of the disease.

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**Key words:** Endometriosis; Colonic perforation; Acute abdomen; Peritonitis; Pregnancy

**Peer reviewers:** Dr. Marty Zdichavsky, MD, Department of General, Visceral and Transplant Surgery, University Hospital Tübingen, Hoppe-Seyler-Str. 3, 72076 Tübingen, Germany; Shanthi V Sitaraman, MD, Division of Digestive Diseases, Room 201-F, 615, Michael Street, Whitehead Research Building, Emory University, Atlanta, Georgia, GA 30322, United States; Philip H Gordon, Professor, Department of Surgery, McGill University, 3755 Cote Ste. Catherine Road, Suite G-314, Montreal, Quebec, H3T 1E2, Canada

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### Abstract

This case report describes a woman with spontaneous rectal perforation from decidualized endometriosis in pregnancy. A 37-year-old woman was admitted to our hospital at 30 wk of pregnancy with symptoms suggestive of pyelonephritis, which persisted until 33 wk of gestation when delivery of a premature male baby was performed through a cesarean section. On postoperative day 2, an abdominal computed tomography showed free air in the peritoneal cavity and a pelvic abscess. Explorative celiotomy revealed a diffuse severe fecaloid peritonitis that originated from a 3-cm wide rectal perforation. A Hartmann operation was then performed. Histopathological findings were

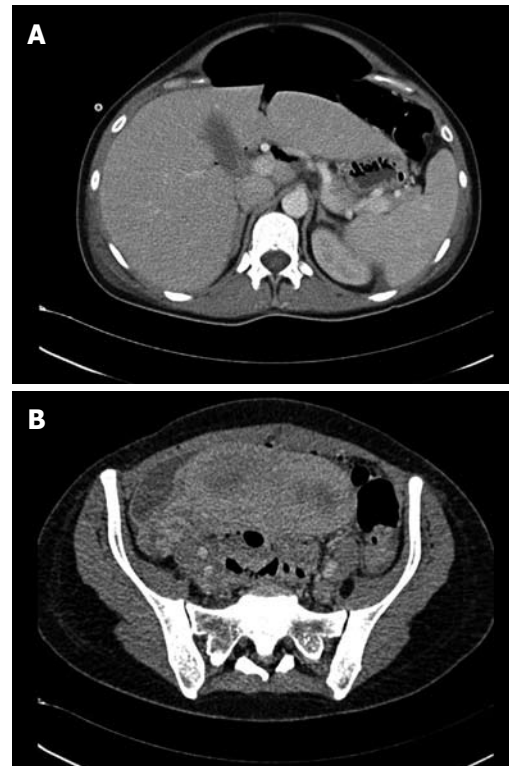
### INTRODUCTION

Endometriosis is defined by the presence of endometrium outside the uterus and usually affects pelvic structures including the bowel. Intestinal involvement occurs in 3%-37% of patients with endometriosis<sup>[1]</sup>. Intestinal endometriosis typically takes the form of asymptomatic serosal implants that occasionally result in intestinal obstruction with recurrent abdominal pain<sup>[2]</sup>. Transmural involvement is not as common, and spontaneous perforation of intestinal endometriosis is a rare complication that generally occurs in pregnancy<sup>[2-4]</sup>.

We report the case of a pregnant woman with spontaneous rectal perforation that originated from a decidualized endometriotic nodule, and we review all cases of intestinal perforation from endometriosis reported in the literature. To the best of our knowledge, this is the first report of spontaneous perforation of the rectal wall in an endometriotic area in pregnancy, which is an extremely rare cause of acute abdomen.

## CASE REPORT

In October 2008, a 37-year-old Caucasian woman was admitted to our hospital because of colicky pain in the right iliac fossa at 30 wk of pregnancy. She had a previous history of endometriosis that was surgically treated. In 2003, she underwent a laparotomy excision of an endometrioma of the left ovary. Later, she was submitted to three subsequent laparoscopies in which left salpingectomy for a sactosalpinx, diathermocoagulation of multiple endometriotic foci, excision of the posterior vaginal fornix, and adhesiolysis mainly between the uterus and sigmoid colon were performed. In 2007, the final laparoscopy showed no endometriosis in the Douglas cul-de-sac. At admission to our hospital, abdominal ultrasound revealed pelvicalyceal dilation on the right side, which improved after double J stent placement. However, symptoms suggestive of pyelonephritis persisted until 33 wk of gestation when delivery of a 1600 g premature male baby was performed through a cesarean section, because of the mother's worsening clinical condition that was caused by developing sepsis that was apparently related to pyelonephritis. Indeed, the clinical diagnosis of pyelonephritis was also supported by detection of pathological levels of *Escherichia coli* in the urine. However, during cesarean section, at the moment of peritoneal incision, a fecaloid smell came from the peritoneal cavity, but unfortunately, this finding was not correctly understood. On postoperative day 2, the patient was pyrexial (40°C), pale and shocked. Blood pressure and pulse rate were 90/50 mmHg and 95 beats/min, respectively. The abdomen was distended, with tenderness and rebound in the lower quadrants, where bowel sounds were absent. Laboratory data were as follows: white blood cell count,  $204 \times 10^9/L$ ; red blood cell count,  $370 \times 10^{12}/L$ ; hemoglobin, 10 g/L; thrombocyte count  $461 \times 10^9/L$ ; fibrinogen, 6.54 g/L. Chest X-ray revealed bilateral pleural effusion, while abdominal plain radiography showed free air in the peritoneal cavity. Enhanced abdominal computed tomography (CT) confirmed these findings (Figure 1A), as well as a low-density area with the features of a pelvic abscess on the right side of the enlarged uterus (Figure 1B). The patient underwent an emergency operation for a presumptive diagnosis of acute diffuse peritonitis. Explorative celiotomy revealed diffuse, severe fecaloid peritonitis that originated from a 3-cm wide rectal perforation in the deep cul-de-sac. Diffuse peritonitis was in an advanced stage and lasted for > 48 h. In addition, the sigmoid colon was adher-



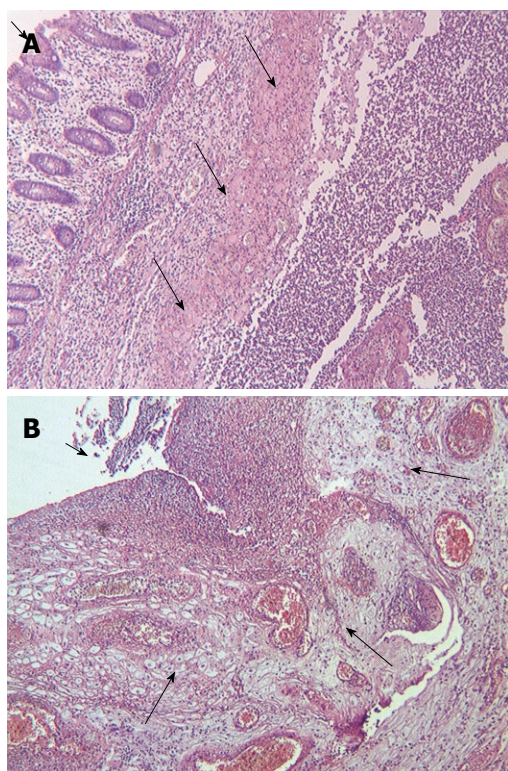
**Figure 1** Axial computed tomography (CT) scan. A: Free air in the abdominal cavity; B: Pelvic abscess at the right side of the enlarged uterus.

ent to the uterus. A Hartmann operation was necessary with end colostomy using the descending colon and closure of the rectal stump, and appendectomy was performed. Bilateral pleural effusion persisted for 14 d after the operation and the patient was finally discharged on postoperative day 19 as a consequence of abdominal wound infection. On gross examination, rectal perforation seemed to originate from an endometriotic nodule. Histopathological findings were consistent with decidualization of the rectal wall (Figure 2A) and vermiform appendix. Decidualized endometriosis was mainly found around the rectal perforation, which was the most likely explanation for this occurrence (Figure 2B). Nursery care was needed for 35 d until the infant reached a stable body weight and was able to feed by mouth. Afterwards, the patient was referred to the gynecologist for further therapy and she was readmitted 3 mo later for Hartmann reversal without any sequelae.

## DISCUSSION

Intestinal endometriosis may affect the ileum, appendix, sigmoid colon and rectum<sup>[4]</sup>. The most common non-genital manifestation is in the rectosigmoid<sup>[3,5]</sup>. The peritoneal implantation of endometrium by retrograde menstruation or the possible metaplasia of peritoneal cells are still the two most accepted etiological theories of endometriosis<sup>[1]</sup>.

Intestinal endometriosis may be found in every layer of the bowel wall but it is most commonly found within the



**Figure 2** The rectal wall. A: Decidualization of the rectal wall (long arrows); mucosa side of the rectal wall (short arrow) (HE,  $\times 40$ ); B: Decidualized endometriosis around the rectal perforation (long arrows); rectal perforation with necrosis at the peritoneal side of the rectal wall (short arrow) (HE,  $\times 100$ ).

subserosa as superficial serosal implants<sup>[2,6]</sup>. Under cyclical hormonal influences, these implants may proliferate and infiltrate the intestinal wall and cause a fibrotic reaction with formation of strictures and adhesions, which may lead to bowel obstruction and recurrent abdominal pain<sup>[4,7]</sup>. On the other hand, transmural bowel wall involvement is not so common and the intestinal mucosa usually remains intact, and perforation of the affected intestinal tract is a very rare complication<sup>[3,6]</sup>. Up till now and including the present report, only 21 cases of intestinal perforation from endometriosis have been reported and among these, nine cases (42.8%) occurred in pregnancy. Sigmoid colon turned out to be the most common site of perforation from endometriosis and accounted for 12 of 21 published cases. The current case is the first report of spontaneous perforation of the rectum (Table 1).

In those patients with perforation, the entire intestinal wall is replaced by endometriotic tissue. In pregnancy, under the effect of progesterone, the area of ectopic endometrium becomes decidualized with a progressive reduction in size<sup>[8,9]</sup>. In our patient, decidualized endometriosis was found mainly around the rectal perforation. Actually, the reduction in size of a transmural endometriotic nodule may lead to perforation, by weakening of the intestinal wall<sup>[2]</sup>, particularly in the third trimester, which is the time of perforation in most reported cases<sup>[2,8]</sup>, as in our patient who had rectal perforation at 33 wk of pregnancy. Moreover, decidualization causes a severe inflammatory response with an increased number of natural killer cells

**Table 1** Literature review of intestinal perforation from endometriosis

Yr	Author	Journal	Site of perforation	n	Pregnancy
1931	Haufler <sup>[11]</sup>	<i>Virchows Arch</i> [in German]	Jejunum	1	Yes
1955	Henriksen <sup>[12]</sup>	<i>Am J Surg</i>	Sigmoid colon	1	No
1977	Clement <sup>[13]</sup>	<i>Br J Obstet Gynaecol</i>	Sigmoid colon	1	Yes
1979	Rud <sup>[14]</sup>	<i>Ugeskr Laeger</i> [in Danish]	Sigmoid colon	1	Yes
1981	Gini <i>et al</i> <sup>[15]</sup>	<i>Br J Obstet Gynaecol</i>	Vermiform appendix	1	Yes
1984	Floberg <i>et al</i> <sup>[16]</sup>	<i>Acta Obstet Gynecol Scand</i>	Sigmoid colon	1	Yes
1987	Nakatani <i>et al</i> <sup>[17]</sup>	<i>Acta Pathol Jpn</i>	Vermiform appendix	1	Yes
1988	Strömberg <i>et al</i> <sup>[18]</sup>	<i>Lakartidningen</i> [in Swedish]	Sigmoid colon	1	No
1988	Ledley <i>et al</i> <sup>[19]</sup>	<i>Am J Gastroenterol</i>	Sigmoid colon	1	No
1990	Goodman <i>et al</i> <sup>[20]</sup>	<i>Gastrointest Radiol</i>	Sigmoid colon	1	No
1992	Bakri <i>et al</i> <sup>[21]</sup>	<i>Int J Gynaecol Obstet</i>	Sigmoid colon	1	No
1993	Yelon <i>et al</i> <sup>[22]</sup>	<i>J Clin Gastroenterol</i>	Vermiform appendix	1	No
1994	Allimant <i>et al</i> <sup>[23]</sup>	<i>J Chir (Paris)</i> [in French]	Ileum	1	No
1995	Abbo <i>et al</i> <sup>[24]</sup>	<i>Minerva Chir</i> [in Italian]	Sigmoid colon	1	No
2000	Bossotti <i>et al</i> <sup>[25]</sup>	<i>Chir Ital</i>	Ileum	1	No
2004	Decker <i>et al</i> <sup>[3]</sup>	<i>Arch Gynecol Obstet</i>	Ileum	1	No
2006	Schweitzer <i>et al</i> <sup>[4]</sup>	<i>Int J Gynaecol Obstet</i>	Sigmoid colon	1	Yes
2008	Faucheron <i>et al</i> <sup>[9]</sup>	<i>Colorectal Dis</i>	Vermiform appendix	1	Yes
2008	Shaw <i>et al</i> <sup>[26]</sup>	<i>Colorectal Dis</i>	Sigmoid colon	1	No
2009	Garg <i>et al</i> <sup>[2]</sup>	<i>World J Gastroenterol</i>	Sigmoid colon	1	No
2010	Pisanu <i>et al</i>	<i>Present report</i>	Rectum	1	Yes
			Total	21	9 Y/12 N

and decidual changes, which are responsible for a higher risk of perforation<sup>[9,10]</sup>. In our case, we believe that perforation was also facilitated by the progressive traction of the enlarged uterus on the strictly adherent sigmoid colon, and consequently, on the decidualized and weakened area of the anterior rectal wall. Moreover, the absence of endometriotic foci in the cul-de-sac at final laparoscopy made rectal perforation unpredictable.

Although endometriosis improves during pregnancy, the current report shows the potential occurrence of serious and unexpected complications of the disease. Both the rareness of the perforation and the symptoms that are suggestive of pyelonephritis or diverticulitis may be misleading and delay the diagnosis. Indeed, the appropriate management of these patients may be challenging and a good outcome is absolutely dependent on a multidisciplinary approach.



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## Primary mixed germ cell tumor of the liver with sarcomatous components

Ai-Min Xu, Shao-Juan Gong, Wei-Hua Song, Xiao-Wei Li, Chun-Hua Pan, Jun-Jun Zhu, Meng-Chao Wu

Ai-Min Xu, Shao-Juan Gong, Wei-Hua Song, Xiao-Wei Li, Chun-Hua Pan, Jun-Jun Zhu, Meng-Chao Wu, Third Department of Intervention Radiotherapy, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200438, China

**Author contributions:** Xu AM and Wu MC designed the research and wrote the manuscript; Gong SJ, Song WH and Li XW performed the research; Pan CH and Zhu JJ analyzed the data.

**Correspondence to:** Ai-Min Xu, MD, Third Department of Intervention Radiotherapy, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, 225 Changhai Road, Shanghai 200438, China. [xuarmy@163.com](mailto:xuarmy@163.com)

Telephone: +86-21-81875181 Fax: +86-21-81875181

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**Peer reviewer:** Miguel Angel Mercado, MD, Surgical Division, National Institute of Medical Sciences and Nutrition, Vasco de Quiroga 15, col. Section 16, Distrito Federal 14000, Mexico

Xu AM, Gong SJ, Song WH, Li XW, Pan CH, Zhu JJ, Wu MC. Primary mixed germ cell tumor of the liver with sarcomatous components. *World J Gastroenterol* 2010; 16(5): 652-656 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i5/652.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i5.652>

### Abstract

Germ cell tumor (GCT) of the liver is extremely rare. Here, we describe a case of hepatic mixed GCT with significant sarcomatous components and elevated serum  $\alpha$ -fetoprotein (AFP) in a 34-year-old man. Histopathologically, the tumor was composed of two GCTs components: yolk sac tumor and immature teratoma. The predominant components of immature teratoma consisted of several types of tissue that represented different germinal layers (endoderm, mesoderm and ectoderm) and showed varying degrees of differentiation with significant sarcomatous components. The yolk sac component showed positivity for AFP and cytokeratin (AE1/AE3). The immature teratoma components showed positivity for varying differentiation markers. Interphase cytogenetic analysis revealed that the yolk sac tumor and immature teratoma were positive for i(12p) and 12p over-representation. In particular, the rhabdomyoblastic components also showed typical i(12p) and 12p overrepresentation. This suggested that sarcomatous components may be associated with dedifferentiation or malignant transformation of certain mesenchymal components within teratoma.

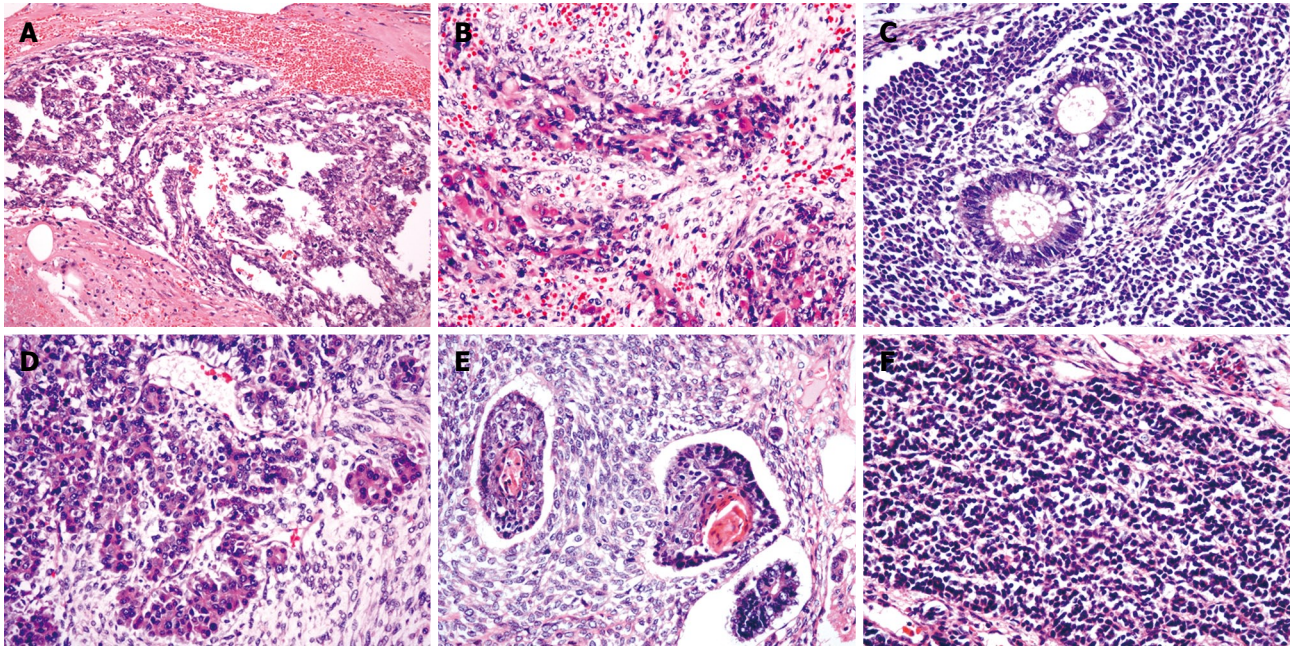
### INTRODUCTION

Germ cell tumors (GCTs) are a diverse family of neoplasms, and they are all presumed to arise from totipotent primordial germ cells<sup>[1]</sup>. This family of neoplasms includes tumors that arise from different anatomic sites, in different age groups, and that vary in clinical behavior. Most GCTs are gonadal in origin, others are extragonadal, which are located mainly along the mid-line of the body. GCT of the liver is extremely rare, and accounts for < 1% of all liver neoplasms<sup>[2]</sup>. Most of them are in children aged < 3 years old, and about half of these tumors are malignant. Approximately 20 cases of malignant GCTs have been reported in the English literature following presentation as teratoma<sup>[3-5]</sup>, choriocarcinoma<sup>[6,7]</sup> or yolk sac tumor<sup>[8,9]</sup> in patients over 21 years of age. The presence of sarcomatous components in gonadal or extragonadal GCTs is also an infrequent phenomenon<sup>[10]</sup>.

Here, we report the light microscopic and cytogenetic findings of a case of hepatic malignant mixed GCT with significant sarcomatous components in a 34-year-old Chinese man.

### CASE REPORT

A 34-year-old man presented with right upper quadrant



**Figure 1** Histological features of mixed GCT of the liver with sarcomatous components. A: Papillary structures resemble yolk sac tumor with Schiller-Duval bodies; B: Rhabdomyoblastic cells in primitive mesenchymal cell components; C: Intestinal-type epithelium embedded in neuroblastoma tissue; D: Acinar structures resembling pancreatic acinar tissue; E: Keratinizing epithelium embedded in primitive mesenchymal tissue; F: Neuroblastoma tissue with rosettes formation [Hematoxylin and eosin (HE) stain; A,  $\times 200$ ; B-F,  $\times 400$ ].

pain in 2006. Ultrasound, magnetic resonance imaging and computed tomography showed a 15 cm  $\times$  12 cm  $\times$  9 cm low density heterogeneous mass in the right liver lobe. Blood tests showed slightly elevated aspartate aminotransferase,  $\gamma$ -glutamyl transferase, alkaline phosphatase and a very high  $\alpha$ -fetoprotein (AFP: 56 500  $\mu$ g/L, normal level  $< 20$   $\mu$ g/L). Other biochemical parameters (renal and liver function) and tumor markers such as carbohydrate antigen 19-9, and carcinoembryonic antigen (CEA) were within normal limits. Results of serology testing showed that serum hepatitis B virus surface antigen and anti-hepatitis C virus antibody were negative. The patient had not received any treatment before operation. No other tumor site was detected by extensive preoperative staging. After the liver tumor was resected, the serum AFP level went down to 512  $\mu$ g/L.

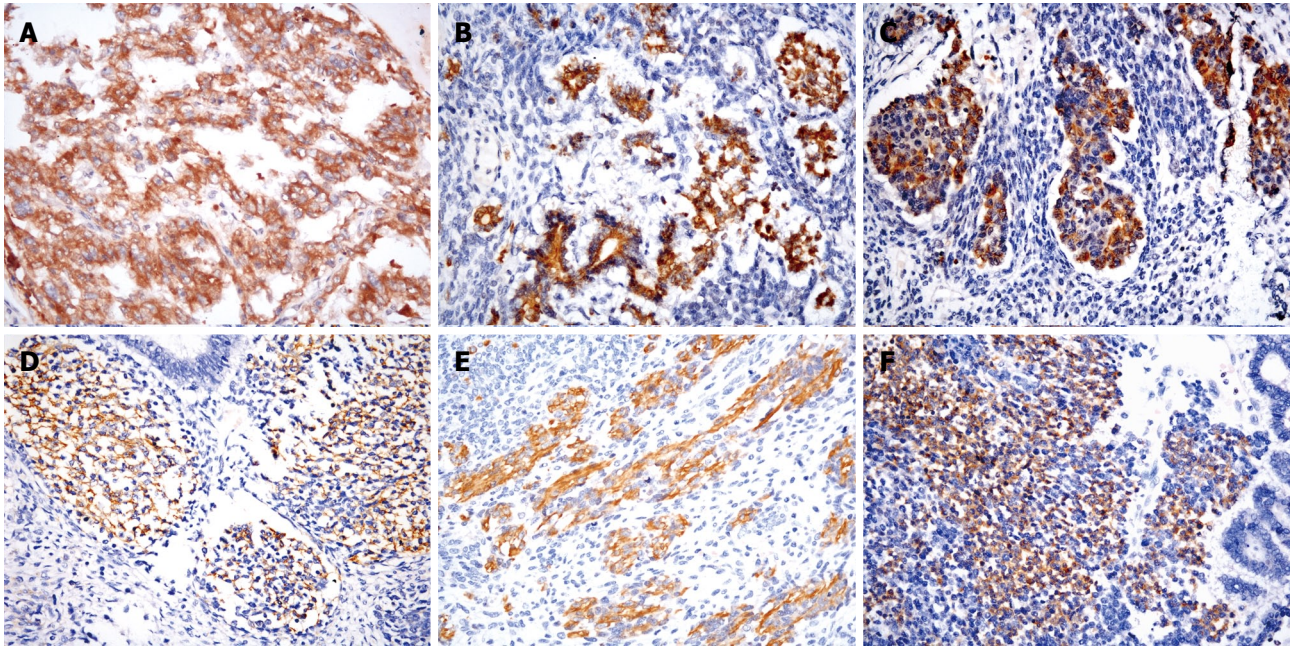
Macroscopically, the partial liver resection specimen from the right lobe contained a single, firm mass with partially necrotic areas. It measured 16 cm  $\times$  12 cm  $\times$  8 cm. The cut surface was reddish-purple, whitish, gray to tan, slightly lobular with fibrous septa, and showed cystic spaces and areas of hemorrhage.

Histologically, the resected tumor was composed of two GCTs components: yolk sac tumor and immature teratoma. The morphological appearance of yolk sac tumor included endodermal sinus-like (with Schiller-Duval bodies), papillary and glandular components (Figure 1A). The predominant components of immature teratoma were composed of several types of tissue that represented different germinal layers (endoderm, mesoderm and ectoderm) and showed varying degrees of differentiation. The endoderm components were primitive mesenchyme

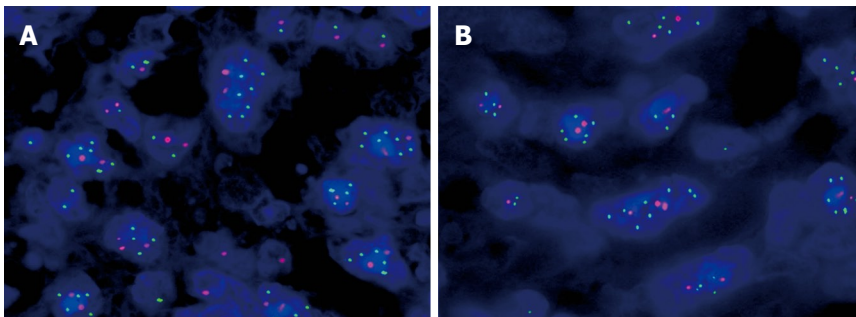
and various mesenchyme-derived tissues, which were composed predominantly of primitive to spindle cells with rhabdomyoblastic (Figure 1B), chondroid and osteoblastic differentiation. The mesoderm components were composed of respiratory, intestinal-type epithelium (Figure 1C), and glandular, ductal or acinar structures that resembled pancreatic acinar tissue (Figure 1D). The ectoderm components were composed of keratinizing or non-keratinizing epithelium (Figure 1E), which resembled neuroblastoma tissue that exhibited rosettes formation (Figure 1F), ganglion differentiation or embryonic neural tube. Mitotic figures were found frequently in various tumorous components. Areas of necrosis were also present. Hepatocellular differentiation was not found in the tumor. Uninvolved liver demonstrated no obvious cirrhosis but slight steatosis. The surgical resection margins were clear.

Immunohistochemically, the yolk sac tumor stained positively for AFP (Figure 2A) and cytokeratin (AE1/AE3). The cells of immature glandular or ductal structures were positive for pCEA, mCEA, AE1/AE3, CK7, CK18 and CK19. Focal hepatocyte marker (HepPar1) protein reaction was seen in the glandular epithelium. The acinar structures that resembled pancreatic acinar cells were positive for AFP (Figure 2B),  $\alpha$ -1-antitrypsin,  $\alpha$ -chymotrypsin (Figure 2C) and AE1/AE3. The primitive mesenchymal spindle cells were stained prominently with vimentin, and focally stained with smooth muscle actin, CD56 (Figure 2D), CD117 and CD34. Desmin and myoglobin were positive in the immature skeletal muscle tissue (Figure 2E). The cells of immature chondroid and osteoblastic components were positive for both vimentin and S-100 protein. The neuroblastoma-like areas and





**Figure 2** Immunohistochemical features of mixed GCT of the liver with sarcomatous components. A: Strong staining for AFP in the yolk sac tumor component; B: Strong staining for AFP in acinar structures resembling pancreatic acinar tissue; C: Strong staining for  $\alpha$ -chymotrypsin in acinar structures resembling pancreatic acinar tissue; D: CD56 positivity in primitive mesenchymal cells; E: Strong staining for desmin in rhabdomyoblastic cells in primitive mesenchymal cell components; F: Strong staining for synaptophysin in neuroblastoma tissue (EnVision Plus stain; A-F,  $\times 400$ ).



**Figure 3** Tumor cells possess isochromosome 12p and 12p overrepresentation, which are characteristic of tumors of germ cell origin. FISH showing cells with 2-7 (green) 12p signals and 1-4 (orange) 12 centromere signals in yolk sac tumor (A), and rhabdomyoblastic cells (B). (green = 12p probe; orange = 12 centromere probe; magnification  $\times 1000$ ).

ganglion cells were positive for vimentin, synaptophysin (Figure 2F), neuron-specific enolase (NSE), glial fibrillary acid protein (GFAP), and focally stained with HMB45, CD99 and CD117. The immature glandular structures and neuroblastoma-like areas showed a high proliferative activity of Ki-67 positivity, whereas proliferative activity in the spindle cell areas was lower. P53 showed strong nuclear expression in all epithelial and rhabdomyoblastic cells. All tumor cells stained negatively for CD30, human chorionic gonadotropin or placental alkaline phosphatase (PLAP). The immunohistochemical profile of tumor in the differential diagnosis of GCT of the liver is shown in Table 1.

Interphase cytogenetic analysis using fluorescence *in situ* hybridization (FISH) revealed that the yolk sac tumor and immature teratoma components were positive for i(12p) and 12p overrepresentation (Figure 3A). In particular, the rhabdomyoblastic cell components also showed typical i(12p) and 12p overrepresentation (Figure 3B). The FISH ratios ranged from 1.21 to 3.30 in various tumor components (mean:  $2.1 \pm 0.8$ ).

Based on the clinical features, histological findings,

immunohistochemical stains and cytogenetic studies, the pathological diagnosis of hepatic mixed tumor with significant sarcomatous components was made. Gonads and retroperitoneal lymph nodes were examined, and did not reveal any abnormality. This patient was cured with a standard protocol of cisplatin, etoposide and bleomycin. Five months after surgery, the patient died of hepatic failure with tumor recurrence and thrombosis of the intrahepatic veins, which indicates that this tumor had the low chemosensitivity. Permission for an autopsy was not granted.

## DISCUSSION

GCTs are a diverse family of neoplasms, and they are gonadal and extragonadal in origin<sup>[1]</sup>. Mixed GCTs include mature/immature teratoma, and one or more malignant germ cell components. Sarcomatous differentiation has been observed previously in primary and metastatic GCTs<sup>[10,11]</sup>. We describe a hepatic mixed GCT with significant sarcomatous components and elevated serum AFP in an adult man. The tumor was composed of two GCT components: yolk sac tumor and immature

Table 1 Immunohistochemical and FISH profiles of tumor that is considered in the differential diagnosis of GCT of the liver

Markers	Yolk sac tumor component	Immature glandular structure	Primitive mesenchymal component	Rhabdomyoblastic component	Chondroid and osteoblastic components	Neuroblastoma component
Antigen						
AFP	+++	+ <sup>1</sup>	-	-	+	-
AE1/AE3	+++	+++	-	-	-	-
Hep par 1	-	+	-	-	-	-
CK7	-	+	-	-	-	-
CK18	+	+++	-	-	-	-
CK19	+++	+++	-	-	-	-
CK20	-	+	-	-	-	-
EMA	-	+	-	-	-	-
$\alpha$ -1-antitrypsin	+	+	-	-	-	-
$\alpha$ -1-chymotrypsin	-	+	-	-	-	-
pCEA	-	++	-	-	-	-
mCEA	-	++	-	-	-	-
Vimentin	-	-	+++	+++	++	+++
Desmin	-	-	-	+++	-	-
Myoglobin	-	-	-	+++	-	-
SMA	-	-	+ <sup>1</sup>	-	-	-
CD30	-	-	-	-	-	-
HCG	-	-	-	-	-	-
GFAP	+	-	-	-	-	++
NSE	-	-	++	-	-	+++
S-100	-	-	-	-	++	++
Synaptophysin	-	-	-	-	-	++
PLAP	-	-	-	-	-	-
CD56	-	-	+	-	-	++
HMB45	-	-	-	-	-	+ <sup>1</sup>
CD117	-	-	++	-	-	-
CD34	-	-	+ <sup>1</sup>	-	-	-
Ki67	+++	+++	+	++	-	+++
P53	+++	+++	-	++	-	++
CD45	-	-	-	-	-	-
FISH	+	+	-	+	-	-

<sup>1</sup>Focal. +: Positive; ++: Strongly positive; -: Negative; FISH: Fluorescence *in situ* hybridization; GCT: Germ cell tumor; AFP:  $\alpha$ -fetoprotein; EMA: Epithelial membrane antigen; CEA: Carcinoembryonic antigen; SMA: Smooth muscle actin; HCG: Human chorionic gonadotropin; GFAP: Glial fibrillary acid protein; NSE: Neuron-specific enolase; PLAP: Placental alkaline phosphatase.

teratoma. The components of immature teratoma included epithelial (keratinizing or non-keratinizing, glandular, ductal or acinar), mesenchymal (rhabdomyoblastic, chondroid and osteoblastic differentiation) and neuroectodermal (neuroblastoma exhibiting rosettes formation, and ganglia) differentiation structures. GCTs in the liver may be primary or metastatic, therefore, it is important to exclude a gonadal tumor before making a diagnosis of malignant GCT. The present case was without any history of pretreatment, because no other tumor site was detected by extensive preoperative staging. This suggested that the tumor was primary to the liver. Therefore, malignant mixed GCT with significant sarcomatous components arising in the liver was diagnosed, based on the clinical features, histological findings, immunohistochemical stains and cytogenetic studies. To the best of our knowledge, this is the first report of hepatic malignant mixed GCT with significant sarcomatous components in adults.

Sarcomatous components are more common in mediastinal GCTs than those of other locations<sup>[10,11]</sup>. Although the pathogenesis of the development of sarcomatous components in GCTs has not been fully elucidated, it has

been proposed to include a dedifferentiation phenomenon; malignant transformation of certain mesenchymal components within teratomas; origin from primitive germ cells; and transformation of the blastematos stroma in yolk sac tumor<sup>[10,11]</sup>. In the present case, the rhabdomyoblastic cell components also showed typical i(12p) and 12p overrepresentation, which supports the origin of the sarcomatous components from malignant transformation of mesenchymal components within the teratoma, and GCTs have the potential to develop almost any type of sarcoma<sup>[10,11]</sup>.

Another interesting finding is the presence of focal pancreatic acinar differentiation, with positive staining for AFP,  $\alpha$ -1-antitrypsin, and chymotrypsin. AFP production is normally a feature of yolk sac, embryonal liver, and embryonal gastrointestinal tract, and it is recapitulated in neoplasms of these structures. Such a phenomenon has been described under the term hepatoid carcinoma in tumors at numerous sites<sup>[12]</sup>. The production of AFP by pancreatic acinar neoplasms is well recognized, and Cingolani *et al*<sup>[12]</sup> have reported a case of such a tumor arising in a mediastinal teratoma, similar to our case but lacking the sarcomatoid components. Therefore, our case



supports the hypothesis that AFP production in pancreatic neoplasms is related to acinar cell differentiation.

In the differential diagnosis, the tumor has to be compared with several entities, including carcinosarcoma, hepatocellular carcinoma or cholangiocarcinoma with sarcomatoid dedifferentiation, yolk sac tumor, mixed hepatoblastoma with teratoid features, lymphoma, and melanoma. The latter two are excluded by negativity of the tumor markers<sup>[13,14]</sup>. Carcinosarcoma of the liver is a malignant tumor that contains an intimate mixture of carcinomatous (either hepatocellular or cholangiocellular) and differentiated sarcomatous elements (such as osteosarcoma, angiosarcoma, rhabdomyosarcoma or malignant schwannoma). Unlike GCT, true carcinosarcoma consists of only a single malignant epithelial component and a single malignant mesenchymal component. They must be distinguished from sarcomatoid carcinoma or spindle-cell carcinoma, morphological variants of hepatocellular carcinoma and/or cholangiocarcinoma. The sarcomatoid part that consists of spindle cells is epithelial marker positive. HepPar1 and pCEA are useful markers for hepatocytes<sup>[15]</sup>. A true immature teratoma does not contain fetal and embryonal hepatoblastoma areas.

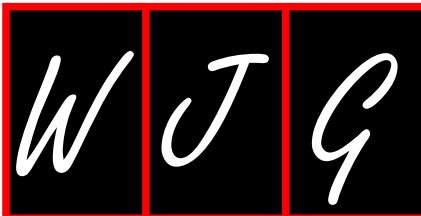
The role of chemotherapy remains speculative. Although the prognosis of GCTs depends on the site and clinical stage, the emergence of sarcomatous components in a GCT portends a worse prognosis, and appears to be highly resistant to the standard combination chemotherapy commonly employed for the treatment of GCT. However, numerous authors have administered chemotherapy to GCTs with sarcomatous differentiation, and some have achieved complete remission<sup>[10,11]</sup>. Our case was also treated with adjuvant chemotherapy after complete surgical excision according to sarcoma protocols, but the effectiveness did not appear to be obvious. Therefore, the role of chemotherapy needs to be clarified in this extremely rare tumor. Addition of sarcoma specific treatment modalities should be explored in such patients to increase the chances for survival.

In summary, primary mixed malignant GCT with sarcomatous components in the liver is extremely rare. The presence of sarcomatous components in GCT is a factor that portends a more aggressive behavior, and appears to be highly resistant to standard chemotherapy. As a result of the extreme rarity of this disease, it appears very important to add additional cases of this pathology to the literature. Further studies are needed to identify prognostic factors, and the role of chemotherapy in the management of GCT.

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## No evidence of Permacol rejection presented by Wotton and Akoh

Neil James Smart

Neil James Smart, Department of General Surgery, Royal Devon & Exeter Hospital, Barrack Road, Exeter, EX2 5DW, United Kingdom

Author contributions: Smart NJ wrote this letter.

Correspondence to: Neil James Smart, PhD, MBBS (Hons), MRCSEd, Department of General Surgery, Royal Devon & Exeter Hospital, Barrack Road, Exeter, EX2 5DW, United Kingdom. [drneilsmart@hotmail.com](mailto:drneilsmart@hotmail.com)

Telephone: +44-1392-411611

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### Abstract

Wotton and Akoh in their previously reported case in this journal postulate that Permacol rejected. This letter provides a detailed critique of that claim and provides an alternative explanation for the histological data provided by the authors. It is also argued that Wotton and Akoh have misrepresented one of the papers in the discussion in their article and a clarification of that referenced paper is given.

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**Key words:** Porcine dermal collagen implant (Permacol); Renal Transplant; Rejection

**Peer reviewer:** Antonio Basoli, Professor, General Surgery "Paride Stefanini", Università di Roma - Sapienza, Viale del Policlinico 155, Roma 00161, Italy

Smart NJ. No evidence of Permacol rejection presented by Wotton and Akoh. *World J Gastroenterol* 2010; 16(5): 657-658 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i5/657.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i5.657>

### TO THE EDITOR

Wotton and Akoh<sup>[1]</sup> have misrepresented one of the cases published in a case series I co-authored<sup>[2]</sup> on the use of Permacol in abdominal wall closure in paediatric renal transplantation in a specious attempt to justify their conclusions.

It is true that one out of five children did suffer a dehiscence of their abdominal wound following the use of Permacol to close the abdominal wall in size mismatched renal transplants. In the particular case discussed, the 9-year-old male had steroid refractory nephrotic syndrome and underwent a bilateral nephrectomy for persistently low albumin levels. That the wound did not heal was much more likely due to chronic steroid use and hypoalbuminaemia than any reaction to the Permacol. It is misleading to use this case as supporting evidence for Wotton and Akoh's claims that Permacol may induce foreign body reaction or rejection.

I see no reliable evidence presented in this case report that Permacol was "rejected". Firstly, we are not told which suture material was used to suture the Permacol in place, but we are informed that the "histology revealed features of acute and chronic inflammation superficially and granulomatous inflammation in the deep layer consistent with a "stitch granuloma". Is it not possible that this "stitch granuloma" could well have been due to the suture material itself rather than Permacol? Secondly, the use of the term "rejection" implies a specific immunopathological entity. "Features of acute and chronic inflammation" is so non-specific that it could be due to any number of factors, but I hypothesize that it was most likely due to the contaminated field. No detailed pathological or immunological evidence (such as characterisation of lymphocytes present, immunofluorescence, immunostaining or electron microscopy) is presented to substantiate the claim of rejection of the Permacol. Since this is alleged to be the first report on rejection of Permacol in humans, the evidence needs to be

more substantial than that presented before the claim can be given credence.

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abdominal wall repair: a case report. *World J Gastroenterol* 2009; **15**: 4331-4333

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**Kyoichi Adachi, MD**, Department of Gastroenterology and Hepatology, Shimane University, School of Medicine Shimane, 89-1 Enya-cho, Izumo-shi Shimane 693-8501, Japan

**Ibrahim A Al Mofleh, Professor**, Department of Medicine, College of Medicine, King Saud University, PO Box 2925, Riyadh 11461, Saudi Arabia

**Alexandra A Alexopoulou, MD**, 2nd Department of Internal Medicine, University of Athens, Medical School, Hippokraton General Hosp, 40 Konstantinoupolos St, 16342 Hilioupolois Athens, Greece

**Fernando Azpiroz, MD**, Digestive System Research Unit, University Hospital Vall d'Hebron, Paseo Vall d'Hebron, 119-129, Barcelona 08035, Spain

**Dario Conte, Professor**, GI Unit - IRCCS Osp. Maggiore, Chair of Gastroenterology, Via F. Sforza, 35, Milano 20122, Italy

**Peter Draganov, Dr.**, Division Gastroenterology, Hepatology and Nutrition, University of Florida, Gainesville, 1600 SW Archer Road PO Box 100214, Florida, FL 32610, United States

**Yasuhiro Fujino, MD, PhD, Director**, Department of Surgery, Hyogo Cancer Center, 13-70 Kitaoji-cho, Akashi, 673-8558, Japan

**Ram Prakash Galwa, Dr., MBBS, MD**, Department of Diagnostic Imaging, The Ottawa hospital, 751 parkdale Avenue, Apartment 803, Ottawa, k1y1j7, Canada

**Mario Guslandi, Professor**, Department of Gastroenterology, S: Raffaele University Hospital, S: Raffaele University Hospital via Olgettina 60, Milano 20132, Italy

**Richard Hu, MD, MSc**, Division of Gastroenterology, Department of Medicine, Olive view-UCLA Medical Center, 14445 Olive View Drive, Los Angeles, CA 91342, United States

**Syed MW Jafri, Professor**, Medicine/Gastroenterology, Aga Khan University, POB 3500, Karachi 74800, Pakistan

**Milan Jirsa, Dr.**, Laboratory of Experimental Medicine - building Z1, Institute for Clinical and Experimental Medicine, Videnska 1958/9, Praha 4, 140 00, Czech

**Boris Kirshtein, MD**, Department of Surgery, "A" Soroka Medical Center, Ben Gurion University of the Negev, POB 151, Beer Sheva, 84101, Israel

**Limas Kupcinskas, Dr., Professor**, Gastroenterology of Kaunas University of Medicine, Mickeviciaus 9, Kaunas LT 44307, Lithuania

**Christopher Mantyh, MD, Associate Professor**, Department of Surgery, Duke University Medical Center, Box 3117, Durham, NC 2771, United States

**Konstantinos Mimidis, MD, PhD, Assistant Professor** in Medicine, First Department of Internal Medicine, University Hospital of Alexandroupolis, Dragana Region, GR-68100, Alexandroupolis, Greece

**Silvio Nadalin, MD, PhD, Director** of Transplant Programm, Department of General, Visceral and Transplant Surgery, University Hospital Tuebingen, Hoppe Seyler Str 3, 72076 Tuebingen, Germany

**Kazunari Tominaga, MD**, Department Of Gastroenterology, Osaka City University, 1-4-3 Asahimachi Abenoku, Osaka 545-8585, Japan

**Sun-Lung Tsai, MD, PhD, Professor, Director**, Hepato-gastroenterology Section, Department of Internal Medicine and Liver Research Unit, Department of Medical Research, Chi Mei Medical Center, 901 Chung Hwa Road, Young-Kang City, Tainan County 710, Taiwan, China

**Takayuki Yamamoto, MD**, Inflammatory Bowel Disease Center, Yokkaichi Social Insurance Hospital, 10-8 Hazuyamacho, Yokkaichi 510-0016, Japan





## Meetings

### Events Calendar 2010

January 25-26  
Tamilnadu, India  
International Conference on Medical  
Negligence and Litigation in Medical  
Practice

January 25-29  
Waikoloa, HI, United States  
Selected Topics in Internal Medicine

January 26-27  
Dubai, United Arab Emirates  
2nd Middle East Gastroenterology  
Conference

January 28-30  
Hong Kong, China  
The 1st International Congress on  
Abdominal Obesity

February 11-13  
Fort Lauderdale, FL, United States  
21th Annual International Colorectal  
Disease Symposium

February 26-28  
Carolina, United States  
First Symposium of GI Oncology at  
The Caribbean

March 04-06  
Bethesda, MD, United States  
8th International Symposium on  
Targeted Anticancer Therapies

March 05-07  
Peshawar, Pakistan  
26th Pakistan Society of  
Gastroenterology & Endoscopy  
Meeting

March 09-12  
Brussels, Belgium  
30th International Symposium on  
Intensive Care and Emergency  
Medicine

March 12-14  
Bhubaneswar, India  
18th Annual Meeting of Indian  
National Association for Study of  
the Liver

March 23-26  
Cairo, Egypt  
14th Pan Arab Conference on  
Diabetes PACD14

March 25-28  
Beijing, China  
The 20th Conference of the Asian

Pacific Association for the Study of  
the Liver

March 27-28  
San Diego, California, United States  
25th Annual New Treatments in  
Chronic Liver Disease

April 07-09  
Dubai, United Arab Emirates  
The 6th Emirates Gastroenterology  
and Hepatology Conference, EGHG  
2010

April 14-17  
Landover, Maryland, United States  
12th World Congress of Endoscopic  
Surgery

April 14-18  
Vienna, Austria  
The International Liver Congress™  
2010

April 28-May 01  
Dubrovnik, Croatia  
3rd Central European Congress  
of surgery and the 5th Croatian  
Congress of Surgery

May 01-05  
New Orleans, LA, United States  
Digestive Disease Week Annual  
Meeting

May 06-08  
Munich, Germany  
The Power of Programming:  
International Conference on  
Developmental Origins of Health  
and Disease

May 15-19  
Minneapolis, MN, United States  
American Society of Colon and  
Rectal Surgeons Annual Meeting

June 04-06  
Chicago, IL, United States  
American Society of Clinical  
Oncologists Annual Meeting

June 09-12  
Singapore, Singapore  
13th International Conference on  
Emergency Medicine

June 14  
Kosice, Slovakia  
Gastro-intestinal Models in  
the Research of Probiotics and  
Prebiotics-Scientific Symposium

June 16-19  
Hong Kong, China  
ILTS: International Liver  
Transplantation Society ILTS Annual  
International Congress

June 20-23  
Mannheim, Germany  
16th World Congress for  
Bronchoesophagology-WCBE

June 25-29  
Orlando, FL, United States  
70th ADA Diabetes Scientific  
Sessions

August 28-31  
Boston, Massachusetts, United States  
10th OESO World Congress on  
Diseases of the Oesophagus 2010

September 10-12  
Montreal, Canada  
International Liver Association's  
Fourth Annual Conference

September 11-12  
La Jolla, CA, United States  
New Advances in Inflammatory  
Bowel Disease

September 12-15  
Boston, MA, United States  
ICAAC: Interscience Conference  
on Antimicrobial Agents and  
Chemotherapy Annual Meeting

September 16-18  
Prague, Czech Republic  
Prague Hepatology Meeting 2010

September 23-26  
Prague, Czech Republic  
The 1st World Congress on  
Controversies in Gastroenterology &  
Liver Diseases

October 07-09  
Belgrade, Serbia  
The 7th Biannual International  
Symposium of Society of  
Coloproctology

October 15-20  
San Antonio, TX, United States  
ACG 2010: American College of  
Gastroenterology Annual Scientific  
Meeting

October 23-27  
Barcelona, Spain  
18th United European  
Gastroenterology Week

October 29-November 02  
Boston, Massachusetts, United States  
The Liver Meeting® 2010--AASLD's  
61st Annual Meeting

November 13-14  
San Francisco, CA, United States  
Case-Based Approach to the  
Management of Inflammatory Bowel  
Disease

December 02-04  
San Francisco, CA, United States  
The Medical Management of HIV/  
AIDS



## Instructions to authors

### GENERAL INFORMATION

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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

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- 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]

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 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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- 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. *HRSA 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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- 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. Wiczorek 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>

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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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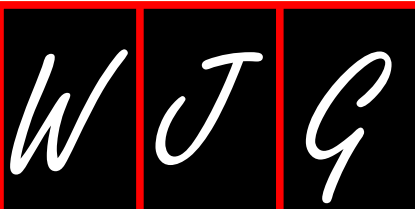
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**EDITING**  
Editorial Board of *World Journal of Gastroenterology*, Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China  
Telephone: +86-10-5908-0039  
Fax: +86-10-8538-1893  
E-mail: [wjg@wjgnet.com](mailto:wjg@wjgnet.com)  
<http://www.wjgnet.com>

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Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China  
Telephone: +86-10-8538-1892  
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Fax: +86-10-8538-1893  
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## Is the DHEAS/cortisol ratio a potential filter for non-operable constipated cases?

AM El-Tawil

AM El-Tawil, Department of Surgery, East Corridor, Ground Floor, University Hospital of Birmingham, Edgbaston, Birmingham, B15 2TH, United Kingdom

Author contributions: El-Tawil AM wrote this paper.

Correspondence to: AM El-Tawil, MSc, MRCS, PhD, Department of Surgery, East Corridor, Ground Floor, University Hospital of Birmingham, Edgbaston, Birmingham, B15 2TH, United Kingdom. [atawil20052003@yahoo.co.uk](mailto:atawil20052003@yahoo.co.uk)

Telephone: +44-121-6978231 Fax: +44-121-4466220

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### Abstract

Constipation is a significant manifestation of a number of psychological disorders. Published papers recommend using self-assessment questionnaires for discriminating psychological from non-psychological constipated patients before operating on them but reports from major surveys revealed that general practitioners failed to diagnose 70% of depressed patients using self-assessment questionnaires. Lower circulating concentrations of progesterone, 17-hydroxyprogesterone, cortisol, testosterone, androstenedione, and dehydroepiandrosterone sulfate (DHEAS) during the follicular phase in constipated young women compared with respective controls were found during the follicular phase of the menstrual cycles. During the luteal phase of the cycle, reductions were identified in estradiol, cortisol and testosterone in the constipated group. Likewise, circulating concentrations of DHEAS were found to be lower in depressed patients than comparable healthy controls. DHEAS/cortisol ratios in morning serum and salivary samples were lower than those retrieved during other times of the day in depressed patients. The idea of recognizing major depression in constipated patients by measuring DHEAS/cortisol ratios in saliva and serum may be plausible but this possibility needs to be confirmed in well-designed studies.

**Key words:** Chronic constipation; Gastrointestinal motility; Colonic inertia; Steroid hormones; Adrenal hormones; Dehydroepiandrosterone; Depression; Anxiety

**Peer reviewers:** Akio Inui, MD, PhD, Professor, Department of Behavioral Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan; Steven D Wexner, MD, Professor of Surgery, The Cleveland Clinic Foundation Health Sciences Center of the Ohio State University, and Clinical Professor, Department of Surgery, Division of General Surgery, University of South Florida College of Medicine, 21st Century Oncology Chair in Colorectal Surgery, Chairman, Department of Colorectal Surgery, Chief of Staff, Cleveland Clinic Florida, 2950 Cleveland Clinic Boulevard, Weston, Florida, FL 33331, United States

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### INTRODUCTION

Constipation is a prominent feature amongst patients with depression<sup>[1-5]</sup>, anorexia nervosa, weight loss, sleep disorders<sup>[6]</sup>, fatigue, and decreased sexual interest, particularly with menarche or after a stressful emotional experience<sup>[7,8]</sup> or surgical operation<sup>[9,10]</sup>.

The outcome of treatment in patients with chronic constipation is unpredictable. This may be a consequence of the lack of effectiveness of such treatment or may reflect heterogeneity within patient subgroups.

### IDIOPATHIC CHRONIC CONSTIPATION AND PSYCHOLOGICAL VARIABLES

A positive link was identified by Martelli *et al*<sup>[11]</sup> between various psychiatric variables and both outlet obstruction and colonic inertia. These results were further confirmed

by others<sup>[12]</sup>. Fisher *et al*<sup>[13]</sup> conducted a survey in 50 patients, 21 of whom complained of chronic severe constipation, 29 had fecal incontinence, and none were receiving psychiatric therapy or had a history of a relevant medical condition or drug therapy that could lead to the development of any of these ailments. The participants were asked to complete the Hospital Anxiety & Depression Scale (HAD) questionnaire and the General Health Questionnaire (GHQ) before and after surgery to assess the surgical outcome. Constipated patients had significantly higher HAD depression scores in comparison with respective controls. Constipated patients who had improvement after surgery had significantly lower pre-operative HAD scores compared with those who had no improvement. Using the same parameters, incontinent patients did not differ from respective controls, but those who had bad results after surgery had significantly higher HAD scores than those who benefited from surgery.

Later on, a similar study was conducted where Ghosh *et al*<sup>[14]</sup> reported the HAD scores determined from a physician-administered questionnaire in patients with chronic constipation, ulcerative colitis, and cancer of the colon, and found a link between the score and the examined clinical symptoms, such as abdominal pain, straining at stool and urgency. The HAD scores were higher in the constipated group in comparison with others, and were significantly higher in those who complained of straining at stool.

However, use of a self-assessment questionnaire as a parameter in patients suffering from chronic disease could not be relied on without other evidence<sup>[15]</sup>. Published reports revealed that general practitioners could not diagnose a significant percentage of psychological cases using self-assessment questionnaires in patients with chronic diseases<sup>[16,17]</sup>.

## PHYSIOLOGY OF STEROIDS HORMONES AND MENSTRUAL CYCLE

Adrenal androgens represent an important component (> 50%) of the circulating androgens in menstruating females<sup>[18-23]</sup>. In males, the adrenal contribution is much less because of the testicular production of androgens. Adrenal secretion of androgens in men is about the same as in women during the follicular phase.

The adult adrenal gland secretes dehydroepiandrosterone (DHEA) at approximately 4 mg/d, DHEA sulfate (DHEAS) at 7-15 mg/d, and androstenedione at 1.5 mg/d<sup>[26]</sup>.

## RELATIONSHIPS BETWEEN STEROID HORMONES AND BRAIN FUNCTIONS

The relationships between steroid hormones and brain functions have mostly been considered within the framework of endocrine mechanisms as genomic responses, elicited by secretory products from steroidogenic endocrine glands, transported through the

bloodstream, and exerting actions on the brain. Ever since the biosynthesis of steroid hormones in the brain<sup>[27]</sup> and their rapid non-genomic actions<sup>[28,29]</sup> were first reported, specific targets for so-called “neurosteroids” in plasma membranes have been postulated.

DHEA and its metabolites, DHEAS and androsterone, have been identified recently as having neurosteroid activity.

DHEAS modulates the actions of the gamma-aminobutyric acid type A (GABAA) receptor, the N-methyl-D-aspartate receptor, and the sigma subtype 1 ( $\sigma_1$ ) receptor<sup>[27,30-35]</sup> among others<sup>[36-38]</sup>. DHEA and DHEAS generally act as noncompetitive antagonists of the GABAA receptor. GABAA receptor-mediated regulation of 5-hydroxytryptamine (5-HT) neuronal firing was found to be sensitive to negative modulation by DHEA and DHEAS, and to positive modulation by androsterone. GABAA receptor-mediated regulation of 5-HT firing may be responsible for some of the reported behavioral and psychological effects of endogenous and exogenous DHEA<sup>[39]</sup>. An assessment of depression ratings in relation to plasma concentrations of several steroid hormones (estradiol, testosterone, estrone, androstenedione, cortisol, DHEA, and DHEAS) in 699 postmenopausal women (aged 50-90 years) who were not taking the contraceptive pill<sup>[40]</sup> found that only DHEAS concentrations were negatively correlated with ratings of depressed mood. Explicitly, higher DHEAS concentrations were associated with less depression, and this association was independent of age, physical activity and weight change. Furthermore, women with categorical diagnoses of depression had significantly lower plasma DHEAS concentrations compared to age-matched non-depressed women<sup>[41]</sup>. Similarly, in a large-scale study of 2855 well-functioning elderly men and women, serum DHEAS concentrations were inversely correlated with depressive symptoms<sup>[42]</sup>. Women whose first onset of major or minor depression occurred during the peri-menopause showed low morning plasma DHEA and DHEAS concentrations<sup>[43]</sup>. Lower plasma DHEA concentrations during pregnancy and during the postpartum period were associated with higher postpartum ratings of depression<sup>[44]</sup>.

## PATHOPHYSIOLOGY OF STEROID HORMONES IN SEVERELY CONSTIPATED PATIENTS

Levels of progesterone, 17-hydroxyprogesterone, cortisol, testosterone, androstenedione, and DHEAS were found to be lower during the follicular phase of the menstrual cycle in patients diagnosed with idiopathic chronic constipation compared with respective healthy controls<sup>[44]</sup>.

A lack of estradiol, cortisol and testosterone was identified during the luteal phase of the cycle in the constipated group<sup>[45]</sup>. The high prevalence of idiopathic constipation in pre-menopausal women is likely a result of the high affinity of progesterone for progesterone receptors together with the non-specific affinity for adrenal androgen receptors, and the lack of a stimulatory effect of estrogen on the wall of the bowel<sup>[45]</sup>.

## MEASUREMENT OF DHEAS/CORTISOL RATIO AND PSYCHOLOGICAL VARIABLES

A deficiency of DHEAS is thus identified in depression and constipation and it would be impossible to rely on it for distinguishing depressed, severely constipated patients. However, estimates of DHEA-to-cortisol ratios in serum and saliva, are likely to be more reliable than concentrations of either hormone alone, with lower morning ratios seen in depression<sup>[46-48]</sup>. The molar DHEAS/cortisol ratio was significantly lower in non-medicated depressed patients than in controls, and the evening salivary DHEA/cortisol ratio was inversely correlated with the length of the current depressive episode<sup>[49]</sup>. Morning salivary DHEA hyposecretion as well as evening cortisol hypersecretion were significantly and independently associated with major depression in young patients<sup>[49]</sup>.

## IMPLICATIONS IN RESEARCH AND IN CLINICAL PRACTICE

This review hypothesizes that measurement of the DHEAS/cortisol ratio in constipated patients could filter out those patients with a psychological disorder and improve the outcome after surgery.

For assessing the credibility of this measurement, a well-designed study needs to be conducted. The recruited constipated patients should have no personal or family background of a major psychological disorder. Participants should be asked to complete a stool diary. In this diary, they would be asked to report on the frequency, shape, consistency of stool, and whether they strained at defecation or not. The starting day would be their first menstruating day of the nearest menstruation cycle and the end would be the commencement of the next one. They would comment on the day and timing of defecation, stool consistency, stool form and the presence or absence of straining at stool.

They would also be asked to give 3 blood samples and 3 samples of saliva in any day during the mid-follicular period (days 7-10). The first samples would be collected early in the morning, the second on the afternoon and the third would be collected early in the evening.

Similarly, the same process would be repeated in any day during the mid-luteal period (days 18-20) to measure the DHEAS/cortisol ratio in serum and in saliva. The whole process would be repeated over the second consecutive menstrual cycle to obtain an average of each measurement. On these 2 d, participants would also be asked to fill in the Hospital Anxiety & Depression Scale questionnaire and General Health Questionnaire.

During the mid luteal period, the value of the DHEAS/cortisol ratio is supposed to be higher than that during the mid-follicular period in purely constipated patients. Because of ovarian secretions during the luteal phase, the concentration of DHEAS would be nearly normal but that of cortisol would still be low. However,

in mixed cases, the ratio would be low, and in particular in the early morning samples.

## CONCLUSION

The use of self-assessment questionnaires for excluding a psychological disorder in severely constipated patients seems insufficient, but the idea of measuring serum and salivary DHEAS/cortisol ratios before embarking upon invasive treatments appears to be more specific. This conclusion warrants confirmation in well-designed studies.

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## Iron: An emerging factor in colorectal carcinogenesis

Anita CG Chua, Borut Klopčič, Ian C Lawrance, John K Olynyk, Debbie Trinder

Anita CG Chua, Borut Klopčič, Ian C Lawrance, John K Olynyk, Debbie Trinder, School of Medicine and Pharmacology, The University of Western Australia, Fremantle Hospital, Fremantle 6160, Western Australia, Australia

Anita CG Chua, John K Olynyk, Debbie Trinder, Western Australian Institute for Medical Research, Fremantle Hospital, Fremantle 6160, Western Australia, Australia

Borut Klopčič, Ian C Lawrance, The Centre for Inflammatory Bowel Diseases, Fremantle Hospital, Fremantle 6160, Western Australia, Australia

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**Correspondence to:** Debbie Trinder, Professor, School of Medicine and Pharmacology, The University of Western Australia, Fremantle Hospital, PO Box 480, Fremantle 6160, Western Australia, Australia. [debbie.trinder@uwa.edu.au](mailto:debbie.trinder@uwa.edu.au)  
Telephone: +61-8-94313640 Fax: +61-8-94312977

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### Abstract

The carcinogenic potential of iron in colorectal cancer (CRC) is not fully understood. Iron is able to undergo reduction and oxidation, making it important in many physiological processes. This inherent redox property of iron, however, also renders it toxic when it is present in excess. Iron-mediated generation of reactive oxygen species *via* the Fenton reaction, if uncontrolled, may lead to cell damage as a result of lipid peroxidation and oxidative DNA and protein damage. This may promote carcinogenesis through increased genomic instability, chromosomal rearrangements as well as mutations of proto-oncogenes and tumour suppressor genes. Carcinogenesis is also affected by inflammation which is exacerbated by iron. Population studies indicate an association between high dietary iron intake and CRC risk. In this editorial, we examine the link between

iron-induced oxidative stress and inflammation on the pathogenesis of CRC.

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**Key words:** Iron; Haem; Colorectal cancer; Oxidative stress; Inflammation; Haemochromatosis

**Peer reviewer:** William Dickey, MD, PhD, Altnagelvin Hospital, Londonderry, BT47 6SB, Northern Ireland, United Kingdom

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### INTRODUCTION

Colorectal cancer (CRC) is the second most common cancer in developed countries. Apart from genetic mutations, environmental factors appear to play a role in intestinal carcinogenesis. Results from numerous population studies support the idea that dietary iron and/or elevated iron levels increase the risk of cancers including CRC, hepatocellular carcinoma (HCC) and lung cancer<sup>[1-6]</sup>. HCC occurs at a higher incidence in hereditary haemochromatosis (HH) patients with hepatic iron overload than in the normal population<sup>[7]</sup> and recently, an increased risk for CRC and breast cancer development in patients with HH has also been demonstrated<sup>[8,9]</sup>. Further support for a role of iron in carcinogenesis comes from animal studies. Multiple injections of iron compounds, such as iron dextran complex, ferric nitrilotriacetate and ferric saccharate in rodents result in the formation of sarcomas, renal cell carcinoma and mesothelioma, respectively<sup>[10-12]</sup>. Iron has also been implicated in intestinal carcinogenesis in rodent models of CRC<sup>[13,14]</sup>.

Iron, whilst indispensable for life, can cause tissue injury through the formation of reactive oxygen species (ROS) and the high oxidative potential of iron and its

participation in oxidative stress-related carcinogenesis have been reviewed in detail elsewhere<sup>[15-17]</sup>. The excessive generation of oxidative stress can lead to carcinogenic events, and this has been the premise for the hypothesis that high iron levels may potentiate the risk of cancer. In addition, iron is a source of sustenance for cancer cell growth and proliferation. Cancer cell growth is enhanced by iron administration and has been shown to be retarded by both dietary iron deprivation<sup>[18]</sup> and treatment with iron chelators<sup>[19,21]</sup>. It is thought that genetic modifications and continual activation of the signalling pathways of cell proliferation by ROS synergistically promote carcinogenesis<sup>[16,22]</sup>. Chronic inflammation also induces cell oxidative stress, which promotes the onset of dysplasia<sup>[23]</sup> and is accompanied by a dysregulation in iron metabolism<sup>[24]</sup>. Nonetheless, the mechanistic link among iron, oxidative stress, inflammation and colorectal carcinogenesis remains to be elucidated.

## CRC

The incidence of CRC varies among countries and this has been mainly attributed to environmental factors, although genetic factors are also important. Environmental risk factors for colorectal carcinogenesis include many dietary factors such as high red meat and alcohol consumption as well as low fibre and vegetable intake<sup>[25,26]</sup>.

The majority of CRCs originate from pre-existing adenomatous polyps of the colonic mucosa<sup>[27]</sup>. These are defined as well demarcated masses of epithelial mucosa with increased crypt proliferation. Eventually, neoplastic cells migrate through the muscularis mucosa and it is once the basement membrane surrounding these cells is breached that the lesions are classified as malignant. These morphological and histopathological changes are accompanied by sequential dysregulation of key molecular pathways of cell division and tissue homeostasis<sup>[28]</sup>. Several syndromes have been described in families with a history of CRC, which involve mutations in components of these pathways<sup>[29]</sup>. Affected persons with familial adenomatous polyposis (FAP) develop hundreds of adenomatous polyps throughout their lifetime, some of which inevitably progress to malignancy. Genetic studies in subjects with FAP led to the discovery of the adenomatous polyposis coli (*APC*) gene, a key gene in the regulation of mucosal epithelial maturation *via* the Wnt signalling pathway<sup>[30,31]</sup>. In contrast, hereditary non-polyposis colorectal cancer (HNPCC) is characterised by an increased risk for developing CRC in the absence of a germ line mutation in the *APC* gene and is typically accompanied by the loss of DNA mismatch repair genes which impacts on other signalling pathways<sup>[32,33]</sup>. Although there is currently no evidence that iron plays a role in the pathology of these syndromes, it is interesting to note that iron can increase Wnt signalling in the absence of *APC*<sup>[34]</sup> and that mutations in the haemochromatosis gene (*HFE*) act as a genetic modifier of HNPCC disease expression<sup>[35]</sup>.

Although there is a strong role for genes in the pathogenesis of CRC in the above-mentioned risk groups,

environmental factors seem to play a more significant role. Population based studies have shown that in immigrant groups, the incidence of CRC changes towards that observed in the host country<sup>[36,37]</sup>. Another indication of the importance of environmental factors is that in Japan, a country with a traditionally low incidence of CRC, the rate has rapidly increased in recent years, a circumstance that has been primarily attributed to changes in life-style in the recent decades<sup>[29]</sup>. It is also interesting to note, that one of the highest incidences of CRC in the United States can be found in Japanese Hawaiians, highlighting the significance of environment *vs* genes<sup>[38]</sup>.

Of the environmental risk factors, the diet is of particular interest since it impacts on the composition of the intestinal luminal contents, which are in direct contact with the colonic mucosa. Diets between countries vary significantly in their iron content and iron-rich food components, suggesting that iron intake could be one of the factors influencing CRC incidence in different populations. Dietary iron as an environmental modifier of CRC has been examined in population-based studies and there is evidence that both dietary iron<sup>[4,5,39]</sup> and/or increased body iron stores<sup>[1,2,40]</sup> enhance the risk of CRC.

## IRON METABOLISM

Iron is a vital trace element participating in numerous biological and cellular processes such as electron transfer, oxygen transport and DNA synthesis as well as cell cycle progression and growth<sup>[41]</sup>. Iron absorption from the diet occurs mainly in the duodenum by a tightly regulated process. Most of the absorbed iron is utilized for erythropoiesis and any excessive iron is stored mainly in the liver<sup>[42]</sup>. Dietary iron occurs in two forms, haem iron from red meat and non-haem iron from plants and dairy products. Both forms of iron are taken up from the intestinal lumen into the enterocyte by different pathways. Haem iron is taken up as an intact metalloporphyrin by a haem transporter, the identity of which has yet to be confirmed. After entering the enterocyte, haem is broken down by haem oxygenase into free iron, biliverdin that is rapidly converted to bilirubin and carbon monoxide<sup>[43,44]</sup>. In contrast, non-haem ferric iron is reduced to ferrous iron by a ferrireductase and is then taken up by divalent metal transporter 1 at the apical surface of enterocytes. The iron from both sources enters a common intracellular iron pool and is stored as ferritin or transferred across the basolateral membrane of the enterocyte into the circulation by ferroportin. Upon release, iron is oxidised by hephaestin and binds to plasma transferrin. Transferrin-bound iron is taken up by cells *via* transferrin receptors. Iron absorption is inversely regulated by body iron levels, increasing during iron deficiency and decreasing in conditions of iron excess. Iron metabolism is regulated by the hepatic hormone, hepcidin, and its expression is controlled by many factors including iron stores, hypoxia, inflammation, anaemia and erythropoiesis<sup>[45,46]</sup>. The regulation of cellular iron metabolism has been extensively reviewed elsewhere<sup>[42,47-49]</sup>.

### Iron and CRC risk

The association between dietary iron and CRC risk has been examined in many population-based studies. A meta-analysis of studies investigating dietary iron intake, body iron stores and CRC demonstrated a positive correlation between iron in the diet and CRC risk<sup>[50]</sup>. Notably, two large prospective cohort studies have found that high iron intake and CRC risk were associated with other factors such as a high fat diet or bile acids<sup>[4,5]</sup> and at least three other case control studies have corroborated the positive correlation between dietary iron and CRC<sup>[39,51,52]</sup>. Of the studies analysing body iron stores and CRC, one large cohort study observed an association between transferrin saturation and CRC risk<sup>[2]</sup> whilst three case control studies found a positive correlation between serum ferritin levels and the formation of colorectal adenomatous polyps<sup>[1,40,53]</sup>. Other studies, however, reported inverse correlations between transferrin saturation<sup>[54]</sup> or ferritin levels<sup>[5]</sup> and CRC risk. The role of body iron stores in CRC appears more complex than that of dietary iron and the influence of genetic factors on body iron stores will be discussed in more detail below.

The effect of high red meat consumption, as a dietary source of iron, on the pathogenesis of CRC has been of considerable interest. Red meat is a major component of the human diet in some societies and contains a high amount of myoglobin and haemoglobin. Both contain haem, a porphyrin structure that contains a central iron atom and it has been suggested that the haem content in red meat promotes colorectal carcinogenesis<sup>[55,56]</sup>. A meta-analysis of 48 studies specifically addressing red meat consumption showed a significantly increased risk of developing CRC in people with a high intake of red meat as well as processed meat in most of the studies<sup>[57]</sup>. Of interest is a recent very large prospective cohort study investigating nutrition and disease that described an increased risk of CRC in people who consumed red meat rich in haem, whilst no increased risk was identified for poultry and an inverse correlation was observed for fish, both of which have a lower haem content<sup>[58]</sup>. Another two prospective cohort studies also reported that haem iron was associated with a higher risk of CRC especially in those who consumed alcohol<sup>[59]</sup> or those with a low intake of chlorophyll<sup>[60]</sup>. It is, however, unclear whether the effects of red meat on colorectal carcinogenesis are due to haem, the iron bound to haem, or a combination of both.

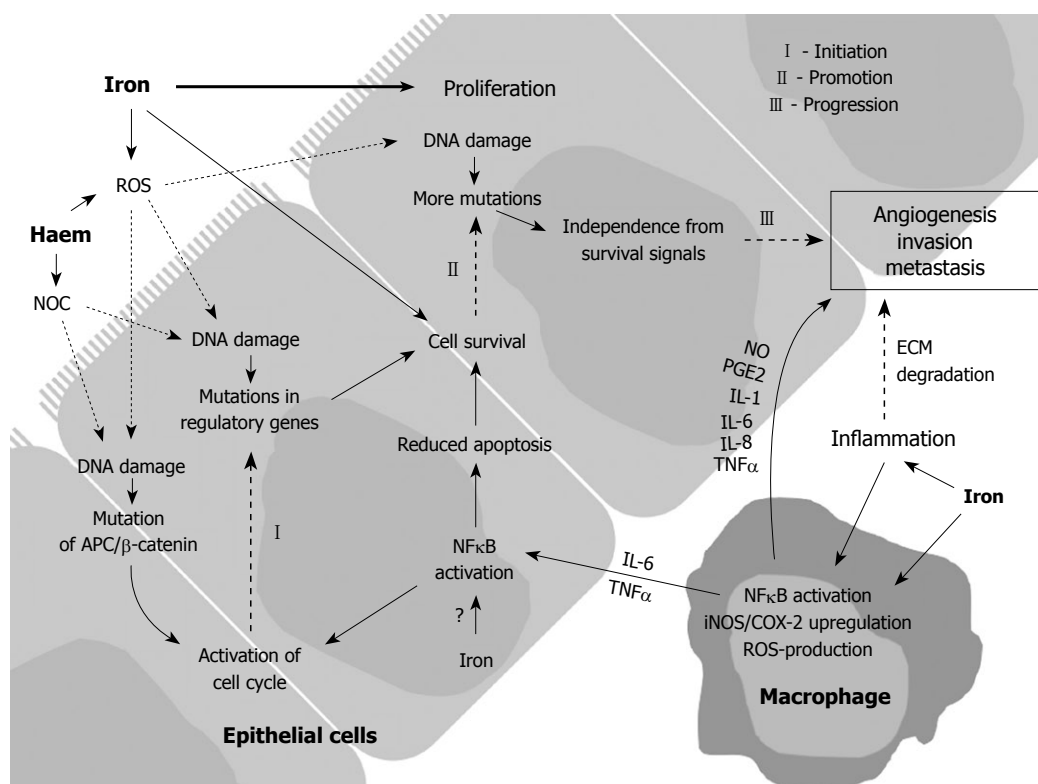
HH is a common disorder of iron metabolism that usually results from a homozygous C282Y mutation in the *HFE* gene. *HFE* protein is a key regulator of hepcidin, and in HH, the *HFE*-mediated regulation of hepcidin is impaired resulting in excessive absorption of iron and increased deposition of iron primarily in the liver<sup>[47,60]</sup>. As mentioned earlier, these individuals have an increased risk of developing CRC. This is exemplified by the recent findings that patients homozygous for the C282Y mutation have a 2.4-fold increased risk of developing CRC<sup>[9]</sup>. This is of particular significance considering that the C282Y mutation is one of the most abundant autosomal mutations in some Western

societies with homozygosity rates ranging from 1/102 in Northern Ireland to less than 1/100 000 in Greece<sup>[61]</sup>. The homozygosity rate of 1/385 in the United States of America calculates as an estimated number of 718 000 affected individuals with an increased risk of CRC<sup>[61]</sup>. Another study describing a 7.7-fold increased risk of developing CRC in patients with homozygous mutations in both *HFE* and *TFR1* genes, further implicates a dysregulation in iron metabolism as a possible mechanism contributing to colorectal carcinogenesis<sup>[62]</sup>. An increased risk for CRC has also been described for compound C282Y/H63D heterozygotes, single C282Y or H63D heterozygotes and H63D homozygotes<sup>[35,63-65]</sup>, but not all studies detected a significant correlation between *HFE* mutations and CRC<sup>[65-73]</sup>. Knekt *et al.*<sup>[2]</sup>, however, reported a 3-fold increased risk for CRC that was associated with a transferrin saturation level exceeding 60% in a large cohort from Finland, which may include subjects with mutations in the *HFE* gene. Furthermore, systemic iron reduction by phlebotomy decreases visceral malignancies and mortality in patients with peripheral arterial disease<sup>[74]</sup> and in blood donors the number of non-haematological malignancies is significantly reduced<sup>[75]</sup> indicating that reduction of iron levels might decrease CRC risk. The availability of mouse models of HH enables future studies to investigate the interaction of dietary iron, regulation of body iron stores and colorectal carcinogenesis.

### Role of luminal iron in colorectal carcinogenesis

There is evidence suggesting that significant iron absorption may occur in the colon<sup>[76,77]</sup>. Increased iron intake results in higher levels of iron in colonic epithelial cells in rats<sup>[78]</sup>, and although divalent metal transporter 1, ferroportin and hephaestin mRNA expression is highest in the duodenum and decreases along with the length of the small intestine<sup>[79]</sup>, their expression is still relatively high in the colon, especially hephaestin and ferroportin. These findings suggest that there may be significant colonic iron transport, which impacts on cell proliferation and cancer development. In a recent study, it was shown that there was increased iron staining in human colorectal tumours<sup>[80]</sup>. The expression of proteins involved in cellular iron uptake such as divalent metal transporter 1 and transferrin receptor 1 was also upregulated. The expression of the iron exporter, ferroportin, was increased but it was located intracellularly whilst hephaestin expression was decreased, suggesting decreased release of iron from the cells. These results suggest that the retention of iron by tumours may facilitate cell proliferation.

Further support for the concept of iron as a risk factor for CRC has been demonstrated in animal studies. Elevated dietary iron levels increased the incidence of tumours in rodent models of CRC induced by inflammatory or carcinogenic agents<sup>[13,14]</sup>. In these experiments, however, iron was supplemented with inorganic carbonyl iron, a form that does not constitute a major component of natural diets. Interestingly, in the inflammatory model, Seril and colleagues demonstrated that systemic iron supplementation did not increase tumour incidence. This



**Figure 1 Potential roles of iron in the development of colorectal cancer.** Luminal iron may cause DNA damage through the generation of reactive oxygen species (ROS) via the Fenton reaction. Haem may also stimulate the production of N-nitroso compounds (NOC) in the colon which are mutagenic. (I) In the initiation phase of tumorigenesis, DNA damage leads to mutations in key genes regulating cell proliferation and survival such as APC or  $\beta$ -catenin of the Wnt pathway; (II) In the promotion phase, the increase in cell proliferation and survival due to the deleterious effects of NOC and ROS leads to further genetic instability and an accumulation of more mutations. Iron is an important nutrient required for proliferation during this phase. Iron also increases intestinal inflammation and the pro-inflammatory cytokines,  $\text{TNF}\alpha$  and IL-6 released from inflammatory cells increase cell survival through inhibition of apoptosis via the activation of  $\text{NF}\kappa\text{B}$ ; (III) In the progression phase, tumour cells gain independence from survival signals and progress towards a malignant phenotype. Iron has been shown to activate  $\text{NF}\kappa\text{B}$  increasing inducible nitric oxide synthase (iNOS) and cyclo-oxygenase (COX)-2 expression in macrophages. Activated macrophages produce more ROS, increase infiltration through degradation of the extracellular matrix (ECM) and promote angiogenesis by release of nitric oxide (NO), prostaglandin E2 (PGE2), IL-1, -6 and -8 as well as  $\text{TNF}\alpha$ , ultimately leading to tissue invasion and metastasis.

suggests that increased luminal iron but not systemic iron levels increase colorectal carcinogenesis in an inflammatory model of CRC<sup>[81]</sup>. In a carcinogen-induced CRC model, the number of preneoplastic lesions increased with the amount of haem in the diet<sup>[82]</sup>. Haem iron is more bioavailable than non-haem iron and has been shown to increase mucosal proliferation and cytotoxicity, indicating that haem may have a greater propensity for inducing malignancy compared with other forms of dietary iron<sup>[55,56]</sup>. Haem has been shown to stimulate the production of endogenous N-nitroso compounds in the large intestine after red meat ingestion, many of which are pro-carcinogenic<sup>[83]</sup>. The genotoxic effect of haem has also been demonstrated in human colonic cells, where DNA damage was induced by haemoglobin and haemin<sup>[84]</sup>. The exact mechanism by which luminal iron (haem and/or non-haem), iron transport, systemic iron levels and their regulation impact the pathogenesis of CRC, however, remains to be elucidated.

### Iron and molecular pathways of colorectal carcinogenesis

Colorectal carcinogenesis is a multi-step process involving the formation of adenomatous polyps and their subsequent

progression to malignancy. At the molecular level, this process is reflected by sequential events of gene mutation and activation of key molecular pathways<sup>[85,86]</sup>. Some of these pathways may be altered by iron and iron-mediated generation of ROS (Figure 1). The *APC* gene was initially identified in patients with FAP and subsequently shown to be mutated in > 80% of human colorectal neoplasia<sup>[85]</sup>. *APC* mutations involved in carcinogenesis led to nuclear accumulation of  $\beta$ -catenin and constitutive activation of the wnt/ $\beta$ -catenin/T cell factor (TCF) signalling pathway. In rodent models of azoxymethane-induced CRC, the majority of colonic tumours harbour mutations in *APC* and/or  *$\beta$ -catenin* genes<sup>[87,88]</sup>. Activation of the wnt/ $\beta$ -catenin/TCF pathway results in increased expression of cyclin D1 and c-myc, both of which are positive regulators of cell proliferation<sup>[89,90]</sup>. Iron has been implicated in *APC* loss<sup>[34]</sup> and iron chelators decrease the expression of cyclin D1 and c-myc<sup>[41]</sup>.

The molecular pathogenesis of ulcerative colitis-associated colorectal carcinogenesis has been extensively studied<sup>[91]</sup>. Events such as chromosomal and microsatellite instability and alterations in tumour suppressor genes (*p53* and *APC* mutations) and DNA mismatch repair genes have been documented<sup>[92,93]</sup>. The inhibitor of  $\text{NF}\kappa\text{B}$  kinase



(IKK $\beta$ )/nuclear factor kappa B (NF $\kappa$ B) signalling pathway constitutes a key molecular link between inflammation and carcinogenesis. NF $\kappa$ B is activated in colorectal carcinogenesis and is influenced by both inflammation and oxidative stress<sup>[94]</sup>. NF $\kappa$ B targets the genes that control cell proliferation, apoptosis, angiogenesis and metastasis<sup>[94,95]</sup>. A direct stimulatory effect of iron on NF $\kappa$ B signalling has also been demonstrated in hepatic macrophages<sup>[96,97]</sup>.

Other pathways involved in colorectal carcinogenesis include cyclo-oxygenase (COX)-2 mediated prostaglandin E2 synthesis and inducible nitric oxide synthase (iNOS)-mediated generation of nitric oxide<sup>[98]</sup>. Prostaglandin E2 is involved in regulating angiogenesis and inhibiting apoptosis<sup>[99]</sup> whilst iNOS activity induces DNA damage and promotes microvascularisation<sup>[100]</sup>. Both COX-2 and iNOS are frequently over-expressed in human CRC<sup>[101,102]</sup> and inhibition of their activity has been shown to decrease tumorigenesis in rodent models of CRC<sup>[103,104]</sup>. Furthermore, iNOS is a target of the wnt/ $\beta$ -catenin/TCF pathway and its production of ROS through nitric oxide is catalysed by iron<sup>[105]</sup>.

## OXIDATIVE STRESS AND COLORECTAL CARCINOGENESIS

Oxidative stress occurs when the body or cell is unable to combat the deleterious effects of overproduction of oxidants or free radicals due to decreased anti-oxidant activity to counterbalance or eliminate them. Oxidative stress is related to many pathological conditions such as infection, inflammation, iron and other transition metal overload. It has also been implicated in carcinogenesis<sup>[15,106,107]</sup>. Although many reactive species and free radicals such as reactive nitrogen species contribute to oxidative stress, the role of ROS in colorectal carcinogenesis will mainly be discussed here. ROS is generated through the partial reduction of oxygen which results in superoxide anion, singlet oxygen, hydrogen peroxide and hydroxyl radical formation. ROS plays a dual role in biological systems. When the balance between oxidant and anti-oxidant activity is maintained, ROS can participate as secondary messengers in intracellular signal transduction cascades, whilst the presence of excessive ROS induces tissue damage.

Iron is a strong oxidant and when present at high levels, it generates ROS *via* the Haber-Weiss-Fenton reaction. Iron-mediated generation of ROS can cause oxidative damage to lipids, nucleic acids or proteins<sup>[86]</sup>. Oxidative damage to proteins and lipids can generate reactive intermediates that can couple to DNA bases resulting in DNA lesions<sup>[86]</sup>. DNA damage as a consequence of prolonged oxidative stress can result in mutation of proto-oncogenes and tumour suppressor genes, microsatellite instability and chromosomal rearrangements as well as a dysregulation in transcription, signal transduction and replication, all of which are associated with carcinogenesis<sup>[115,86,108]</sup>. Haem is also an oxidant<sup>[109]</sup>, and despite being essential for many biological processes and enzyme systems,

excessive free haem catalyses ROS production, resulting in oxidative stress<sup>[110]</sup>. The degradation of haem by haem oxygenase-1 alleviates oxidative stress<sup>[111]</sup> and bilirubin, a by-product of haem breakdown, is anti-oxidative and has been shown to scavenge peroxy radicals in plasma<sup>[112,113]</sup>. In mice lacking copper- and zinc-containing superoxide dismutase, oxidative damage is pervasive and the rate of liver cancer development is increased later in life<sup>[114]</sup> whilst, mice with decreased manganese-containing superoxide dismutase activity have an increased risk for lymphoma and adenocarcinoma<sup>[115]</sup>. These results suggest that reduced anti-oxidant activity can lead to cancer.

Oxidative stress is enhanced in neoplastic tissue from the colonic mucosa of CRC patients<sup>[116,117]</sup>. In these patients, lipid peroxidation is increased in colonic tumours compared with normal mucosa<sup>[116]</sup>. In addition, there is a greater extent of DNA strand breakage in colonic mucosal cells isolated from neoplastic tissues compared with normal tissues from cancer patients<sup>[117]</sup>. Oxidative damage is also more evident in the earlier stages of CRC than in the more advanced stages of cancer. The accumulation of iron in a human colon cancer cell line has been shown to correlate with increased oxidative protein and DNA damage<sup>[118]</sup>. In rodent studies, mice and rats fed a diet high in iron<sup>[119-122]</sup> and haem<sup>[82]</sup> exhibited greater lipid peroxidation activity in the colon and increased colonic aberrant crypt foci, which are pre-neoplastic lesions<sup>[82,123,124]</sup>. Oxidative damage markers are increased in the colons of *Hfe* knockout mice<sup>[125]</sup>, indicating an increased presence of ROS in these iron-loaded mice. Oxidative stress due to high iron and/or haem levels may, therefore, be instrumental in mediating colorectal carcinogenesis.

## INFLAMMATION AND CRC

The relationship between inflammation and tumour development has been a major focus in recent cancer research. Persistent inflammation as a result of infection promotes carcinogenesis; for example, infection with hepatitis B and C and human papilloma viruses are associated with HCC and cervical cancer, respectively, whilst *Helicobacter pylori* infection is linked to gastric cancer<sup>[126]</sup>. Furthermore, subjects with inflammatory bowel disease such as ulcerative colitis and Crohn's disease suffer from recurring inflammation in the colonic mucosa and are at an increased risk of developing CRC<sup>[127]</sup>.

Chronic inflammation and metabolites from phagocytic processes result in formation of excessive ROS and nitric oxide<sup>[98,128]</sup>, which as mentioned above, can directly cause damage to DNA, protein or lipids. Inflammatory cells active in chronic inflammation are also present within a tumour and its surrounding tissue. This suggests that the presence of oxidative stress and the network of inflammatory cytokines and chemokines in a tumour microenvironment may perpetuate carcinogenesis by promoting genotoxicity, proliferation and survival as well as angiogenesis, cell invasion and metastasis<sup>[129]</sup>. Cytokines that are frequently associated with carcinogenesis include TNF $\alpha$  and IL-6, which promote cell proliferation and

survival<sup>[130]</sup>. Angiogenesis, invasion and metastasis are influenced by the cytokines, TNF $\alpha$ , IL-1, -6 and -8<sup>[129]</sup>.

CRC occurs in approximately 4% of patients with ulcerative colitis<sup>[131]</sup> where the risk for CRC has been reported to be approximately 10-fold higher than in the normal population<sup>[127,132]</sup>. The risk for cancer increases with longer duration and the extent of colon affected by this disease, and how well the inflammation is controlled, indicating that it is the prolonged inflammatory stimulus that directly affects the pathogenesis of CRC in these patients. In addition to the blood loss and iron deficiency due to the chronic intestinal inflammation, patients with ulcerative colitis and Crohn's disease may also develop iron deficiency anaemia secondary to inflammation and reduced mobilization of bone marrow iron and are frequently treated with oral iron supplementation. Hence, the effects of iron on colitis-associated colorectal carcinogenesis have also been examined. Chronic inflammation induced in mice treated with dextran sodium sulphate resulted in colorectal tumorigenesis which became worse with dietary iron supplementation, indicating the tumour-promoting role of iron when inflammation was present<sup>[13]</sup>. This was accompanied by the increased presence of enhanced nitrotyrosine and iNOS expression, which implicates a role for oxidative stress in inflammation-associated carcinogenesis. Further evidence comes from experimental models where colitis is attenuated when anti-oxidant activity is increased<sup>[133,134]</sup>. In addition, the formation of pro-oxidants due to increased activity of phagocytic leukocytes in the colons of ulcerative colitis patients has been reported<sup>[135]</sup>. These findings suggest that oxidative stress induced by both inflammation and iron plays a major role in inflammation-associated colorectal carcinogenesis.

Better understanding about the relationship between inflammation and iron metabolism has been achieved since the identification of hepcidin. Inflammation affects iron homeostasis by inducing hepcidin through an IL-6-mediated pathway<sup>[45]</sup>. Increased hepcidin levels caused decreased iron absorption<sup>[136,137]</sup> as well as iron retention by reticulo-endothelial macrophages, which may result in hypoferraemia (low serum iron concentration)<sup>[138]</sup>. Hypoferraemia is associated with the anaemia of chronic disease, also known as anaemia of inflammation. Haem, like iron, is pro-inflammatory and increases the expression of inflammatory adhesion molecules in endothelial cells<sup>[109]</sup>. Haem oxygenase 1 knockout mice suffer from anaemia, tissue iron loading and severe inflammation, having enlarged spleens and lymph nodes, vasculitis and inflammatory cell infiltrates in the liver<sup>[139]</sup>. Carbon monoxide, a by-product of haem degradation, ameliorates inflammation in a mouse model of colitis<sup>[140]</sup>. High dietary iron and/or haem levels are likely to contribute to the pathogenesis of inflammation-associated colorectal carcinogenesis.

## FUTURE PERSPECTIVES

Population-based studies as well as animal studies point to a role for dietary and/or systemic body iron levels in

colorectal carcinogenesis. The effect of high iron levels on regulatory pathways of iron metabolism through HFE and hepcidin as well as the increased production of ROS in the presence of iron provide potential mechanisms. The interference of high iron levels with ROS and/or inflammation and their effects on pathways involved in colorectal carcinogenesis remains poorly understood. Future studies in mouse models of HH, dietary iron overload and colorectal carcinogenesis will provide valuable insights into this fascinating aspect of iron biology and CRC.

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Yusuf Bayraktar, Professor, Series Editor

## Recent developments in palliative chemotherapy for locally advanced and metastatic pancreas cancer

Soley Bayraktar, Ulas Darda Bayraktar, Caio Max Rocha-Lima

Soley Bayraktar, Ulas Darda Bayraktar, Caio Max Rocha-Lima, Division of Hematology/Oncology, Sylvester Comprehensive Cancer Center, University of Miami, Miami, FL 33136, United States

Author contributions: Bayraktar S, Bayraktar UD and Rocha-Lima CM equally contributed to the preparation of this manuscript.

Correspondence to: Soley Bayraktar, MD, Division of Hematology/Oncology, Sylvester Comprehensive Cancer Center, University of Miami, 1475 NW 12th Ave St 3300, Miami, FL 33136, United States. sbayraktar@med.miami.edu

Telephone: +1-305-4580999 Fax: +1-305-5851145

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### Abstract

In spite of advances made in the management of the other more common cancers of the gastrointestinal tract, significant progress in the treatment of pancreatic cancer remains elusive. Nearly as many deaths occur from pancreatic cancer as are diagnosed each year reflecting the poor prognosis typically associated with this disease. Until recently, the only treatment with an impact on survival was surgery. In the palliative setting, gemcitabine (Gem) has been a standard treatment for advanced pancreatic cancer since it was shown a decade ago to result in a superior clinical benefit response and survival compared with bolus 5-fluorouracil. Since then, clinical trials have explored the pharmacokinetic modulation of Gem by fixed dose administration and the combination of Gem with other cytotoxic or the biologically "targeted" agents. However, promising trial results in small phase II trials have not translated into survival improvements in larger phase III randomized trials in the advanced disease setting. Two trials have recently reported modest survival improvements with the use of combination treatment with Gem and capecitabine (United Kingdom National Cancer Research GEMCAP trial) or erlotinib (National Cancer Institute of Canada

Clinical Trials Group PA.3 trial). This review will focus on the use of systemic therapy for advanced and metastatic pancreatic cancer, summarizing the results of several recent clinical trials and discuss their implications for clinical practice. We will also discuss briefly the second-line chemotherapy options for advanced pancreatic cancer.

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**Key words:** Adjuvant therapy; Chemotherapy; Palliative therapy; Pancreas cancer; Radiotherapy

**Peer reviewers:** Jorg Kleeff, MD, Consultant Surgeon, Department of Surgery, Klinikum rechts der Isar, Technical University of Munich, Ismaninger Str. 22, 81675 Munich, Germany; Edward L Bradley III, MD, Professor of Surgery, Department of Clinical Science, Florida State University College of Medicine, 1600 Baywood Way, Sarasota, FL 34231, United States

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### INTRODUCTION

Pancreatic cancer is responsible for approximately 5% of cancer-related deaths and is the eighth most common cause of cancer-related death for both genders combined worldwide<sup>[1]</sup>. Recent estimates indicate that approximately 42000 new cases and deaths are expected to occur in the United States during 2009. For all the stages combined, the 1- and 5-year survival rates are only 23% and 5%, respectively<sup>[2]</sup>.

The prognosis is even poorer for patients with advanced pancreatic cancer. At the time of diagnosis, approximately half of the patients have metastases, and their median overall survival (OS) with treatment is around 6 mo; whe-

reases approximately one third of patients diagnosed with locally advanced disease have an OS ranging between 6 and 9 mo<sup>[3]</sup>. Only 15%-20% of patients are eligible for surgery at diagnosis<sup>[3]</sup>. Only about 20% of surgically resected patients with localized disease will survive 5 years. There is a clear need for better systemic treatments.

This review will summarize and discuss the various clinical trials of chemotherapy for locally advanced and metastatic pancreatic cancer, including the more recent trials, which have investigated the novel targeted agents.

## PALLIATIVE CHEMOTHERAPY FOR ADVANCED PANCREATIC CANCER

Patients with metastatic or locally advanced inoperable pancreatic cancer are enrolled in clinical trials as a group, although these patients have different prognoses. The role of radiation therapy for patients with locally advanced disease remains controversial. In addition to a survival benefit, the palliative role of chemotherapy in addition to best supportive care compared to supportive care alone has been demonstrated in advanced pancreatic cancer<sup>[4-6]</sup>. Patients treated with 5-fluorouracil (5-FU) based chemotherapy had an OS of 6-10 mo compared with 2-3.5 mo in patients who did not receive chemotherapy. Glimelius *et al*<sup>[4]</sup> also reported that quality of life (QOL) was better, and quality-adjusted survival time was longer, for patients who were randomized to chemotherapy (median of 4 mo *vs* 1 mo,  $P = 0.01$ ) since gemcitabine (Gem) was established as a standard therapeutic agent.

### Single agent Gem

The improvement in survival with 5-FU-based chemotherapy compared to best supportive care, and of Gem compared to bolus 5-FU has established Gem as the standard treatment in advanced or metastatic pancreatic cancer<sup>[7]</sup>. In phase II studies, single-agent Gem has shown modest response rates (RR) of 6%-11% with disease stabilization occurring in a further 19%-32%<sup>[8]</sup>. The toxicities observed with Gem include bone marrow suppression, lethargy, a flu-like syndrome, nausea and vomiting, and peripheral edema. Several trials have attempted to improve upon the efficacy of Gem.

**Fixed dose Gem:** The administration of Gem usually involves a fixed dose rate (FDR) of 10 mg/m<sup>2</sup> per min. Gem is a pro-drug that is converted to its active tri-phosphate form intracellularly. FDR infusion maximizes the intracellular concentrations of the phosphorylated forms of Gem<sup>[9]</sup>.

In a randomized phase II trial<sup>[10]</sup>, Gem at FDR infusion led to a higher RR and better survival, although the primary end point of time to treatment failure (ITF) was similar for both arms. (2.1 mo for FDR Gem *vs* 1.8 mo,  $P = 0.09$ ). The median survivals were 8.0 and 5.0 mo, and the 1-year survivals were 28.8 and 9%, for both arms, respectively. The incidence of hematological toxicity, particularly grade 3-4 neutropenia, was higher in the FDR Gem arm (48.8% *vs* 26.5%).

However in a phase III trial by the Eastern Cooperative Oncology Group (ECOG)<sup>[11]</sup>, the FDR of Gem or

**Table 1** Progression-free and overall survival analyses from the ECOG 6201 trial<sup>[11]</sup>

Parameter	PFS		OS	
	Median (mo)	Log-rank <i>P</i>	Median (mo)	Log-rank <i>P</i>
All eligible patients ( <i>n</i> = 824)	2.9		5.6	
Gem ( <i>n</i> = 275)	2.6		4.9	
FDR Gem ( <i>n</i> = 277)	3.5	0.09	6.2	0.15
GemOx ( <i>n</i> = 272)	2.7		5.7	

PFS: Progression-free survival; OS: Overall survival; ECOG: Eastern Cooperative Oncology Group; Gem: Gemcitabine; GemOx: Gem and oxaliplatin.

GemOx [Gem and oxaliplatin (Ox)] did not meet the survival superiority endpoint of the trial compared to standard infusion Gem. Table 1 shows the efficacy results from this trial.

### Gem-based combination chemotherapy

Despite promising phase II trials, the combination of Gem with other cytotoxic drugs has not been proved to be superior to Gem alone in survival (Table 2).

**Gem and FU:** Phase III trials of Gem plus FU compared with single-agent Gem in patients with advanced disease have not shown any benefit in terms of survival<sup>[12,13]</sup>. In a phase III ECOG trial, 322 patients with advanced pancreatic cancer were randomized to Gem alone *vs* Gem combined with FU. OS was 5.4 mo for Gem alone and 6.7 mo for Gem plus FU ( $P = 0.09$ ). Progression-free survival (PFS) for Gem alone was 2.2 mo, compared with 3.4 mo for Gem plus FU ( $P = 0.022$ ).

**Gem and capecitabine:** The combination of capecitabine and Gem (GemCap) has shown promising clinical activity in phase I and II clinical studies in advanced pancreatic cancer patients<sup>[14,15]</sup>. A phase III trial conducted by Herrmann *et al*<sup>[16]</sup> also showed positive results for good performance status (PS) patients. Of 319 patients in the study, median OS, the primary end point, was 8.4 and 7.2 mo in the combination and Gem alone arms, respectively ( $P = 0.234$ ). In addition, there was no statistically significant difference in PFS between the arms (4.8 mo *vs* 4.0 mo,  $P = 0.0207$ ). Only the subgroup analysis of patients with good performance status [Karnofsky performance status (KPS) score of 90-100] have shown significant prolongation of median OS in the GemCap group compared with the control group (10.1 mo *vs* 7.4 mo, respectively,  $P = 0.014$ )<sup>[16]</sup>.

In the more recently reported United Kingdom Phase III trial<sup>[17]</sup> (UK NCRI study), a higher dose intensity of Gem and capecitabine was used than in the previous trial. Capecitabine dose was approximately 44% higher, and the Gem dose in the combined arm was approximately 12% higher. The dose of Gem in the control arm was identical in the two trials. Median OS was shown to be significantly superior in the GemCap group compared with the Gem group (7.4 mo *vs* 6 mo, respectively,  $P = 0.014$ ), as were the ORR (14% *vs* 7%, respectively,  $P = 0.001$ ) and



Table 2 Phase III trials of gemcitabine doublets

Phase III trial	Combination	OS (mo)	P	PFS (mo)	P
Berlin <i>et al</i> <sup>[12]</sup> (n = 322)	Gem + FU	6.7	0.09	3.4	0.022
	Gem	5.4		2.2	
Herrmann <i>et al</i> <sup>[16]</sup> (n = 319)	Gem + Cap	8.4	0.234	4.8	0.0207
	Gem	7.2		4.0	
Heinemann <i>et al</i> <sup>[19]</sup> (n = 219)	Gem + Cisplatin	7.6	0.12	5.3	0.053
	Gem	6.0		3.1	
Colucci <i>et al</i> <sup>[20]</sup> (n = 107)	Gem + Cisplatin	6.9	0.48	5	0.048
	Gem	4.6		2	
Louvvet <i>et al</i> <sup>[23]</sup> (n = 326)	GemOx	9.0	0.13	5.8	0.04
	Gem	7.1		3.7	
Poplin <i>et al</i> <sup>[11]</sup> (n = 824)	GemOx	5.7	0.09	2.7	0.15
	Gem	4.9		2.6	
	Gem FDR	6.2		3.5	
Rocha Lima <i>et al</i> <sup>[27]</sup> (n = 342)	IRINOXEM	6.3	0.789	3.5	0.352
	Gem	6.6		3.0	
O'Reilly <i>et al</i> <sup>[29]</sup> (n = 339)	Gem + Exatecan	6.7	0.52	3.9	0.22
	Gem	6.2		4.0	

FU: Fluorouracil.

the 1-year survival rates (26% and 19%, respectively). Although, higher doses of chemotherapy were used in this study, there was no significant difference in the frequency of grade 3 or 4 adverse events between the two trials. These data were presented in 2005. However, the final results of this trial have not been reported and a full manuscript has not been produced.

Additionally, Bernhard *et al*<sup>[18]</sup> assessed the clinical benefit response (CBR) and QOL in patients treated with GemCap or Gem alone. CBR was defined as improvement from baseline for 4 consecutive weeks in pain (pain intensity or analgesic consumption) and KPS, stability in one but improvement in the other, or stability in pain and performance status but improvement in weight. Of 319 patients, 19% of patients treated with the combination regimen and 20% of patients treated with Gem alone experienced a CBR, with a median duration of 9.5 and 6.5 wk, respectively ( $P = 0.02$ ). There was no treatment difference in QOL ( $n = 311$ ) between the two treatment arms. Regardless of their initial condition, some patients experienced an improvement in QOL on chemotherapy by symptom control; however, this was followed by a worsening 1-2 mo before treatment failure (all  $P < 0.05$ )<sup>[18]</sup>.

**Gem and platinum:** A recent randomized phase III trial evaluating Gem with or without cisplatin in patients with advanced pancreatic cancer demonstrated a trend toward increased OS (7.6 mo *vs* 6.0 mo,  $P = 0.12$ ) and PFS in the combination arm relative to the control arm but these differences were not statistically significant<sup>[19]</sup>. Also, there was no significant difference in QOL between the arms and only nausea and vomiting were significantly increased in the combination arm (22.2% *vs* 5.8%,  $P = 0.002$ ). Similarly, another randomized study did not show a benefit in survival for combination treatment (6.9 mo *vs* 4.6 mo,  $P = 0.48$ ) despite a marked improvement in response rate (26.4% *vs* 9.2%,  $P = 0.02$ )<sup>[20]</sup>.

On the basis of published preclinical *in vitro* synergy data between Gem and Ox<sup>[21]</sup>, the French Multidisciplinary

Clinical Research Group in Oncology (GERCOR) has conducted a phase II study in 64 patients with advanced or metastatic pancreatic cancer<sup>[22]</sup>. The encouraging results observed with GemOx in this phase II study has prompted the initiation of a phase III trial, conducted by both GERCOR and the Italian Group for the Study of Gastrointestinal Tract Cancer (GISCAD). In this phase III study, GemOx was superior in terms of PFS (5.8 mo *vs* 3.7 mo,  $P = 0.04$ ), RR (26.8% *vs* 17.3%,  $P = 0.04$ ) and clinical benefit (38.2% *vs* 26.9%,  $P = 0.03$ )<sup>[23]</sup> in both the metastatic and locally advanced population. The 1-year survivals observed in both arms of the study were impressive (34.7% and 27.8%, respectively,  $P = 0.22$ ). However, median OS did not significantly improve (9.0 mo *vs* 7.1 mo,  $P = 0.13$ ). For patients with locally advanced disease, median OS was identical in both arms (10.3 mo), whereas in patients with metastatic disease, median OS was 8.5 and 6.7 mo for GemOx and Gem alone, respectively ( $P = 0.17$ )<sup>[23]</sup>. The identical OS in patients with locally advanced disease and failure of this study to demonstrate the statistical significance of its primary end point has been attributed to the assignment of some patients to chemoradiotherapy after 3 mo of chemotherapy. Thirty percent and 32% of patients in the Gem and GemOx arms, respectively, presented with locally advanced disease. Chemoradiotherapy was recommended after 3 mo of chemotherapy in the case of stable disease or response, at the discretion of each investigator. Sixteen out of 40 (40%) and 11 out of 33 (33.3%) patients in the GemOx and Gem arms, respectively, received chemoradiotherapy. The incidence of grade 3-4 thrombocytopenia, vomiting and peripheral sensory neuropathy was increased in the combination arm<sup>[23]</sup>. An ECOG study<sup>[11]</sup> was designed to compare the survival impact of single-agent Gem *vs* Gem FDR or GemOx in metastatic or locally advanced pancreatic cancer, and performance status 0 to 2. Of 824 patients enrolled, there was no significant difference in median survival and PFS among the 3 treatment arms (Table 1). A meta-analysis of 5 randomized trials (two oxaliplatin-based and three

cisplatin-based Gem combinations) by Heinemann *et al*<sup>[24]</sup> demonstrated a significant improvement in ORR and PFS in 2 trials, while the level of significance was not reached in the other 3 trials. The platinum-based combination regimens consistently prolonged OS. However, none of the individual trials showed a statistically significant superiority compared to Gem alone. A significant improvement in OS was detected only when a combined analysis of the five trials was performed (HR = 0.85,  $P = 0.010$ ).

**Gem and topoisomerase inhibitors:** Irinotecan alone has a response rate of 9% in advanced pancreatic cancer<sup>[25]</sup>. In a phase II, multicenter, single-arm study with irinotecan and Gem (IRINOgem), 11/45 patients (24%) had 50% or greater reductions in tumor area with a RR of 20% (95% CI, 8%-32%). CA 19-9 was found to decrease during therapy in 50% of patients and was reduced by  $\geq 50\%$  in 30% of patients<sup>[26]</sup>. There were significant ( $P < 0.001$ ) correlations between proportional changes in CA 19-9 and radiographic changes in the tumor area. Median TTP, median survival and 1-year survival rate were modest at 2.8 mo, 5.7 mo and 27%, respectively. Severe toxicities were uncommon and primarily limited to grade 4 neutropenia (2%), grade 4 vomiting (2%), and grade 3 diarrhea (7%)<sup>[26]</sup>. This phase II data was followed by a phase III randomized study conducted by Rocha Lima *et al*<sup>[27]</sup> to compare the OS of 180 patients randomly assigned to IRINOgem ( $n = 173$ ) *vs* Gem ( $n = 169$ ). Unfortunately, the combination was not found to improve OS (6.3 mo *vs* 6.6 mo,  $P = 0.789$ ), although the combination had a significantly better tumor RR (16.1% *vs* 4.4%,  $P < 0.001$ )<sup>[27]</sup>. Median TTP was 3.5 mo for the IRINOgem group *vs* 3.0 mo for the Gem group ( $P = 0.352$ ). However, subset analyses in patients with locally advanced disease suggested a TTP advantage with IRINOgem *vs* Gem (7.7 mo *vs* 3.9 mo). CA 19-9 progression was positively correlated with tumor progression as shown in the previous phase II trial conducted by the same author. The incidence of grade 3 diarrhea was higher in the IRINOgem group but grade 3 to 4 hematologic toxicities and QOL measures were similar<sup>[27]</sup>.

Another topoisomerase inhibitor exatecan (DX-8951f) was studied in a randomized phase III trial and was shown to be inferior to Gem in RR and improvement in QOL<sup>[28]</sup>. Furthermore, the combination of exatecan and Gem failed to show any significant survival benefit over Gem alone in a phase III study (6.7 mo *vs* 6.2 mo,  $P = 0.52$ )<sup>[29]</sup>. Patients in the combination treatment arm experienced significantly more grade 3-4 toxicity, in particular neutropenia (30% *vs* 15%,  $P = 0.001$ ), thrombocytopenia (17% *vs* 5%,  $P = 0.004$ ) and vomiting (11% *vs* 5%,  $P = 0.04$ )<sup>[29]</sup>.

The oral topoisomerase I inhibitor rubitecan (9NC) has also been tested in pancreatic cancer in phase I / II trials<sup>[30,31]</sup>. In the phase II trial of 19 enrolled patients, an objective response was documented in 4 of the 14 evaluable patients (28.6%). Overall median survival was 21 wk and the 1-year survival was 16.7%. Toxicity leading to temporary discontinuation of 9NC was encountered in seven patients (36.8%), all related to a prior dose increase, while milder toxicity was observed in eight patients (42.1%)<sup>[31]</sup>.

**Gem and taxanes:** Although the taxanes (docetaxel and paclitaxel) have both single-agent activity and activity in combination chemotherapy in advanced pancreatic cancer, they are associated with significant toxicity, particularly myelosuppression. In a phase I / II study of Gem with docetaxel, the dose-limiting toxicity was grade 3-4 neutropenia<sup>[32]</sup>. Subsequent phase II combination studies have reported RR of 12%-18% and median survivals of 4.7-8.9 mo<sup>[33-35]</sup>. The incidence of grade 3-4 neutropenia was improved by the addition of prophylactic G-CSF (31%) or ciprofloxacin (48%), although these studies still reported an incidence of febrile neutropenia in 12% of patients<sup>[33,34]</sup>.

**Gem with other agents:** Gem has also been investigated in a multidrug combination chemotherapy regimen. A very small randomized study comparing the combination of cisplatin, epirubicin, FU and Gem (PEFG regimen,  $n = 51$ ) to Gem alone ( $n = 46$ ) showed better 4-mo PFS (primary end point) (60% *vs* 28%) and RR (38.5% *vs* 8.5%,  $P = 0.008$ )<sup>[36]</sup> in the PEFg group than in the control group. Both the 1-year OS (38.5% *vs* 21.3%,  $P = 0.119$ ) and the median OS (5.4 mo *vs* 3.3 mo,  $P = 0.0033$ ) were impressive in the PEFg group. There was no significant difference in QOL between the treatment arms, although there was a higher CBR in the PEFg arm (65% *vs* 25%,  $P = 0.0139$ ). However, grade 3-4 neutropenia (43% *vs* 14%,  $P = 0.0001$ ) and thrombocytopenia (30% *vs* 1%,  $P = 0.0001$ ) occurred more frequently in the PEFg arm. Subsequently, this regimen was modified by increasing the dose intensity of cisplatin and epirubicin (both at 30 mg/m<sup>2</sup> every 14 d) and of Gem (at 800 mg/m<sup>2</sup> every 14 d) in an attempt to further improve activity and efficacy, to reduce toxicity and to yield a schedule more suitable to the patient<sup>[37]</sup>. When compared with 84 patients treated with classical PEFg at the same institution, dose-intense PEFg was not inferior in terms of PFS at 6 mo (63% *vs* 57%), 1-year OS (48% *vs* 42%) and RR (49% *vs* 49%); it allowed an increase in dose intensity for Gem of 32%, for cisplatin and epirubicin of 36% (FU reduced by 3%) which significantly reduced grade 3-4 hematological toxicity (neutropenia: 26% *vs* 86%,  $P < 0.00001$ ; thrombocytopenia: 4% *vs* 58%,  $P < 0.00001$ ) and reduced the number of outpatient accesses by one-third<sup>[37]</sup>.

### Emerging role of the novel targeted agents in pancreatic cancer

A better understanding of the biology of cancer has led to the development of novel agents targeting pathways of cancer cell survival. Since Gem has been considered a standard treatment for advanced pancreatic cancer for the past decade, clinical trials have explored the combination of Gem and biological "targeted" agents. However, despite their promise in preclinical studies, most of the clinical trials with the newer agents have not shown survival advantage when compared with standard Gem. A questionable exception is the combination of Gem and erlotinib which showed superiority in median survival compared to Gem alone, but only a net gain of two weeks

**Table 3** Phase I / III trials of gemcitabine in combination with novel targeted therapies

	Combination	OS (mo)	P	PFS (mo)	P	ORR (%)	SD (%)
Phase II trial							
Xiong <i>et al</i> <sup>[41]</sup> (n = 61)	Gem + Cetuximab	7.1		3.8		12.2	63.4
Fogelman <i>et al</i> <sup>[47]</sup> (n = 50)	GemOx + BEV	12.1		NR		NR	39
Kim <i>et al</i> <sup>[48]</sup> (n = 82)	GemOx + BEV	8.1		5.7		11.3	NR
Ko <i>et al</i> <sup>[49]</sup> (n = 57)	Gem + Cetuximab + BEV	NR		3.5		10.7	29
	Cetuximab + BEV			1.8		0	24
Kindler <i>et al</i> <sup>[50]</sup> (n = 139)	Gem + BEV + Erlotinib	7.8		5.0		23	49
	Gem + BEV + Cetuximab	7.2		5.1		18	45
Phase III trial							
Moore <i>et al</i> <sup>[40]</sup> (n = 569)	Gem + Erlotinib	6.37	0.038	NR	0.004		
	Gem	5.91					
Philip <i>et al</i> <sup>[43]</sup> (n = 735)	Gem + Cetuximab	6.5	0.14	3.5	0.058		
	Gem	6.0		3.0			
Kindler <i>et al</i> <sup>[45]</sup> (n = 602)	Gem + BEV	5.7	NS	4.8	NS		
	Gem	6.0		4.3			
Vervenne <i>et al</i> <sup>[46]</sup> (n = 607)	Gem + BEV	7.1	NS	4.6	0.0002		
	Gem	6.0		3.6			

ORR: Overall response rate; SD: Stable disease; NR: Not reported; NS: Not significant; BEV: Bevacizumab; Gem: Gemcitabine; Gem FDR: Gemcitabine fixed-dose rate; GemOx: Gemcitabine 1000 mg/m<sup>2</sup> iv over 100 min on day 1 plus oxaliplatin 100 mg/m<sup>2</sup> on day 2 every 14 d.

was observed, questioning the true clinical significance of this superiority<sup>[3]</sup> (Table 3).

### Gem based chemotherapy with novel targeted agents

**Gem and erlotinib:** Preclinical synergy with Gem and erlotinib in inducing apoptosis in pancreatic xenograft models was demonstrated<sup>[38]</sup>, and a phase I study established the dose of erlotinib for single-agent daily dosing to be 150 mg/d, with which the incidence of severe diarrhea and/or skin rash was unacceptably high<sup>[39]</sup>. In a phase III trial by Moore *et al*<sup>[40]</sup>, 569 patients with locally advanced and metastatic pancreatic cancer were randomly assigned to receive erlotinib plus Gem *vs* Gem alone. The study showed statistically significant improvements in OS (6.37 mo in the erlotinib arm and 5.91 mo in the control arm,  $P = 0.038$ ) and PFS ( $P = 0.004$ ). Median survival in the erlotinib group was 6.24 mo and the 1-year survival rate was 23% compared with 5.91 mo and 17% in the control arm. There was a slight increase in the incidence of grade 3-4 skin rash and diarrhea (6% *vs* 1%) in the group receiving erlotinib, although there was no overall difference in QOL between the arms. As in studies of anti-EGFR agents in colorectal cancer, the presence of rash was associated with a higher likelihood of achieving disease control<sup>[40]</sup> ( $P = 0.05$ ). This study did not require EGFR positivity to be demonstrated prior to study entry and the overall rate of EGFR expression observed was 57%, which was lower than has been reported in previous studies<sup>[41,42]</sup>. A subgroup analysis by EGFR status suggested a trend towards benefit from erlotinib regardless of EGFR status, but there was inadequate power to show statistical significance.

**Gem and cetuximab:** In a phase II study<sup>[41]</sup>, 41 patients with EGFR expressing advanced pancreatic cancer were treated with the combination of Gem and cetuximab. A reasonable RR of 12.2% was reported, with a further 63.4% of patients achieving disease stabilization. These

results led to a randomized phase III trial<sup>[43]</sup> undertaken by The South Western Oncology Group (SWOG, S0205), the results of which were presented at the 43rd American Society of Clinical Oncology (ASCO) Annual Meeting in 2007 and did not show any survival benefit<sup>[43]</sup>. Seven hundred and thirty five patients were enrolled between January 2004 and April 2006. The median survival was 6 mo in the Gem arm and 6.5 mo in the Gem plus cetuximab arm for an overall HR of 1.09 (95% CI: 0.93-1.27,  $P = 0.14$ ). The corresponding PFS was 3.0 and 3.5 mo, for the Gem and Gem-cetuximab arms, respectively (HR: 1.13; 95% CI: 0.97-1.30,  $P = 0.058$ ). The unconfirmed responses yielded 14% in the Gem arm and 12% in the Gem-cetuximab arm.

**Gem and bevacizumab:** Another targeted agent with promising efficacy in pancreatic cancer is bevacizumab which was studied in combination with Gem in a phase II trial that resulted in a RR of 19%<sup>[44]</sup>. However, the results of the US Cancer and Leukemia Group B (CALBG) phase III randomized trial of Gem with or without bevacizumab did not reveal any improvement in survival upon addition of bevacizumab<sup>[45]</sup>. Median OS in the bevacizumab arm *vs* the control arm was 5.7 mo *vs* 6.0 mo (95% CI: 4.9-6.5 mo *vs* 5.0-6.9 mo) and PFS of 4.8 mo *vs* 4.3 mo, respectively (95% CI: 4.3-5.7 mo *vs* 3.8-5.6 mo). This result did not prevent completion of a similar, Roche sponsored trial, AVITA, in which 607 patients with metastatic pancreatic cancer were randomized to Gem and erlotinib with or without bevacizumab<sup>[46]</sup>. There was no significant prolongation of survival with the addition of bevacizumab, although disease-free survival (DFS) was statistically significantly improved (from 3.6 to 4.6 mo). Bevacizumab was reported to be safe in this combination, despite an increase in the incidence of epistaxis, hypertension and proteinuria. Interestingly, there was no reported increase in thrombotic events with bevacizumab<sup>[3]</sup>. The AVITA study suggests that antiangiogenic strategies may have merit in the treatment of

advanced pancreatic cancer, although the margin of benefit with bevacizumab is modest.

Two phase II trials were presented at the 2009 ASCO Gastrointestinal Cancer Symposium which evaluated the efficacy of Gem in combination with biologic agents. Fogelman *et al*<sup>[47]</sup> reported the final results of a 3-drug combination consisting of Gem, Ox and bevacizumab in 50 patients with advanced pancreatic cancer. This triple drug combination achieved 1 and 2-year survival rates of 40% and 16%, respectively with a high response rate of 39%<sup>[47]</sup>. In addition, this regimen demonstrated a higher RR and longer median survival compared to a previously reported Gem and Ox study<sup>[23]</sup>. Of note, there was a correlation between CA 19-9 levels and median survival. Another phase II trial<sup>[48]</sup> assessing the combination of GemOx plus bevacizumab included 82 patients with advanced pancreatic cancer. This study showed 6-mo survival of 65.0% (95% CI: 53.5%-75.3%), median survival of 8.1 mo (95% CI: 6.5-9.3 mo) and median TTP of 5.7 mo (95% CI: 4.4-6.4 mo).

On the other hand, Gem with a dual monoclonal antibody regimen was disappointing. Ko *et al*<sup>[49]</sup> designed a phase II trial to evaluate the efficacy of dual EGFR/VEGF monoclonal antibodies cetuximab and bevacizumab with or without Gem. Fifty-seven patients received dual antibodies. Overall RR was only 10.7% in the Gem arm and OS data has not been presented yet<sup>[49]</sup>. The above results were confirmed by another phase II trial by Kindler *et al*<sup>[50]</sup>. One hundred and thirty-nine patients with locally advanced pancreatic cancer received Gem, bevacizumab and erlotinib or Gem, bevacizumab and cetuximab<sup>[50]</sup>. Interestingly, a correlation between early hypertension and response to treatment was observed. There was no significant difference between the two arms in either OS or PFS. Therefore, cetuximab or bevacizumab is not recommended for the treatment of advanced pancreatic cancer in the current clinical setting outside of an investigational trial.

### Other combined regimens

There have been very few attempts to address the role of alternative cytotoxic agents other than Gem in the first-line setting which may represent better platforms for the addition of targeted therapies. One such study conducted by Ducreux *et al*<sup>[51]</sup> evaluated the efficacy of oxaliplatin alone (OXA), infusional FU alone (FU) and an oxaliplatin/infusional 5-FU combination (OXFU) in the phase II setting. 90% of patients had metastatic disease (81% with liver metastases) and 83% of patients had PS 0-1. Median TTP and OS were higher in the combination arm (4.2 and 9.0 mo, respectively) than either of the single-agent arms (OXA, 2.0 and 3.4 mo; FU, 1.5 and 2.4 mo, respectively). Response rate was 10% in the OXFU arm and the safety profile was encouraging<sup>[51]</sup>.

In the FFCD 0301 trial<sup>[52]</sup>, a large phase III trial presented in the first-line setting, 202 patients with advanced pancreatic cancer were randomized to either FU and leucovorin plus cisplatin followed by Gem or *vice versa*. Patients received therapy until progression after which they could cross to the opposite arm. After a median follow-up

of 44 mo, the majority of patients ( $n = 192$ ) died. There was no significant difference in survival between the two arms. One-year and two-year survival figures were also identical between the Gem and FU plus cisplatin arms. Although it is unlikely that FU and cisplatin will replace Gem due to toxicity concerns, these data provided the rationale for non-Gem containing regimens in the first-line setting. One may consider a pharmacogenomic profile in the future to select either therapy.

EndoTAG-1 is a novel cationic liposomal formulation of paclitaxel. It increases the microvascular permeability probably due to vascular damage. Manipulation of the blood-tumor barrier with EndoTAG-1 can increase the effectiveness of conventional chemotherapy. The combination of Gem plus liposomal paclitaxel at three different dose levels (11, 22, or 44 mg/m<sup>2</sup>) was compared to Gem alone in 200 patients with metastatic pancreatic cancer<sup>[53]</sup>. Preliminary results were presented at the 2009 ESMO meeting. This regimen achieved a disease control rate of 53%-69% depending on the dosage of paclitaxel. Median PFS was 18, 20, and 19 wk, respectively, in the Gem/EndoTAG-1 low, medium, and high dose groups, compared with 12 wk in the Gem monotherapy group. Median OS was 7.2 mo with Gem alone *vs* 8.4, 8.7, and 9.4 mo with Gem plus low, medium, and high dose EndoTAG-1. Twelve-month survival rates were 17% with Gem alone *vs* 22%, 36% and 33% for Gem plus low, medium and high dose EndoTAG-1.

The results of a randomized phase II trial of 3 different regimens in patients with advanced pancreatic cancer suggested that capecitabine plus Ox is comparable to Gem combined with either capecitabine or Ox<sup>[54]</sup>. A phase II trial conducted by Burtneiss *et al*<sup>[55]</sup> confirmed the activity of another non-Gem regimen. Ninety-two patients with advanced pancreatic cancer were randomly assigned to receive irinotecan/docetaxel (Arm A) or irinotecan/docetaxel/cetuximab (Arm B). Median OS were reported to be 6.5 (95% CI: 4.8-8.6 mo) and 7.4 mo (95% CI: 4.4-10.7 mo) in Arm A and B, respectively. However, this triple regimen was associated with high rates of grade 3-4 neutropenia and diarrhea<sup>[55]</sup>.

### Other agents

Numerous studies employing other novel targeted agents are currently being developed. The CALGB presented the results of a single-arm phase II study of sunitinib for patients with advanced pancreatic cancer who had previously been treated with Gem-based therapy. No responses were reported in 77 treated patients, and stable disease in only 7 patients<sup>[56]</sup>. The California consortium reported similar disappointing results with sorafenib when combined with Gem<sup>[57]</sup>. In this randomized study, chemo-naïve pancreatic cancer patients received sorafenib as a single agent or in combination with Gem. No responses resulted with sorafenib alone and the median survival in the Gem plus sorafenib arm was only 6 mo. Wolpin *et al*<sup>[58]</sup> treated 31 Gem-refractory pancreatic cancer patients with everolimus, an oral mTOR inhibitor. Although the agent was tolerable, there was no response and disease stability



was uncommon. These targeted agents do not merit further study in pancreatic cancer due to their insufficient anti-tumor activity. Other targeted agents, which have been tested in pancreatic cancer and not found to add any survival benefit, include the farnesyl transferase inhibitor tipifarnib<sup>[59,60]</sup> and the matrix metalloproteinase inhibitors marimastat<sup>[61-63]</sup> and BAY 12-9566<sup>[64]</sup>.

Another new agent, AMG 655, is a fully humanized monoclonal antibody that targets human death receptor 5 (DR5), activates caspases, and induces apoptosis in sensitive tumor cells. In pancreatic cancer xenografts, the anti-tumor activity of AMG 655 was enhanced by adding Gem. In a phase I trial<sup>[65]</sup>, patients with metastatic pancreas cancer were enrolled into sequential cohorts of 3- or 10-mg/kg AMG 655 iv on days 1 and 15 plus Gem 1000 mg/m<sup>2</sup> iv on days 1, 8, and 15 every 28 d. Best overall tumor response assessed by RECIST criteria showed that 3 (23%) patients had partial responses, 6 (46%) had stable disease (range 15-34+ wk), and 4 (31%) patients had progressive disease. Four of 7 patients (57%) with baseline CA19-9 > 100 U/mL had a  $\geq 70\%$  decrease on study. The median PFS was 5.3 mo and the 6-mo survival rate was 76.2% (42.7%-91.7%)<sup>[65]</sup>. A randomized phase II trial of 10 mg/kg AMG 655 every 2 wk plus Gem has been completed in patients with metastatic pancreatic cancer and the results are forthcoming.

## SECOND-LINE CHEMOTHERAPY FOR ADVANCED PANCREATIC CANCER

There is no standard second-line regimen for advanced pancreatic cancer after Gem failure and there is a paucity of trials in this setting. Gem may offer palliative benefits in the second-line setting in patients that have not been treated with Gem previously<sup>[66]</sup>, and results from a phase II study ( $n = 30$ ) suggest that FDR Gem and Ox may have activity in patients who become refractory to standard Gem therapy<sup>[67]</sup>. All patients received at least one cycle of GemOx (median 5). Response in 31 evaluable patients was as follows: Partial response: 7/31 (22.6%),  $\geq 8$  wk: 11/31 (35.5%), s.d. < 8 wk: 1/31 (3.2%), Progressive disease: 12/31 (38.7%). Median duration of response and TTP were 4.5 and 4.2 mo, respectively. Median survival was 6 mo (range 0.5-21 mo). The CONKO-3 study<sup>[68]</sup> randomized 168 patients who had Gem-refractory pancreatic cancer to 5-FU, LV and oxaliplatin (OFF) or 5-FU and LV (FF). The study showed an improved OS by 2 mo in the OFF arm (4.8 mo *vs* 2.3 mo respectively,  $P = 0.0077$ ). Both regimens were tolerable, with the exception of higher neuropathy in the OFF arm. There was also a significant prolongation of PFS in the treatment arm (13 wk *vs* 9 wk)<sup>[68]</sup>. After those significant results, this regimen has been regarded as an appropriate second-line regimen for Gem refractory pancreatic cancer patients.

In a phase III study patients with advanced pancreatic cancer who had failed at least one line of chemotherapy were randomized to rubitecan or physicians' choice of treatment<sup>[69]</sup>. Eighty-five percent of patients in both arms had previously received Gem; 70% and 73% had

received FU; 60% and 63% had received both drugs in combination, respectively. The study was unable to show a statistically significant improvement in OS (3.7 mo *vs* 3.1 mo,  $P = 0.626$ ), and PFS was only marginally improved (1.9 mo *vs* 1.6 mo,  $P = 0.001$ ).

In a phase II trial by Cartwright *et al*<sup>[70]</sup>, 42 patients were treated with oral capecitabine 1250 mg/m<sup>2</sup> administered twice daily in 3-weekly cycles consisting of 2 wk of treatment followed by 1 wk without treatment. Twenty-four percent of patients experienced a significant CBR as evidenced by improvement in pain intensity, analgesic consumption, and/or KPS. Three (7.3%) of the 41 patients with measurable disease had an objective partial response. The median time to objective response was 85 d (range, 47 to 91 d) and duration of response was 208, 260, and 566 d for the three responding patients. One patient with non-measurable but assessable disease had improved residual disease with a positive CBR. For a total of 4 responders among the 42 assessable patients, the OS rate was 9.5%<sup>[70]</sup>. Of note, the capecitabine dose (1000 mg/m<sup>2</sup> *po* twice daily) recommended in the guidelines was less than the dose described by Cartwright *et al*<sup>[70]</sup>, because the higher dose has been associated with increased toxicity (diarrhea, hand and foot syndrome).

In another phase II trial<sup>[71]</sup>, pancreatic cancer patients were administered capecitabine (1000 mg/m<sup>2</sup> twice daily for 14 d) combined with Ox (130 mg/m<sup>2</sup> given on day 1 for 14 d) every 21 d (patients aged > 65 years or with an ECOG PS of 2 received Ox 110 mg/m<sup>2</sup> on day 1 and capecitabine 750 mg/m<sup>2</sup> twice daily for 14 d). The treatment was repeated every 3 wk. Of the 39 evaluable patients, 1 patient had a partial response and 10 patients demonstrated stable disease. The median OS was 23 wk and PFS was 9.9 wk. The 6-mo and 1-year survival rates were 44% and 21%, respectively. The most common grade 3-4 non-hematologic toxicity was fatigue<sup>[71]</sup>.

Currently, it is recommended that physicians enroll their patients in a clinical trial if they progress on first-line therapy; however, when investigational therapy is not available, alternatives for good PS patients include capecitabine with or without Ox or OFF.

## CONCLUSION

In the first-line setting, Gem with or without erlotinib has been the standard treatment for pancreatic cancer since 1997, despite low response rates and short survival outcome. The recent introduction of targeted therapies in the therapeutic armamentarium against cancer raised hopes in the treatment of patients with advanced or metastatic cancers. Unfortunately, the target agents studied to date have fallen short of these expectations. Knowledge of the molecular events occurring in the malignant transformation processes should allow the development of more efficient targeted therapies. Metastatic and locally advanced pancreatic cancers have consistently been observed as independent predictors of outcome in randomized clinical trials. One should study these two different pancreatic cancer populations separately.

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Yusuf Bayraktar, Professor, Series Editor

## Experience of a single center with congenital hepatic fibrosis: A review of the literature

Ali Shorbagi, Yusuf Bayraktar

Ali Shorbagi, Yusuf Bayraktar, Faculty of Medicine, Department of Internal Medicine, Gastroenterology Clinic, Hacettepe University, 06100 Sıhhiye, Ankara, Turkey

**Author contributions:** Shorbagi A was responsible for the literature review and preparation of the manuscript; Bayraktar Y provided patient details and photographs which were used in describing the experience of a single center, as well as finalizing the final draft for submission.

**Correspondence to:** Ali Shorbagi, MD, Faculty of Medicine, Department of Internal Medicine, Gastroenterology Clinic, Hacettepe University, 06100 Sıhhiye, Ankara, Turkey. [shorbagi@hacettepe.edu.tr](mailto:shorbagi@hacettepe.edu.tr)

Telephone: +90-312-3051712 Fax: +90-312-4429429

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### Abstract

Congenital hepatic fibrosis (CHF) is an autosomal recessive inherited malformation defined pathologically by a variable degree of periportal fibrosis and irregularly shaped proliferating bile ducts. It is one of the fibropolycystic diseases, which also include Caroli disease, autosomal dominant polycystic kidney disease, and autosomal recessive polycystic kidney disease. Clinically it is characterized by hepatic fibrosis, portal hypertension, and renal cystic disease. CHF is known to occur in association with a range of both inherited and non-inherited disorders, with multiorgan involvement, as a result of ductal plate malformation. Because of the similarities in the clinical picture, it is necessary to differentiate CHF from idiopathic portal hypertension and early liver cirrhosis, for which a liver biopsy is essential. Radiological tests are important for recognizing involvement of other organ systems. With regards to our experience at Hacettepe University, a total of 26 patients have been diagnosed and followed-up between 1974 and 2009 with a diagnosis of CHF. Presentation with Caroli syndrome was the most common diagnosis, with all such patients presenting with symptoms of recurrent

cholangitis and symptoms related to portal hypertension. Although portal fibrosis is known to contribute to the ensuing portal hypertension, it is our belief that portal vein cavernous transformation also plays an important role in its pathogenesis. In all patients with CHF portal vein morphology should be evaluated by all means since portal vein involvement results in more severe and complicated portal hypertension. Other associations include the Joubert and Bardet-Biedl syndromes.

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**Key words:** Congenital hepatic fibrosis; Fibropolycystic disorders; Portal hypertension; Bardet Biedl syndrome

**Peer reviewer:** Fabrizio Montecucco, MD, Assistant Professor, Division of Cardiology, Department of Internal Medicine, University of Geneva, Avenue de la Roseraie 64, 1211 Geneva, Switzerland

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### INTRODUCTION

Congenital hepatic fibrosis (CHF) is an autosomal recessive inherited malformation defined pathologically by a variable degree of periportal fibrosis and irregularly shaped proliferating bile ducts. The hepatic manifestations of CHF were first described in 1856<sup>[1]</sup>. The term CHF, with its varied clinical manifestations, was recognized in 1960<sup>[2]</sup>, and later on elaborated in 1961<sup>[3]</sup>. CHF is one of the fibropolycystic diseases, which also include Caroli disease, autosomal dominant polycystic kidney disease (AD-PKD), and autosomal recessive polycystic kidney disease (ARPKD). Clinically it is characterized by hepatic fibrosis,

portal hypertension, and renal cystic disease.

The exact incidence and prevalence of CHF are not known, but it is a rare disease. By 1988, only 200 patients with CHF had been reported in the literature<sup>[4]</sup>. In most patients, the first manifestations of the disease are signs or symptoms related to portal hypertension, especially splenomegaly and varices, often with gastrointestinal bleeding<sup>[5]</sup>. The clinical manifestations of CHF are, however, nonspecific, making the diagnosis of this disorder extremely difficult. Onset of symptoms and signs is highly variable and ranges from early childhood to the 5th or 6th decade of life, although this disorder is diagnosed in most patients during adolescence or young adulthood<sup>[6]</sup>. The late appearance of symptoms and their clinical evolution suggest that CHF is a dynamic and progressive condition.

## ASSOCIATED SYNDROMES

CHF occurs in association with a range of both inherited and non-inherited disorders, with multiorgan involvement (Table 1). Several gene mutations have been established for more commonly encountered conditions that have been better investigated (e.g. Joubert syndrome, Bardet-Biedl syndrome). In these conditions, hepatic fibrosis has been reported to occur to varying degrees; however, in most cases, the main cause of morbidity and mortality is involvement of other organ systems, particularly the kidneys and central nervous system. Patients rarely reach adulthood, and in many cases death occurs intrauterine or in early childhood.

## PATHOPHYSIOLOGY

It has been established that congenital hepatic fibrosis, and indeed Caroli's disease closely resemble each other pathophysiologically, in that both occur as a result of ductal plate malformation. The ductal plate is a cylindrical layer of cells that surround a branch of the portal vein, and is the embryonic precursor of the intrahepatic bile ducts, as both interlobular and intralobular bile ductules develop from the ductal plate. Progressive remodeling starts at 12 wk of gestation, and full maturation is usually complete by 20 wk. Arrest of maturation and the lack of remodeling of the ductal plate that occurs as a result leads to the persistence of an excess number of immature embryonic duct structures. This abnormality has been termed the ductal plate malformation. The persistence of these immature duct elements stimulates the formation of portal fibrous tissue, and it is this periportal fibrosis that contributes to the clinical picture of recurrent cholangitis or portal hypertension and associated symptoms (Figure 1). Although long standing portal hypertension is known to result in secondary portal vein thrombosis, and eventually portal vein cavernous transformation (PVCT), it is firmly believed that PVCT is actually a component of the disorder, present at the onset rather than developing at a later stage. Embryologically speaking, the development of bile ducts and hepatic vasculature are closely related. The

ductal plate malformation has been shown to be associated with a "pollard willow" malformation of the portal vein, which results in too many small and closely branched portal veins, which supports the idea that PVCT may be congenital. Histologically, enlarged portal tracts containing immature ductal plates surrounding several hypoplastic or even obliterated portal vein branches are observed<sup>[7]</sup>. In one report, PVCT was observed in almost 50% of patients with congenital hepatic fibrosis, and such patients had relatively larger splenomegaly than those without PVCT, as well as suffering from more frequent bleeding episodes from esophageal varices<sup>[8]</sup>.

Furthermore, depending on the stage of arrest of maturation, either the small interlobular bile ducts (congenital hepatic fibrosis), or the medium intrahepatic bile ducts (Caroli's disease) may be involved. Involvement of both simultaneously results in what is known as Caroli's syndrome. In this context, the clinical picture of Caroli's disease (recurrent cholangitis) may be so predominant that co-existing congenital hepatic fibrosis may easily be overlooked. A liver biopsy is therefore warranted in all patients with suspected Caroli's disease to confirm the presence or absence of Caroli's syndrome<sup>[9,10]</sup>.

The hepatic stellate cell (HSC) is at the center of the hepatic fibrotic process associated with liver disease, and has also been shown to play a role in the progression of the disease in congenital hepatic fibrosis. It is widely accepted that transforming growth factor (TGF)- $\beta$  is a potent growth inhibitory and profibrotic cytokine which plays a pivotal role in the physiological process of wound healing as well as in the pathogenesis of organ fibrosis<sup>[11]</sup>. TGF- $\beta$  expression has been shown to be increased in a wide range of fibrotic diseases. Initiation of HSC activation is primarily induced by TGF- $\beta$ 1 derived from Kupffer cells. TGF- $\beta$ 1 mediates its profibrotic actions by stimulating fibroblasts and related cell types, including the HSC in the liver, to secrete a wide range of extracellular matrix proteins. In pathological conditions this leads to accumulation of fibrotic matrix or in a more physiological context to the efficient healing of wounds<sup>[12-14]</sup>. Latent TGF- $\beta$  is also activated by MMP-9, another product of Kupffer cells. TGF- $\beta$  has other important actions, namely its immunomodulatory properties and its antiproliferative effects on epithelial cells, including hepatocytes.

Several studies have attempted to establish the pathophysiological mechanism behind the abnormal and excessive fibrotic response associated with CHF. Degradation of the basement membrane and extracellular matrix (ECM) constituents, and the remodeling of the ECM are important processes of embryonic development. Basal laminar components such as laminin and type IV collagen along with the coordinated expression of proteolytic enzymes are thought to be essential for the normal development of intrahepatic bile ducts<sup>[15-18]</sup>. Most of the proteolytic enzymes involved in these processes belong to the matrix metalloproteinases (MMPs) and the serine proteinases, in particular the plasminogen activator (PA)/plasmin system<sup>[19,20]</sup>. Both tissue PA (tPA) and urokinase type PA have been shown to contribute to the plasminogen-

Table 1 Syndromes with associated congenital hepatic fibrosis

Associated disorder	Genetic anomaly [chromosome (gene)]	Characteristic clinical features
Caroli syndrome	6p21.1-p12 ( <i>PKHD1</i> gene)	Caroli's disease - ectasia or segmental dilatation of the larger intrahepatic ducts
Polycystic kidney disease	6p21.1-p12 ( <i>PKHD1</i> gene)	Progressive cystic dilation of the renal tubule (resulting in renal failure), hepatic cysts, cerebral aneurysms, cardiac valvular abnormalities
Joubert syndrome	9q34.3; 11p12-q13; 6q23 ( <i>AHI1</i> gene); 2q13 ( <i>NPHP1</i> gene); 12q21.32 ( <i>CEP290</i> or <i>NPHP6</i> gene); 8q21 ( <i>TMEM67</i> gene); 16q12.2 ( <i>RPGRIP1L</i> gene)	Cerebellar vermis hypoplasia retinitis pigmentosa, nystagmus, ataxia
Senior-Loken syndrome	2q13 ( <i>NPHP1</i> , <i>NPHP4</i> , <i>NPHP5</i> genes); 3q22 ( <i>NPHP3</i> gene)	Cerebellar ataxia and skeletal abnormalities, nephronophthisis, retinal dystrophy, sensorineural hearing loss
COACH syndrome	4p15.3 ( <i>CC2D2A</i> gene)	Cerebellar vermis hypo/aplasia, oligophrenia, ataxia, coloboma, polydactyly
Cogan syndrome	2q13 ( <i>NPHP1</i> gene)	Oculomotor apraxia, nephronophthisis, cerebellar ataxia
Arima syndrome	Not yet established	Cerebellar vermis hypoplasia, renal abnormalities, psychomotor retardation
Meckel syndrome	17q23 ( <i>MKS1</i> gene); 8q ( <i>TMEM67</i> gene); 12q ( <i>CEP290</i> gene); 16q12.2 ( <i>RPGRIP1L</i> gene); 4p15 ( <i>CC2D2A</i> gene); 11q	Microcephaly, renal cystic disease, hypoplastic or ambiguous genitalia, polydactyly, congenital heart defect, cleft palate, ocular defects
Bardet-Biedl syndrome	11q13; 16q21; 3p12-q13 ( <i>ADP-ribosylation factor</i> gene); 15q22.3; 2q31; 20p12 ( <i>MKS</i> gene); 4q27; 14q32.11 ( <i>tetratricopeptide repeat domain-containing</i> gene); 7p14; 12q; 9q33.1; 4q27; 17q23 ( <i>MKS1</i> gene); 12q21.3 ( <i>CEP290</i> gene)	Rod-cone dystrophy (atypical retinitis pigmentosa), postaxial polydactyly, central obesity, mental retardation, hypogonadism, and renal dysfunction
Alstrom syndrome	2p13 ( <i>ALMS1</i> gene)	Childhood obesity congenital retinal dystrophy, sensorineural hearing loss, endocrinopathies, cardiomyopathy, renal failure
Oral-Facial-Digital type IV - Not yet established		Lobulated tongue, pseudo-cleft of lip, hyperplastic frenula, polydactyly, severe bilateral deafness
Mohr-Majewski syndrome		

dependent lysis of basement membrane laminin in human carcinoma cell lines. Furthermore, plasmin contributes to the activation of MMP-9 and MMP-13 which also play an important role in the degradation of basement membrane components including type IV collagen. In a recent study by Yasoshima *et al.*<sup>[21]</sup>, it was postulated that biliary overexpression of plasminogen and tPA leads to the generation of excessive amounts of plasmin, and subsequent plasmin dependent lysis of the ECM molecules which may contribute to biliary dysgenesis in CHF.

Overexpression of the osteopontin gene has also been implicated in the pathophysiology of biliary atresia, as well as congenital cholestatic syndromes such as CHF and Caroli's disease. Osteopontin is a stimulant of fibroinflammation, and its overexpression has been shown to be regulated by the presence of excessive amounts of regulatory factors such as NF- $\kappa$ B and TGF- $\beta$ 1<sup>[22]</sup>.

In an effort to establish how the presence of excessive immature bile ducts contributes to the process of fibrosis, Sato *et al.*<sup>[23]</sup> managed to demonstrate in a rat model that in the presence of TGF- $\beta$ 1, cholangiocytes acquire mesenchymal features, thus resembling fibroblasts. They speculated that excess production of extracellular matrix molecules by these transformed cells may contribute to the progressive periportal/hepatic fibrosis.

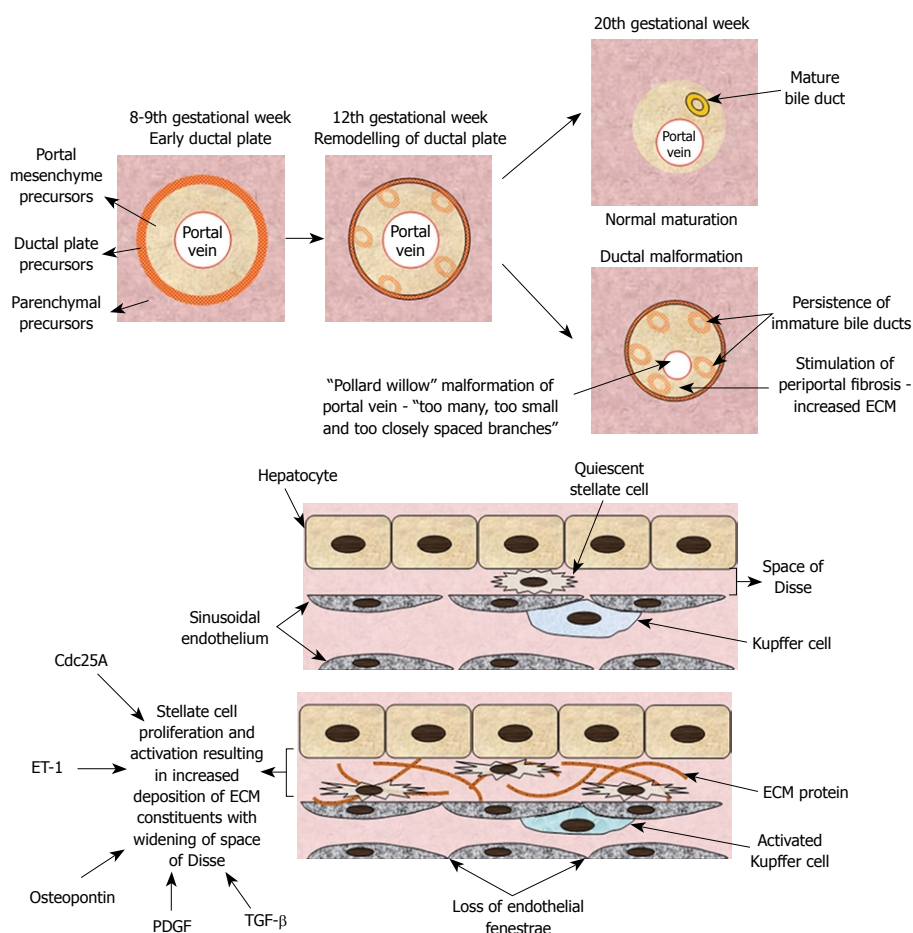
On a different note, a possible role of microRNA has been postulated in the pathogenesis of fibropolycystic disorders involving both the liver and the kidneys. Chu *et al.*<sup>[24]</sup> demonstrated decreases in the levels of the microRNA miR15a in the livers of patients with ARP-KD, ADPKD and CHF. They reported that this resulted in an increase in the expression of a cell-cycle regulator

known as cell division cycle 25A gene product (Cdc25A), which is directly responsible for cellular proliferation and cystogenesis *in vitro*.

## CLINICAL PICTURE

The age of onset of presentation and the severity of symptoms varies greatly, with patients usually being diagnosed in childhood or early adulthood, although presentations as late as in the fifth decade have been reported. Although patients usually present with symptoms involving other organ symptoms (e.g. renal, central nervous system, *etc.*), cases referred for gastroenterologic/hepatologic consultation generally have complaints attributed to CHF. Four clinical forms have been defined<sup>[25]</sup>: (1) Portal hypertension (most common; more severe in the presence of portal vein abnormality); (2) Cholangitic - cholestasis and recurrent cholangitis; (3) Mixed; and (4) Latent - presentation at a late age.

Most patients are asymptomatic, while some may complain of mild right upper quadrant pain. Patients with a predominant portal hypertensive picture may present with upper gastrointestinal variceal bleeding. Physical examination findings include hepatomegaly, with predominant involvement of the left lobe, splenomegaly and nephromegaly. The liver is firm, with a mildly nodular surface. Laboratory workup may reveal mild elevations in liver enzymes. Patients with a predominantly cholangitic clinical picture may have marked elevations in alkaline phosphatase (ALP),  $\gamma$ -glutamyl transpeptidase (GGT) and bilirubin. Varying cytopenias (leukopenia, thrombocytopenia) secondary to hypersplenism may be seen on a blood count. Abnormal renal func-



**Figure 1 Pathogenesis of congenital hepatic fibrosis.** Embryological and molecular perspective. ET-1: Endothelin 1; PDGF: Platelet-derived growth factor; TGF: Transforming growth factor; ECM: Extracellular matrix; Cdc25A: Cell division cycle 25A gene product (cell-cycle regulator).

tions tests are associated with extensive cystic renal disease, which may even progress to end-stage renal failure<sup>[26]</sup>.

## COMPLICATIONS

### **Cholangiocellular carcinoma**

The association of cholangiocellular carcinoma (CCC) with congenital cystic malformation of the biliary tree, as seen in Caroli's disease and Caroli's syndrome, has been well established, ranging from 2.5%-16% of afflicted individuals. However, pure congenital fibrosis has also been reported to result in CCC<sup>[27,28]</sup>.

## DIAGNOSIS

### **Role of radiology**

Ultrasonography (US) is generally regarded as the first line modality used in the diagnostic process with its high utility, lack of radiation exposure and its capability of detecting the bile duct and liver parenchymal abnormality. In particular, its unique capability of detecting the parenchymal heterogeneity and the associated kidney abnormalities accentuates its role in the diagnosis. Findings include hypertrophy of the left lateral segment and caudate, normal or hypertrophic left medial segment, atrophic right lobe, presence of hepatosplenomegaly, dilatation of the intrahepatic and extrahepatic bile ducts with concomitant focal cystic or solid lesions such as regenerative liver nodules and periportal thickening, dilated intrahepatic bile ducts

and stones in the ducts (Caroli's disease), hepatic and renal cysts, and portal vein cavernous transformation (Figure 2).

Computed tomography (CT) offers an advantage to US in that it provides a better depiction of gross morphology of the liver with accurate volume measurements and imaging of liver vasculature, as well as demonstrating any changes in the biliary tree (Figure 3A and B). Periportal cuffing, indicative of the fibrotic process, may also be easily detected with CT. Moreover, imaging of the central nervous system, particularly by CT is essential in the differential diagnosis of syndromes with associated congenital hepatic fibrosis.

Magnetic resonance imaging (MRI), on the other hand, is an attractive alternative, especially since it does not involve the use of radiating energy. Magnetic resonance cholangiopancreatography (MRCP) allows for detailed and thorough evaluation of the biliary tree and renal abnormalities, where lesions that were missed by US may even be detected. Some authors advocate that with the advent of newer technologies like half-fourier-acquisition single-shot turbo spin-echo (HASTE) it may even be possible to quantify the extent of parenchymal fibrosis. Brain MRI is also essential to identify cerebellar malformations associated with disorders such as the Arima, Joubert and COACH syndromes (Figure 4).

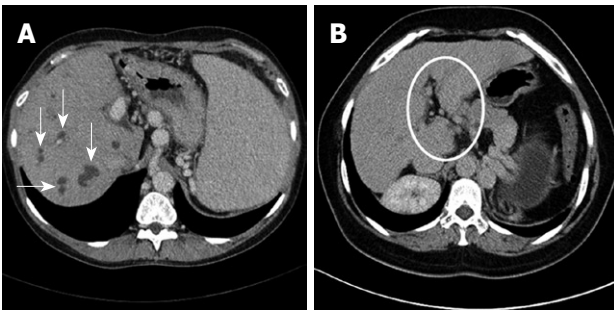
### **Histopathological findings**

A unequivocal diagnosis of congenital hepatic fibrosis can only be made by a examination of a liver biopsy. The





**Figure 2** Ultrasound image of a patient with congenital hepatic fibrosis (CHF). Heterogenous appearance of hepatic parenchyma. The circled area depicts the presence of portal vein cavernous transformation.

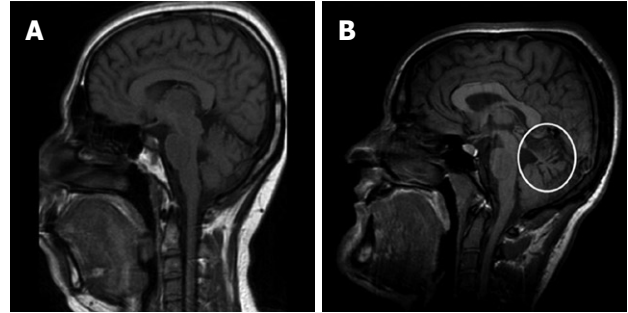


**Figure 3** Abdominal computerized tomography (CT) scans of two patients with CHF. A: White arrows depict cystic dilations of the biliary tree associated with Caroli's syndrome; B: Circled area shows portal vein cavernous transformation in a patient with Bardet-Biedl syndrome.

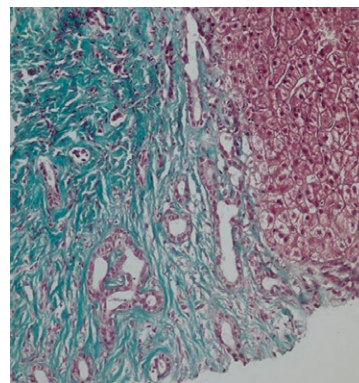
classical histological findings of this disorder are varying degrees of hepatic fibrosis with nodular formation, which may become extensive as the disease progresses. In the eyes of inexperienced pathologists, histopathological findings may easily be mistaken for cirrhosis. In CHF, widened fibrous bands may be encountered in the portal tract containing an increased number of irregularly shaped proliferating bile ducts lined by normal cuboidal epithelium. Unlike cirrhosis, hepatic lobules are usually normal with normal hepatocyte morphology, particularly in the early stages (Figure 5). Signs of cholestasis may be observed in the setting of associated cholangitis. Other findings include cystic dilatation of bile ducts (Caroli's disease), and hypoplasia of the portal vein branches in association with supernumerous hepatic artery branches. In fact, congenital absence of the portal vein has been reported in a pediatric patient with CHF<sup>[29]</sup>. Similarly, considering the close association of portal vein cavernous transformation with CHF, it has been postulated that such a portal vein anomaly may be a component of the disorder, rather than a consequence, since bile ducts and portal veins share embryonic origins<sup>[7]</sup>.

## TREATMENT

As yet, no treatment modality has been shown to actually stop or even reverse the pathological process in con-



**Figure 4** Brain magnetic resonance imaging (MRI) scans of two patients with congenital hepatic fibrosis. A: A patient with Bardet-Biedl syndrome with normal findings; B: The circled area depicts cerebellar vermis atrophy manifested by more prominent folds/sulci, associated with Joubert syndrome.



**Figure 5** Liver biopsy of a patient with CHF. The left side of the image depicts a portal area with extensive fibrosis and the presence of several bile ducts with cuboidal epithelium that have arrested at different stages of the maturation process. On the right, hepatocytes with normal morphology may be seen (× 230, trichrome stain).

genital hepatic fibrosis, and it remains a progressive and debilitating condition. However, extensive research has been underway into the pathogenesis of fibrosis of the liver, particularly in the setting of chronic liver diseases, and some of the treatment options available may in fact be extended to CHF patients. Instead, for CHF, treatment is directed at the management of its complications.

## Anti-fibrotic therapy

Several agents have been studied, particularly in the setting of chronic liver disease. Although results have been promising, especially in animal studies, the clinical impact on humans has failed to live up to expectations. For example, colchicine is a plant alkaloid that inhibits polymerization of microtubules, and is believed to be antifibrotic, preventing collagen secretion and deposition. It had been shown to effectively inhibit collagen synthesis and fibrosis in experimental animal models, however almost all clinical trials, as well as several meta analyses, failed to show any benefits in humans, and current recommendations do not include colchicine as an antifibrotic agent<sup>[30]</sup>.

The angiotensin II system, on the other hand, represents an extremely attractive antifibrotic target, as overproduction of angiotensin II has been shown to stimulate stellate cell activation and fibrogenesis in the liver.

**Table 2** Several antifibrotic agents studied in the setting of hepatic fibrosis of different etiologies<sup>[33-43]</sup>

Authors	Antifibrotic agent studied	Patient group (n)	Dose used	Therapeutic efficacy
Kershenobich <i>et al</i> <sup>[33]</sup> , 1988	Colchicine	Cirrhosis - all causes (100)	0.6-1.8 mg/d	5-yr survival rates: colchicine 75%, placebo 34%; Histological improvement: colchicine 30%, placebo 0%
Pockros <i>et al</i> <sup>[34]</sup> , 2007	IFN- $\gamma$	Cirrhosis - HCV-related (488)	IFN- $\gamma$ 1b 100 mg and 200 mg	Histological improvement in select group with IFN-inducible T cell a chemoattractant (I-TAC),
Weng <i>et al</i> <sup>[35]</sup> , 2005	IFN- $\gamma$	Cirrhosis - HBV-related (99)	50 mg IFN- $\gamma$ intramuscularly on a daily basis for 3 mo	Histological improvement: treatment group 63%, control group 24.1%
Debernardi-Venon <i>et al</i> <sup>[36]</sup> , 2007	Angiotensin II receptor blockers (candesartan)	Cirrhosis - all causes (47)	8 mg/d	Has been shown to decrease hepatic venous pressure gradient in patients with cirrhotic portal hypertension; studies investigating histological improvement still underway
Neuschwander-Tetri <i>et al</i> <sup>[37]</sup> , 2003	PPAR ligands (rosiglitazone)	NASH associated fibrosis (30)	4-8 mg/d	Significant improvements in zone 3 perisinusoidal fibrosis
Armendáriz-Borunda <i>et al</i> <sup>[38]</sup> , 2006	Pirfenidone	HCV-related fibrosis (15)	1200 mg/d	Histological improvement in 30% of patients
Ferenci <i>et al</i> <sup>[39]</sup> , 1989	Silymarin	Cirrhosis - all causes (170)	140 mg three times daily	4-yr survival rate: silymarin 58%, placebo 39%; no histopathological studies available
Nelson <i>et al</i> <sup>[40]</sup> , 2003	Interleukin-10	HCV related fibrosis (30)	subcutaneously at a daily or thrice weekly dose of 8 pg/kg or a thrice weekly dose of 4 pg/kg	Significant improvements in histology
Poupon <i>et al</i> <sup>[41]</sup> , 2003	Ursodeoxycholic acid	PBC (367)	15-20 mg/kg per day	Significantly delayed progression of histopathological changes
Lieber <i>et al</i> <sup>[42,43]</sup> , 2003	Polyenyl-phosphatidylcholine	Chronic alcoholics (789)	1.5 g three times daily	No improvement in fibrosis; new study underway

HCV: hepatitis C virus; HBV: Hepatitis B virus; IFN- $\gamma$ : Interferon- $\gamma$ ; PPAR: Peroxisome proliferator-activated receptor; NASH: Non-alcoholic steatohepatitis; PBC: Primary biliary cirrhosis.

Angiotensin II may also play a role in the pathogenesis of portal hypertension, thus providing an added benefit to attempts inhibiting the system. The data in humans, however, has been mixed, with no conclusive evidence supporting the use of angiotensin receptor blockers for the prevention of liver fibrosis<sup>[30]</sup>.

Pirfenidone, another promising antifibrotic agent, whose mechanism of action is not clearly understood, was found to be useful in the management of idiopathic pulmonary fibrosis. Its benefit on liver fibrosis has yet to be sufficiently investigated. Other potential antifibrotic agents have been listed in Table 2<sup>[30]</sup>.

### Endoscopic therapy

Endoscopic treatment is the mainstay for primary and secondary prophylactic management of esophageal and gastric varices, as well as in the setting of acute bleeding. Similarly, for the management of recurrent cholangitis attacks associated with Caroli's syndrome, besides the use of antibiotics, drainage and stone extraction using endoscopic retrograde cholangiopancreatography (ERCP) may be indicated.

### Radiological intervention

Transjugular intrahepatic portosystemic shunts are considered for patients not amenable to sclerotherapy, and is particularly valuable in treating patients with refractory bleeding to buy time until liver transplantation.

### Surgery

Surgical shunts may also be indicated with the aim of portal decompression in patients with variceal bleeding not

satisfactorily managed endoscopically. Procedures of choice include nonselective total portosystemic shunts, nonselective partial portosystemic shunts that maintain some antegrade blood flow to the liver and selective portosystemic shunts, which decompress the gastroesophageal junction and the spleen through the splenic vein to the left renal vein. On the other hand, for Caroli's disease with recurrent bouts of cholangitis, partial liver resection may be indicated in case of extensive heterogenous involvement of a segment of the liver.

### Liver transplantation

Liver transplantation is the only known cure for CHF, and is indicated at the later stages of the disease, with the development of signs of liver failure. In Caroli's syndrome, frequent recurrence of cholangitis with diffuse involvement of the liver is also an indication for transplantation<sup>[9,10,31]</sup>. In 2008, Rossi *et al*<sup>[32]</sup> reported on three patients who had co-incidental hepatic failure due to CHF and end-stage renal failure as a result of polycystic kidney disease. All three underwent successful liver and kidney transplantation (1 simultaneous and 2 sequential) with excellent long term results.

## HACETTEPE EXPERIENCE

Throughout the 35 years between 1974-2009 in the history of the Department of Gastroenterology at Hacettepe University, Ankara, a total of 26 patients, 16 female and 10 male, with an average age of presentation of 28.4 years, have been diagnosed with congenital hepatic fibrosis. While up to the year 1985 only 3 patients were diagnosed,

the remaining 23 patients were diagnosed in the 1990s and particularly after the year 2000. This may be attributed to better recognition of the disorder by both clinicians and pathologists.

The most common presenting symptom was abdominal distention (11/26 - 42.3%) attributed to hepatosplenomegaly, with a history of recurrent cholangitis present in 6/26 (23%) of patients. In only two patients (7.7%) bleeding from esophageal varices was the presenting finding.

In 8/26 patients (31%) CHF was found to be in association with Caroli's disease (a combination otherwise known as Caroli syndrome). Incidentally, all patients who presented with signs of cholangitis suffered from Caroli's disease, where cholangitis is an expected manifestation. Joubert's syndrome with associated cerebellar vermis anomalies was diagnosed in 2 patients. In 2008, three siblings were referred to the department with common findings including mental retardation, blurred vision, nystagmus, truncal obesity, optic fundal and neurological abnormalities. Further investigation into the possible etiology of co-incidental hepatosplenomegaly resulted in a diagnosis of CHF after liver biopsy. All three were diagnosed as suffering from Bardet Biedl syndrome.

All but three of the patients under follow-up in our department are alive and well. Two patients died after contracting cholangiocellular cancer, while the third patient with Caroli's disease who had undergone several endoscopic gall stone extraction procedures died of biliary sepsis in 2002. Three patients, all of whom had Caroli's syndrome, underwent successful liver transplantation.

## CONCLUSION

CHF is a very rare disorder usually occurring in association with other fibropolycystic disorders, including renal involvement. Thus, pure/isolated CHF is very rare. It is necessary to differentiate it from idiopathic portal hypertension and early liver cirrhosis. After a diagnosis of CHF is established, the physician must investigate other organ systems, particularly for neuromuscular or renal involvement. A liver biopsy is essential in the diagnosis and differential diagnosis of CHF, as the presence of small bile duct dilatation and proliferation would rule out other metabolic disorders of the liver.

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Yusuf Bayraktar, Professor, Series Editor

## How useful is rectal endosonography in the staging of rectal cancer?

Taylan Kav, Yusuf Bayraktar

Taylan Kav, Yusuf Bayraktar, Division of Gastroenterology, Department of Medicine, Hacettepe University School of Medicine, Sıhhiye, Ankara 06100, Turkey

Author contributions: Kav T and Bayraktar Y contributed equally to this work.

Correspondence to: Taylan Kav, MD, Division of Gastroenterology, Department of Medicine, Hacettepe University School of Medicine, Sıhhiye, Ankara 06100, Turkey. [tkav@hacettepe.edu.tr](mailto:tkav@hacettepe.edu.tr)

Telephone: +90-312-3051712 Fax: +90-312-4429429

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### Abstract

It is essential in treating rectal cancer to have adequate preoperative imaging, as accurate staging can influence the management strategy, type of resection, and candidacy for neoadjuvant therapy. In the last twenty years, endorectal ultrasound (ERUS) has become the primary method for locoregional staging of rectal cancer. ERUS is the most accurate modality for assessing local depth of invasion of rectal carcinoma into the rectal wall layers (T stage). Lower accuracy for T2 tumors is commonly reported, which could lead to sonographic overstaging of T3 tumors following preoperative therapy. Unfortunately, ERUS is not as good for predicting nodal metastases as it is for tumor depth, which could be related to the unclear definition of nodal metastases. The use of multiple criteria might improve accuracy. Failure to evaluate nodal status could lead to inadequate surgical resection. ERUS can accurately distinguish early cancers from advanced ones, with a high detection rate of residual carcinoma in the rectal wall. ERUS is also useful for detection of local recurrence at the anastomosis site, which might require fine-needle aspiration of the tissue. Overstaging is more frequent than understaging, mostly due to inflammatory changes. Limitations of ERUS are operator and experience

dependency, limited tolerance of patients, and limited range of depth of the transducer. The ERUS technique requires a learning curve for orientation and identification of images and planes. With sufficient time and effort, quality and accuracy of the ERUS procedure could be improved.

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**Key words:** Rectal cancer; Colorectal cancer; Staging; Endorectal ultrasonography; Endorectal ultrasound; Accuracy; Tumor invasion; Nodal metastases; Other rectal tumors; Diagnostics

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### INTRODUCTION

Colorectal cancer is the most common gastrointestinal malignancy and the second most common cause of cancer-related deaths in Western countries<sup>[1]</sup>. Nearly 30% of these cancers arise in the rectum<sup>[2]</sup>. It is essential to determine prognostic factors in a patient before primary therapy is instituted. If examination has been delayed, it might be too late to influence the survival of a patient because of the lost opportunity to downstage the tumor before surgery. Primary surgery is no longer the only treatment due to recent advances in oncology and availability of therapeutic options. The potential advantages of preoperative treatment are to shrink the tumor size and thereby enhance the resectability rate

and facilitate sphincter-saving surgery, to reduce local recurrences, and possibly to improve long-term survival<sup>[3]</sup>. The prognosis of rectal cancer is closely related to several factors, including depth of tumoral invasion, number of metastatic lymph nodes, and involvement of the circumferential margin. Assessment of the cancer invasion through the bowel wall (T stage) remains the primary and most important factor in treatment of patients with rectal cancer<sup>[1-4]</sup>.

The TNM system for staging cancer of the colon and rectum to guide treatment and prognosis corresponds with the Dukes system: Stage I, Dukes A; stage II, Dukes B; and stage III, Dukes C. Stage IV corresponds to the presence of distant metastases<sup>[5]</sup>. Survival rates differ between T stages, and identifying poor prognostic groups within each stage has been the object of research. Early rectal cancers (T0) have a high, five-year survival rate of 95%. T3N0M0 and T4N0M0 lesions are stage II. Invasion of one or two lymph nodes but no distant metastasis (T1-4N1-2M0) with any T level represents stage III disease. Stage IV disease is the most severe, with distal metastasis (T1-4N1-2M1). The five-year survival rate for stage IV disease is poor (Table 1). There is a marked improvement in survival with early disease. A number of authors have shown a relationship between survival and the depth of extramural spread that is independent of other prognostic factors, including the circumferential margin status<sup>[6-8]</sup>.

The presence of lymph node (LN) involvement is important for the clinical decision, as early and locally advanced disease are managed differently. Endorectal ultrasound (ERUS) is a safe diagnostic method that allows both tumor invasion and lymph node metastatic involvement to be staged, and it contributes significantly to the selection of an adequate surgical strategy in patients with rectal cancer<sup>[7-9]</sup>.

Lesions confined to the wall may be resected by transanal excision or low anterior resection. Lesions involving, or in close proximity to, the anus might need abdominoperineal resection (APR). Patients with locoregionally-advanced lesions (extension onto the perirectal fat and/or perirectal or pelvic adenopathy) should be considered for neoadjuvant chemoradiotherapy. Neoadjuvant therapy has been shown to reduce local recurrence and permit an increased likelihood of a sphincter-sparing operation, with less toxicity compared with postoperative regimes. Thus, unlike more proximal colon cancers, the optimal method of management of rectal carcinoma is critically dependent on accurate preoperative staging of the disease<sup>[9,10]</sup>.

These therapeutic strategies appear to reduce local recurrence rates, increase sphincter-preserving surgeries, and possibly improve overall survival. Surgeries, and possibly improve overall survival. Therefore, staging of rectal cancer is important for selecting patients for adequate management prior to disturbing the tumor bed and potentially disseminating the disease. In daily practice we have been using newly developed and improved technologies that enable us to assess the extent of rectal cancer, which in turn influences choice of therapy. At

present, existing modalities for the preoperative staging of rectal cancer include computed tomography (CT); magnetic resonance imaging (MRI) with traditional body, endorectal, or phased-array coils; ERUS with rigid or flexible probes; and positron emission tomography (PET) with and without CT. The choice of modality is often influenced by local expertise and availability. This article reviews the current literature on the usefulness of ERUS in the staging of rectal cancer.

## ENDORECTAL SONOGRAPHY

Endorectal sonography was introduced to clinical practice in 1983 and has been successfully used in clinical practice for the evaluation of both the prostate and the rectum. In 1985, Hildebrant and Feifel introduced endorectal ultrasound as a means of staging rectal carcinoma<sup>[11]</sup>. In the last decade ERUS has become a widely accepted tool for staging of gastrointestinal cancers. Availability of ERUS in developing countries is limited, and there is a variation in availability and use of ERUS across Europe; the United Kingdom being the country in which ERUS is most widely used<sup>[12]</sup>. When ERUS is available, oncologists usually prefer to use it for staging of rectal cancer, which is the second most common cause of consultation with endosonographic examination indicated by surveyed oncologists. Most oncologists (89.5%) thought ERUS made an important impact on the management of patients with rectal cancer<sup>[13]</sup>.

Primary rectal adenocarcinoma is a common cause of a rectal mass on imaging. Other, less-common, lesions of the anorectum and perirectal tissues might resemble an adenocarcinoma. Transrectal sonography has proved to be a fast, safe, and accurate initial method for the staging of known rectal cancers or masses, although not for the screening of suspected rectum tumors, and is widely accepted as the diagnostic modality of first choice<sup>[14,15]</sup>. Imaging of the anorectum and perirectal tissues is technically challenging and can be difficult to interpret, as fecal material might be present, rectal lesions can be mobile or large, and general orientation is difficult<sup>[16]</sup>. The technique of transrectal sonography requires a learning curve for orientation and identification of ultrasound images and planes of rectal tumors. With sufficient effort, time, and meticulous technique, however, the rectum can be easily examined<sup>[15,16]</sup>.

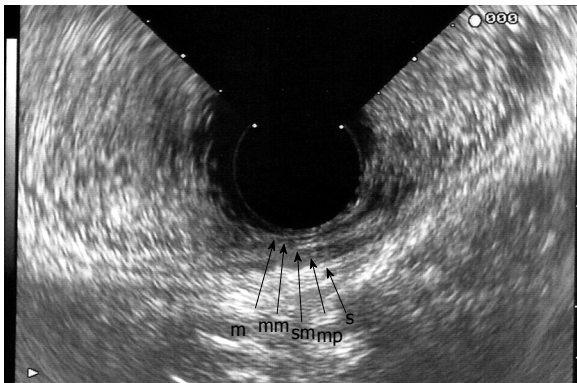
### Technique

To perform ERUS it is preferable to have an empty rectum, because fecal material can distort the images obtained. Laxative enemas are usually sufficient for rectal lesions, but standard colonoscopy preparation, even for rectal end sigmoid lesions, could optimize imaging so that it is free of artifacts. For endosonographic examination of proximal colonic lesions, such a preparation is a prerequisite. Pre-examination sigmoidoscopy should be routinely performed to ensure the lumen is clear of debris. The procedure is well tolerated and can be performed without sedation. Intraluminal rectal ultrasound examination of

**Table 1** Tumor stage on endorectal ultrasound to determine the management strategy, and corresponding survival rates<sup>[2,10]</sup>

Stage	T and N groups	Management	Five year survival
I	T 1-2, N0, M0	Snare polypectomy, EMR-	> 90%
II	T3 - 4, N0, M0	ESD, TAEX, LAR, APR	60%-85%
III	T1-4, N1, M0	LAR, RT followed by APR	25%-60%
IV	T1-4, N0-2, M1	RT-CT followed by APR	5%-7%

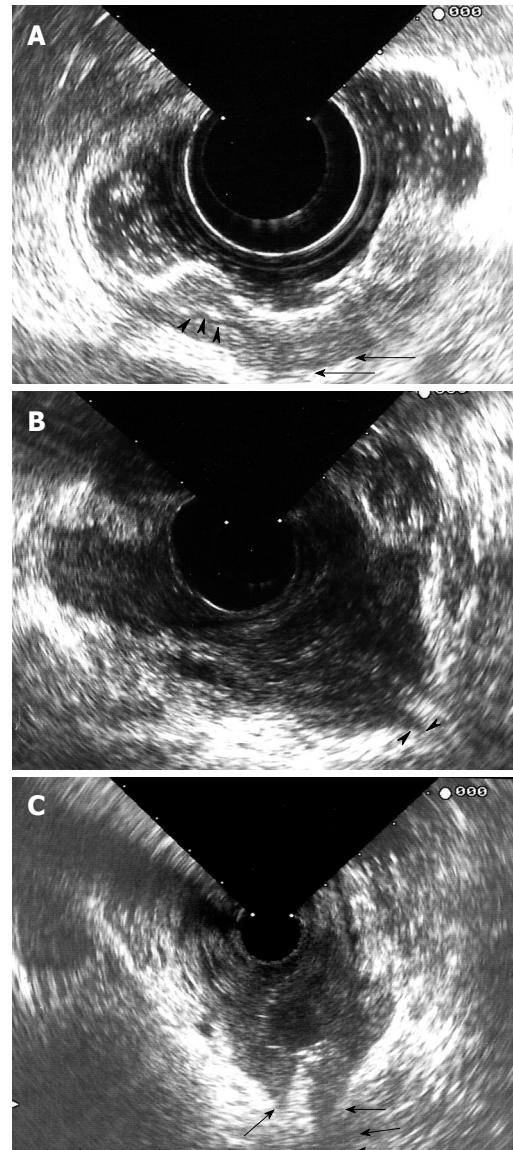
N0: No regional lymph node metastasis; N1: Metastasis in one to three regional lymph nodes; N2: Metastasis in four or more regional lymph nodes; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; TAEX: Transanal excision; LAR: Low anterior resection; APR: Abdominoperineal resection; RT: Neoadjuvant radiotherapy; RT-CT: Neoadjuvant radiochemotherapy.



**Figure 1** Normal endorectal sonogram image acquired by flexible echo-endoscope. The layers of the rectum are as follows: hyperechoic mucosa (m), hypoechoic muscularis mucosa (mm), hyperechoic submucosa (sm), hypoechoic muscularis propria (mp), and hyperechoic serosa (s).

rectal lesions can be done with a rigid probe or a flexible echoendoscope with a radial transducer. At our institution, we use a front-viewing upper echoendoscope, which can be advanced under direct vision to the level of the lesion. Linear echoendoscopes are normally used for fine-needle aspiration in case tissue sampling is needed, but could be used for routine ERUS<sup>[9]</sup>. For the purpose of this discussion, both techniques are considered as ERUS. Commonly, a dedicated blind rectal probe is used for ERUS. The probe is inserted and advanced into the rectum, where a water-filled balloon at the tip of the probe is inflated for evaluation of the rectum. High-frequency miniprobe are available and can be used with standard endoscopes to image the gastrointestinal wall and focal lesions under endoscopic vision. ERUS accurately visualizes the layers of the rectal wall and the precise localization of the layers of the rectal wall disrupted by the tumor, and the presence of perirectal lymph node metastases can be established<sup>[17]</sup>.

Endosonographically, the bowel wall is seen as five alternating hyper- and hypoechoic layers (Figure 1)<sup>[9,17-19]</sup>, as a result of differences in acoustic impedance, corresponding to histological layers. The first (hyperechoic) layer is the interface between the superficial mucosa and water or a water-filled balloon; the second (hypoechoic) layer represents the mucosa and muscularis mucosae; the



**Figure 2** Endorectal ultrasound (ERUS) image. A: A rectal carcinoma that appears to be T1 (penetration into submucosa) in one part (arrowheads show intact muscularis propria) and T2 (penetration into muscularis propria-arrows) in another part; B: A T3 rectal adenocarcinoma. Arrowheads show that the lesion penetrated into perirectal fat; C: A locally invasive cervical cancer, which invaded the rectum (arrows show tumor breach).

third (hyperechoic) layer denotes the submucosa and its interfaces; the fourth (hypoechoic) layer represents the muscularis propria; and the fifth (hyperechoic) layer is the interface between the serosa and perirectal fat.

Carcinomas are hypoechoic, and the degree to which they disrupt and penetrate the rectal wall layers suggests the local stage<sup>[4]</sup>. Ultrasonographic staging of tumor depth is denoted by the prefix “u”. The ultrasonographic staging corresponds to the TNM classification<sup>[5]</sup>. A uT1 tumor does not penetrate the muscularis propria. A uT2 tumor penetrates the muscularis propria (Figure 2A). A uT3 tumor proceeds beyond the muscularis propria, infiltrating the perirectal fat to a variable degree (Figure 2B). A uT4 tumor infiltrates surrounding organs<sup>[10,18]</sup>. As the tumor stage is advanced, a marked decrease in survival is



observed. ERUS, however, cannot reliably visualize the mesorectal fascia and thus cannot indicate whether the planned surgical circumferential resection margin will be successful<sup>[1-5]</sup>.

The sonographic criteria for identifying involved lymph nodes consist of size greater than 5 mm, mixed signal intensity, irregular margins, and spherical rather than ovoid or flat shape. ERUS can distinguish the different anatomic layers of the bowel, and thus it appears to have advantages over both CT and MRI in assessing mural penetration, and is invaluable in assessing patients considered for local resection<sup>[10,20]</sup>.

Indications for ERUS in rectal cancer are as follows<sup>[21]</sup>:

(1) to choose endoscopic mucosal resection or transanal excision in case of a large polyp or small rectal cancer (lesion is T1 by ERUS); (2) to determine whether preoperative chemotherapy and radiation is needed; and (3) surveillance after surgery for rectal cancer.

### T and LN staging

As outlined above, appropriate staging guides the treatment. Many other modalities, including CT and MRI of the abdomen, have been utilized to correctly determine the TNM stage. In 80 consecutive patients with newly diagnosed rectal cancer who were prospectively evaluated, Harewood *et al.*<sup>[22]</sup> reported T staging accuracy of 91%, compared to 71% for CT, and N staging accuracy of 82%, compared to 76% for CT.

The accuracy of ERUS for assessing local depth of invasion of rectal carcinoma (T stage) ranges from 80% to 95%, compared to 65%-75% for CT and 75%-85% for MRI<sup>[20]</sup>. ERUS has been demonstrated to be very accurate for staging superficial rectal tumors, with accuracy in evaluating tumor ingrowth into rectal wall layers ranging from 69% to 97%<sup>[23,24]</sup>.

A recent meta-analysis evaluating all ERUS studies from 1980 to 2008 showed that accuracy was high (88%-95%). The sensitivity and specificity of ERUS to diagnose stage T1 cancer were 87.8% and 98.3%, respectively. For stage T2, ERUS had a sensitivity and specificity of 80.5% and 95.6%, respectively. For stage T3, ERUS had a sensitivity and specificity of 96.4% and 90.6%, respectively. In diagnosing stage T4 cancer, ERUS had a sensitivity of 95.4% and specificity of 98.3%<sup>[25]</sup>. One common finding is a lower accuracy for T2 tumors. Several reasons have been suggested, including the difficulty in distinguishing those tumors that have deep invasion into the muscularis propria from those with microscopic invasion into the perirectal fat. This could raise problems with sonographic T3 cancers that have been overstaged, because there is an increased tendency to give preoperative radiotherapy to T3 cancers.

Zorcolo *et al.*<sup>[26]</sup> evaluated the accuracy of ERUS for the distinction of early vs advanced rectal lesions before transanal endoscopic microsurgery and they found ERUS differentiated early and advanced rectal lesions with 96% sensitivity, 85% specificity, and 94% accuracy. Similarly, another retrospective series reached 89.2% accuracy for staging of early rectal carcinomas<sup>[27]</sup>.

ERUS is also helpful in determining the presence of residual cancer in the rectal wall. A retrospective series with 63 patients showed the presence of residual cancer in patients who underwent surgery ( $n = 30$ ) with 54% accuracy. Authors stated that ERUS was more useful than morphological or histological criteria for determining residual cancer<sup>[28]</sup>.

Transanal endoscopic microsurgery (TEM) and endoscopic submucosal dissections have been becoming more popular because of they offer function-preserving resections. An important problem that has arisen in this setting is the assessment of the tumor breach to the submucosa, which changes the mode of surgery. Other imaging modalities are known to be poor at staging in early cancers. According to a prospective study involving 156 patients, of whom 62 underwent TEM, no understaging was observed with an accuracy of 95%, and only 5% were overstaged. ERUS is accurate at predicting early disease<sup>[29]</sup>.

ERUS was useful in detecting cancer recurrence at the anastomosis site. This often requires serial examination to differentiate postoperative scars from local recurrences. In sonographically equivocal cases, tissue characterization and sampling *via* FNA make ERUS very accurate; although the surveillance period was not assessed, a recommendation was made of every 3-6 mo during the first two years after low anterior resection<sup>[20]</sup>.

Assessment for nodal metastases is less accurate than that for tumor depth. According to a recent meta-analysis of 35 studies by Puli *et al.*<sup>[30]</sup>, which involved more than 2700 patients, the sensitivity of ERUS in diagnosing nodal involvement in rectal cancer was 73.2% and it had a specificity of 75.8%.

Discrepancies in accuracies could be partly due to the variable criteria used for defining nodal metastases. For rectal cancer in particular, over half of the metastatic nodes secondary to rectal cancer are  $\leq 5$  mm and are located within 3 cm of the primary tumor<sup>[31]</sup>. In a large trial, lymph node metastatic disease was shown to predict local recurrence. There is a wide variation in accuracy for metastatic nodal detection with ERUS (62%-87%), CT (22%-73%), and MRI (39%-95%)<sup>[32]</sup>. ERUS criteria are a lack of ovoid morphology and central echogenic nidus, but its limited field of view is a major limitation<sup>[4]</sup>. Data from pooled analyses, as well as from recent smaller studies, reveal that the sensitivity of ERUS in detecting LN metastasis ranges from 50% to 83%, which is comparable with that of MRI (sensitivity 45% to 79%)<sup>[9,10]</sup>. Assessment of nodal metastases is difficult because most small lymph nodes are not easily observed with ERUS, and 18% of lymph nodes less than 5 mm harbor metastases<sup>[17]</sup>. More recent studies suggest that multiple criteria should be used to improve accuracy. Gleeson *et al.*<sup>[20]</sup> conducted a study with ERUS guided FNA to identify nodal echo characteristics and size for prediction of malign infiltration, and to determine if any combination of standard nodal criteria had sufficient predictive value to preclude FNA. Nodal hypoechogenicity and short axis  $\geq 5$  mm were independent factors for malignancy. If all four



malignant nodal echo features of node were present, it distinguished the malignant from the benign node. These US features were node size, echogenicity, shape, and the border. A long axis length greater than 9 mm was 95% specific for the presence of malignancy.

Accuracies of ERUS might vary with different tumor stages. Overstaging is more frequent than understaging. As with MRI, overstaged T2 lesions are the most common causes of inaccuracy. ERUS cannot reliably or precisely differentiate an irregular outer rectal wall due to peritumoral inflammation or real transmural tumor extension<sup>[7-9,33]</sup>. Staging of the stenotic lesions might also be difficult; they are probably suboptimally staged because of the inability of the probe to traverse the lesion. This problem is greater with rigid probes. Flexible probes have the ability to evaluate the iliac region for adenopathy, which is clinically important because these nodes are retained in standard resection with total mesorectal excision. In one study, up to 28% of lymph node-positive distal tumors showed iliac adenopathy, with 6% of patients having only iliac adenopathy. Thus, failure to evaluate this region could lead to inadequate surgical margins in up to 6% of patients with low rectal lesions. Lymph nodes > 5 mm in size have a 50% to 70% chance of being malignant compared with only 20% of nodes < 4 mm. ERUS-guided FNA allows confirmation of malignancy in suspicious nodes during the same examination, as long as the primary tumor does not lie in the path of the needle<sup>[33]</sup>.

Preoperative chemoradiation is a main reason for lower staging accuracy rate. Napoleon *et al.*<sup>[34]</sup> found a variation in the accuracy of T staging from 86% (in patients referred directly to surgery) to 46% (in patients after neoadjuvant radiation therapy).

Overstaging is mainly caused by inflammatory and associated reactive changes in the rectum wall after preoperative radiotherapy. They are presented as hypo-echoic lesions and can be confused with carcinoma. However, radiotherapy affects the wall thickness but does not change the five-layered image. In one particular study, comparison of postradiation ERUS correlated with histopathology findings revealed that ultrasound was actually assessing the fibrosis that had replaced the tumor; therefore, after radiotherapy, what is staged by ERUS is no longer the tumor but the extent of fibrosis in the rectal wall. A histopathological examination showed that the residual tumor, when present, was always within the fibrosis, never outside or separate from it<sup>[33,35]</sup>.

In general, ERUS is better at detecting lymph nodes in the distal and middle thirds of the rectum<sup>[21,33]</sup>. The overstaging of lymph node status is primarily caused by the presence of reactive swollen lymph nodes that could be considered as malignant. The small blood vessels, urethra, and seminal vesicle are known to be mistaken for metastatic lymph nodes. Blood vessels can simulate malignant nodes, but they can be differentiated by moving the transducer to outline the linear or branching course of the vessel and by power Doppler. The main reasons for nodal status understaging are difficulty in

detecting very small involved nodes (less than 2 mm) and nodes outside the perirectal tissue<sup>[21,33]</sup>.

The three-dimensional reconstruction is also thought to improve visualization of subtle protrusions of tumors infiltrating into adjacent tissues and organs, allowing for improved T and N staging. A study of 25 patients undergoing three-dimensional ERUS, two-dimensional ERUS, and MRI showed no significant difference in T- or N-stage accuracy, but it was thought that MRI and three-dimensional ERUS improved understanding of the spatial relationship of the tumor due to their ability to obtain multiplanar images<sup>[36]</sup>.

The limitations of ERUS are that it is heavily operator dependent; it has poor patient acceptability; it has limited depth of penetration; it cannot be performed in stenotic tumors<sup>[16,21]</sup>; and it is unable to visualize tumors located in the upper rectum with a rigid probe, detect lymph nodes outside the range of the transducer, or visualize mesorectal fascia because of its limited field of view. In addition, accuracy is affected by postbiopsy peritumoral inflammation, hemorrhage, and villous or pedunculated tumors<sup>[22,31]</sup>. Overstaging of tumor depth frequently occurs as a result of paraneoplastic inflammation, as ultrasound cannot clearly differentiate between inflammatory and neoplastic tissue<sup>[22,31]</sup>.

Several authors suggest that obstructive tumors interfere with accurate staging. In such tumors, inadequate probe contact perpendicular to the tumor makes it more likely to be mis-staged. Some authors reported better accuracy rates for high compared to low rectal tumors, while others found the opposite<sup>[32]</sup>.

The tumor margin cannot be assessed accurately, which in turn causes mis-staging, because of inadequate bowel preparation and bulky tumors that lie outside the focal length of the transducer.

There is a learning curve with operator variability<sup>[33]</sup>. Badger *et al.*<sup>[37]</sup> found that experience does not affect the T and N staging accuracy, suggesting that there was no learning curve. Others supported the effect of experience and appropriate training for accurate staging<sup>[38]</sup>. Inexperience has been cited as contributing to many of the poor accuracies in tumor depth infiltration<sup>[17]</sup>.

Orrom *et al.*<sup>[39]</sup> reported an increase in diagnostic accuracy of ultrasound from 59.3% to 95% over a period of three years when ERUS was performed by several operators and a single skilled operator performed the later exams. More time and meticulous training are required for improvements in the accuracy of ERUS, a statement supported by different studies showing a progress from 50% to over 90%<sup>[39-42]</sup>. It has also been suggested that centralization of ERUS could provide more caseloads to experienced operators and result in a high-quality service<sup>[41]</sup>.

Studies suggest a learning curve of up to 50 cases for tumor depth and more than 75 cases for accurate node status assessments<sup>[41]</sup>. Interpretation is often more difficult after a partial excision or neoadjuvant chemoradiation, which can result in a hematoma or local inflammation with obliteration of sonographic layers of the rectal wall.

Presence of inflammatory changes, desmoplastic changes, and hypervascularity could lead to overstaging because the echogenicity of tumors is similar to that of muscularis propria and inflammatory infiltrate<sup>[42]</sup>.

## OTHER MALIGNANCIES OF THE RECTUM

Transrectal sonography is useful to differentiate extramural lesions, extrinsic compression, vascular lesions, and solid tumors. Other types of malignancies resembling rectal adenocarcinoma include neuroendocrine tumors, which usually manifest as small, mobile, submucosal nodules or focal areas of submucosal thickening, and primary squamous cell carcinomas, which seem to be frequently locally invasive and involve regional lymphatic vessels. Lymphomas are rare, and can be a primary lesion or a secondary infiltration of the large intestine, which characteristically involves the deeper layers of the intestinal wall. Anorectal melanoma is another rare rectal tumor. Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor that originates in the alimentary tract, but it rarely involves the anorectal region. The tissue of origin is the muscle layer of the bowel wall, and the size can be variable. On sonography, a GIST appears as a hypoechoic mass<sup>[15]</sup>. ERUS is also helpful for determining local invasion of the rectum by other pelvic malignancies (Figure 2C).

## CONCLUSION

ERUS is a safe and accurate technique for the local staging of rectal carcinoma with reported high accuracy rates for T and N staging. Although availability is limited, it has been implemented into clinical practice in clinical decision making regarding treatment modality. A growing body of expertise has confirmed the clinical impact. ERUS is also helpful in assessing recurrence of rectal cancer and evaluation of subepithelial masses. Technological improvements in ultrasound might improve accuracy and reduce the overstaging problem.

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## Capillaria hepatica in China

Chao-Ding Li, Hui-Lin Yang, Ying Wang

Chao-Ding Li, Hui-Lin Yang, Department of Orthopedics, First Affiliated Hospital of Soochow University, Soochow 215006, Jiangsu Province, China

Ying Wang, Department of Hematology, First Affiliated Hospital of Soochow University, Soochow 215006, Jiangsu Province, China

Author contributions: Li CD, Yang HL and Wang Y all contributed to this paper.

Correspondence to: Hui-Lin Yang, Professor, Department of Orthopedics, First Affiliated Hospital of Soochow University, Soochow 215006, Jiangsu Province, China. hlyangsd@126.com  
Telephone: +86-512-67780429 Fax: +86-512-67780999

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### Abstract

*Capillaria hepatica* (*C. hepatica*) is a parasitic nematode causing hepatic capillariasis in numerous mammals. Ecologic studies showed that the first hosts of *C. hepatica* were rodents, among which rats had relatively high infection rates, which explains why *C. hepatica* spreads globally. Anatomical studies showed that the liver was the principal site of colonization by these parasites and physical damage tended to occur. Although *C. hepatica* might lead to serious liver disorders, relevant clinical reports were rare, because of the non-specific nature of clinical symptoms, leading to misdiagnosis. This review mainly focuses on the biological characteristics and epidemiology of *C. hepatica* in China and histopathologic changes in the liver, with expectation of gaining a better understanding of the disease and seeking more effective treatment.

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**Key words:** *Capillaria hepatica*; Enoplida infections; Liver diseases; Host-parasite interactions; Diagnosis; Treatment

**Peer reviewer:** Dr. Assy Nimer, Assistant Professor, Liver Unit, Ziv Medical Centre, Box 1008, Safed 13100, Israel

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### INTRODUCTION

*Capillaria hepatica* (*C. hepatica*) is a nematode parasite of wild rodents and other mammals and has worldwide distribution<sup>[1-8]</sup>. Adult worms colonize the liver of the host<sup>[6,9-11]</sup>. They can cause hepatic capillariasis, a serious liver disorder, which may be found both in humans and animals<sup>[11-14]</sup>. These parasites could be accidentally transmitted to humans by ingestion of embryonated eggs. Up to the year 2000, 37 cases of human infections had been documented<sup>[15]</sup>. However, there are few reports of the pathology of the infection, which results in serious effects in subjects because of the special anatomic area in which *C. hepatica* congregates. Clinical symptoms of hepatic capillariasis were non-specific with manifestations of persistent fever, hepatomegaly, eosinophilia and, more seriously, death.

### MORPHOLOGY AND BIOLOGICAL FEATURES

#### Morphology

A typical adult *C. hepatica* takes the shape of a slender nematode, with the anterior part of the body narrow, and the posterior part gradually swelling. The females measure about 53-78 mm × 0.11-0.20 mm, but males are approximately 24-37 mm × 0.07-0.10 mm. The esophagus is long, occupying half of the body of the female and a third of the male body. The cauda of *C. hepatica* bears a copulatory spicule and sheath. The eggs of *C. hepatica* resemble those of *Trichuris trichiura*, but differ in size. The *C. hepatica* egg is about 48-66 μm × 28-36 μm, and numerous minipores can be seen in the outer shell<sup>[16]</sup>.

#### Biological features

*C. hepatica* parasites live in liver parenchyma, where they



Table 1 Three cases of hepatica capillariasis in China

Reporter	Date	Area in China	Diagnostic basis
Bing-Kun Xu <sup>[27]</sup>	1979	Guangdong Province	<i>Capillaria hepatica</i> ( <i>C. hepatica</i> ) detected by liver biopsy
Xi-Meng Lin <sup>[28]</sup>	2003	Tangzhuang, Xinxiang City	Persistent fever (40°C), hepatomegaly eosinophilia, and adult <i>C. hepatica</i> detected by liver biopsy
Jia-Nin Huang <sup>[29]</sup>	2003	Fuzhou City, Fujian Province	Persistent fever, anemia, hepatomegaly, eosinophilia, and eggs detected by liver biopsy

Table 2 Infection rate in distinct areas with different rodent species

Reporter	Date	Area in China	Investigated species	Infection rate (%)
Zhou <i>et al.</i> <sup>[33]</sup>	1990	Wuhan City, Hubei Province	Norway rat	61.90
			<i>Rattus flavipectus</i>	61.90
			<i>Mus musculus</i>	19.10
Liu <i>et al.</i> <sup>[40]</sup>	1997	Shandong Province	Various rodent species including those of rodent-shaped animals	27.36 (Norway rat dominant)
Zhou <i>et al.</i> <sup>[34]</sup>	1998	Kunming City, Yunnan Province	Norway rat	66.67
			Yellow breasted rat	65.13
			<i>Mus musculus</i>	21.11
Yuan <i>et al.</i> <sup>[36]</sup>	1998	Ningde City, Fujian Province	Chestnut rat	55.56
			Norway rat	66.67
			<i>Rattus flavipectus</i>	44.33
			<i>Rattus losea</i>	38.94
			<i>Rattus confucianus</i>	30.00
Xue <i>et al.</i> <sup>[37]</sup>	1998	Fuqing City, Fujian Province	<i>Rattus flavipectus</i>	13.11
			Norway rat	12.34
			Shrew	5.29
			<i>Mus musculus</i>	4.59
			Norway rat	46.15
Zhang <i>et al.</i> <sup>[35]</sup>	2002	Jiangle Location, Fujian Province	<i>Rattus flavipectus</i>	66.67
Shen <i>et al.</i> <sup>[39]</sup>	2003	Dali City, Yunnan Province	Commensal Mus	76.83
			Norway rat	77.01
			<i>Rattus flavipectus</i>	77.46
			Wild Mus	4.47
			<i>Rattus rattus sladeri</i>	38.81
Lin <i>et al.</i> <sup>[41]</sup>	2007	Henan Province	Norway rat	25.83
			<i>Rattus flavipectus</i>	12.90
			<i>Mus musculus</i>	10.00
			Various species of rodents	49.50
Tung <i>et al.</i> <sup>[38]</sup>	2009	Taichung		

become biologically mature, then lay eggs in this site. Eggs are immature when produced in the first 4 wk, and these eggs will develop into larvae under favorable conditions of appropriate temperature and moisture. When embryonated, eggs can be ingested by a predator, their larvae then hatch and invade the intestinal mucosa, transporting themselves *via* the mesenteric vein and portal vein to the liver. The first ecdysis takes place 3–4 d after their arrival in the liver, followed by the second, third (5–7 d) and fourth (9–16 d) larval stages. In the fourth stage, sexual differentiation starts. After sexual differentiation (male, 18 d; female, 20 d), they will experience their final ecdysis and become fifth-stage larvae. The life-span of the female lasts about 59 d, with 40 d for males<sup>[17]</sup>. It is worthy of note that eggs produced by females in the liver are metabolically active for a prolonged period of time, but remain immature. The host which has ingested these immature eggs displays a “spurious infection”. In contrast, “true infection” occurs when the host ingests embryonated eggs, which will result in the production of larvae that can invade the intestine wall and lead to hepatica capillariasis.

## EPIDEMIOLOGY IN CHINA

### *Epidemiology in the human population*

Reports of the 37 cases of hepatica capillariasis indicate they were scattered predominantly in Japan, India, America, Canada, Brazil, Germany, Italy, Korea and Czechoslovakia<sup>[15,18–26]</sup>. While only 3 cases of “true infection” had been confirmed in China<sup>[27–29]</sup>, those few cases found in China do not necessarily encompass the overall actual morbidity, as the final diagnosis would have to rely on biopsy or necropsy<sup>[30,31]</sup>, so both the rate of misdiagnosis and missed diagnosis could be higher. Table 1 shows the 3 cases with detailed clinical symptoms.

### *Epidemiology in the animal population*

The chief hosts of *C. hepatica* are various rodents, including more than 70 species, and the principal hosts include *Tamias striatus*, squirrel, mole, shrew, opossum, weasel and skunk<sup>[32]</sup>. In mainland China, the total infection rate of hepatica capillariasis in rodent species ranges widely. Table 2 highlights the infection rate in distinct areas with different rodent species<sup>[33–41]</sup>.

## **PATHOLOGY OF HEPATICA CAPILLARIASIS**

*C. hepatica* primarily invade the sinus hepaticus, where they experience maturation and egg-laying. Both the worms and their eggs cause focal chronic inflammation in the liver, and around these worms and eggs appear diverse inflammatory cells, including macrophages, eosinophils, and some multinucleate giant cells. Inflammatory infiltration may persist until the final formation of encapsulation or calcification of dead worms. After the focal parasitic necroinflammatory lesions, septal fibrosis occurs. Although the pathological course of the formation of fibrosis has not been well established, it was speculated that the slow and continuous release of disintegrated products from encapsulated parasitic lesions activated the Kupffer cells, which then promoted the development of fibrosis in the liver<sup>[42]</sup>. Whether there is a relationship between the focal parasitic hepatic lesions and septal fibrosis remain to be resolved. In the experiment of Gomes *et al.*<sup>[12]</sup>, rats were first infected with 600 embryonated eggs, and then injected with a corticoid and *C. hepatica* antigen. After treatment, focal inflammation ceased, but there was no evident alteration in the formation of septal fibrosis. These findings indicated that, although focal lesions and septal fibrosis were both caused by *C. hepatica* infection, they played different roles in the pathological course of the infection. Further studies should be conducted to explore the pathological course of hepatic fibrosis.

## **DIAGNOSIS**

Hepatica capillariasis is an exceptionally rare infection in humans with non-specific clinical manifestations, and frequent misdiagnoses have been made<sup>[43]</sup>. More importantly, the main difficulties interfering with correct diagnosis were related to the unique biological characteristics of the parasite. Apart from those cases of “spurious infection”, both worms and eggs could not be detected in the peripheral blood and stools of infected hosts, so routine laboratory tests of blood and stools invariably showed negative results. Although liver biopsy was a precise and quick method in confirming *C. hepatica* infection, it was not the most appropriate one, as biopsy was a traumatic diagnostic approach. With introduction of immuno-techniques, the detection of *C. hepatica* became more convenient and efficient. Assis and colleagues<sup>[12]</sup> employed an indirect immunofluorescence test to diagnose hepatica capillariasis successfully. Huang *et al.*<sup>[44]</sup> developed a diagnostic test for experimental rat hepatica capillariasis using an enzyme-linked immunosorbent assay, with high sensitivity and specificity, which was specific for *C. hepatica* infection. The tests described above have been considered practical, reliable and sensitive. A sensitive immunological test is useful for particular clinical situations, but it is essential to take account of the epidemiological surveys in local areas, which may help lead to a more comprehensive diagnosis.

The differential diagnosis should include accidental tissue infection by nematodes, including *Toxocara cati*, *Toxocara canis*, *Fasciola hepatica*, and *Schistosoma japonicum*, hepatitis B virus, hepatitis C virus and visceral larva migrants<sup>[45-48]</sup>.

## **TREATMENT**

Pereira *et al.*<sup>[30]</sup> reported a case of hepatica capillariasis in Brazil where the male subject with massive *C. hepatica* infection survived after treatment with prednisone, disophenol, and pyrantel tartrate. Thanks to marked eosinophilia in the peripheral blood and hepatic lesions, the patient underwent initial therapy with prednisone (60 mg/d) for a session of 10 d and sequential maintenance by 10 mg every other 10 d. To kill the parasites, or at least to prevent the production of eggs, the patient was treated with disophenol (2-6-diiodo-4-nitrophenol) intramuscularly in a single dose of 7.5 mg/kg body weight and with pyrantel tartrate orally in a single dose of 30 mg/kg body weight. Three years after the treatment, a needle biopsy of the liver, showed sparse portal fibrosis but it was otherwise normal, and the patient remained well during an 8-year follow-up. Also, medication with albendazole was generally effective<sup>[31]</sup>.

Other than chemical treatment, partial hepatectomy or some distinct surgical intervention proved therapeutically effective in animal experiments in rats. The results revealed morphologically that the fibrosis was unaffected, but its relative quantity within the microscopic field appeared significantly decreased, as a consequence of the increased liver tissue mass following regeneration<sup>[49]</sup>.

## **RESEARCH ACHIEVEMENTS**

While prevalence of *C. hepatica* is dominant in rodents, other mammalian species showed slight resistance to this infection even in laboratory conditions. Yang *et al.*<sup>[50]</sup> examined the predisposition to *C. hepatica* between rats and cats by injecting each animal with embryonated eggs at high density. The long-term investigation revealed that every rat became infected with *C. hepatica*, while, as was expected, the liver biopsy from cats showed negative results. To further confirm whether there were some differences between rats and mice in the course of the formation of hepatic fibrosis, Andrade *et al.*<sup>[13]</sup> infected both rats and mice with embryonated eggs, and he found that, although rats and mice both had the same pathological changes in the first stage, there were distinct features in the development of hepatic fibrosis. Researching into the immunological mechanisms of hepatica capillariasis, Kim *et al.*<sup>[51]</sup> measured cytokine mRNA expression in mice spleen cells and mesenteric lymph node cells. In the earlier stages, expression of T-helper, Th1 and Th2, cells were at a high level, as well as the expression of immunoglobulin G1 and G2. Expression in functional cells in the spleen was relatively higher than in mesenteric lymph node cells, which indicated that the spleen was the main location of the

response to the infection rather than the mesenteric lymph node. With the density of egg production, expression of interferon- $\gamma$  became stronger, suggesting that it had significant importance in the defense against infection.

## CONCLUSION

*C. hepatica* can cause a serious liver disorder in its hosts including humans and animals. More simple and accurate diagnostic methods and more effective treatment measures need to be further developed. A better understanding of *C. hepatica* and hepatic capillariasis would help humans to better combat the disease.

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## ***Schistosoma mansoni* proteins attenuate gastrointestinal motility disturbances during experimental colitis in mice**

Nathalie E Ruysers, Benedicte Y De Winter, Joris G De Man, Natacha D Ruysers, Ann J Van Gils, Alex Loukas, Mark S Pearson, Joel V Weinstock, Paul A Pelckmans, Tom G Moreels

Nathalie E Ruysers, Benedicte Y De Winter, Joris G De Man, Natacha D Ruysers, Ann J Van Gils, Paul A Pelckmans, Tom G Moreels, Laboratory of Experimental Medicine and Pediatrics, Division of Gastroenterology, University of Antwerp, 2610 Antwerp, Belgium

Alex Loukas, Mark S Pearson, Division of Infectious Diseases and Immunology, Queensland Institute of Medical Research, 4006 Brisbane, Australia

Joel V Weinstock, Division of Gastroenterology-Hepatology, Tufts New England Medical Center, Boston, MA 02111, United States

**Author contributions:** Ruysers NE, De Winter BY, De Man JG, Ruysers ND and Van Gils AJ performed the experiments; Ruysers NE analyzed the data; Loukas A, Pearson MS and Weinstock JV provided the proteins and the analytic tools and were also involved in editing the manuscript; De Winter BY, De Man JG, Pelckmans PA and Moreels TG coordinated the work and were also involved in editing the manuscript; Ruysers NE wrote the paper.

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**Correspondence to:** Tom G Moreels, Professor, Division of Gastroenterology and Hepatology, Antwerp University Hospital, Wilrijkstraat 10, 2650 Antwerp, Belgium. [tom.moreels@uza.be](mailto:tom.moreels@uza.be)  
Telephone: +32-3-8213323 Fax: +32-3-8214478

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### **Abstract**

**AIM:** To investigate the therapeutic effect of *Schistosoma mansoni* (*S. mansoni*) soluble worm proteins on gastrointestinal motility disturbances during experimental colitis in mice.

**METHODS:** Colitis was induced by intrarectal injection of trinitrobenzene sulphate (TNBS) and 6 h later, mice were treated ip with *S. mansoni* proteins. Experiments were performed 5 d after TNBS injection. Inflammation

was quantified using validated inflammation parameters. Gastric emptying and geometric center were measured to assess *in vivo* gastrointestinal motility. Peristaltic activity of distal colonic segments was studied *in vitro* using a modified Trendelenburg set-up. Cytokine profiles of T-lymphocytes isolated from the colon were determined by real time reverse transcriptase-polymerase chain reaction.

**RESULTS:** Intracolonic injection of TNBS caused severe colitis. Treatment with *S. mansoni* proteins significantly ameliorated colonic inflammation after 5 d. TNBS did not affect gastric emptying but significantly decreased the geometric center and impaired colonic peristaltic activity 5 d after the induction of colitis. Treatment with *S. mansoni* proteins ameliorated these *in vivo* and *in vitro* motility disturbances. In addition, TNBS injection caused a downregulation of effector T cell cytokines after 5 d, whereas a *S. mansoni* protein effect was no longer observed at this time point.

**CONCLUSION:** Treatment with *S. mansoni* proteins attenuated intestinal inflammation and ameliorated motility disturbances during murine experimental colitis.

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**Key words:** *Schistosoma mansoni*; Helminth proteins; Colitis; Peristalsis; Crohn's disease; Gastrointestinal motility; Trinitrobenzene sulphate

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## INTRODUCTION

Crohn's disease and ulcerative colitis, the two most common forms of inflammatory bowel diseases (IBD), are idiopathic inflammatory disorders of the intestine. The current hypothesis states that IBD results from an uncontrolled immune response against intraluminal bacterial antigens in genetically predisposed individuals<sup>[1,2]</sup>. Genetic factors as well as environmental factors contribute to the development of the inappropriate immune response<sup>[3]</sup>.

The incidence of Crohn's disease is highest in well-developed countries<sup>[4]</sup>. According to the hygiene hypothesis, this is directly related to the higher hygienic standards in these countries<sup>[5]</sup>. It is suggested that the lack of exposure to intestinal parasites (e.g. helminths) contributes to the susceptibility to Crohn's disease<sup>[6-8]</sup>. Several experimental and clinical studies showed the beneficial effect of helminth infections in IBD<sup>[9-12]</sup>. Current research is focusing on identifying helminth molecules with immunomodulatory function that exert this protective effect<sup>[13]</sup>.

Patients with Crohn's disease often suffer from disturbed gastrointestinal motility leading to symptoms such as abdominal pain, cramps, diarrhea, weight loss, rectal bleeding and malnutrition<sup>[14,15]</sup>. It is well established that inflammation of the gut results in functional and structural changes of the enteric nervous system<sup>[16,17]</sup> and changes in smooth muscle contractility<sup>[18,19]</sup>. For instance, patients with ulcerative colitis have increased propagating contractions with lower peak amplitudes coupled with variable transit<sup>[20]</sup>, whereas delayed gastric emptying and prolongation of orocecal transit time have been reported in patients with Crohn's disease<sup>[21-23]</sup>. Dysmotility can also occur in non-inflamed sites of the gastrointestinal tract. Gastroparesis often occurs in patients with inflammation restricted to the small or large intestine<sup>[14,24]</sup>. Motility disturbances may also persist in the period following an episode of gastrointestinal inflammation, resulting in the development of irritable bowel syndrome or functional dyspepsia<sup>[14,25]</sup>.

The aim of this study was to investigate the therapeutic potential of *Schistosoma mansoni* (*S. mansoni*) soluble worm proteins (SmSWP) on gastrointestinal motility disturbances 5 d after induction of experimental colitis in mice both *in vivo* and *in vitro*. In addition, the inflammatory reaction was quantified and the balance between different T cell subsets was investigated based on their cytokine profile to elucidate the underlying immunological pathways.

## MATERIALS AND METHODS

### TNBS-induced colitis

Colitis was induced by intraluminal injection of 2,4,6-

trinitrobenzene sulfonic acid (TNBS) as previously described<sup>[26]</sup>. Briefly, male Swiss mice (weight 26-28 g, Charles River, France) were fasted for 24 h and subsequently anesthetized by ketamine (90 mg/kg, ip) and xylazine (10 mg/kg, ip). Next, 100  $\mu$ L of a 10 mg TNBS in 30% ethanol solution was injected intrarectally. Ethanol is required to break the intestinal epithelial barrier, whereas TNBS is a haptenating agent that immunogenizes autologous proteins. Control animals received an intrarectal injection of 100  $\mu$ L saline. Afterwards, mice were held upside-down for 1 min to prevent leakage of TNBS solution. The Medical Ethical Committee on animal experimentation of the University of Antwerp, Belgium, approved all experiments.

### Preparation of antigen mixtures

SmSWP were prepared as described previously<sup>[27]</sup>. Briefly, *S. mansoni* adult worms were recovered from mice (housed at the Queensland Institute of Medical Research, Brisbane, Australia), washed and homogenized in PBS and soluble proteins were extracted by centrifugation. Mice were treated with 25  $\mu$ g SmSWP ip. Proteins were diluted to a final volume of 100  $\mu$ L in PBS. Control animals were injected ip with 100  $\mu$ L PBS.

### Experimental protocol

In a previous study we investigated the time course of inflammation during TNBS colitis and found that inflammation peaked at day 3 and that colitis was self-limiting with near complete remission after 1 wk. In the present study, we wanted to evaluate the effect of helminth protein treatment after the peak of inflammation but when overt signs of colitis were still present.

In a first set of experiments, we scored the therapeutic effect of SmSWP on colonic inflammation 5 d after the induction of colitis. Six hours after TNBS injection, mice were treated once ip with 25  $\mu$ g *S. mansoni* proteins or phosphate-buffered saline (PBS). Five days later, mice were sacrificed and inflammation was scored based on 5 parameters: clinical disease activity, macroscopic and microscopic inflammation score, extent of colonic inflammation and myeloperoxidase activity. Two different groups were studied: TNBS mice treated with PBS (TNBS-PBS) after 5 d and TNBS mice treated with 25  $\mu$ g SmSWP (TNBS-SmSWP) after 5 d ( $n = 8-10$  in each group).

In a second set of experiments, we investigated the effect of SmSWP treatment on *in vivo* gastrointestinal motility and *in vitro* colonic peristalsis 5 d after induction of colitis. Four different groups were studied: control-PBS mice, control mice treated with 25  $\mu$ g SmSWP (control-SmSWP), TNBS-PBS mice and TNBS-SmSWP mice ( $n = 7-10$  in each group). In preliminary experiments we also investigated the effect of colitis on gastrointestinal motility disturbances 3 d after the induction of colitis, and the effect of SmSWP treatment as these experiments were not performed previously.

In a third set of experiments, we investigated the

cytokine profile of colonic T cells 5 d after induction of colitis. Cytokine profiles were studied in 4 different groups: control-PBS, control-SmSWP, TNBS-PBS, TNBS-SmSWP ( $n = 5-8$  in each group, for each  $n$ , colonic tissue of 3 mice was pooled).

### Inflammatory scores

Briefly, the clinical disease score (0-8) was based on the following characteristic parameters (0-2 score each): weight loss, piloerection, immobility and blepharitis<sup>[27]</sup>.

After sacrifice, the colon was removed and opened to score colonic damage macroscopically. Four parameters were taken into account: presence of adhesions, degree of colonic ulcerations, wall thickness, and degree of mucosal edema. The total score ranged from 0 to 12<sup>[28]</sup>. The extent of inflammation in the colon was also measured and expressed in cm. Tissue samples were harvested for histological assessment of the inflammatory infiltrate and for myeloperoxidase (MPO) assay. Colonic segments were fixed in 4% formaldehyde and embedded in paraffin for hematoxylin-eosin staining. Microscopic inflammation score ranged from 0 to 10 based on the following parameters: inflammatory infiltrate, number of gut wall layers infiltrated, loss of mucosal architecture, and edema<sup>[27]</sup>. MPO activity was measured to monitor the degree of myeloid cell infiltration in the colon. Colonic MPO activity was assayed according to published methods<sup>[29]</sup> and expressed as units MPO per gram tissue.

### In vivo measurement of gastrointestinal motility: Evans blue technique

Mice were fasted for 18 h and *in vivo* semi-liquid meal motility was assessed according to published methods<sup>[30]</sup>. Briefly, mice received an intragastric injection of 0.1 mL Evans blue (50 mg/mL + 0.5% methylcellulose) *via* an orogastric cannula. Fifteen minutes later, mice were anesthetized and a laparotomy was performed. The stomach and small intestine were resected and the small intestine was divided into 5 segments of equal length. The amount of Evans blue in the segments was measured spectrophotometrically to assess gastric emptying (GE) and geometric center (GC):  $\%GE = [\Sigma A_{565} (\text{intestine 1-5}) / \Sigma A_{565} (\text{stomach} + \text{intestine 1-5})] \times 100$ ;  $GC = \Sigma (A_{565} \text{ of Evans blue per segment} \times \text{segment number}) / \text{total } A_{565}$ .

### In vivo measurement of gastrointestinal motility: Solid beads technique

Mice were fasted for 18 h and *in vivo* solid meal motility was assessed as previously described<sup>[31]</sup>. Mice received an intragastric gavage of 25 green glass beads (0.4-0.5 mm in diameter) together with 0.5 mL H<sub>2</sub>O solution *via* an orogastric cannula and were transferred to a wired bottom cage to prevent coprophagy<sup>[32]</sup>. Subsequently, 30, 120 and 360 min after gavage, mice were anesthetized, the gastrointestinal tract was resected and divided into

different segments: stomach, 5 small intestinal segments, cecum, 2 colonic segments and feces. The number of beads in each segment was counted under a stereomicroscope and GE and GC were calculated by the following equations:  $\%GE = [\text{number of beads (small intestine 1-5} + \text{cecum} + \text{colon 1-2} + \text{feces}) / \text{total number of beads}] \times 100$ ;  $GC = \Sigma (\text{beads per segment} \times \text{segment number}) / \text{total number of beads}$ .

### In vitro evaluation of colonic peristaltic activity

Assessment of colonic peristalsis was performed as previously described<sup>[31]</sup>. Briefly, mice were anesthetized, the colon was removed, flushed and put in cold aerated Krebs-ringer solution. The distal colon segment (3 cm in length) was mounted horizontally in an organ bath. For each segment, the oral end was connected to a perfusion pump for intraluminal infusion of Krebs solution and the other end was attached to a pressure transducer and a vertical tube of which the outlet could be raised in height. After 30 min of equilibration, the outlet was increased from 0 to 7.5 cm. Under these circumstances, spontaneous peristaltic contractions occurred. This activity was associated with regular pressure increases which were recorded by the pressure transducer and analyzed by a data-acquisition system (CED 1401, Cambridge Electronic Design, Cambridge, UK). After an equilibration period of 20 min, the mean amplitude (cmH<sub>2</sub>O) of 3 consecutive peristaltic contractions as well as the mean time interval(s) between 4 subsequent peristaltic contractions were calculated and compared.

### Investigation of T cell cytokine profiles

Colonic lamina propria mononuclear cells were isolated based on a 30%:70% gradient Percoll column as previously described<sup>[27,33]</sup>. Colonic lamina propria T cells were subsequently isolated by positive selection using the EasySep enrichment procedure employing antibody-coated, magnetic particles as described by the manufacturer (Stem Cell Technologies, Vancouver, Canada). Total RNA was extracted from isolated colonic T cells by using the Absolutely RNA microprep kit as described by the manufacturer (Stratagene, La Jolla, CA, USA).

Using real time reverse transcriptase-polymerase chain reaction (RT-PCR), we performed a quantitative analysis of the mRNA expression of different cytokines to determine the balance between T helper (Th) 1, Th17, Th2 and Treg (regulatory T) cells in colonic tissue. TaqMan Gene Expression assays (Applied Biosystems, Lennik, Belgium) specific for IFN $\gamma$  produced by Th1 cells, IL17 produced by Th17 cells, IL-5 produced by Th2 cells and IL-10 produced by Treg cells were performed on a ABI Prism 7300 sequence detector system (Applied Biosystems, Lennik, Belgium) in 25  $\mu$ L reaction volumes containing One step Universal PCR master mix (Applied Biosystems, Lennik, Belgium) as previously described<sup>[27]</sup>.

## Drugs

NaCl 0.9% (Plurule®, Baxter, Lessines, Belgium); 2,4,6 trinitrobenzene sulfonic acid solution (Fluka, Neu-Ulm, Germany); PBS (GIBCO BRL, Merelbeke, Belgium); diethyl ether, ethanol absolute, 30% hydrogen peroxide, methanol absolute, potassium dihydrogen phosphate, dipotassium hydrogen phosphate trihydrate (Merck, Darmstadt, Germany); xylazine (Rompun®, Bayer, Brussels, Belgium); ketamine (Ketalar®, Pfizer, Brussels, Belgium); hexadecyltrimethylammonium bromide, *o*-dianisidine dihydrochloride, FCS, collagenase, Percoll, Evans blue (Sigma Chemical, St. Louis, Missouri, USA); RPMI 1640, EDTA, HBSS, HEPES, L-glutamine,  $\beta$ -mercaptoethanol, sodium pyruvate, penicillin, streptomycin (Invitrogen, Merelbeke, Belgium), glass beads (0.4-0.5 mm in diameter) (VWR international, Leuven, Belgium) were purchased from the respective companies mentioned in parentheses. Helminth protein preparation was described earlier.

## Presentation of results and statistical analysis

Data are presented as mean  $\pm$  SE. Statistical analysis was performed in SPSS 16.0 for Windows. Analyses of the non-parametric data (clinical disease score, macroscopic and microscopic inflammation score) were performed by Mann-Whitney *U* tests. Parametric data (extent of inflammation, MPO, GE, GC, amplitude, interval and RT-PCR results) were analyzed by Student's *t*-tests or by two-way ANOVA (with TNBS colitis as factor 1 and protein treatment as factor 2). When the interaction was significant one-way ANOVA and Student-Newman-Keuls post hoc analysis was performed. *P* values  $\leq$  0.05 were considered to be significant.

## RESULTS

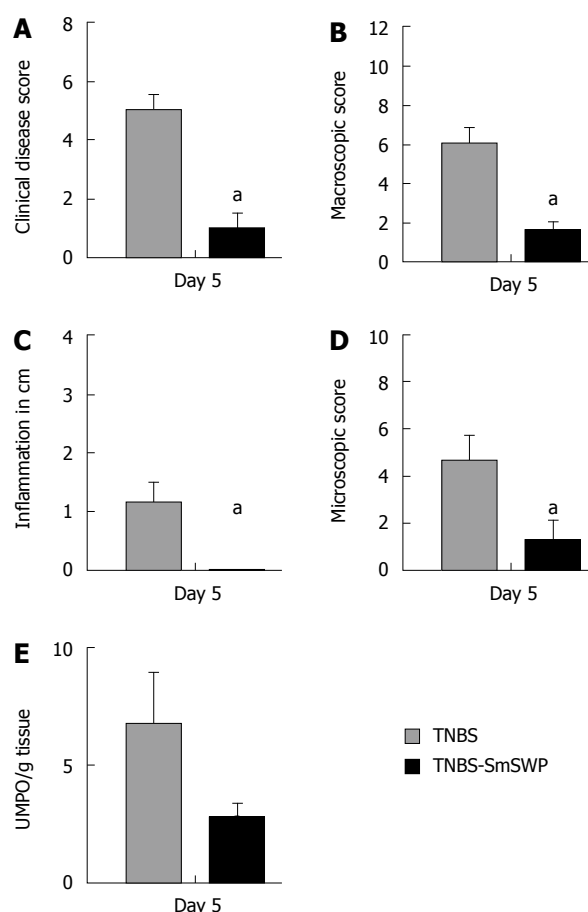
### Effect of SmSWP on TNBS-induced colitis after 5 d

The injection of TNBS caused an increase in all inflammatory parameters (Figure 1A-E) as compared to control mice that did not show any signs of inflammation (data not shown).

Treatment of TNBS-injected mice with SmSWP caused a significant decrease in clinical disease score (Figure 1A), macroscopic inflammation score (Figure 1B), extent of colonic inflammation (Figure 1C), microscopic inflammation score (Figure 1D) and a tendency to decrease the MPO activity (Figure 1E) as compared to TNBS-PBS mice.

### Effect of TNBS-induced colitis per se on gastrointestinal motility

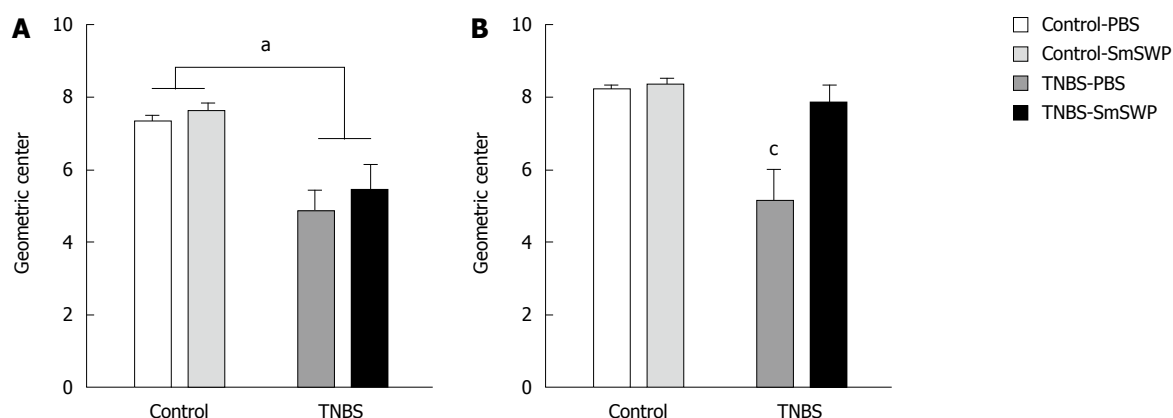
In preliminary experiments, 3 d after the induction of colitis, GE and GC of a semi-liquid Evans blue solution were not significantly different between control and TNBS mice: GE was  $43\% \pm 9\%$  in controls and  $48\% \pm 10\%$  in TNBS colitis mice and GC was  $2.1 \pm 0.3$  in controls and  $2.2 \pm 0.3$  in TNBS colitis mice ( $n = 7-9$ ). We also evaluated



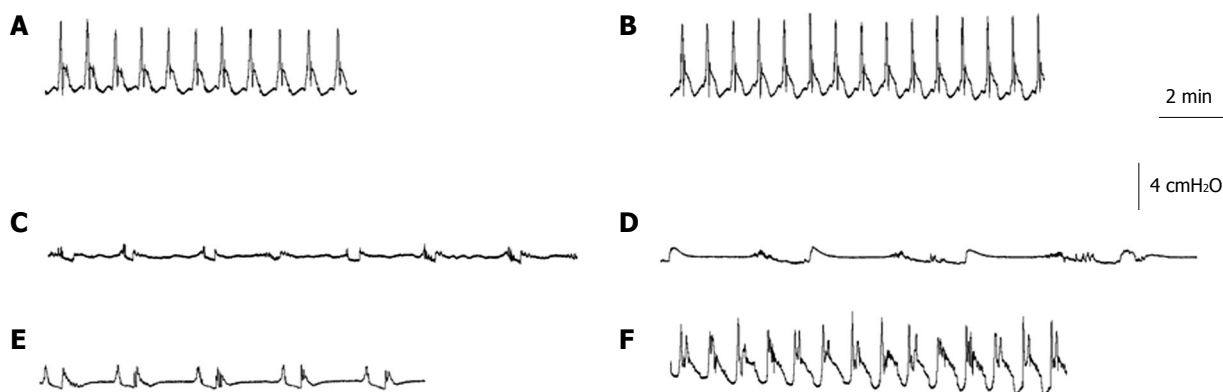
**Figure 1** Effect of 25  $\mu$ g *Schistosoma mansoni* soluble worm (SmSWP) proteins on clinical disease score (A), macroscopic score (B), extent of inflammation (C), microscopic score (D) and myeloperoxidase (MPO) activity (E) 5 d after trinitrobenzene sulphate (TNBS)-induced colitis. Grey bars represent phosphate-buffered saline (PBS)-treated TNBS mice; black bars represent SmSWP-treated TNBS mice. Data are presented as mean  $\pm$  SE. Non-parametric data (A, B, D) were analyzed by the Mann-Whitney *U* test, parametric data (C, E) were analyzed by the Student's *t*-test;  $n = 8-10$ ;  $^aP \leq 0.05$ , significant effect of SmSWP treatment.

the effect of colitis on gastrointestinal motility after intragastric gavage of 25 glass beads 3 d after the injection of TNBS. Experiments were performed 30 min, 120 min and 360 min after intragastric gavage of the marker: GE progressed over time in control mice (from  $32\% \pm 12\%$  to  $61\% \pm 14\%$  and  $100\% \pm 0\%$ , respectively) and in TNBS mice (from  $42\% \pm 13\%$  to  $81\% \pm 9\%$  and  $97\% \pm 2\%$ , respectively) but no significant differences between the control and TNBS groups were observed. The GC also increased over time from  $1.5 \pm 0.2$  (30 min) to  $3.2 \pm 0.7$  (120 min) and to  $7.3 \pm 0.2$  (360 min) in control mice. This time-dependent increase in GC was also observed in mice with colitis (from  $1.7 \pm 0.3$  to  $2.9 \pm 0.5$  and  $5.5 \pm 0.6$ ). When measured 360 min after gavage of the beads, GC in mice with colitis ( $5.5 \pm 0.6$ ) was significantly lower as compared to control mice ( $7.33 \pm 0.2$ ). Based on these preliminary results, further measurements studying the effect of worm protein treatment on GC were performed 360 min after intragastric gavage of 25 glass beads.





**Figure 2** Effect of 25  $\mu$ g *S. mansoni* proteins on geometric center 3 d (A) and 5 d (B) after the induction of colitis. Data were analyzed by two-way ANOVA with the Student-Newman-Keuls (SNK) post hoc test;  $n = 7-10$ ;  $^aP \leq 0.05$ , significant colitis effect;  $^cP \leq 0.05$ , post hoc analysis showed a statistically significant difference from the other 3 groups.



**Figure 3** Peristaltic tracings as recorded in the control-PBS group (A), the control-25  $\mu$ g SmSWP group (B), the TNBS-PBS group on day 3 (C), the TNBS-PBS group on day 5 (D), the TNBS-25  $\mu$ g SmSWP group on day 3 (E) and the TNBS-25  $\mu$ g SmSWP group on day 5 (F).

### Effect of SmSWP treatment on delayed gastrointestinal transit during TNBS colitis

Experiments were performed 3 d (Figure 2A) and 5 d (Figure 2B) after TNBS injection. Treatment of control mice with SmSWP had no effect *per se* on GC at both time points (Figure 2A and B). TNBS-colitis significantly reduced GC 360 min after intragastric gavage of the beads both at day 3 and at day 5 (Figure 2A and B). Treatment of colitis mice with SmSWP had no effect on GC 3 d after the injection of TNBS (Figure 2A). However, treatment of colitis mice with SmSWP reversed the TNBS-induced decrease in GC at day 5 (Figure 2B).

### Effect of SmSWP treatment on colonic peristalsis

Distention-induced peristaltic contractions were recorded (Figure 3) and, subsequently, the amplitude and the interval between the peristaltic waves were measured.

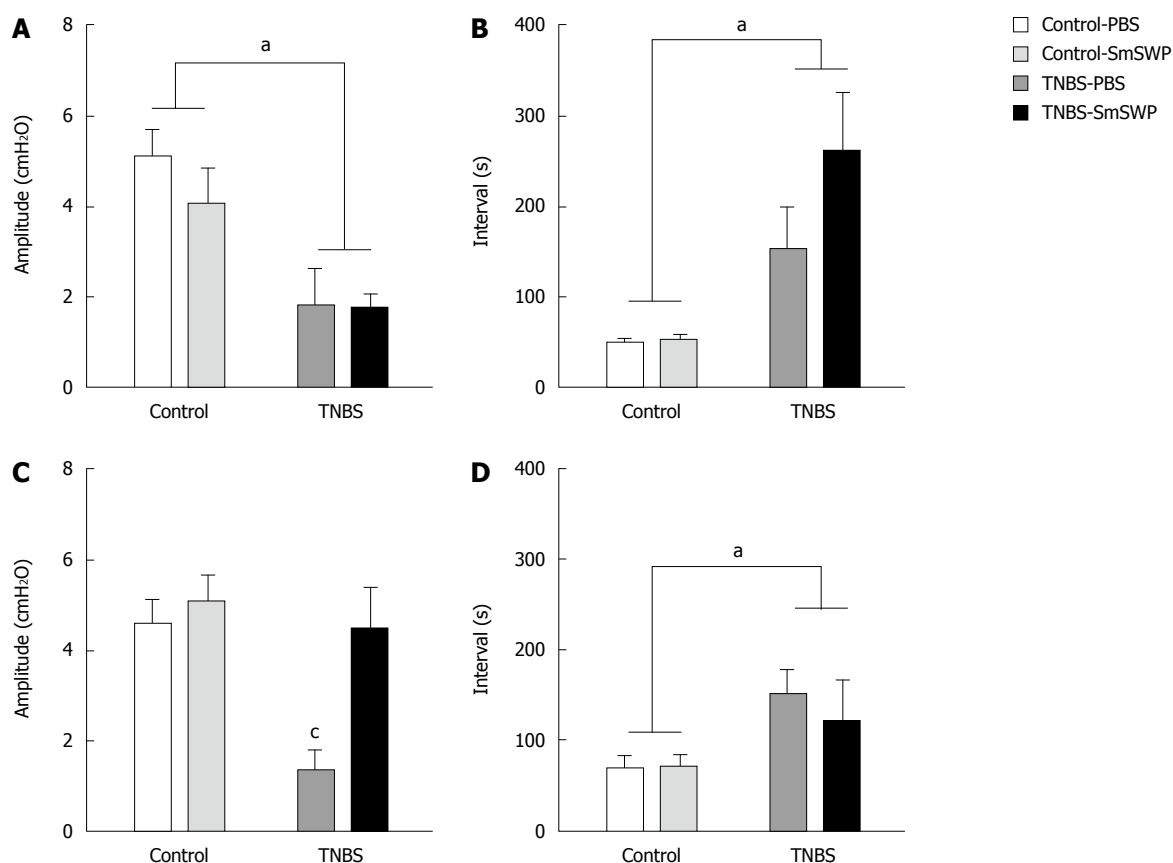
Peristaltic activity of distal colonic segments was measured 3 d (Figure 4A and B) and 5 d (Figure 4C and D) after TNBS enema. Treatment of control mice with SmSWP had no significant effect on colonic peristaltic activity at the two different time points (Figure 3B and Figure 4A-D). The induction of colitis caused significant impairment of peristaltic activity as shown by a significant

decrease in amplitude and an increase in interval between the waves. These TNBS-induced disturbances in peristalsis were significant both on day 3 and on day 5 (Figure 3C and D, Figure 4A-D). Furthermore, it is important to note that in 4 of 8 TNBS-PBS mice on day 3 we were not able to measure any peristaltic activity whereas this was only the case in 1 of 8 TNBS-SmSWP mice.

Treatment with SmSWP did not ameliorate the disturbed peristaltic activity caused by intestinal inflammation after 3 d (Figure 3E, Figure 4A and B). However, 5 d after the induction of colitis the amplitude of the distention-induced peristaltic contractions was significantly increased to normal control values when mice were treated with SmSWP (Figure 3F and Figure 4C). At this time point, the mean interval between the waves remained increased after treatment with SmSWP as compared to control animals (Figure 4D).

### Measurement of cytokine profiles in colonic T cells

We recently showed the importance of the differential roles of Th1, Th17, Th2 and Treg cells in colonic tissue 3 d after the induction of TNBS colitis and the effect of SmSWP treatment on these T cell subsets<sup>[27]</sup>. In this study we investigated the cytokine profiles of



**Figure 4** Effect of 25 µg *S. mansoni* proteins on the amplitude and interval of peristaltic waves 3 d (A, B) and 5 d (C, D) after the induction of colitis. Data are presented as mean ± SE. Data were analyzed by two-way ANOVA with SNK post hoc test;  $n = 7-9$  (except for the TNBS-PBS group on day 3  $n = 4$ );  $^aP \leq 0.05$ , significant colitis effect;  $^cP \leq 0.05$ , post hoc analysis showed a significant difference from the other 3 groups.

T cells isolated from colonic tissue on day 5 after the induction of colitis. As shown in Figure 5A, interferon (IFN)- $\gamma$  mRNA expression was not significantly altered in colonic T cells 5 d after the induction of colitis. On the other hand, we found a significant downregulation of interleukin (IL)-17 and IL-5 expression 5 d after the induction of colitis in both PBS- and SmSWP-treated mice (Figure 5B and D). Investigating the Treg response, we found that injection of TNBS and treatment with SmSWP had no significant effect on IL-10 mRNA expression 5 d after TNBS injection (Figure 5C).

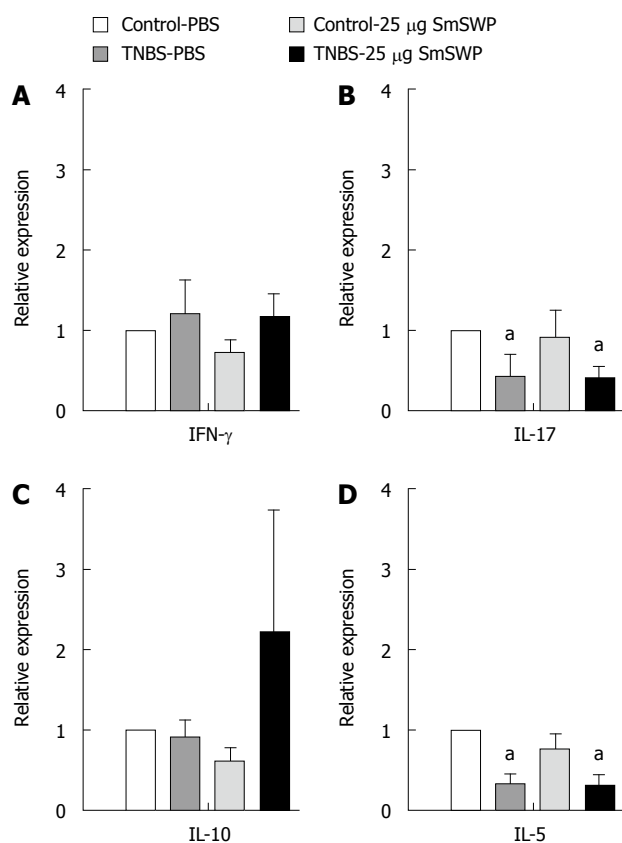
## DISCUSSION

In this study we showed that treatment with SmSWP ameliorated *in vivo* and *in vitro* motility disturbances in a murine model of TNBS-induced colitis after 5 d.

Experimental and clinical data support the idea that helminths provide protection against IBD<sup>[34]</sup>. To avoid the possible disadvantages of a therapy with living parasites, current research is now focusing on the identification and characterization of helminth-derived immunosuppressive molecules that contribute to the protective effect<sup>[13]</sup>. Furthermore, it is well established that gut inflammation leads to disturbed gastrointestinal motility<sup>[14]</sup>. The model of TNBS-induced colitis is widely

used to investigate motility disturbances occurring in the inflamed colon. We previously showed that contractility of colonic longitudinal smooth muscle strips was time-dependently decreased during TNBS colitis in rats and concurrent infection with *S. mansoni* abrogated these TNBS-induced contractility disturbances<sup>[10]</sup>.

In a previous study we showed that TNBS colitis caused clear signs of inflammation 3 d after the induction of colitis and that our model of TNBS colitis was self-limiting with near complete remission after 1 wk<sup>[27]</sup>. In this study we focused on a later phase during murine TNBS-induced colitis. We showed that 5 d after TNBS injection, treatment with SmSWP also caused a significant decrease in clinical disease score, macroscopic inflammation score, extent of colonic inflammation and microscopic inflammation score at this later time point along the course of colitis. There was however no significant difference in MPO activity between TNBS-PBS and TNBS-SmSWP mice at this time point although a clear tendency of inhibition was observed. Taken together, these results indicate that treatment with helminth proteins ameliorated colonic inflammation leading to accelerated healing of colitis. A similar beneficial effect has been described previously by our group: rats with TNBS colitis showed spontaneous and complete healing of inflammation 4 wk after the induction of colitis and this was reduced to 2 wk



**Figure 5** Interferon (IFN) and interleukin (IL) mRNA expression of T helper (Th) 1 (A), Th17 (B), regulatory T (Treg) (C) and Th2 (D) cells isolated from colonic tissue at day 5. Data are expressed as relative expression and the control-PBS group was chosen as calibrator. Data are presented as mean  $\pm$  SE. Data were analyzed by two-way ANOVA with SNK post hoc test when appropriate;  $n = 5-8$  (except for IL-17  $n = 2-5$ );  $^*P \leq 0.05$ , significant colitis effect, no significant effect of worm protein treatment was shown at day 5.

in rats infected with *S. mansoni*<sup>[10]</sup>.

Investigation of the effect of TNBS colitis on gastrointestinal motility failed to show an effect on gastrointestinal transit of a semi-liquid meal. In other words, colitis did not affect gastric emptying in our murine model. Nevertheless, delayed gastric emptying has been described in a clinical setting<sup>[21,23]</sup> as well as in the rat TNBS model<sup>[35]</sup>. Literature on TNBS-induced motility disturbances in mice is scarce and gastric emptying disturbances have not been reported so far. In addition to species differences, this lack of effect of colitis on gastric emptying in mice might be linked to the type and severity of inflammation induced and to the time point chosen to perform motility experiments.

On the other hand, assessment of gastrointestinal transit of a solid meal showed that the geometric distribution of solid beads in the gastrointestinal tract was significantly decreased 3 d after the induction of colitis and this decrease was still evident after helminth protein treatment by day 3. Five days after the induction of colitis, the geometric distribution was still significantly altered in mice with colitis but treatment with worm proteins significantly reversed transit to normal values at this time point. These results indicate that although the

healing process of intestinal inflammation is ongoing in untreated TNBS mice by day 5, gastrointestinal motility of the gastrointestinal tract remains disturbed. Only when inflammatory signs are almost completely absent, as in the SmSWP-treated TNBS mice on day 5, was *in vivo* gastrointestinal motility of the distal gastrointestinal tract restored.

Comparable results were found on *in vitro* colonic peristalsis. The amplitude of distension-induced pressure waves as well as the interval between the waves were significantly altered in the colon of mice with TNBS-induced colitis, both at day 3 and day 5. Treatment with SmSWP did not have any ameliorating effect after 3 d whereas the amplitude was significantly increased to normal control values after 5 d. The interval between the waves was nevertheless still significantly augmented as compared to controls at day 5. This suggests that some signs of disturbed peristalsis persisted although inflammation is resolving and that the disturbed interval of *in vitro* peristaltic waves 5 d after colitis and worm treatment did not have any repercussion on *in vivo* colonic motility which, at that time point, was normalized in these mice.

With regard to the clinical setting, treatment of IBD patients with *Trichuris suis* ova caused clinical amelioration of both Crohn's disease activity index and ulcerative colitis disease activity index<sup>[11,12]</sup>. This decrease in clinical disease scores might indicate that symptoms such as diarrhea and abdominal pain are less frequent after treatment with helminths. It is well known that infection with intestinal helminths can alter gastrointestinal motility thus contributing to worm expulsion<sup>[36]</sup>. The role of T cells in those circumstances was previously investigated, leading to the understanding that infection-induced intestinal muscle hypercontractility is CD4+ T cell-dependent<sup>[37]</sup>.

Cytokines produced by mucosal leucocytes can also mediate neurogastrointestinal function. We previously showed that the pro-inflammatory cytokine IL-1 $\beta$  modulates gastrointestinal neuromuscular function<sup>[38]</sup>. In addition, Th2 cytokines IL-4 and IL-13 contribute to intestinal muscle hypercontractility<sup>[39]</sup>. Treatment with exogenous IL-10 has been shown to abrogate the delayed gastrointestinal transit during postoperative ileus<sup>[40]</sup>. Gastrointestinal inflammation during Crohn's disease is mediated *via* Th1 lymphocytes as well as through the recently described Th17 cells<sup>[41]</sup>. On the other hand, it is well established that helminths have the potential to evoke strong regulatory T cell responses with immunosuppressive properties<sup>[42]</sup>. In this way, we might hypothesize that infection with helminths induce Th2 and Treg immune responses that contribute to the amelioration of motility disturbances during colitis.

As such, we measured the cytokine profile of colonic T cells. We previously showed that a Th1 response (upregulation of IFN- $\gamma$ ) in the colon was evident 3 d after induction of TNBS colitis. This Th1 response

was significantly suppressed after administration of *S. mansoni* proteins. Treatment with SmSWP also caused an upregulation of regulatory T cell cytokines in the colon after 3 d<sup>[27]</sup>. In this study we identified the balance between the different T cell subsets in the colon at a later time point along the course of colitis, 5 d after the injection of TNBS. Our results showed there was no longer a significant effect on IFN- $\gamma$  mRNA expression after the induction of colitis, indicating that the Th1 response seen on day 3 in the colon of TNBS-PBS mice had subsided by day 5. Furthermore, injection of helminth proteins decreased the expression of IL-17 after 3 d, both in control mice and in TNBS mice<sup>[27]</sup>. After 5 d we found decreased IL-17 mRNA expression due to a colitis effect instead of a protein effect. These differential results on IL-17 mRNA expression at both time points are interesting: at day 3 the effect on IL-17 expression was related to helminth protein, whereas it was colitis mediated at day 5. Although we did not detect a significant effect of colitis or worm protein treatment on IL-5 expression after 3 d, a significant downregulation of IL-5 expression on day 5 was apparent both in the PBS treated group and in the helminth protein treated group. One might hypothesize that the naturally occurring healing response leads to the production of regulatory cytokines which are able to suppress cytokines produced by T effector cells including IL-17 and IL-5. This coincides with the attenuation of inflammatory parameters at this time point as described above.

Experiments performed 3 d after the induction of colitis showed a significant upregulation of the mRNA expression of regulatory cytokines IL-10 and transforming growth factor- $\beta$  after treatment with SmSWP that had subsided by day 5. Our results showed that the immunological effect of helminth protein treatment on Th1 and Treg cells, which is present after 3 d as shown previously<sup>[27]</sup>, has diminished after 5 d. This might be explained by the fact that proteins were only injected once 6 h after TNBS or PBS injection and not repeatedly until day 5. Nevertheless, this single injection with helminth proteins evoked a protective effect that was almost immediate, leading us to assume that these proteins might also have an effect on innate immunity. It was previously reported that infection with *S. mansoni* prevented experimental colitis in mice by a mechanism dependent on macrophages<sup>[43]</sup>. Furthermore, dendritic cells are key regulators in the immune defence of the gut and are also influenced by helminth infections<sup>[44,45]</sup>. Investigation on how helminth proteins affect cells of the innate immune system might contribute to a better understanding of the immunological pathways by which helminth proteins suppress ongoing colonic inflammation.

In this study, we provide evidence that treatment with helminth proteins contributes to amelioration of gastrointestinal motility disturbances. Inhibition of inflammation and amelioration of motility disturbances

after treatment with helminth proteins both appear at the same time. However, whether the beneficial effect of helminth protein treatment on gastrointestinal motility is directly or indirectly related to amelioration of inflammation needs to be further established. If helminth proteins provoke a reaction that not only leads to a reduction in inflammation but also influences the enteric nervous system and/or smooth muscle cells directly, these proteins might be useful in the treatment of gastrointestinal motility disturbances.

Taken together, we showed that treatment with *S. mansoni* proteins significantly attenuated the course of TNBS-induced colitis leading to reversal of *in vivo* gastrointestinal motility disturbances and amelioration of *in vitro* colonic peristalsis 5 d after induction. We conclude that SmSWP have therapeutic potential in gut inflammation leading to a marked reduction in inflammation and in gastrointestinal motility disturbances, accelerating the natural course of remission.

## COMMENTS

### Background

Gastrointestinal inflammation during inflammatory bowel diseases (IBD) results from an uncontrolled immune response against intraluminal antigens in genetically predisposed persons and might lead to motility disturbances with related symptoms. The lack of exposure to helminth infections, as a result of improved living standards and medical conditions, has contributed to the increased incidence of IBD in the developed world.

### Research frontiers

Epidemiological, experimental and clinical data support the idea that helminths provide protection against IBD. However, treatment with living helminths may have serious drawbacks such as infection and/or invasion of the parasite to other tissues in the human host where they might cause pathology. Therefore, in this study the authors evaluated the therapeutic potential of helminth-derived proteins on inflammation and associated motility disturbances.

### Innovations and breakthroughs

This study investigates the effect of TNBS colitis and exposure to *Schistosoma mansoni* proteins on murine gastrointestinal motility. This is a novel pursuit. The effects of inflammation and therapeutic interventions on gastrointestinal motility are largely ignored but critically important. The authors showed that treatment of experimental colitis with helminth proteins restored gastrointestinal motility.

### Applications

Treatment with helminth soluble proteins attenuates inflammation and ameliorates motility disturbances during experimental colitis. These results suggest that helminth soluble proteins represent an attractive therapeutic option in the management of IBD.

### Peer review

This is an interesting study dealing with the effect of *Schistosoma mansoni* proteins on inflammatory and motility response in a rat model of inflammatory bowel disease. The experimental methods are described comprehensively and the interpretations and conclusions justified by the results.

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## Involvement of PI3K and ERK1/2 pathways in hepatocyte growth factor-induced cholangiocarcinoma cell invasion

Apaporn Menakongka, Tuangporn Suthiphongchai

Apaporn Menakongka, Tuangporn Suthiphongchai, Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

**Author contributions:** Menakongka A performed the experiments, analyzed the data and wrote the manuscript; Suthiphongchai T designed the study, analyzed the data and wrote the manuscript.

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**Correspondence to:** Tuangporn Suthiphongchai, Associate Professor, Department of Biochemistry, Faculty of Science, Mahidol University, 272 Rama 6 Road, Bangkok 10400, Thailand. [scsc@mahidol.ac.th](mailto:scsc@mahidol.ac.th)

Telephone: +662-2015609 Fax: +662-3547174

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### Abstract

**AIM:** To investigate the role of hepatocyte growth factor (HGF) in cholangiocarcinoma (CCA) cell invasiveness and the mechanisms underlying such cellular responses.

**METHODS:** Effects of HGF on cell invasion and motility were investigated in two human CCA cell lines, HuCCA-1 and KKU-M213, using Transwell *in vitro* assay. Levels of proteins of interest and their phosphorylated forms were determined by Western blotting. Localization of E-cadherin was analyzed by immunofluorescence staining and visualized under confocal microscope. Activities of matrix degrading enzymes were determined by zymography.

**RESULTS:** Both CCA cell lines expressed higher Met levels than the H69 immortalized cholangiocyte cell line. HGF induced invasion and motility of the cell lines and altered E-cadherin from membrane to cytoplasm localization, but did not affect the levels of secreted matrix metalloproteinase (MMP)-2, MMP-9 and

urokinase plasminogen activator, key matrix degrading enzymes involved in cell invasion. Concomitantly, HGF stimulated Akt and extracellular signal-regulated kinase (ERK)1/2 phosphorylation but with slightly different kinetic profiles in the two cell lines. Inhibition of the phosphoinositide 3-kinase (PI3K)/Akt pathway by the PI3K inhibitor, LY294002, markedly suppressed HGF-stimulated invasion of both CCA cell lines, and inhibition of the ERK pathway by U0126 suppressed HGF-induced invasion of the KKU-M213 cell line but had a moderate effect on HuCCA-1 cells.

**CONCLUSION:** These data indicate that HGF promotes CCA cell invasiveness through dys-localization of E-cadherin and induction of cell motility by distinct signaling pathways depending on cell line type.

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**Key words:** Hepatocyte growth factor; Invasion; Cholangiocarcinoma; Phosphoinositide 3-kinase; Extracellular signal-regulated kinase

**Peer reviewers:** Hong Joo Kim, MD, PRO, Department of Internal Medicine, Sungkyunkwan University Kangbuk Samsung Hospital, 108, Pyung-Dong, Jongro-Ku, Seoul, 110-746, South Korea; Yu-Yuan Li, Professor, Department of Gastroenterology, First Municipal People's Hospital of Guangzhou, Guangzhou Medical College, Guangzhou 510180, Guangdong Province, China

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### INTRODUCTION

Cholangiocarcinoma (CCA) is a malignant tumor of the

biliary epithelium associated with a high metastatic and mortality rate<sup>[1]</sup>. Incidence of this cancer has increased worldwide<sup>[2]</sup>, and in Thailand the highest incidence is in the northeastern region, where *Opisthorchis viverrini* infection is also prevalent<sup>[3]</sup>. Although the exact molecular mechanisms of cholangiocarcinogenesis are still under investigation, alterations in important growth factor pathways, such as hepatocyte growth factor (HGF)/Met and ErbB2, have been suggested as being involved<sup>[4]</sup>.

Overexpression and deregulation of Met, a receptor tyrosine kinase, have been reported in many types of cancers<sup>[5]</sup>. Met is activated *via* binding to its ligand, HGF, also known as scatter factor (SF), a soluble factor first identified as a growth factor for hepatocytes and a dissociation factor for epithelial cells<sup>[6]</sup>. Hitherto there have been a limited number of investigations into the role of Met in cholangiocarcinoma. Several reports have demonstrated a correlation between Met expression and CCA<sup>[7-10]</sup>. Immunohistochemical data indicate high expression of Met in well-differentiated CCA and hyperplastic bile ducts of nontumorous liver surrounding CCA, whereas Met expression is low in poorly differentiated tumor<sup>[7,8]</sup>. Met expression is increased in early developmental stages of CCA, suggesting a role in cholangiocarcinogenesis<sup>[9]</sup>. Moreover, there is a correlation between Met expression and CCA invasion through adjacent connective tissues<sup>[11]</sup>. HGF level has been shown also to correlate with CCA differentiation stages in both human and rat models<sup>[10,12]</sup>.

HGF/Met activation induces a variety of biological processes, including cell scattering, invasion, proliferation and survival<sup>[13-15]</sup>. Among the various cellular responses induced by HGF, cell invasion and metastasis have been implicated strongly in numerous cancer types. HGF has been reported to promote the main requirements of tumor invasion, namely, disruption of cell-cell adhesion complex, cell adhesion to extracellular matrix (ECM), cell motility and production of matrix degrading enzymes, such as matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA)<sup>[15-18]</sup>. Phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPKs/ERKs) are the main intracellular signaling pathways implicated in HGF-induced invasion<sup>[19,20]</sup>.

The present study focuses on the role of HGF/Met in CCA cell invasion and the mechanisms underlying cellular responses. Here, we demonstrate that Met is overexpressed in human CCA cell lines and that HGF stimulation induces CCA cell invasion, motility and E-cadherin translocation, but has no effect on MMPs or uPA activity. Use of inhibitors of MEK and PI3K indicate that HGF induces invasion in two different CCA cell lines *via* distinct signaling pathways.

## MATERIALS AND METHODS

### Cell culture

Human CCA cell lines HuCCA-1 and KKU-M213 were kindly provided by Professor S Sirisinha (Mahidol University, Bangkok, Thailand)<sup>[21,22]</sup> and Associate Professor

B Sripa (Khon Kaen University, Khon Kaen, Thailand)<sup>[23,24]</sup>, respectively. Cholangiocyte H69 cell line was kindly provided by Professor G Alpini (Texas A&M University, TX, USA) and Professor G Gores (Mayo Clinic, MN, USA). CCA cells were grown in HAM/F12 medium (Gibco Invitrogen Co., Auckland, NZ) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin G sodium, 100 µg/mL streptomycin sulfate, 0.25 µg/mL amphotericin B (Invitrogen Co., Auckland, NZ) and 15 mmol/L HEPES (USB Co., OH, USA) at 37°C under a humidified 50 mL/L CO<sub>2</sub> atmosphere. H69 cells were cultured in DMEM/F12 and DMEM (1:1) (Gibco Invitrogen Co., Auckland, NZ) supplemented with hormones, epidermal growth factor and 10% FBS as previously described<sup>[25]</sup>.

### Western blotting analysis

Levels of Met, ERK1/2 and Akt and their phosphorylated forms, and E-cadherin, were determined by Western blotting. Cells ( $2 \times 10^5$ ) were cultured in 30-mm plates for two days, then incubated with 50 ng/mL recombinant NSO-produced human HGF (R&D Systems, Inc., MN, USA) in serum-free media for 15, 60 and 360 min in the presence or absence of LY294002 (Calbiochem, CA, USA) or U0126 (Tocris Bioscience, MO, USA). Cells were then lysed with 1 × SDS loading buffer (50 mmol/L Tris-HCl pH 6.8, 2% SDS, 10% glycerol and 100 mmol/L β-mercaptoethanol) and lysate proteins were separated by 8% SDS polyacrylamide gel-electrophoresis. Proteins were transferred to nitrocellulose membrane (Hybond ECL, GE healthcare, Buckinghamshire, UK), which was incubated with antibodies specific for Akt, ERK1/2 and their phospho-forms (Cell Signaling Technology, Danvers, MA) or with anti-Met, anti-E-Cadherin, anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-phospho-Met (Cell Signaling Technology, Danvers, MA) antibodies, followed by HRP-conjugated secondary antibodies. Signals were developed using Enhance Chemiluminescence kit (GE Healthcare, Buckinghamshire, UK) and detected with FluorChem SP (Alpha Innotech Corporation, San Leandro, CA). Band densities were quantitated using AlphaEaseFC software (Alpha Innotech Corporation, San Leandro, CA). The data were presented in the relative band density when compared to those at zero time points.

### Invasion and motility assay

HGF-induced CCA cell invasiveness was determined by Matrigel Transwell *in vitro* invasion assay as described by Albini *et al.*<sup>[26]</sup> with some modification. In brief, the upper chamber of a Transwell unit (6.5-mm diameter polycarbonate membrane with 8-µm pore size) (Corning Incorporated Life Science, Corning, NY), was coated with 30 µg of Matrigel (BD Biosciences, Bedford, MA). Cells (80% confluent) were harvested using TrypLE Express (Invitrogen, Co., Grand Island, NY) and resuspended in serum-free media in the presence or absence of 50 and 100 µmol/L LY294002 or 1 and 5 µmol/L U0126. A 200 µL aliquot of cell ( $10^5$ ) suspension was added to the upper chamber. The lower chamber was filled with 600 µL of serum-free media containing 10, 50 or



100 ng/mL human HGF as chemoattractant. BSA (0.1% in serum-free medium) was used as negative control. After 6 h of incubation at 37°C under CO<sub>2</sub> atmosphere, non-invading cells in the upper chamber were removed and cells that invaded the Matrigel and had attached to the lower surface of the Transwell membrane were fixed with 25% methanol for 30 min and stained with 0.5% crystal violet. Invaded cells were counted in 5 random fields under light microscope at 100 × magnification. The reported values represent mean ± SE of the results obtained from three independent experiments.

Motility assay was performed using the Transwell chamber in the same manner as in the invasion assay but Matrigel coating was omitted.

#### **Determination of gelatinase and urokinase plasminogen activator activities**

Gelatinase (MMP-2 and MMP-9) and uPA levels secreted into conditioned media were determined by gelatin and plasminogen gelatin zymography under non-reducing conditions. Cells (80% confluent) were incubated with serum-free media in the presence of HGF (0, 10, 50 and 100 ng/mL) for 6 h. For gelatinase activity assay, 20 × concentrated conditioned media was mixed with SDS loading buffer in the absence of sulfhydryl reducing agent and electrophoresed in 7.5% SDS-polyacrylamide gel containing 1 mg/mL gelatin. uPA zymography was performed in a similar manner except that 10 µg/mL plasminogen and 1 mg/mL gelatin were copolymerized with 10% SDS-polyacrylamide gel and conditioned media was not concentrated. Gels were washed twice with 2.5% TritonX-100 for 1 h to remove SDS, then incubated for 18 h in reaction buffer (for gelatinase: 50 mmol/L Tris-HCl pH 7.5, 10 mmol/L CaCl<sub>2</sub>, 1 µmol/L ZnCl<sub>2</sub> and 1% TritonX-100; for uPA: 100 mmol/L Tris-HCl pH 7.8, 150 mmol/L NaCl and 1% Triton X-100). Gels were stained for 2 h with 0.25% Coomassie blue and destained with 45% methanol and 10% acetic acid. Unstained bands in gelatin gel with estimated molecular weight of 65 and 85 kDa corresponded to MMP-2 and MMP-9 respectively, and that of 45 kDa in plasminogen-gelatin gel corresponded to uPA.

#### **Immunofluorescence analysis**

CCA cells ( $3 \times 10^5$ ) were grown on sterile coverslips for two days. Then the monolayer cells were treated with 0-100 ng/mL HGF for 6 h. Cells were washed twice with PBS, fixed in solution containing 3% paraformaldehyde and 2% sucrose, permeabilized with 0.5% Triton X-100 and incubated with 10% FBS, 0.1% Triton X-100 in PBS. Cells were then incubated overnight at 4°C with mouse anti-E-cadherin monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), followed by fluorescent Alexa Fluor® 568-conjugated goat anti-mouse IgG secondary antibodies (Molecular Probes, Eugene, OR). After washing with PBS, the coverslips were mounted with 0.01% para-phenylenediamine dihydrochloride (Sigma Aldrich, Inc., St. Louis, MO) in 70% glycerol, and visualized under a confocal laser scanning microscope

(Olympus FV1000; Olympus Co. Tokyo, Japan) equipped with Olympus FV10-ASW 1.7 software.

#### **Statistical analysis**

Invasion and motility results are expressed as mean ± SE. Multiple comparisons were performed using one-way analysis of variance (ANOVA) with *P* value < 0.05 considered statistically significant.

## **RESULTS**

#### **Met expression and phosphorylation in CCA cells**

Western blotting analysis of both CCA cell lines (HuCCA-1 and KKU-M213) showed higher Met expression than in normal cholangiocytes (H69) (Figure 1A). Stimulation of cells by exogenous HGF resulted in induction of tyrosine phosphorylation at the critical autophosphorylation sites (pY1234/1235) in the catalytic domain of Met, but with a slight difference in the kinetics of Met activation between the two CCA cell lines; i.e. HGF stimulated a more rapid Met phosphorylation in HuCCA-1 cells (reaching a maximum at 15 min) than in KKU-M213 (maximum at about 15-60 min) (Figure 1B and C).

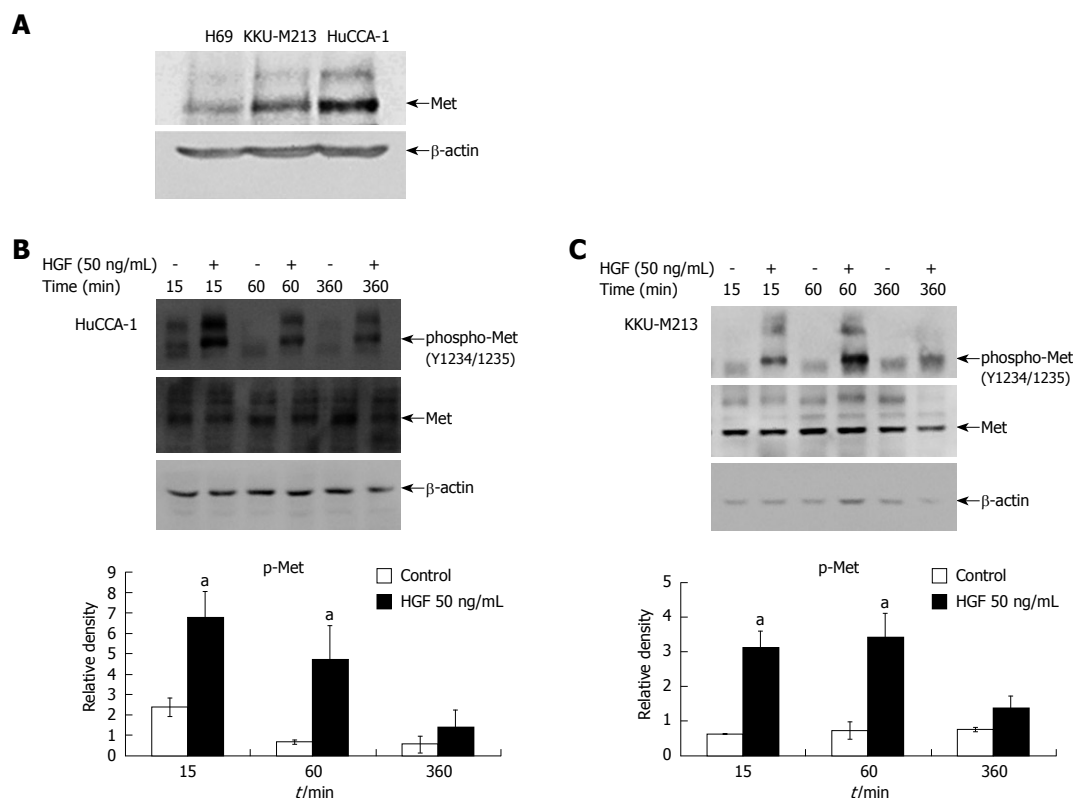
#### **Effects of HGF on CCA cell invasiveness and motility**

HGF has been reported as being able to induce invasion of several cancer cell types<sup>[27]</sup>. Here, CCA cell invasiveness and motility in response to HGF were investigated using a Transwell *in vitro* invasion/motility assay. In the absence of HGF, CCA cells showed abilities to migrate and invade, which were stimulated further by HGF in a dose-dependent manner over the concentration range of 10-100 ng/mL (Figure 2). Although basal migration and invasion abilities of HuCCA-1 were relatively low when compared to that of KKU-M213, they were dramatically stimulated by HGF to levels comparable to those of HGF-induced KKU-M213.

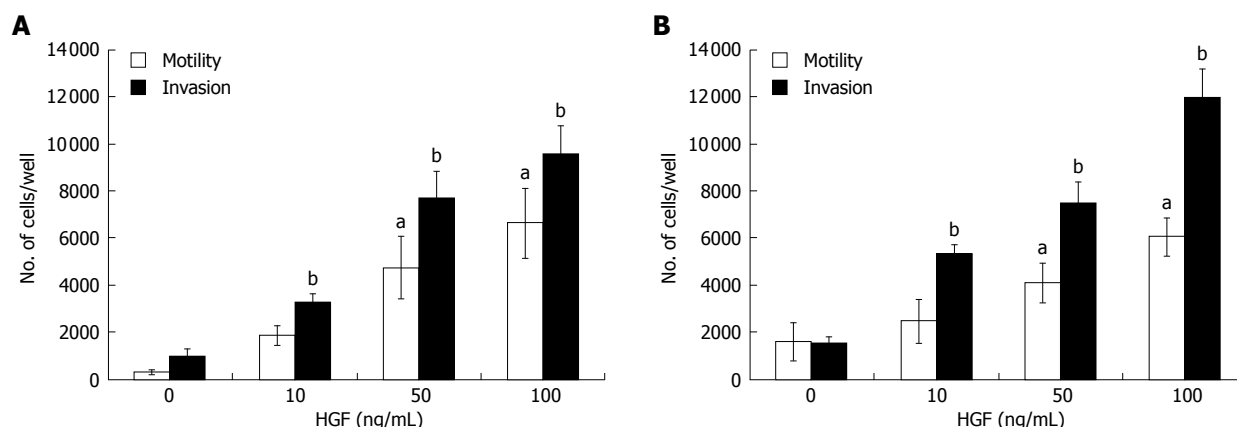
H69 cells, immortalized cholangiocytes, possessed very low invasive ability. Of  $10^5$  cells added to the upper compartment of the Transwell chamber, only  $70 \pm 21$  cells invaded in the control and  $335 \pm 72$  cells invaded upon HGF treatment. Although the HGF could induce H69 invasion, the level of invasion was marginal when compare to those of CCA cell lines.

#### **Effects of HGF on E-cadherin expression and localization and matrix metalloproteinase and uPA secretion**

HGF is able to induce changes in expression and localization of E-cadherin resulting in cell movement in several type of cancers<sup>[28-30]</sup>. To investigate the possibility of an involvement of E-cadherin in HGF-induced CCA cell migration, we determined the effects of HGF on E-cadherin expression by Western blotting and on localization by immunofluorescence staining. E-cadherin protein level did not change within 6 h of HGF treatment (Figure 3A). However, immunofluorescence demonstrated that HGF altered E-cadherin localization from the cell boundary to the cytoplasmic compartment



**Figure 1** Steady state level of Met expression in cholangiocarcinoma cell lines and activation by hepatocyte growth factor (HGF). Cell lysates from 80% confluent cells cultured in 10% fetal bovine serum (FBS) medium were examined for Met expression by Western blotting analysis (A). Lysates from HuCCA-1 (B) and KKU-M213 (C) cells treated with or without 50 ng/mL HGF for various times were analyzed by Western blotting for levels of Met and phospho-Met (pY1234/1235). The graphs show band densities of phospho-Met relative to those at zero time points. Data are presented as mean  $\pm$  SE of results obtained from three independent experiments. <sup>a</sup> $P < 0.05$  vs untreated control.



**Figure 2** HGF induction of cholangiocarcinoma motility and invasiveness. *In vitro* invasion and motility assays of HuCCA-1 (A) and KKU-M213 (B) cells were conducted in a Transwell unit coated with and without Matrigel. Cells ( $10^5$ ) in serum-free medium were plated in the upper chamber of a Transwell unit and 0-100 ng/mL HGF added to the lower chamber. After 6 h of incubation, cells invading to the lower compartment of the Transwell unit were stained and counted. The numbers of invaded/motile cells are presented as mean  $\pm$  SE of results obtained from three independent experiments. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , vs untreated control.

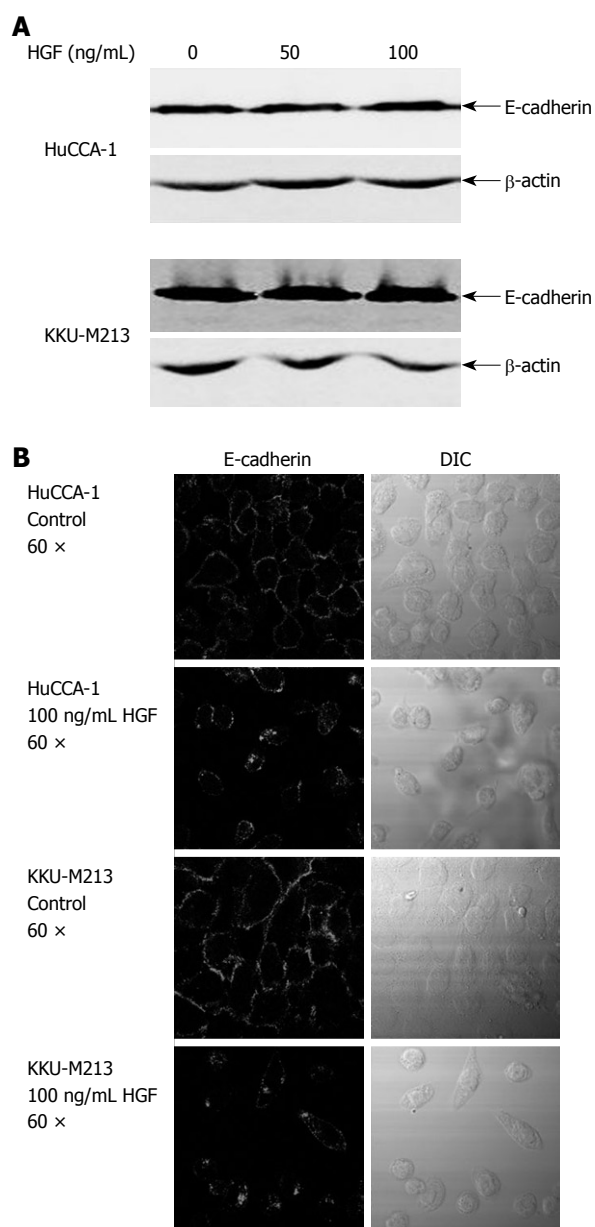
(Figure 3B and C).

The effect of HGF on secretion of matrix degrading enzymes, a major factor contributing to cell invasiveness, was investigated by gelatin zymography. Zymograms from conditioned media of HuCCA-1 cells showed a clear band indicating MMP-2 activity, while those of KKU-M213 cells revealed both MMP-2 and MMP-9 activities (Figure 4A), demonstrating that the two CCA cell lines constitutively expressed high amounts of

MMP-2 and/or MMP-9 at basal levels. However, these enzyme activities were not increased following HGF treatment (Figure 3A). Similarly, high basal activity of uPA was found in both CCA cell lines, which was not affected by the presence of HGF (Figure 4B).

#### Involvement of ERK1/2 and PI3K signaling pathways in HGF-induced CCA cell invasiveness

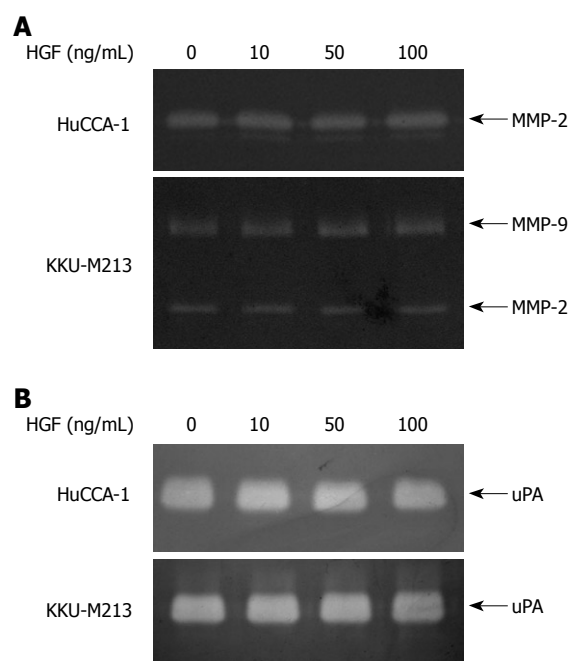
The mechanism responsible for HGF-induced inva-



**Figure 3** Effects of HGF on E-cadherin expression and localization. A: cholangiocarcinoma (CCA) cells were treated with HGF for 6 h, then cell lysate was analyzed by Western blotting with anti-E-cadherin and -β-actin monoclonal antibodies; B: After treatment with 0 and 100 ng/mL HGF for 6 h, cells were analyzed by immunofluorescence using anti-E-cadherin antibody and visualized under confocal laser scanning microscopy (60 × objective magnification plus 2 × digital magnification).

siveness of CCA cell lines was investigated by examining the signaling pathways of ERK1/2 and PI3K. HGF (50 ng/mL) stimulated both HuCCA-1 and KKKU-M213 phosphorylation of ERK1/2 and Akt, with the latter being the major downstream effector of PI3K (Figure 5A and B). However, different time response profiles were observed between these two cell lines in HGF-induced ERK1/2 and Akt activation. In KKKU-M213 cells, HGF significantly induced activation of ERK1/2 and Akt at up to 360 min, whereas in HuCCA-1 cells, after 360 min of induction, activation decreased to nearly those of unstimulated levels.

To confirm the roles of these two signaling pathways

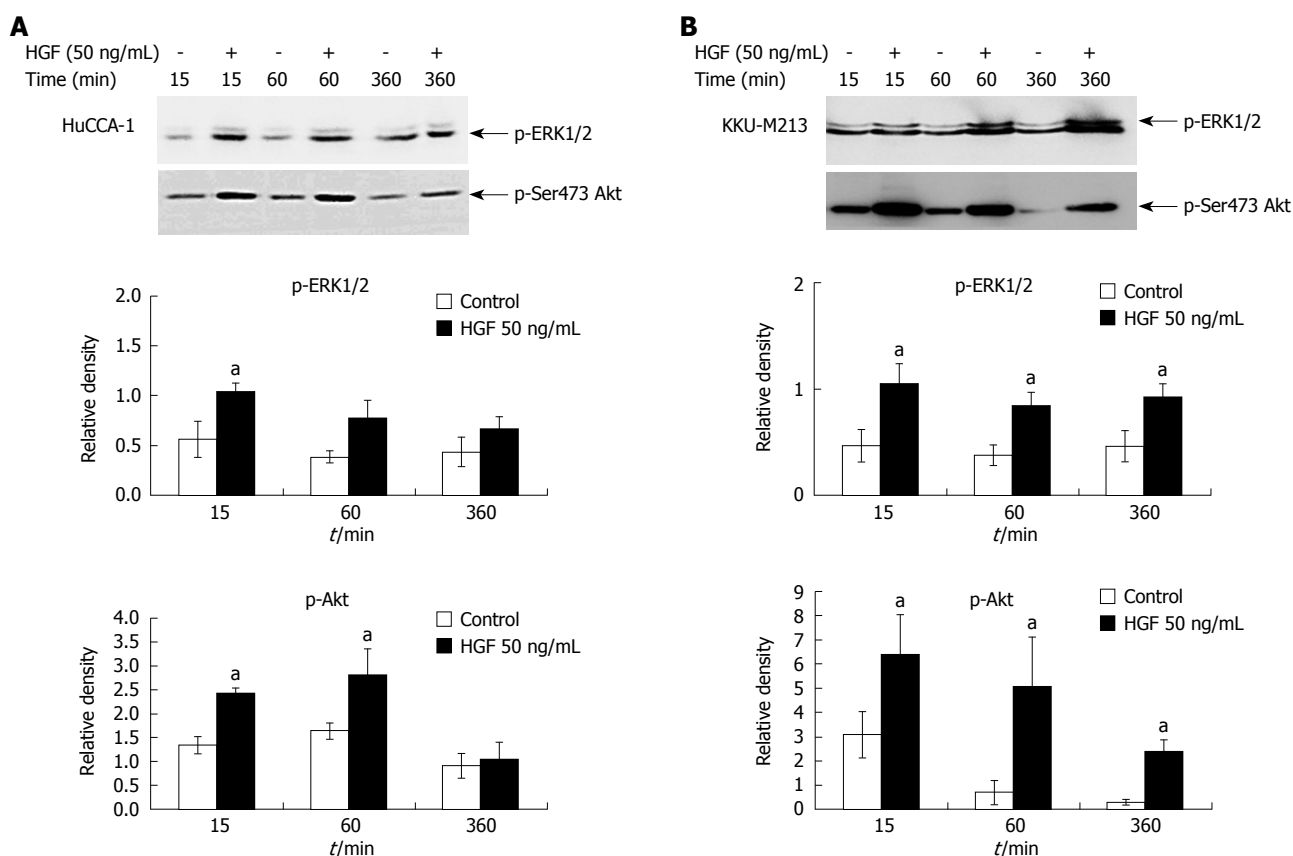


**Figure 4** Effect of HGF on levels of secreted matrix degrading enzymes from cholangiocarcinoma HuCCA-1 and KKKU-M213 cell lines. Cells were treated with various concentrations of HGF (0-100 ng/mL) in serum-free medium for 6 h. Conditioned media were then analyzed for MMP-2 (approximate 65 kDa) and MMP-9 (approximate 85 kDa) gelatinolytic activity by gelatin zymography (A) and for uPA by plasminogen-gelatin zymography (B).

in response to HGF stimulation, we tested the antagonistic effect of U0126 and LY294002; a MEK1 and a PI3K inhibitor, respectively. LY294002 (50 μmol/L) inhibited HGF-stimulated phosphorylation of Akt in both CCA cell lines to an undetectable level (Figure 6A) and markedly inhibited HGF-induced cell invasion, but did not have any significant effect on the invasion of non HGF-stimulated cells (Figure 6B and C). U0126 (1 and 5 μmol/L) reduced HGF-induced invasion of KKKU-M213 cells (to 29% and 18% of untreated control, respectively) (Figure 7C). However, U0126 only had a marginal inhibitory effect on HGF-induced invasion of the HuCCA-1 cell line (Figure 7B). Nevertheless, U0126 completely inhibited ERK1/2 phosphorylation of HuCCA-1 cells, whereas phospho-ERK1/2 was still detectable in KKKU-M213 cells even at the highest U0126 concentration used (Figure 7A).

## DISCUSSION

Overexpression of Met has been reported in CCA and is correlated with progression and invasion of this type of cancer<sup>[9,11]</sup>. In this study, we demonstrated that HGF induced cell invasion, motility and change in E-cadherin localization in two human CCA cell lines, HuCCA-1 and KKKU-M213, both of which overexpress Met; but without affecting secretion of the matrix degrading enzymes, MMP-2, MMP-9 and uPA. However, the signaling pathways underlying HGF-induced invasiveness of the two cell lines were different, with ERK1/2 activation being more important for HGF-induced KKKU-M213 cell invasion than for HuCCA-1 cell invasion.



**Figure 5** HGF induction of ERK1/2 and Akt phosphorylation in cholangiocarcinoma HuCCA-1 and KKU-M213 cell lines. About 80% confluent cells were treated with 50 ng/mL HGF in serum-free medium for 15, 60, 360 min. Lysates from HuCCA-1 (A) and KKU-M213 (B) cells were assessed for total and phosphorylated forms of ERK1/2 and Akt by Western blotting assay. The graphs showed band densities of phospho-ERK1/2 and phospho-Akt relative to those at zero time points. Data are presented as mean  $\pm$  SE of results obtained from three independent experiments. <sup>a</sup> $P < 0.05$  vs untreated control.

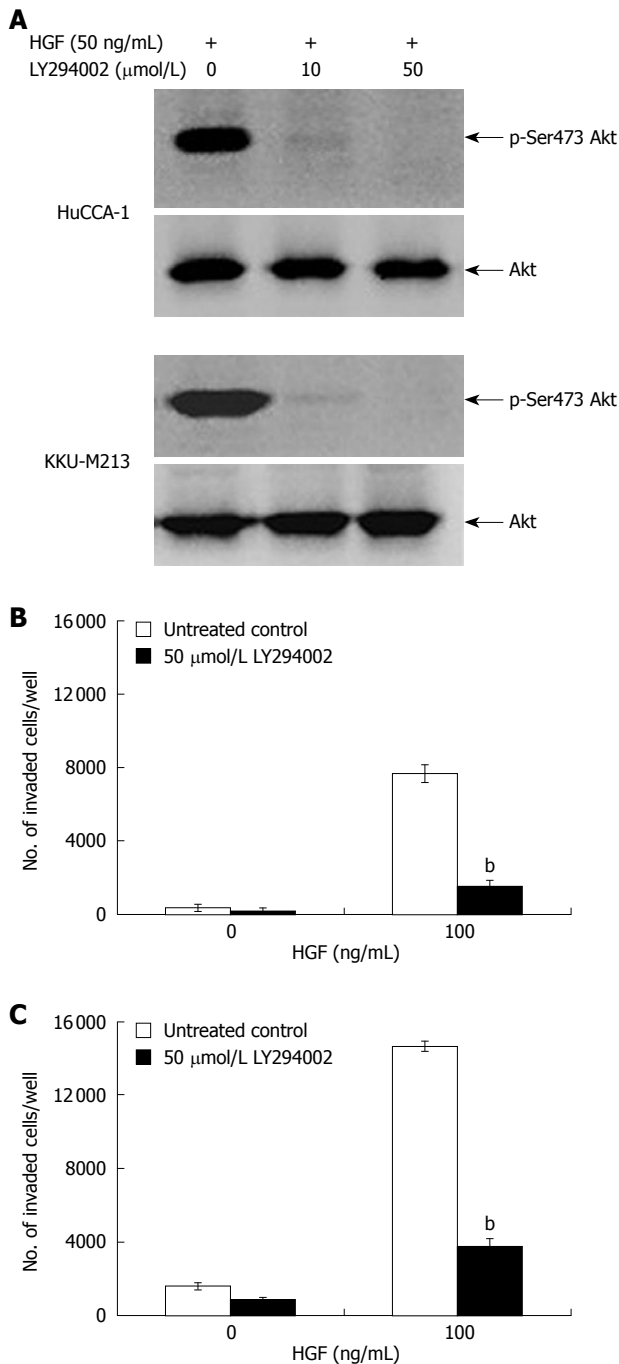
Two major factors contributing to an increase in cancer cell invasiveness are enhancement of extracellular matrix degradation and activation of cell motility. The effects of HGF on induction of these phenomena vary with different cell types. For instance, HGF enhances cell motility but not MMP-9 or uPA activities in breast cancer MDA-MB-231 cell line<sup>[16]</sup>, while it induces both motility and matrix degrading enzyme expression in colon cancer Caco-2, prostate cancer PC-3 and DU-145 cells<sup>[31,32]</sup>. In our study, HGF induced invasion of both CCA cell lines by increasing motility but not MMP-2, MMP-9 or uPA levels. As the expression of the basal levels of these matrix degrading enzymes was already high in both CCA cell lines, this may be sufficient for providing cellular transmigration. Therefore, induction of cell motility alone by HGF, without augmenting extracellular matrix degrading enzyme levels, appears to be sufficient for cell invasiveness. Alterations of only some process(es) required for cell invasion have been reported as being able to alter cell invasiveness. For instance, inhibitors of ERK1/2<sup>[33]</sup> and myosin light chain kinase<sup>[34]</sup> suppress prostate cancer cell invasion by decreasing cell motility but not matrix degrading enzyme activity.

E-cadherin is the key mediator of cell-cell adhesion. Cell scattering induced by HGF results from disruption of E-cadherin function, either by reducing expression or changing its cellular localization<sup>[29]</sup>. In this study, we found

that HGF caused E-cadherin to move from membrane to cytoplasm but had no effect on amount. These results are consistent with previous studies in a keratinocyte cell line, in which HGF reduced E-cadherin at cell-cell boundaries without changing its protein level<sup>[35,36]</sup>. Although we did not investigate the mechanism of HGF-disrupted E-cadherin function, previous reports have implicated the involvement of Ras-RIN2-Rab5 and  $\beta$ -catenin in this process. Kimura *et al*<sup>[37]</sup> demonstrated in a cell free system that HGF activates Ras which binds and activates RIN2, a Rab5-GEF (guanine nucleotide exchange factor of Rab5), leading to Rab5 activation. This active Rab5, a small G protein regulating endocytosis, in turn promotes E-cadherin endocytosis. In addition, Shibamoto *et al*<sup>[36]</sup> showed that HGF promotes tyrosine phosphorylation of  $\beta$ -catenin and decreases E-cadherin at the cell-cell boundaries resulting in the reduction of cell-cell adhesion mediated by E-cadherin.

Basement membrane normally acts as a barrier for tumor cell invasion; therefore, it is generally expected that the rate of invasion at which a cell degrades this barrier should be slower than or equal to the rate of cell migration. However, with the HuCCA-1 cell line, the basal cell invasion rate (with no HGF stimulation) was higher than that of migration. This suggests that some component(s) in Matrigel may have a role in inducing HuCCA-1 cell invasion. In support of this notion,

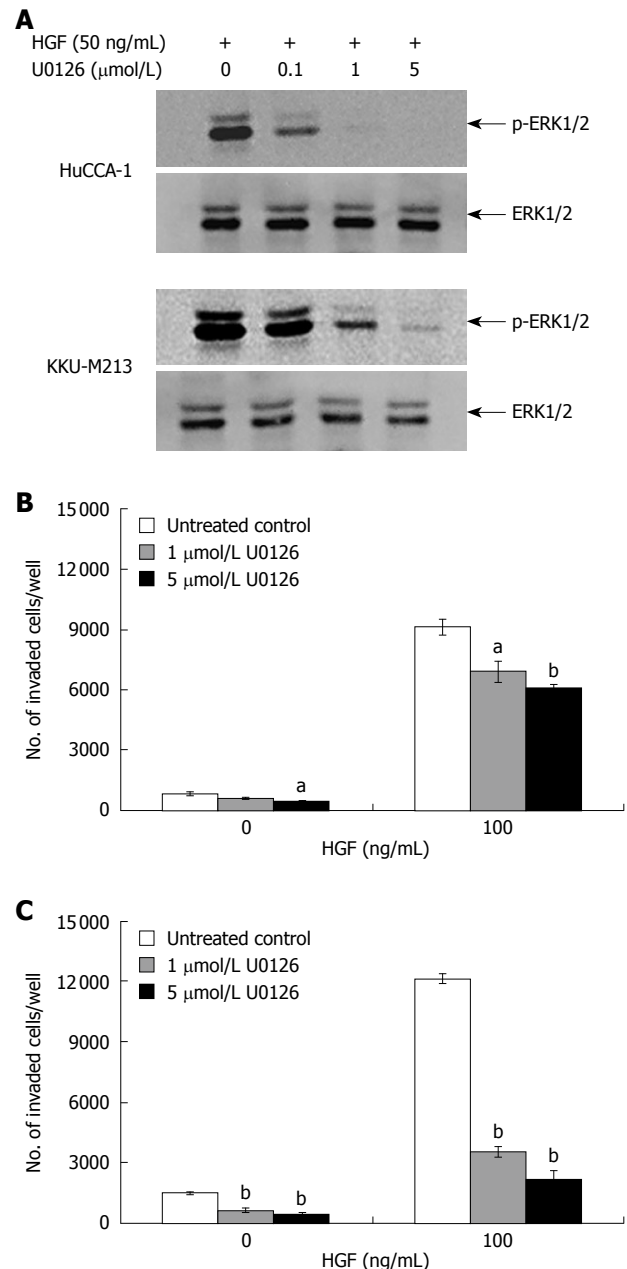




**Figure 6** Suppression of HGF-induced cholangiocarcinoma cell invasiveness by PI3-kinase inhibitor, LY294002. HuCCA-1 and KKU-M213 cells were treated with 50 ng/mL HGF in the absence (control) or presence of 10 and 50  $\mu\text{mol/L}$  LY294002 for 6 h, and subsequently Akt phosphorylation was determined by Western blotting (A). *In vitro* invasion of HuCCA-1 (B) and KKU-M213 (C) cells was evaluated in the absence or presence of HGF with or without 50  $\mu\text{mol/L}$  LY294002. Numbers of invaded cells are presented as mean  $\pm$  SE of results obtained from three independent experiments. <sup>a</sup> $P < 0.01$  vs control.

Chintala *et al.*<sup>[38]</sup> have shown that Matrigel and components of ECM (namely, type IV collagen and fibronectin) induce migration and invasion of many glioma cell lines.

In the KKU-M213 cell line, HGF was better at inducing invasion than migration, and this was not related to the stimulation of secretion of matrix degrading enzymes. A possible explanation is the existence



**Figure 7** Suppression of HGF-induced cholangiocarcinoma cell invasiveness by MEK1 inhibitor, U0126. HuCCA-1 and KKU-M213 cells were treated with 50 ng/mL HGF in the absence (control) or presence of 0.1, 1 and 5  $\mu\text{mol/L}$  U0126 for 6 h, and subsequently ERK1/2 phosphorylation was determined by Western blotting (A). *In vitro* invasion of HuCCA-1 (B) and KKU-M213 (C) cells was evaluated in the absence or presence of HGF with or without 1 and 5  $\mu\text{mol/L}$  U0126. Numbers of invaded cells are presented as mean  $\pm$  SE of results obtained from three independent experiments. <sup>a</sup> $P < 0.05$  and <sup>b</sup> $P < 0.01$  vs control.

of a synergism between HGF and extracellular matrix component(s) in the Matrigel. A combination of HGF and Matrigel induced higher motility than HGF alone (data not shown). Cooperation between HGF and ECM component(s) to promote cell migration could occur by enhancing the function of integrins<sup>[16,39]</sup>, adhesion molecules regulating a variety of cellular properties including adhesion and migration by binding to ECM components. HGF induces cell scattering and migration by

increasing integrin  $\alpha 2$  expression in MDCK cells<sup>[39]</sup> and also promotes breast cancer MDA-MB-231 cell invasion and adhesion by inducing integrin aggregation at lamellipodia, thereby enhancing avidity of integrins to their ligands in ECM and increasing association of integrin to actin, which may participate in cell migration<sup>[16]</sup>.

A variety of signaling pathways are involved in HGF-induced cell invasiveness, including PI3K, ERK1/2 and Src<sup>[40]</sup>. In CCA, Src, FAK<sup>[41]</sup> and ERK1/2<sup>[42]</sup> are involved in HGF-induced HuCCA-1 cell invasion. Here, we showed that HGF induced Met activation concomitant with the promotion of both ERK1/2 and Akt phosphorylation in these two CCA cell lines. To reveal the involvement of ERK and PI3K pathways in HGF-induced invasion, inhibitors of specific signaling transduction pathways were used. PI3K inhibitor (LY294002) significantly inhibited both HuCCA-1 and KKKU-M213 cell invasion stimulated by HGF, while basal invasion was marginally affected. As for the ERK pathway, U0126, a specific inhibitor of MEK1, drastically reduced HGF-promoted KKKU-M213 cell invasion, while slightly reducing HGF-induced HuCCA-1 invasion, even though it inhibited ERK1/2 phosphorylation of the latter cell line to a greater extent than in the former. The insensitivity of HGF-stimulated HuCCA-1 invasion to U0126 treatment suggests a reduced dependence of this CCA cell line on the ERK signaling pathway, whereas HGF-induced KKKU-M213 invasion is dependent on both PI3K and ERK1/2 activation.

ERK1/2 activation is known to regulate a variety of cellular functions, such as proliferation, differentiation, migration, and invasion in response to diverse extracellular stimuli<sup>[43]</sup>. Duration of ERK1/2 activation is one of the factors determining a particular cellular response<sup>[39,44]</sup>. McCawley *et al.*<sup>[45]</sup> showed that EGF and HGF have the ability to induce SCC-12F keratinocyte migration. These two growth factors induce sustained ERK1/2 activation, which is associated with enhanced MMP-9 expression and SCC cell migration<sup>[45,46]</sup>. In MDCK cells, HGF induces sustained ERK1/2 activation, promoting cell scattering and migration *via* the enhancement of integrin- $\alpha 2$  expression, whereas EGF induces transient ERK1/2 activation, which has no effect on cell scattering<sup>[39]</sup>. Our data indicated that prolonged ERK1/2 activation was crucial for HGF-induced invasion of KKKU-M213 cells, but was not necessary for HuCCA-1 cells in which HGF rapidly and transiently activated ERK. Thus, sustained ERK activation provides a possible explanation for the difference in downstream signaling pathways observed in HGF-induced invasion of the two CCA cell lines. Moreover, this sustained ERK1/2 activation may be responsible for a synergism between HGF and Matrigel in KKKU-M213 cells by inducing integrin expression, as in MDCK cells<sup>[39]</sup>.

In summary, this study provides evidence for the contribution of a HGF signaling pathway to the induction of CCA cell invasion. HGF promoted invasion *via* stimulation of cell motility, but not MMP or uPA secretion. HGF regulated invasiveness of two independent CCA cell lines by different signaling pathways, with PI3K being a com-

mon pathway underlying HGF-induced invasiveness in both cell lines, whereas the importance of ERK1/2 was determined by the duration of ERK1/2 activation. However, the mechanisms regulating temporal ERK1/2 activation and possible synergism between HGF and matrix in inducing invasion remains to be elucidated. Understanding the signaling mechanism responsible for CCA invasiveness will be valuable to help identify better targets for cancer therapy, such as that associated with a common rather than a cell specific pathway.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Cholangiocarcinoma (CCA) is a malignant tumor of the biliary epithelium associated with a high metastatic and mortality rate. Incidence of this cancer has increased worldwide, and the highest incidence occurs in northeast Thailand. Overexpression of Met has been reported in CCA and is correlated with progression and invasion of this type of cancer.

### Research frontiers

Hepatocyte growth factor (HGF)/Met activation induces a variety of biological processes, including cell scattering, invasion, proliferation and survival. Although several reports have demonstrated a correlation between Met expression and CCA, hitherto there have been only a limited number of detailed investigations into the role of Met in cholangiocarcinoma.

### Innovations and breakthroughs

HGF induced cell invasion and motility and altered E-cadherin localization in two human CCA cell lines overexpressing Met, without affecting the matrix degrading enzymes, matrix metalloproteinase (MMP)-2, MMP-9 and urokinase plasminogen activator (uPA). This is the first report of a difference in the signaling pathways responsible for the HGF-induced invasiveness of the two human CCA cell lines, in that extracellular signal-regulated kinase (ERK)1/2 activation is more important for HGF-induced invasion of one cell line than of the other.

### Applications

Understanding the role of HGF/Met in CCA invasiveness and the molecular mechanisms underlying this process provides valuable information to help identify targets for future treatment of CCA patients.

### Terminology

Phosphoinositide 3-kinase (PI3K) and ERK are signaling molecules downstream of many receptor tyrosine kinases including Met. These proteins have been shown to play an important role in cell invasion, a crucial factor of cancer metastasis. In this study HGF is shown to stimulate cell invasion and motility of CCA cell lines through PI3K and/or ERK pathways.

### Peer review

CCA is a common malignant tumor with a high metastatic and mortality rate. Investigation into its molecular mechanism is important for understanding the pathogenesis of CCA. This study focused on the role of HGF/Met in CCA cell invasion and the mechanisms underlying cellular responses. Although a number of papers on this field have been published, this study still adds some new information into the knowledge already documented.

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## Endoscopic findings and clinicopathologic characteristics of colonic schistosomiasis: A report of 46 cases

Jun Cao, Wen-Jia Liu, Xin-Yun Xu, Xiao-Ping Zou

Jun Cao, Wen-Jia Liu, Xiao-Ping Zou, Department of Gastroenterology, Nanjing Gulou Hospital Affiliated to Medical School of Nanjing University, Nanjing 210008, Jiangsu Province, China

Xin-Yun Xu, Department of Pathology, Nanjing Gulou Hospital Affiliated to Medical School of Nanjing University, Nanjing 210008, Jiangsu Province, China

Author contributions: Cao J designed the study and wrote the paper; Liu WJ collected the data; Xu XY was responsible for pathology of schistosomal colonic disease; Zou XP designed mainly the study and revised the manuscript.

Correspondence to: Xiao-Ping Zou, MD, PhD, Department of Gastroenterology, Nanjing Gulou Hospital Affiliated to Medical School of Nanjing University, 321 Zhongshan Road, Nanjing 210008, Jiangsu Province, China. [zouxiaoping795@hotmail.com](mailto:zouxiaoping795@hotmail.com)  
Telephone: +86-25-88304616-20601 Fax: +86-25-88042292

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colitis, 1 as Crohn's disease, and 7 as ischemic colitis. The segments of rectum and sigmoid colon were involved in 29 patients (63.0%). Intact *Schistosoma* ova were deposited in colonic mucosa accompanying infiltration of eosinocytes, lymphocytes, and plasma cells in acute schistosomal colitis patients. Submucosal fibrosis was found in chronic schistosomal colitis patients. Among the 17 patients with a signal polyp, hyperplastic polyp, canalicular adenoma with a low-grade intraepithelial neoplastic change, tubulovillous adenoma with a high-grade intraepithelial neoplastic change were observed in 10, 5, and 2 patients, respectively. Eight out of the 46 patients were diagnosed as colonic carcinoma.

**CONCLUSION:** Endoscopy contributes to the diagnosis of colonic schistosomiasis although it is nonspecific. A correct diagnosis of colonic schistosomiasis can be established by endoscopy in combination with its clinicopathologic characteristics.

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**Key words:** Colonic schistosomiasis; Colonoscopy; Diagnosis; Pathology

**Peer reviewer:** Patrick O'Dwyer, MB, BCh, BAQ, FRCS, MCh, FRCS (Glasg), Professor, University Department of Surgery, Western Infirmary, Glasgow, G11 6NT, United Kingdom

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### Abstract

**AIM:** To make a retrospective analysis of endoscopy findings and clinicopathologic characteristics of colonic schistosomiasis in order to further improve our understanding of the disease and decrease its misdiagnosis.

**METHODS:** Endoscopy findings and clinicopathologic characteristics of 46 intestinal schistosomiasis patients were retrospectively analyzed. All the patients underwent colonoscopy and all biopsy specimens stained with hematoxylin and eosin were observed under a light microscope.

**RESULTS:** Of the 46 colonic schistosomiasis patients, 1 was diagnosed as acute schistosomal colitis, 16 as chronic schistosomal colitis and 29 as chronic active schistosomal colitis according to their endoscopic findings and pathology. Not all patients were suspected of or diagnosed as colonic schistosomiasis. Of the 12 misdiagnosed patients, 4 were misdiagnosed as ulcerative

### INTRODUCTION

Colonic schistosomiasis is defined as a specific acute or chronic inflammatory reaction of *Schistosoma* ova that

are deposited mainly in colorectal mucosa. The majority of humans are infected with *Schistosoma japonicum*, *hematobium* and *mansoni*. *Schistosoma japonicum* and *mansoni* often lead to intestinal disease<sup>[1]</sup>. Chinese people are commonly infected with *Schistosoma japonicum*. In the 1950s, schistosomiasis was epidemic at a large scale in regions along the Yangtze River and in more than 400 counties in South China<sup>[2-4]</sup>. Because of the effective prevention and cure measures taken in China in recent years, schistosomiasis has been eliminated in most epidemic regions. However, its spread is not yet completely controlled and schistosomiasis occurs every year in a small number of people in the epidemic regions of China<sup>[3,4]</sup>. Since the number of colonic schistosomiasis patients is small, physicians know little about it, thus often misdiagnosing intestinal schistosomiasis. In the present study, we made a retrospective analysis of endoscopy findings and clinicopathologic characteristics of 46 colonic schistosomiasis patients in order to further improve our understanding of the disease and decrease its misdiagnosis.

## MATERIALS AND METHODS

From May 2005 to May 2009, 46 patients with colonic schistosomiasis were admitted to Endoscopy Center, Affiliated Gulou Hospital of Medical School of Nanjing University (Nanjing, China). Their endoscopy findings and clinicopathologic characteristics were retrospectively analyzed. The patients gave their written informed consent before colonoscopy (Olympus CF-240I or CF-H260AZI, Tokyo, Japan). When a lesion was detected at colonoscopy, tumor tissue samples were taken and fixed in 4% buffered paraformaldehyde, embedded in paraffin, and stained with hematoxylin-eosin. Two pathologists independently examined the tumor tissue sections under a light microscope (Olympus, Tokyo, Japan).

## RESULTS

### Clinical characteristics

Of the 46 colonic schistosomiasis patients (32 men and 14 women) at the age of  $65.4 \pm 10.8$  years (range 40-80 years), 31 were from the epidemic areas of schistosomiasis and 15 from the non-epidemic areas with a history of contacting water containing *Schistosoma* ova.

The time from onset of symptoms to visit of a doctor ranged 4 d to 7 years (mean 4.5 years). The common symptoms were repeated fever and hematochezia. Among the symptoms occurred in 46 patients, diarrhea was found in 31 (67.3%), bloody stool in 8 (17.3%), abdominal pain in 35 (76.1%), incomplete intestinal obstruction in 3 (4.7%), turgescence spleen in 6 (8.4%) and hepatosplenic schistosomiasis in 1 patients (1.5%), respectively.

### Endoscopy features

All the patients underwent colonoscopy with a success in 41 patients and a failure in 5 patients. The whole colon, right colon, and left colon were involved in 4 (8.7%), 4

Table 1 Location of affected colon

Location of colonic injury	<i>n</i>	Percent
Cecum	2	4.3
Whole colon	4	8.7
Ascending + transverse colon	1	2.2
Descending colon	8	17.4
Descending + transverse colon	1	2.2
Hepatic flexure of colon	1	2.2
Sigmoid colon	3	6.5
Sigmoid colon + rectum	9	19.6
Rectum	17	37.0

(8.7%), and 38 (82.6%) patients, respectively (Table 1). Among the 38 patients with their left colon involved, only descending colon, descending and transverse colon, only sigmoid colon, sigmoid colon and rectum, and only rectum were involved in 8, 1, 3, 9, and 17 patients, respectively. The lesion was mainly located in rectum and sigmoid colon of 29 patients (63.0%).

Among the 46 patients, acute submucosal colitis, chronic submucosal colitis, chronic active submucosal colitis were diagnosed in 1, 16, and 29 patients, respectively. Friable or edematous mucosa with more mucus exudates, scattered petechial hemorrhage, invisible submucosal blood vessels, erythema and granularity of mucosa with irregular ulcerations could be observed in acute submucosal colitis patients by colonoscopy. Chronic submucosal colitis was characterized by pale intestinal mucosa, confused vascular net with more flat or elevated yellow nodules, even intestine stricture, single polyp or more polyps. Acute and chronic inflammation reactions occurred simultaneously in the same or different segments of colon, and a clear dividing line emerged between the two types of inflammation in chronic active submucosal colitis patients. Acute inflammation was often observed in the right colon and chronic inflammation usually occurred in the left colon. Acute and chronic inflammation was also observed in the same segment of colon (Figure 1).

### Pathology characteristics

Intact *Schistosoma* ova were deposited in lamina propria with infiltration of eosinocytes and neutrophilic granulocytes in acute schistosomal colitis patients. *Schistosoma* ova were calcified and deposited with infiltration of lymphocytes and plasma cells in submucosa, lamina propria in chronic schistosomal colitis patients. Atrophy of intestinal mucosa epithelium, reduction of intestinal glands, submucosal hyperplasia and different degrees of fibrosis were also observed in chronic schistosomal colitis patients. Two different types of *Schistosoma* ova were found in chronic and acute schistosomal colitis patients. In addition, *Schistosoma* oviposition was associated with the clinical and histopathological changes in colonic schistosomiasis (Figure 2).

Colonoscopy showed that a single polyp in 17 out of the 46 patients. Among the 17 patients, hyperplastic polyps, canalicular adenoma with a low-grade intraepithelial neoplastic change and tubulovillous adenoma with a high-

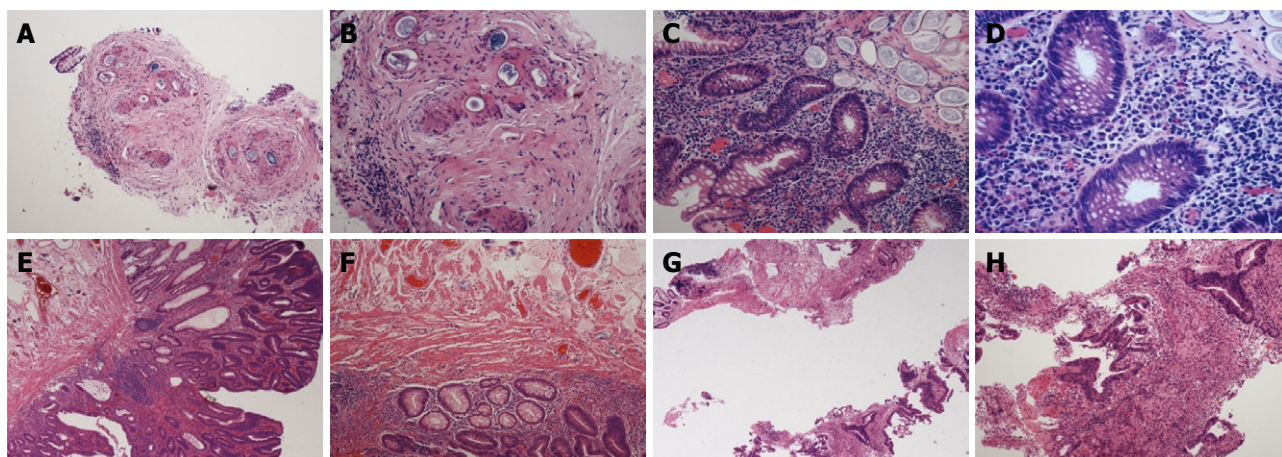


**Figure 1 Endoscopic findings of schistosomal colonic disease.** A: Congestive, edematous mucosa in rectum with purulent secretion in mixed colitis; B: Congestive and edematous mucosa of sigmoid colon and intestinal stricture in mixed colitis; C: Mucosal erosion, superficial ulcer and granular change in descending colon with invisible submucosal blood vessels in mixed colitis; D: Congestive, edematous and erosive mucosa in rectum with invisible submucosal blood vessels in mixed colitis; E: Coarse, congestive, ulcerative mucosa and intestinal stricture in descending colon in mixed colitis; F: Patchy congestion and vague vascular net in mucosa of sigmoid colon in mixed colitis; G: Vascular net like map of sigmoid colon in chronic colitis; H: Cobwebbed vessels in rectum in chronic colitis; I: Giant flat, lobulated polypus in rectum in chronic colitis; J: Giant polypus in sigmoid colon in chronic colitis.

grade intraepithelial neoplastic change were detected in 10, 5 and 2 patients, respectively. Of the 46 patients, 8 were diagnosed as colonic carcinoma, including papillary

adenocarcinoma in 2, mucinous adenocarcinoma in 1, signet-ring cell carcinoma in 1, and tubular adenocarcinoma in 4 patients, respectively.





**Figure 2** Pathology of schistosomal colonic disease (HE staining). A: Chronic inflammation in rectal mucosa and calcified schistosomal ova around fibroplasia and foreign-body giant cell reaction in submucosa (original magnification  $\times 100$ ); B: Same view as A, at a different magnification (original magnification  $\times 200$ ); C: Chronic inflammation accompanying acute activity and deposited schistosomal ova in submucosa (original magnification  $\times 200$ ); D: Same view as A, at a different magnification (original magnification  $\times 400$ ); E: Canalicular adenoma accompanying low-grade intraepithelial neoplastic change and more deposited schistosomal ova in rectum (original magnification  $\times 40$ ); F: Same view as A, at a different magnification (original magnification  $\times 100$ ); G: Rectal adenocarcinoma and deposited schistosomal ova in rectum (original magnification  $\times 40$ ); H: Same view as A, at a different magnification (original magnification  $\times 100$ ).

### Therapy

High frequency electric snare of colonic polyps was performed under a colonoscope for 17 colonic schistosomiasis patients. Among these patients, colonic carcinoma was radically removed in 8 patients, schistosomiasis was treated with antischistosomiasis drug (praziquantel) in 1 patient and the other patients received symptomatic treatment.

### DISCUSSION

Schistosoma ova are mainly parasitized in the inferior mesenteric and portal vein when one is infected with them. Intestinal schistosomiasis occurs due to deposition of Schistosoma ova in submucosa producing a granulomatous reaction<sup>[5]</sup>. Mucosal edema, hemorrhage and ulceration may occur in bowel wall at its early stage, while thickened bowel wall, polyps, or enteric cavity stricture, *etc.*, can be detected at its advanced stage<sup>[6]</sup>. In our study, colonic schistosomiasis was divided into acute schistosomalous colitis, chronic schistosomalous colitis, and chronic active schistosomalous colitis according to the inflammation reaction in colon. In the present study, 46 colonic schistosomiasis patients included 1 acute schistosomalous colitis, 16 chronic schistosomalous colitis and 29 chronic active schistosomalous colitis patients. The difference between acute and chronic schistosomalous colitis depends on whether Schistosoma ova are intact. A Schistosoma ovum or several Schistosoma ova are deposited in submucosa and lamina propria in acute schistosomalous colitis patients with infiltration of eosinocytes. Schistosoma ova are calcified and deposited in submucosa and lamina propria with infiltration of lymphocytes and epithelioid cells in chronic schistosomalous colitis patients. Fibroplasia could be observed in colonic submucosa of chronic schistosomalous colitis patients. The deposition sites of Schistosoma ova are sigmoid colon, upper segment of rectum, descending colon, transverse colon, cecum and ascending colon. In our study, the

lesion was located in the rectum and sigmoid colon of 29 patients (63.0%) and in the colon segments of 17 patients (37.0%).

Although nonspecific, colonoscopy may provide valuable information for the diagnosis of colonic schistosomiasis. Colonoscopy can show edematous, congestive mucosa and petechial hemorrhage in acute schistosomalous colitis patients, and confused vascular net with more close-set flat or elevated yellow nodules, polyps and intestine stricture in chronic schistosomalous colitis patients. Acute and chronic inflammation could be observed in colon segments of chronic active schistosomalous colitis patients. The most characteristic finding is the gray-yellow or yellowish white schistosomalous nodules similar to those of pseudomembranous enterocolitis. In our study, colonoscopy showed schistosomalous nodules in only 6 patients (16.2%), which may be the reason why physicians cannot make a correct diagnosis of the disease based only on colonoscopic findings. Schistosomalous oviposition is the golden diagnostic standard for colonic schistosomiasis. Schistosoma ova are deposited in lamina propria and/or in submucosa<sup>[7]</sup> with infiltration of eosinocytes and neutrophilic granulocytes in acute schistosomalous colitis patients. Schistosoma ova are calcified or ruptured with infiltration of lymphocytes and plasma cells in submucosa and lamina propria of chronic schistosomalous colitis patients. Submucosal hyperblastosis and fibrosis could also be found in chronic schistosomalous colitis patients. Two types of Schistosoma ova can be detected in chronic acute schistosomalous colitis patients.

In our study, a definite diagnosis was not made only based on endoscopic findings. Four patients were misdiagnosed as ulcerative colitis, 1 as Crohn's disease, and 7 as ischemic colitis, indicating that physicians know little about the disease.

Colorectal cancer is one of the most common malignant gastrointestinal tumors and its occurrence has in-



creased in recent years. Its pathogenesis remains unclear, thus requiring further study. *Schistosoma japonicum* infection is considered a significant risk factor for colonic cancer in Asia although it is still controversial<sup>[5]</sup>. A total of 454 colorectal carcinoma specimens have been studied in China, showing that 289 of them are associated with *Schistosoma japonicum* infection<sup>[8]</sup>. Kaw *et al*<sup>[9]</sup> studied 1277 colonic carcinoma patients and found that schistosomiasis is often accompanied with rectal carcinoma. Mei *et al*<sup>[10]</sup> studied 352 colonic carcinoma patients and found that 14.3% of them have the complication of schistosomiasis. These colonic carcinomas are moderately-differentiated tubular and mucinous adenocarcinomas. In the present study, 8 colonic schistosomiasis patients (17.3%) had complication of colonic carcinoma, and 2 had complications of a high-grade intraepithelial neoplastic change and precancerous lesion. However, the mechanism of *Schistosoma japonicum* infection leading to carcinoma is unclear, which may be associated with chronic inflammation, ulceration and mucosa repair due to *Schistosoma* ova. Tumorigenesis may result from gene mutations in epithelial cells of glands due to the long time stimulation of mucosa by *Schistosoma* ova.

The incidence of colonic schistosomiasis has been greatly declined. However, many complications may occur if it is not early diagnosed and treated. Colonic schistosomiasis should be diagnosed based on its clinical symptoms and signs, colonoscopic findings and pathologic characteristics. If *Schistosoma* ova are found in biopsy, it can be diagnosed. If *Schistosoma* ova are not observed in biopsy, the near-normal crypts with excess mucus and diffuse or focal infiltration of eosinophilic granulocytes may be highly suggestive of colonic schistosomiasis<sup>[11]</sup>.

## COMMENTS

### Background

Schistosomiasis was epidemic at a large scale in the regions along the Yangtze River and more than 400 counties in South China 50 years ago. Thanks to the effective prevention and cure measures taken in recent years, schistosomiasis has been eliminated in most epidemic regions. However, its spread is not completely controlled in several regions and schistosomiasis still occurs in a small number of people in its endemic region, which threatens their health. Since the number of patients with still suffer from schistosomiasis has greatly declined, physicians know little about it and often misdiagnose it.

### Research frontiers

Colonic schistosomiasis is seldom reported at present. In this study, the endoscopy findings and clinicopathologic characteristics of 46 colonic schistosomiasis patients were retrospectively analyzed, showing that *Schistosoma japonicum* infection may be a risk factor for colonic cancer.

### Innovations and breakthroughs

The endoscopy findings and clinicopathologic characteristics of 46 colonic schistosomiasis patients were analyzed. The disease was classified into acute schistosomal colitis, chronic schistosomal colitis, and chronic active schistosomal colitis. The results indicate that *Schistosoma japonicum* infection may be a risk factor for colonic cancer and schistosomal polyps should be removed under an endoscope.

### Applications

The endoscopy findings and clinicopathologic characteristics of colonic schistosomiasis were described, which may improve our further understanding of the disease and decrease its misdiagnosis.

### Terminology

Colonic schistosomiasis: An acute and chronic specific inflammatory reaction due to *Schistosoma* ova in colonic and rectal mucosa. Pseudomembranous enterocolitis: An infection of the colon with *Clostridium difficile*, characterized by diarrhea, fever, vomiting and abdominal pain.

### Peer review

This paper is interesting and should be published. The authors, however, need to highlight the high incidence of cancer in their populations.

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## Endoscopic ultrasound-guided celiac plexus neurolysis using a reverse phase polymer

Keith L Obstein, Fernanda P Martins, Gloria Fernández-Esparrach, Christopher C Thompson

Keith L Obstein, Christopher C Thompson, Gastroenterology Division, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, United States

Fernanda P Martins, Federal University of São Paulo, Rua Botucatu, 640 2º andar, São Paulo 04023-062, Brazil

Gloria Fernández-Esparrach, Department of Gastroenterology, Endoscopy Unit, Hospital Clinic, Villarroel 170, Barcelona 08036, Spain

Author contributions: Obstein KL, Martins FP, Fernández-Esparrach G and Thompson CC contributed equally to this work; Obstein KL, Martins FP, Fernández-Esparrach G and Thompson CC performed the research; Obstein KL, Martins FP, Fernández-Esparrach G and Thompson CC analyzed the data; Obstein KL, Martins FP, Fernández-Esparrach G and Thompson CC wrote the paper.

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Correspondence to: Christopher C Thompson, MD, MSc, FACP, FASGE, Gastroenterology Division, Brigham and Women's Hospital, 75 Francis Street, Thorn 1123, Boston, MA 02115, United States. [ccthompson@partners.org](mailto:ccthompson@partners.org)

Telephone: +1-617-5258266 Fax: +1-617-2646342

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**RESULTS:** EUS-guided CPN was successfully performed in all 6 pigs without immediate complication. Methylene blue was identified throughout the peritoneal and retroperitoneal cavity in group 1. The blue colored poloxamer was found in the retroperitoneal cavity immediately adjacent to the aorta, in the exact location of the celiac plexus in group 2.

**CONCLUSION:** EUS-guided CPN using a reverse phase polymer in a non-survival porcine model was technically feasible. The presence of a poloxamer gel at the site of the celiac plexus at necropsy indicates a precise delivery of the neurolytic agent.

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**Key words:** Celiac plexus neurolysis; Celiac plexus blockade; Endoscopic ultrasound; Polymer

**Peer reviewer:** Dr. Massimo Raimondo, Division of Gastroenterology and Hepatology, Mayo Clinic, 4500 San Pablo Road, Jacksonville, FL 32224, United States

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### Abstract

**AIM:** To assess the feasibility of endoscopic ultrasound (EUS)-guided celiac plexus neurolysis (CPN) using a poloxamer.

**METHODS:** In this prospective evaluation, six Yorkshire pigs underwent EUS-guided CPN. Three received an injection of 10 mL of 0.25% Lidocaine plus methylene blue (group 1) and three received an injection of 10 mL of 0.25% Lidocaine plus blue colored poloxamer (PS137-25) (group 2). Necropsy was performed immediately after the animals were sacrificed. The abdominal and pelvic cavities were examined for the presence of methylene blue and the blue colored poloxamer.

### INTRODUCTION

Pancreatic cancer and chronic pancreatitis commonly cause pain that is difficult to control<sup>[1-3]</sup>. Opioids are frequently used in an attempt to mitigate pain, however, tolerance, nausea, constipation and other side effects develop<sup>[4,5]</sup>. Non-pharmacologic therapies are often employed to improve pain control and quality of life while reducing drug-related side effects. Celiac plexus blockade (CPB) using steroids or celiac plexus neurolysis (CPN) using alco-

hol has been utilized and considered safe. Endoscopic ultrasound (EUS)-guided CPB and CPN have demonstrated safety and efficacy through real-time imaging and anterior access to the celiac plexus from the posterior gastric wall, thereby avoiding complications related to the puncture of spinal nerves, arteries and the diaphragm.

Unfortunately, EUS-guided CPN and CPB provide limited benefit in terms of degree and duration of pain relief<sup>[3]</sup>. While benefit duration of EUS CPN diminishes after 8-12 wk, the etiology remains unknown<sup>[6,7]</sup>. One theory is that the neurolytic or blockade agent washes away from the celiac plexus injection site due to its liquid free-flowing form and does not remain in the ideal anatomical location. Thus, if a neurolytic or blockade agent could be delivered in an alternate phase (solid or gel), it could offer the potential for enhanced efficacy and safety<sup>[8]</sup>.

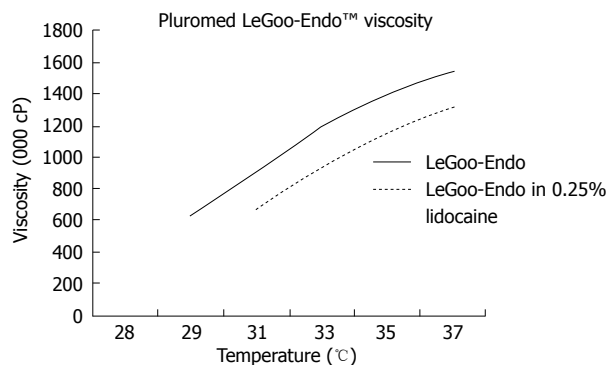
Recently, non-ionic surfactant triblock (ABA) copolymers of polyethylene oxide<sub>a</sub>-polypropylene oxide<sub>b</sub>-polyethylene oxide<sub>a</sub> (PEO<sub>a</sub>-PPO<sub>b</sub>-PEO<sub>a</sub>), also termed as poloxamers, have been widely used in industrial and medical applications<sup>[9-14]</sup>. Certain poloxamers have demonstrated rapid reverse phase thermosensitive properties at certain concentrations. Purified poloxamers PS138-25, PS107-20 and PS137-25 (Pluromed Inc., Woburn, MA, USA) are thin liquids at room temperature while at body temperature they are solid gel plugs (Figure 1). Therefore using a neurolytic or blockade agent as an additive in a purified poloxamer will potentially form a solid gel plug at the exact location of injection at the celiac plexus with enhancement of efficacy and safety. This study will assess the feasibility of EUS-guided CPN/CPB using a poloxamer in a non-survival porcine model.

## MATERIALS AND METHODS

Six Yorkshire pigs (25-30 kg) were food restricted for 24 h prior to the procedure. Intravenous (iv) Telazol (4.4 mg/kg), Atropine sulfate (0.04 mg/kg) and Xylazine (2.2 mg/kg) were used for anesthesia induction followed by inhaled Isoflurane (1% to 3%) on a semi-closed circuit for anesthesia maintenance after endotracheal intubation.

EUS-guided CPN was then performed using a linear echoendoscope (GF-UC140P, Olympus, Tokyo, Japan). Once the location of the celiac plexus was identified by its position relative to the celiac artery, a 19-gauge needle (Wilson-Cook Medical, Inc., Winston-Salem, NC, USA) was introduced under direct EUS visualization (Figure 2). The needle was flushed with 2 mL normal saline and aspiration was performed to evaluate for vessel penetration prior to additional injections.

Three pigs were randomly assigned to receive a single injection of 10 mL of Lidocaine (0.25%) plus methylene blue (group 1) and three pigs randomly received a single injection of 10 mL of Lidocaine (0.25%) plus blue colored poloxamer PS137-25 (LeGoo-endo™, Pluromed, Inc., Woburn, MA, USA) (group 2). Due to the increased viscosity of the poloxamer, a greater amount of force was required for injection. This was easily overcome with the use of a Controlled Radial Expansion (CRE) balloon dilator inflation hand pump system (Boston



**Figure 1** Temperature profile of LeGoo-Endo™ (PS137-25) that transits from liquid to gel at body temperature. The viscosity of LeGoo-Endo™ with Lidocaine is slightly higher than LeGoo-Endo™ at body temperature.



**Figure 2** Illustration of endoscopic ultrasound (EUS)-guided celiac plexus neurolysis (CPN). Figure from Arcidiacono PG, Rossi M. Celiac Plexus Neurolysis. *J Pancreas* 2004; 5: 315-321.

Scientific, Natick, MA, USA). During the procedure, the blood pressure, heart rate, temperature, ventilation, and oxygenation status of the pigs were continuously monitored by the professional veterinary team of the Animal Research at Children's Hospital (ARCH) (Boston, MA, USA). The investigators were not blinded to the injection group.

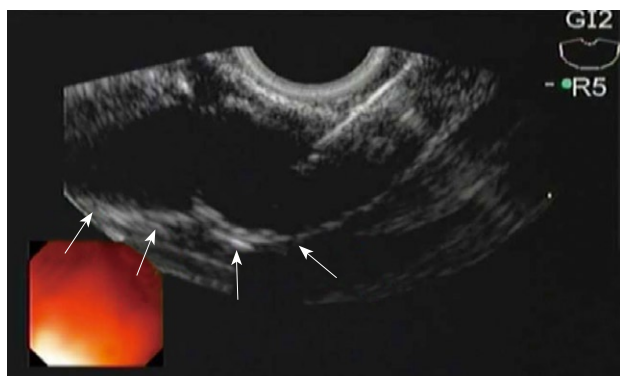
Using Fatal Plus (86 mg/kg), the group 1 pigs were immediately sacrificed after the procedure and the group 2 pigs were sacrificed at 60 min after the procedure. Necropsy was performed immediately upon death of the pigs and close examinations of the peritoneal and retroperitoneal cavities were made. Photographic and video records were obtained. This study was approved by the Animal Research Committee of the ARCH and complied with the National Academy of Sciences Guide for the Care and Use of Laboratory animals.

## RESULTS

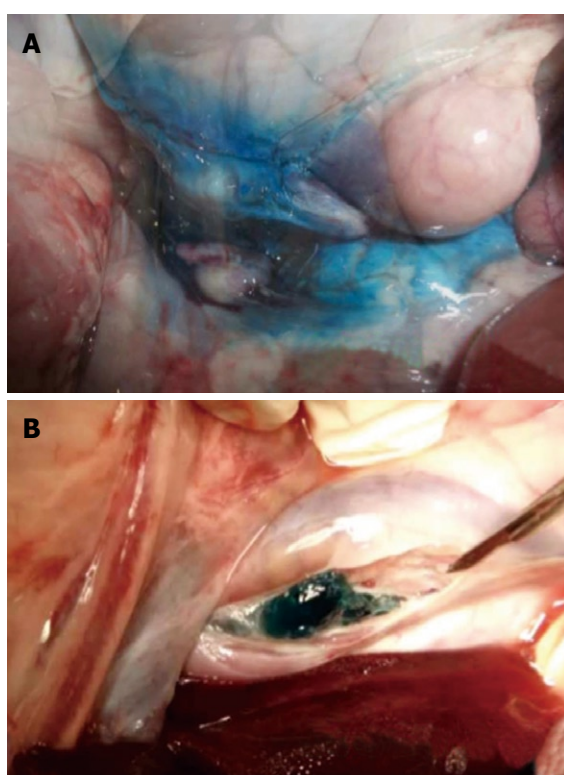
All the six pigs tolerated the procedure well with no immediate complications. EUS-guided CPN was successfully performed in both groups. An echodense smudge was endosonographically visualized after injection of Lidocaine plus methylene blue and Lidocaine plus blue colored poloxamer PS 137-25 (Figure 3).

At necropsy, methylene blue was identified spread throughout the peritoneal and retroperitoneal cavities in group 1. In group 2, a blue gel plug was identified in the retroperitoneal cavity, adjacent to the aorta, in the exact





**Figure 3** EUS-guided injection of Lidocaine plus blue colored poloxamer PS 137-25. The outer margin of the hypoechoic poloxamer is visualized upon injection into the celiac plexus (arrows).



**Figure 4** Group 1 (A) and group 2 (B) at necropsy.

location of the celiac plexus without evidence of any blue gel elsewhere in the peritoneal or retroperitoneal cavity (Figure 4).

## DISCUSSION

EUS-guided CPN and CPB are safe and effective methods for pain control in patients with pancreatic neoplasms and chronic pancreatitis<sup>[3-6,15-18]</sup>. Unfortunately, the degree and duration of therapeutic effect vary. Gress *et al.*<sup>[18]</sup> performed EUS-guided CPB in 90 patients with chronic pancreatitis and found that 55% of patients had decreased pain scores at a mean follow-up of 8 wk. This included a reduction in pain medication requirements as reported by the patients in the study. Persistent benefit was found

in 26% of patients at week 12 and in 10% at week 24. Gunaratnam *et al.*<sup>[6]</sup> performed EUS-guided CPN in 58 patients with pancreatic cancer, 78% of whom experienced a decline on a continuous 11-point visual analog pain scale 2 wk after the procedure. Only 54% experienced a decline of greater than 2 points after EUS-guided CPN and the efficacy diminished 8-12 wk after the procedure in those not receiving adjuvant therapy. Those authors also found that opioid administration increased throughout the study, however this increase was not statistically significant. Levy *et al.*<sup>[3]</sup> administered EUS-guided direct ganglia injection in 33 patients with pancreatic cancer and chronic pancreatitis, 94% and 80% of the patients reported pain relief with alcohol injection at 2-4 wk after the procedure, however long-term follow-up data was not recorded.

In a report in 1996, fluoroscopic evaluation of the abdomen was conducted in patients who underwent EUS-guided CPN. Of those examined, all were noted to have injected material spread in a periaortic distribution with dye spread anterior and lateral to the aorta with extension in both the cranial and caudal direction<sup>[16]</sup>. This study supports our findings in group 1 pigs where the injected methylene blue was identified spread throughout the peritoneal and retroperitoneal cavities.

While short-term reduction in pain has been indicated, long-term benefit with this technique is limited. This may be explained by the fact that until recently, the celiac ganglia was unable to be directly visualized, leading to a less precise delivery of therapy<sup>[3,19,20]</sup>. Other possibilities include interference with direct visualization due to the echodense smudge and potential alterations in anatomy after injecting one side of the aorta with the therapeutic agent using the double injection technique. Additionally, dispersion of the therapeutic agent away from the desired location may act to influence the efficacy and duration.

The goal of this study was to evaluate the feasibility of EUS-guided CPN using a poloxamer that would potentially remain in the desired target location. This may yield a safer and more durable therapeutic result. In group 2, where the PS137-25 was injected, a gel plug was successfully created at the intended location of the celiac ganglia. The gel maintained the therapeutic agent in the celiac plexus and potentially released the drug slowly over time, thereby optimizing long-term therapeutic results.

A limitation of this study is that it is an acute, non-survival evaluation without long-term follow-up. It is therefore unclear how the injected gel plug interacts with the porcine model over time. Additionally, the study was performed using Lidocaine instead of a steroid or alcohol as it was simpler to integrate into the PS137-25. Steroid or alcohol integration may influence the temperature phase transition point of the poloxamer, however, the formation of a gel at body temperature and liquid at room temperature would remain intact. Lastly, the quantity and rate of drug release from the gel plug was unable to be determined in this current study.

In conclusion, EUS-guided CPN using a reverse phase polymer is feasible in a non-survival porcine model. The formation of a gel plug at the exact location of the celiac ganglia



may avoid dispersion of the injected therapeutic agent and increase the duration of analgesic effect. A survival study is now necessary to determine the duration and breakdown of the gel plug within the body. This would provide useful information on potential complications related to the plugs presence and allow for assessment of the therapy from the gel plug over time. Future studies will also integrate the use of steroids and alcohols into the poloxamer.

## COMMENTS

### Background

Pancreatic cancer and chronic pancreatitis commonly cause pain that is difficult to control. Nonpharmacologic therapies are often employed to improve pain control and quality of life while reducing drug-related side effects. Endoscopic ultrasound (EUS)-guided celiac plexus blockade (CPB) using steroids or celiac plexus neurolysis (CPN) have demonstrated safety and efficacy. Unfortunately, the neurolytic or blockade agent may wash away from the celiac plexus injection site due to its liquid free-flowing form and does not remain in the ideal anatomical location. Therefore, using a neurolytic or blockade agent as an additive in a reverse thermodynamic phase poloxamer will potentially form a solid gel plug at the exact location of injection at the celiac plexus with enhancement of efficacy and safety.

### Research frontiers

Expanded research utilizing reverse phase poloxamers for additional applications in medical practice. Survival studies utilizing reverse phase poloxamers to determine the duration and breakdown of the gel plug within the body. Enhancement of the efficacy and safety of CPB and CPN.

### Innovations and breakthroughs

Recently, non-ionic surfactant triblock (ABA) copolymers of polyethylene oxide<sub>n</sub>-polypropylene oxide<sub>m</sub>-polyethylene oxide<sub>n</sub> (PEO<sub>n</sub>-PPO<sub>m</sub>-PEO<sub>n</sub>), also termed as poloxamers, have been widely used in industrial and medical applications. Certain poloxamers have demonstrated rapid reverse phase thermosensitive properties at certain concentrations. Purified poloxamers PS138-25, PS107-20 and PS137-25 (Pluromed Inc., Woburn, MA, USA) are thin liquids at room temperature while at body temperature they are solid gel plugs. Therefore, the novel use of a neurolytic or blockade agent as an additive in a purified poloxamer will potentially form a solid gel plug at the exact location of injection at the celiac plexus.

### Applications

EUS-guided CPN using a reverse phase polymer is feasible in a non-survival porcine model. The formation of a gel plug at the exact location of the celiac ganglia may avoid dispersion of the injected therapeutic agent and increase the duration of analgesic effect. A survival study is now necessary to determine the duration and breakdown of the gel plug within the body. Future studies will also integrate the use of steroids and alcohols into the poloxamer.

### Terminology

Poloxamer: A non-ionic surfactant triblock (ABA) copolymer of polyethylene oxide<sub>n</sub>-polypropylene oxide<sub>m</sub>-polyethylene oxide<sub>n</sub> (PEO<sub>n</sub>-PPO<sub>m</sub>-PEO<sub>n</sub>). Reverse phase thermosensitivity: When a material or substance is in its liquid phase at cold temperature and in its solid phase at hot temperature.

### Peer review

Dr. Thompson *et al* present an animal experience on the use of reverse phase polymer to increase the efficacy of EUS-guided celiac plexus injection for pancreatic pain control. This represent an interesting experiment outlining the possible improvement of the procedure by using a compound which facilitate the retention of the injectate at the local level (celiac plexus).

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## ABCG5-positivity in tumor buds is an indicator of poor prognosis in node-negative colorectal cancer patients

Isabel Hostettler, Inti Zlobec, Luigi Terracciano, Alessandro Lugli

Isabel Hostettler, Inti Zlobec, Luigi Terracciano, Alessandro Lugli, Institute for Pathology, University Hospital of Basel, Schoenbeinstrasse 40, Basel, CH-4031, Switzerland

**Author contributions:** Hostettler I and Zlobec I contributed equally to the research; Hostettler I collected and interpreted the data; Lugli A designed the study, conceptualized the project and interpreted the data; Zlobec I analyzed and interpreted the data and drafted the manuscript; Terracciano L provided the material and administrative support; All authors played a role in the editing, revision and final approval of this manuscript.

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**Correspondence to:** Dr. Alessandro Lugli, MD, Institute for Pathology, University Hospital of Basel, Schoenbeinstrasse 40, Basel, CH-4031, Switzerland. [alugli@uhbs.ch](mailto:alugli@uhbs.ch)

Telephone: +41-61-2652390 Fax: +41-61-2653194

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### Abstract

**AIM:** To analyze the expression of 8 putative cancer stem cell (CSC) markers within colorectal cancer tumor buds and to determine their prognostic impact in patients with this disease.

**METHODS:** Immunohistochemistry was performed on 101 colorectal cancer resections for CK22 (to identify tumor buds) as well as CD133, CD166, CD24, CD44s, CD90, EpCAM, ALDH1, and ABCG5, and their expression within tumor buds was evaluated.

**RESULTS:** CD90, CD44s, and CD133 expression in tumor buds was found in less than 5% of all cases. ALDH1, CD24, CD166 were expressed in 16.5%, 16.2%, and 34% cases, respectively, while ABCG5 and EpCAM expression was more frequent and found in 35% and 69% of cases, respectively. Of the 8 markers studied, EpCAM and ABCG5 positivity in tumor buds were significantly associated with poor prognosis ( $P = 0.023$ ,

$P = 0.038$ , respectively) in multivariable analysis with pT and pN classification [ $P = 0.048$ ; hazard ratio (HR): 2.64; 95% CI: 1.0-6.9, for EpCAM and  $P = 0.029$ ; HR: 2.22; 95% CI: 1.0-4.5, for ABCG5]. Poor survival time was particularly striking for lymph node-negative patients with ABCG5-positive buds ( $P < 0.001$ ).

**CONCLUSION:** Expression of putative stem cell markers EpCAM and ABCG5 within the tumor buds of colorectal cancer are frequently noted and are associated with poor prognosis.

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**Key words:** Colorectal cancer; Cancer stem cells; Tumor budding; ABCG5; Prognosis

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### INTRODUCTION

In 1985, Gabbert and colleagues described a peculiar feature at the invasive border of differentiated colonic tumors: neoplastic glands irregularly arranged into small strands or single cells without junctional complexes and often missing even rudimentary basement membranes<sup>[1,2]</sup>.

Their observation of the tumor front of differentiated adenocarcinomas focally acquiring the phenotype of undifferentiated tumors is credited for pioneering the concept commonly referred to today as epithelial mesenchymal transition (EMT) and represented in colorectal cancer by its histological hallmark “tumor budding”.

Defined as single cells or clusters of up to 4 or 5 cells at the invasive tumor front, tumor budding can easily be spotted using pan-cytokeratin stains and is highly associated with an infiltrating tumor border configuration<sup>[3]</sup>. The adverse prognostic impact of tumor budding in colorectal cancer has consistently been reported and recognized by the American Joint Committee on Cancer/Union International Contre le Cancer (AJCC/UICC) as an additional prognostic factor to complement Tumor Node Metastases (TNM) staging<sup>[4-13]</sup>. Moreover, tumor budding is frequently linked to high-grade tumors, lymph node positivity, vascular and lymphatic invasion, as well as to both local tumor recurrence and distant metastasis<sup>[11,14-17]</sup>.

Several lines of evidence seem to suggest that tumor buds may, to some extent, represent malignant colorectal cancer stem cells (CSC) because of their potential for migration and re-differentiation locally and at sites of metastasis<sup>[18]</sup>. “Pseudopodia-like” cytoplasmic protrusions have been described in tumor buds, which seem to be in direct contact with adjacent interstitial tissue suggesting their formation during cell migration<sup>[2,19,20]</sup>. Previous studies on EMT and events occurring at the invasive tumor front implicate, in particular, Wnt pathway signaling in the process of tumor budding<sup>[21]</sup>. This is evidenced by increased  $\beta$ -catenin immunohistochemical staining in tumor buds, a concomitant loss of E-cadherin, as well as overexpression of laminin5 $\gamma$ 2 along with activation of transcriptional repressors SLUG, and ZEB1<sup>[19,22,23]</sup>. Other groups have described changes in the expression of several matrix metalloproteinases (MMP-2, MMP-7, MMP-9), and extensive staining of  $\beta$ (III)-tubulin, a major constituent of microtubules, all suggestive of invasion and migration potential of tumor buds<sup>[24-26]</sup>. Together with loss of epithelial-like properties and cell-cell adhesion, in addition to the ability to re-differentiate at distant sites, the hypothesis that tumor buds could represent putative migrating stem cells is not far-fetched.

Phenotypic characterization of colorectal CSC is still debated although putative CSC populations have been identified in several solid tumors based on functional stem cell-like properties and expression of specific markers. Recently, 4 such markers have been proposed for colorectal cancer; CD133, a glycoprotein expressed on CD34+ stem and progenitor cells in fetal liver, endothelial precursors and fetal neural stem cells; CD44s, an adhesion molecule with roles in signaling, migration, and homing, EpCAM, a homophilic Ca<sup>2+</sup>-independent cell adhesion molecule expressed on the basolateral surfaces of most epithelial cells; and CD166 or activated leukocyte cell adhesion molecule (ALCAM) known as a mesenchymal stem cell marker<sup>[27]</sup>. Other putative stem cell markers have also generated interest in other tumor types including ABCG5, a member of the ATP binding cassette family involved in

transport of sterol and other lipids, ALDH1, a member of the aldehyde dehydrogenase family of enzymes with roles in proliferation, differentiation, and survival, CD24, an adhesion molecule and ligand for P-selectin, and CD90, a mediator of thymocyte adhesion to thymic stroma<sup>[28]</sup>.

Considering the apparent stem cell-like properties of tumor buds and adverse effect of budding on clinical outcome, we hypothesized that expression of a subset of these 8 putative stem cell markers could have significant implications for prognosis in patients with positive tumor budding. Thus, the aim of this study was to determine the impact of CD166, CD44s, EpCAM, ALDH1, CD133, CD24, CD90, and ABCG5 expressed within tumor buds on prognosis in patients with colorectal cancer.

## MATERIALS AND METHODS

### Patients

Three hundred patients with pre-operatively untreated tumors who underwent tumor resection between 1987 and 1996 at the University Hospital of Basel, Switzerland were initially included in this study. These patients were randomly selected from a larger previously described cohort of 938 colorectal cancer patients with full clinicopathological information<sup>[29]</sup>. Histopathological features were re-reviewed from the corresponding hematoxylin and eosin slides by an experienced gastrointestinal pathologist (LT) and included histological subtype, pT classification, pN classification, tumor grade, and vascular invasion. Tumor border configuration and peritumoral lymphocytic inflammation were diagnosed according to Jass *et al.*<sup>[30]</sup>. Clinical data were retrieved from patient reports including age at diagnosis, tumor diameter, and tumor location. The clinical endpoint of interest was cancer-specific survival time. Censored observations included patients who died for reasons other than colorectal cancer, who were alive or who were lost to follow-up. The study design is outlined in Figure 1.

### Specimen characteristics

The paraffin-embedded colorectal cancer resection specimens for all 300 patients were retrieved from the archives of the Institute of Pathology, University Hospital of Basel as well as at the Institute of Clinical Pathology, Basel, Switzerland. The use of material for this study was approved by the local ethics committee of the University of Basel.

### Assay methods

**Immunohistochemistry for CK22 staining:** All 300 specimens were cut at 4  $\mu$ m and underwent immunostaining for CK22, a marker of epithelial cells that served to highlight areas of tumor budding, and which is routinely performed in our laboratories for diagnostic purposes. Briefly, tissues were de-waxed and re-hydrated in dH<sub>2</sub>O. Following pressure cooker-mediated antigen retrieval in 0.001 mol/L ethylenediaminetetraacetic acid pH 8.0, endogenous peroxidase activity was blocked using 0.5% H<sub>2</sub>O<sub>2</sub>. Sections were incubated with 10% normal



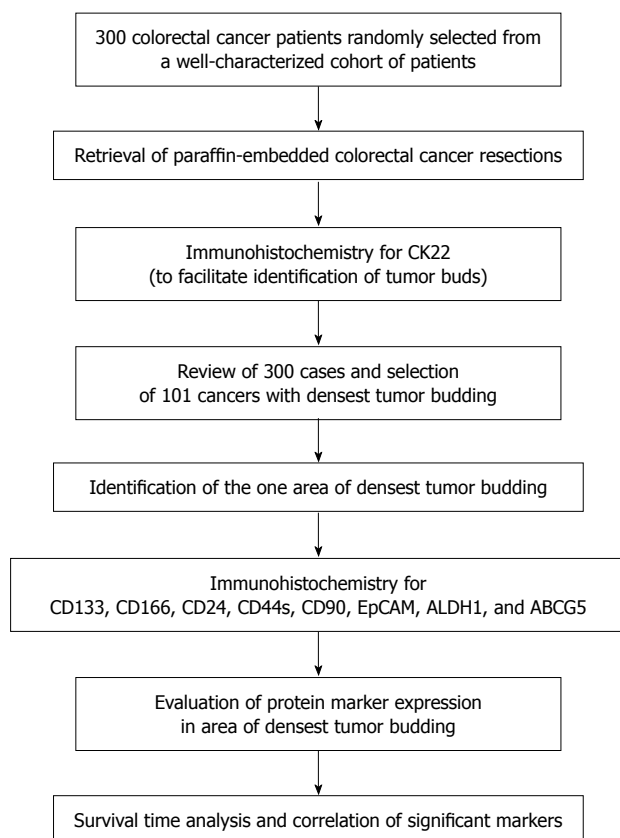


Figure 1 Study design.

goat serum for 20 min. After incubation with primary antibody (CK22 polyclonal, Genetex, Inc., 1:100), sections were incubated with horseradish peroxidase-conjugated secondary antibody (DakoCytomation) for 30 min at room temperature, immersed in amino-ethylcarbazole (DakoCytomation) for 30 min, and counterstained with hematoxylin.

**Selection of densest budding cases:** All 300 cases were evaluated using a 10 × magnification for the presence of tumor budding (AL). Since this study was designed to focus on expression of putative stem cell markers within the tumor buds themselves, cases with the densest number of budding cells were selected for the analysis ( $n = 101$ ). These 101 cases were then carefully re-scored for tumor budding according to the method proposed by Ueno *et al.*<sup>[11]</sup>. Briefly, the tumor border was scanned at 10 × power and the area of most dense budding identified. In the center of this area, tumor buds (single cells or clusters of up to 5 cells) were counted at 20 × magnification. In order to locate this same region of dense budding on serial sections, the area was circled with a felt-tip pen. The clinico-pathological features for these 101 patients are outlined in Table 1.

**Immunohistochemistry for putative stem cell markers:** Following a similar protocol as described above, the 101 cases with densest tumor budding were immunostained for CD166 (clone 110G/07; 1:200; Novocastra), CD44s (clone DF1485; 1:50; Dako), EpCAM (clone VU-1D9; 1:200; Cell

Table 1 Patient characteristics ( $n = 101$ )

Clinico-pathological features		Frequency $n$ (%)
Gender ( $n = 101$ )	Female	63 (62.4)
	Male	38 (37.6)
Tumor location ( $n = 101$ )	Left-sided	64 (63.4)
	Right-sided	37 (36.6)
Histological subtype ( $n = 101$ )	Mucinous	7 (6.9)
	Non-mucinous	94 (93.1)
pT classification ( $n = 99$ )	pT1-2	16 (16.2)
	pT3-4	83 (83.8)
pN classification ( $n = 100$ )	pN0	52 (52.0)
	pN1-2	48 (48.0)
Tumor grade ( $n = 99$ )	G1-2	92 (92.9)
	G3	7 (7.1)
Vascular invasion ( $n = 99$ )	Absence	80 (80.8)
	Presence	19 (19.2)
Tumor border configuration ( $n = 99$ )	Pushing	23 (23.2)
	Infiltrating	76 (76.8)
Peritumoral lymphocytic inflammation ( $n = 99$ )	Absent	79 (79.8)
	Present	20 (20.2)
Age ( $n = 101$ )	Mean (range)	67.4 (41-89)
Tumor diameter ( $n = 101$ )	Mean (range)	54.9 (20-170)
5-year survival rate ( $n = 101$ )	% (95% CI)	69.3 (59-78)

Signaling), ALDH1 (isoform  $\alpha 1$ , clone Polyclonal; 1:500; AbCam), CD133 (clone 24139; 1:100; Cell Signaling), ABCG5 (1:200, Sigma-Aldrich), CD90 (clone 5E10, 1:100, BD Pharmingen), CD24 (clone SN3B, Neomarkers, 1:100). CD133, CD166, CD44, CD24, CD90, EpCAM, and ABCG5 were evaluated for both membrane and cytoplasmic staining; ALDH1 was exclusively evaluated in the cytoplasm of tumor buds. The number of CD166, CD44s, EpCAM, ALDH1, CD133, CD24, CD90, and ABCG5 positive tumor buds was then evaluated in the area of densest tumor budding as determined by CK22 staining.

### Statistical analysis

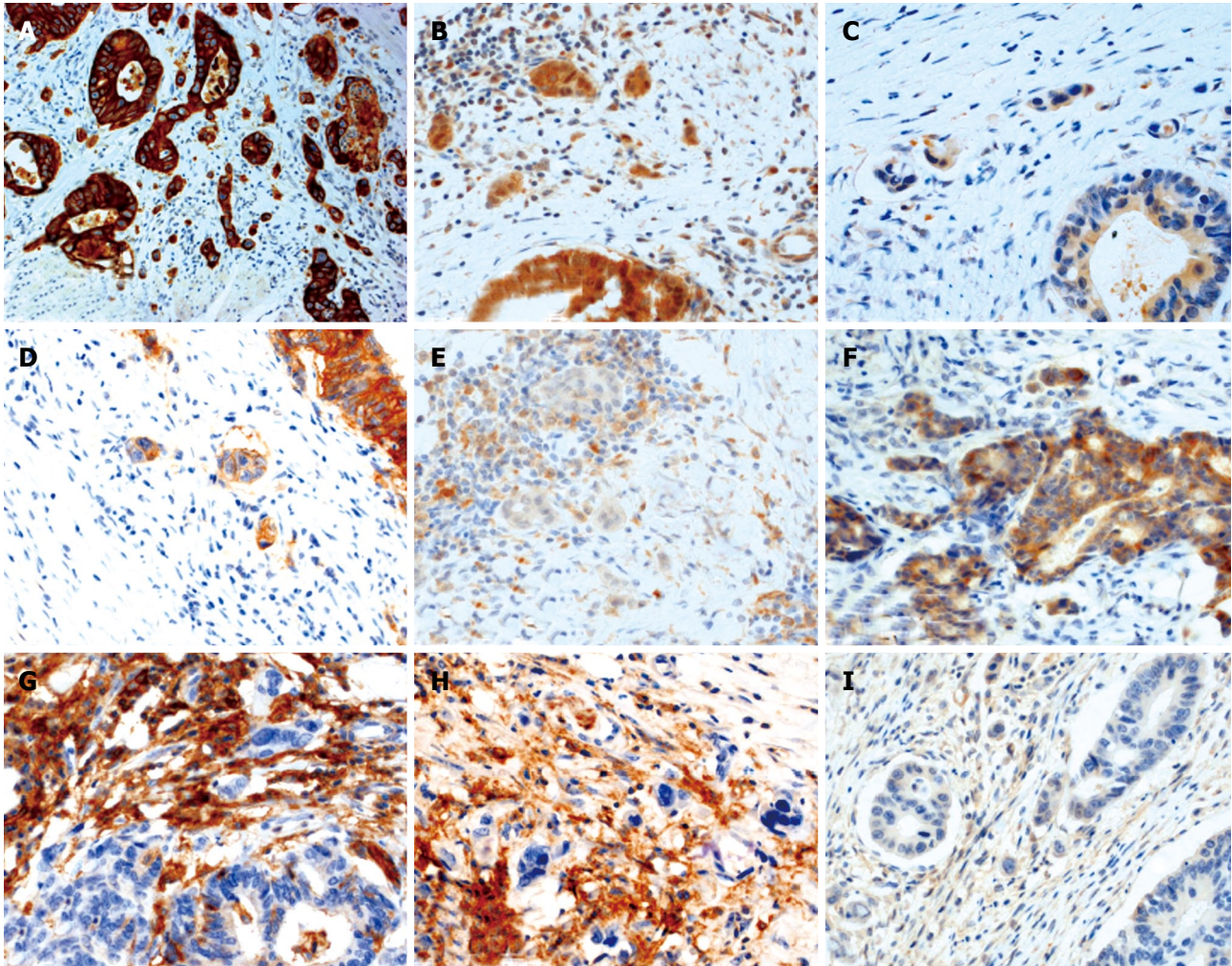
Univariate survival analysis was carried out using the Kaplan-Meier method and log rank test. Two multivariable Cox regression analyses were performed. First, to test the independent prognostic value of tumor budding, the effects of pT stage, pN stage, tumor grade, and vascular invasion were adjusted for. Subsequently, because of the small number of positive cases, only 2 variables could be entered into the multivariable Cox regression analysis along with positive expression of the protein in tumor buds, hence pT classification and pN classification were selected. The assumption of proportional hazards was verified prior to this analysis. Hazard ratios (HR) and 95% CI were obtained to determine the prognostic effect of positive cases adjusting for pT and pN. Kendall's correlation coefficient ( $r$ ) was obtained for correlation analysis of markers.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Prognostic value of tumor budding

In order to confirm the prognostic value of tumor budding in our series, cases were divided into 3 groups





**Figure 2** Immunohistochemical expression of putative cancer stem cell markers by tumor buds in colorectal cancer. A: Cytokeratin 22 staining highlighting the presence of tumor buds in low power magnification (5 ×); B-I: 40 × magnification. Positive expression of ABCG5 (B), ALDH1 (C), and EpCAM (D) in tumor buds with scattered positive staining of stromal cells, absence of staining of CD133 in tumor buds with positive staining of stromal cells (E), positive expression of CD166 in tumor buds with occasional positivity of stromal cells (F), absence of CD24 (G), CD44s (H), and CD90 staining (I) in tumor buds with positive stromal cell expression.

based on the distribution of number of tumor buds: those with < 40 buds, between 41-60 buds, and finally those with > 60 buds per 20 × field. The greater the number of tumor buds the more unfavorable was the prognosis both in univariate ( $P < 0.001$ ) and multivariable analysis with pT, pN, tumor grade, and vascular invasion (HR: 1.6, 95% CI: 1.2-2.1).

#### Expression of putative stem cell markers within tumor buds

CK22 staining was used to identify regions of densest tumor budding with epithelial cells exclusively immunoreactive for the protein. Staining for ABCG5, ALDH1, CD133, CD166, CD24, and CD44s could be observed in both tumor cells and inflammatory or stromal cells. EpCAM staining was predominantly limited to expression in tumor cells whereas CD90 was almost always expressed by stromal cells and only in 3 cases in the tumor itself.

Marker expression was then evaluated in the area of densest budding. Representative immunostains for all markers are shown in Figure 2. Only one case (1.03%)

was positive for CD90, while 5 (5.1%) and 6 (6.1%) cases were positive for CD44s and CD133, respectively. On the other hand, a considerably larger number of positive cases was found to express ALDH1 (16/97, 16.5%), CD24 (16/99, 16.2%) and CD166 (34/100, 34%). Finally, ABCG5 and EpCAM staining were frequent events with 39/97 (40.2%) and 69/100 (69%) positive cases, respectively (Figure 3).

#### Prognostic differences with putative stem cell marker expression in tumor buds

No relationship between survival time and ALDH1, CD24 and CD166 was observed. Patients with positive EpCAM or ABCG5 within tumor buds had a significantly poorer outcome in comparison to patients with no expression of these markers ( $P = 0.023$  and  $P = 0.038$ , respectively) (Figure 4). Multivariable analysis was performed for EpCAM and ABCG5 along with pT and pN classification. EpCAM maintained its significant association with a negative effect on outcome (HR: 2.64, 95% CI: 1.0-6.9,  $P = 0.048$ ), adjusted for pT and pN classification, a result

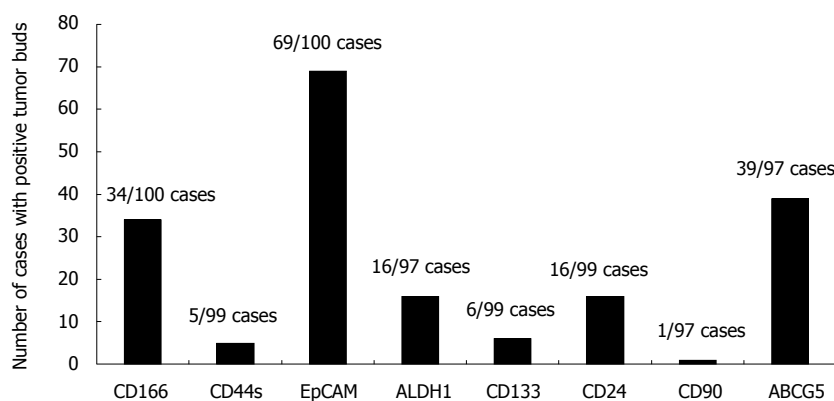


Figure 3 Histogram showing the number of cases with any degree of positive staining for the 8 putative stem cells markers.

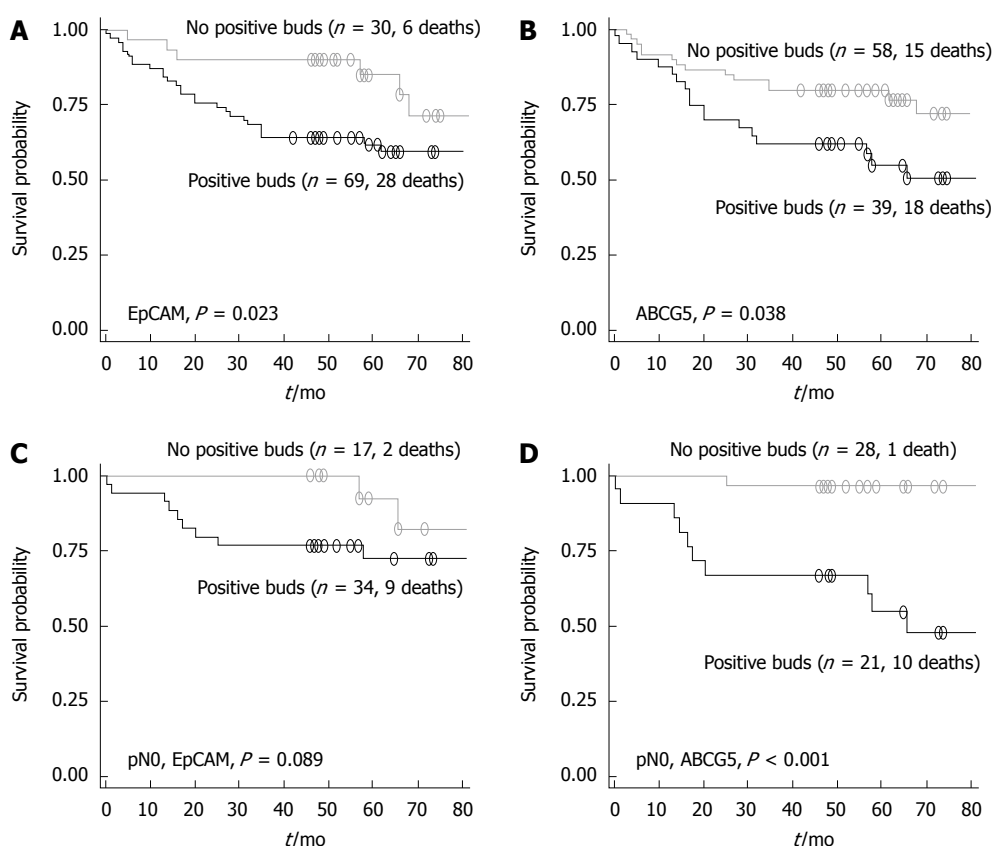


Figure 4 Kaplan-Meier survival curves illustrating the prognostic differences in patients with or without positive staining of EpCAM (A) and positive staining of ABCG5 (B) in tumor buds; differences in prognosis are further analyzed for lymph node-negative patients differing in EpCAM (C) and ABCG5 (D) expression in tumor buds.

which was also pronounced in patients with lymph node-negative disease. Similarly, positive ABCG5 expression in tumor buds was again associated with a poor patient prognosis ( $P = 0.029$ ) underlined by a relative risk of death of 2.22 (95% CI: 1.0-4.5) compared to patients lacking expression of ABCG5. ABCG5-positive patients with lymph node-negative cancers had a particularly poor outcome in comparison to their node-negative and ABCG5-negative counterparts ( $P < 0.001$ ).

#### Correlation between EpCAM and ABCG5 expression

In order to determine whether the same cases expressed

both EpCAM and ABCG5, the correlation between these markers was tested. The correlation coefficient  $r = 0.17$  and  $P = 0.08$ , indicated a positive but non-significant trend in the expression of these markers. Of the 96 patients evaluable for both EpCAM and ABCG5, 31 (32.3%) were positive and 21 (21.9%) were negative for both markers. We subsequently tested whether the combination of these markers could additionally stratify patients into prognostic subgroups. Prognosis was worse in patients positive for both EpCAM and ABCG5 ( $P = 0.013$ ) with a relative risk of death of 2.39 (95% CI: 1.2-4.7) compared to patients negative for both. In comparison to



the relative risk of death for either EpCAM or ABCG5 alone, the combination of both markers does not suggest a superior discrimination of patients into better and worse prognostic subgroups. A negative but statistically non-significant correlation between CD44s and EpCAM ( $r = -0.15$ ,  $P = 0.145$ ) and ABCG5 ( $r = -0.1$ ,  $P = 0.328$ ) was observed.

## DISCUSSION

In this study we evaluated 8 of the most promising putative cancer stem cell markers CD166, CD44s, EpCAM, ALDH1, CD133, CD24, CD90, and ABCG5 and their expression in colorectal tumor buds using 101 whole tissue sections from a well-characterized cohort of patients. Our main findings suggest that positive expression of EpCAM and ABCG5 within tumor buds is a frequent event and may confer a significant and adverse prognosis in patients with colorectal cancer, particularly in lymph node-negative patients expressing ABCG5.

Several of these putative CSC markers have previously been evaluated in tumor buds. Horst *et al.*<sup>[31]</sup> assessed CD133 in colorectal cancers using 3 different antibodies. They reported pronounced expression of CD133 in tumor glands close to the invasive margin but restricted to glandular differentiated cells and a general lack of CD133 in the tumor buds themselves. They further found that nuclear  $\beta$ -catenin expression and CD133 were not correlated and that the 2 protein markers may stain different, yet overlapping populations of tumor cells<sup>[32]</sup>. Our results of only a few CD133-positive tumor budding cases and no prognostic differences between patients with CD133-positive and -negative tumor budding are in line with these findings. Investigating rectal cancers, Gosens *et al.*<sup>[33]</sup> found strong membranous EpCAM staining in the tumor center and a progressive loss at the tumor front associated with high tumor grade, tumor budding, and a poor local and distant recurrence-free survival. This was also accompanied by a concomitant increase in cytoplasmic EpCAM staining as well as overexpression of  $\beta$ -catenin. We also observed a pronounced loss of EpCAM toward the invasive tumor front, particularly in tumors with infiltrating margins, as well as a shift in localization of EpCAM expression from membrane to cytoplasm. The findings of this study indicate that despite this loss towards the border, patients with EpCAM-positive tumor buds have a most unfavorable survival time, a result which was maintained in multivariable analysis. Although EpCAM, like CD44, is known for its cell-adhesion function, it seems to have versatile roles in signaling, cell migration, proliferation, and differentiation depending on the microenvironment<sup>[34]</sup>. In the normal epithelium, EpCAM supports adhesion, whereas in carcinoma it seems to prevent strong cell-cell adhesion, enabling cell migration and metastasis similar to E-cadherin. The intracellular localization of EpCAM and its identification by immunohistochemistry may represent differential roles of this protein in colorectal cancer

progression and partially explain why, despite loss of expression from normal at tumor center to tumor border, the positive expression in buds is linked to a poorer patient outcome.

Masaki *et al.*<sup>[35]</sup> have also described associations between membranous CD44 and CD44v6 expression and a higher degree of tumor budding. However, it is unclear from these studies whether expression was evaluated in the tumor center, then correlated with tumor budding or whether expression was evaluated in buds themselves. Our group has also previously found that loss of membranous expression of both CD44s and CD44v6 within the tumor center is highly correlated with an infiltrating tumor border configuration, a result which is in line with the findings of this study showing only rare cases expressing CD44s in tumor buds, too few in fact for adequate survival analysis.

ABCG5 is a member of the ATP-binding cassette subfamily G and plays a role in the efflux transport of cholesterol<sup>[36,37]</sup>. Its expression has been correlated with clinical melanoma progression and it is hypothesized to contribute to the refractoriness of metastatic cancer to chemotherapy<sup>[38]</sup>. Indeed, specific targeting of ABCG5 with monoclonal antibodies appears to significantly inhibit cell growth. To date, ABCG5 does not appear to have been investigated in colorectal cancer, and moreover in tumor buds. However, our findings of ABCG5 expression in a considerable number of colorectal cancer tumor buds as well as an adverse prognosis in particular in patients with lymph node-negative disease suggests that the role of ABCG5 in colorectal pathogenesis warrants further investigation.

Our results of adverse prognosis in EpCAM-positive and ABCG5-positive patients may be to some extent affected by the lack of information regarding cancer treatment. Despite this limitation, the unfavorable outcome associated with EpCAM and, particularly with ABCG5-positivity was maintained in patients with lymph node-negative colorectal cancers who, by today's treatment guidelines, are not generally considered for adjuvant chemotherapy<sup>[39]</sup>. The findings of this study regarding the prognostic value and expression of EpCAM and ABCG5 within colorectal tumor buds should be considered preliminary and require validation on independent patient cohorts.

To summarize, in contrast to CD133, CD166, CD24, CD44s, CD90, and ALDH1, the expression of putative stem cell markers EpCAM and ABCG5 within the tumor buds of colorectal tumors are frequent events indicating poor prognosis. In particular, patients with lymph node-negative disease expressing EpCAM or ABCG5 have a particularly unfavorable prognosis suggesting that the immunohistochemically analyzed EpCAM and ABCG5 in tumor buds may be useful biomarkers of poor outcome in this subgroup of patients. Further studies are necessary to address the important issue of whether EpCAM- or ABCG5-positive tumor buds indeed represent migrating colorectal CSC.

## COMMENTS

### Background

Tumor budding at the invasive tumor front of colorectal cancer is recognized as an important independent prognostic factor. Several lines of evidence seem to suggest that tumor buds may to some extent represent malignant colorectal cancer stem cells because of their potential for migration and re-differentiation locally and at sites of metastasis.

### Research frontiers

Phenotypic characterization of cancer stem cells is still debated although at least 8 putative stem cell markers have been suggested including CD166, CD44s, EpCAM, ALDH1, CD133, CD24, CD90, and ABCG5. The research hotspot is how the expression of putative cancer stem cell markers can be potentially used as prognostic biomarkers in patients with colorectal cancer.

### Innovations and breakthroughs

Considering the apparent stem cell-like properties of tumor buds and adverse effect of budding on clinical outcome, in this study the authors performed immunohistochemical staining of 8 promising putative cancer stem cell markers, namely CD166, CD44s, EpCAM, ALDH1, CD133, CD24, CD90, and ABCG5 and assessed their expression within tumor buds to determine their frequency and potential prognostic significance in patients with colorectal cancer.

### Applications

The study results suggest that, in contrast to CD133, CD166, CD24, CD44s, CD90, and ALDH1, the expression of putative cancer stem cell markers EpCAM and ABCG5 within the tumor buds of colorectal cancer are frequent events associated with poor prognosis.

### Terminology

Tumor budding: single cells or clusters of up to 4 or 5 cells at the invasive tumor front of colorectal cancer which are diagnosed at high magnification and highly associated with an infiltrating tumor growth pattern. Cancer stem cells: tumorigenic cell populations with the potential to self-renew and differentiate.

### Peer review

The study is characterized technically by an excellent application of immunohistochemistry and provides interesting evidence to aid in understanding the correlation between cancer stem cell markers in the invasive front of colorectal cancer and prognosis.

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## Pericardiocentesis with cisplatin for malignant pericardial effusion and tamponade

Takatsugu Oida, Kenji Mimatsu, Hiso Kano, Atsushi Kawasaki, Youichi Kuboi, Nobutada Fukino, Sadao Amano

Takatsugu Oida, Kenji Mimatsu, Hiso Kano, Atsushi Kawasaki, Youichi Kuboi, Nobutada Fukino, Department of Surgery, Social Insurance Yokohama Central Hospital, 268 Yamashita-cho, Naka-ku, Yokohama 231-8553, Japan

Sadao Amano, Department of Surgery, Nihon University School of Medicine, 30-1 Kami-cho, Oyaguchi, Itabashi-ku, Tokyo 177-0023, Japan

**Author contributions:** Oida T, Mimatsu K, Kano H, Kawasaki A, Kuboi Y and Fukino N carried out the operation and were consultants overseeing the patient's care; Oida T wrote the manuscript; Amano S drafted the manuscript and revised it critically.

**Correspondence to:** Takatsugu Oida, MD, PhD, Department of Surgery, Social Insurance Yokohama Central Hospital, 268 Yamashita-cho, Naka-ku, Yokohama 231-8553, Japan. [ooida.takatsugu@yokochu.jp](mailto:ooida.takatsugu@yokochu.jp)

Telephone: +81-45-6411921 Fax: +81-45-6719871

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was  $7.7 \pm 2.7$  d (range, 5-13 d). No fluid reaccumulation was observed. Mean survival time was  $120 \pm 71$  d (range, 68-268 d).

**CONCLUSION:** Pericardiocentesis along with catheter drainage appears to be a safe and effective for pericardial malignant effusion and tamponade, and cisplatin instillation prevents recurrence.

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**Key words:** Malignant pericardial effusion; Cardiac tamponade; Esophageal cancer; Pericardiocentesis; Cisplatin

**Peer reviewers:** Nadia Peparini, MD, PhD, Department of General Surgery "Francesco Durante", La Sapienza University, Viale del Policlinico, 155, 00161 Rome, Italy; Mark Bloomston, MD, FACS, Assistant Professor, Division of Surgical Oncology, The Ohio State University, N924 Doan Hall, 410W. 10th Avenue, Columbus, OH 43082, United States

### Abstract

**AIM:** To evaluate the role and outcome of pericardiocentesis with intrapericardial cisplatin instillation for malignant pericardial effusion resulting from esophageal cancer.

**METHODS:** We retrospectively studied 7 patients who underwent pericardiocentesis with intrapericardial cisplatin instillation for malignant pericardial effusion resulting from esophageal cancer. After pericardiocentesis, we performed catheterization of the pericardial space under ultrasonogram guidance. Malignant etiology of the pericardial fluid was confirmed by cytological examination. Subsequently, cisplatin (10 mg in 20 mL normal saline) was instilled into the pericardial space.

**RESULTS:** The mean total volume of the aspirated effusion fluid was  $782 \pm 264$  mL (range, 400-1200 mL). The drainage catheter was successfully removed in all patients, and the mean duration of pericardial drainage

Oida T, Mimatsu K, Kano H, Kawasaki A, Kuboi Y, Fukino N, Amano S. Pericardiocentesis with cisplatin for malignant pericardial effusion and tamponade. *World J Gastroenterol* 2010; 16(6): 740-744 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i6/740.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i6.740>

### INTRODUCTION

The most frequent causes of spontaneous pericardial tamponade are neoplastic invasion, idiopathic or infectious pericarditis, and uremia. Pericardial effusion is a known complication of many advanced malignancies, and has a strong impact on both the quality of life and prognosis. Malignant pericardial effusion and tamponade is a rare medical-surgical emergency that impairs cardiac function and causes death. To facilitate the diagnosis, it is necessary to identify the clinical features of this condition, and

design adequate management strategies. Esophagectomy with reconstruction of the esophagus is associated with many fatal complications such as infection, anastomotic leakage, and respiratory and hemodynamic instability. The clinical syndrome of pericardial tamponade after esophagectomy has been relatively well reported. Acute distension of the stomach<sup>[1]</sup>, herniation of the omentum<sup>[2]</sup>, mediastinal bleeding<sup>[3]</sup> and even volvulus of the interposed colon<sup>[4]</sup> have been implicated as the clinical syndromes of pericardial tamponade. Moreover, the development of cardiac tamponade due to intra-pericardial fluid accumulation after esophagectomy has also been reported<sup>[5,6]</sup>.

Here, we report the outcome of intrapericardial instillation of cisplatin (CDDP) for the treatment of malignant pericardial effusion and tamponade resulting from esophageal carcinoma.

## MATERIALS AND METHODS

### Patients

We retrospectively studied 7 male patients who underwent pericardiocentesis with intrapericardial instillation of CDDP for the treatment of esophageal cancer at the Department of Surgery, Social Insurance Yokohama Central Hospital, Yokohama, Japan, between March 1997 and April 2009. Their mean age was  $67 \pm 3.4$  years (range, 61-71 years). Of the 7 patients, 5 underwent subtotal esophagectomy *via* a standard right thoracoabdominal approach along with three-field lymphadenectomy and reconstruction was performed using gastric pull-up with cervical anastomosis *via* the poststernal route, and the remaining 2 patients had undergone chemoradiotherapy previously.

### Diagnosis and definition of severity of pericardial effusion and tamponade

Echocardiography was used for the diagnosis of pericardial effusion and tamponade. When the diastolic echo-free space between the left ventricular posterior wall and the pericardium was  $< 10$  mm, the condition was classified as mild; when the space was 10-20 mm, the condition was classified as moderate; and when the space was  $> 20$  mm, the condition was classified as severe pericardial effusion<sup>[7]</sup>. Cardiac tamponade was defined according to the clinical and echocardiographic criteria<sup>[8]</sup>. The presence of the classic tamponade symptoms such as tachycardia, dyspnea, or tachypnea with clear lungs, or signs of increased systemic venous pressure, hypotension, or pulsus paradoxus, or accompanied by echocardiographic findings was accepted as cardiac tamponade<sup>[7]</sup>. We performed pericardiocentesis on the patients with cardiac tamponade and moderate to severe pericardial effusion.

### Percutaneous pericardiocentesis and cytology

Echocardiography with fluoroscopy-guided subxiphoid pericardiocentesis using an 8-French pigtail drainage catheter with multiple side holes (Aspiration Seldinger Kit/Nippon Sherwood Medical Industries LTD) was

performed under local anesthesia with mild sedation. After the catheter was placed into the pericardial space, we aspirated the fluid. Cytological examination was performed using the aspirate culture.

### Instillation of CDDP

After the cytological examination-based confirmation of the malignant etiology of the pericardial fluid and the complete drainage of the fluid, we administered 10 mg of CDDP into the pericardial space during each pericardiocentesis *via* the catheter; subsequently, the catheter was clamped. The catheter was declamped the following day, and the fluid was re-aspirated. When the volume of the re-aspirated fluid was more than 30 mL, 10 mg CDDP was re-administered. When the volume of the re-aspirated fluid was less than 30 mL, the catheter was removed.

## RESULTS

Echocardiography with fluoroscopy-guided pericardiocentesis using extended catheter drainage was performed in 7 patients with malignant cardiac tamponade resulting from esophageal cancer. Table 1 shows the patients' characteristics. Staging of esophageal cancer was performed according to guidelines of the International Union Against Cancer Classification of Malignant Tumors (UICC), and the distribution of the various stages among the patients was as follows: II B, 1/7 (14.3%); III, 3/7 (42.8%); and IV A, 3/7 (42.8%). The following clinical symptoms were observed in the patients; dyspnea was observed in all the patients (100%), and tachycardia was observed in 5 patients (71.4%).

Table 2 lists the outcomes of the patients who underwent pericardiocentesis with CDDP instillation. Total volume of the aspirated effusion fluid ranged from 400 to 1200 mL (median,  $782 \pm 264$  mL). Pericardiocentesis with CDDP instillation was required twice in 2 patients (28.6%), 3 times in 4 patients (57.1%), and 5 times was in 1 patient (14.3%), (median, 3 times). The drainage catheter was successfully removed in all the patients. The duration of pericardial drainage ranged from 5 to 13 d (median,  $7.7 \pm 2.7$  d). None of the patients showed fluid accumulation. Nausea, as a side effect of pericardiocentesis with CDDP instillation, was observed in 2 patients (28.6%); however, hematologic and renal toxicity did not develop in any of the patients. After pericardiocentesis with CDDP instillation, we performed systemic chemotherapy [5-fluorouracil (5-FU) + CDDP] in 3 patients (43%). The overall median survival time for these 7 patients was  $120 \pm 71$  d (range, 68-268 d) (Table 3).

## DISCUSSION

Cardiac tamponade can occur in any type of pericarditis case, but it is more commonly observed in neoplastic, tuberculous, and purulent pericarditis cases than in viral or idiopathic pericarditis cases. Pericardial effusion is a known complication of many advanced malignancies,

Table 1 Characteristics of the patients

Patient No.	Age	Sex	Stage of esophageal cancer	Former treatment	Symptoms
1	68	M	T2, N1, M0: Stage II B	Esophagectomy + chemotherapy	Dyspnea, tachycardia
2	69	M	T3, N1, M0: Stage III	Esophagectomy + chemotherapy	Dyspnea, tachycardia
3	64	M	T2, N1, M0: Stage III	Esophagectomy + chemotherapy	Dyspnea
4	70	M	T3, N1, M1a: Stage IVA	Esophagectomy + chemotherapy	Dyspnea, tachycardia
5	71	M	T2, N1, M1a: Stage IVA	Chemoradiotherapy	Dyspnea, tachycardia
6	65	M	T2, N1, M0: Stage II B	Esophagectomy + chemotherapy	Dyspnea, tachycardia
7	62	M	T2, N1, M1a: Stage IVA	Chemoradiotherapy	Dyspnea

Table 2 Outcomes of intrapericardial instillation of cisplatin

Patient No.	Amount of fluid (mL)	CDDP instillation (mg) × times	Duration of drainage (d)	Side effect	Reaccumulation of fluid
1	580	10 × 2	5	-	-
2	400	10 × 2	5	-	-
3	760	10 × 3	8	-	-
4	980	10 × 3	8	Nausea	-
5	1200	10 × 5	13	Nausea	-
6	685	10 × 3	8	-	-
7	870	10 × 3	8	-	-

CDDP: Cisplatin.

Table 3 Outcomes of the patients

Patient No.	Additional therapy (systemic)	Survival (d)	Cause of death
1	5-Fu + CDDP	126	Lung and pleural metastases
2	5-Fu + CDDP	268	Pleural metastases
3	5-Fu + CDDP	137	Lung and bone metastases
4	-	68	Lung and pleural metastases
5	-	61	Lung and bone metastases
6	-	104	Lung and pleural metastases
7	-	77	Pleural metastases

5-Fu: 5-fluorouracil.

and it has a strong impact on both the quality of life and prognosis. To facilitate the diagnosis of patients with malignant disease, it is necessary to identify the clinical features of cardiac tamponade and design adequate management strategies. Cardiac tamponade, observed in up to 15% of patients with cancer, can develop because of the malignant involvement of the pericardium under metastatic disease conditions, contiguous extension, or primary involvement<sup>[9]</sup>. Pericardial effusion in patients with esophageal carcinoma is most commonly associated with radiation and/or chemotherapy, and rarely with esophago-pericardial fistula<sup>[10]</sup>. All our 7 patients had advanced esophageal cancer; of them, 5 patients underwent esophagectomy and chemotherapy and the remaining 2 had undergone chemoradiotherapy previously.

The following are the typical signs of acute cardiac tamponade; a decrease in the arterial blood pressure, increase in the central venous pressure, and a small, quiet heart. The diagnosis of pericardial effusion and cardiac tamponade was confirmed using echocardiography and according to the clinical and echocardiographic criteria<sup>[8]</sup>. Because all our patients showed symptoms of dyspnea with or without tachycardia, it was necessary to confirm the diagnosis using echocardiography.

Accumulation of fluid in the pericardial space in patients is often not evident until the development of cardiac tamponade. Pericardiocentesis is generally performed as an initial treatment for symptomatic pericardial effusion and cardiac tamponade; however, re-accumulation of the fluid after pericardiocentesis is often observed. Hence, alternative procedures to pericardiocentesis, including insertion of a pleuropericardial window<sup>[11]</sup>, total or partial pericardiectomy<sup>[12]</sup>, external radiotherapy<sup>[13]</sup>, local instillation of a chemotherapeutic agent<sup>[14]</sup>, local instillation of a sclerosing agents<sup>[15]</sup>, and systemic chemotherapy<sup>[16]</sup>, are often performed. A review article that summarized the results of previous studies showed that the overall success rate of pericardiocentesis was 44.4%; indwelling pericardial catheters, 76.3%; and intrapericardial administration of sclerosing or cytotoxic agents, 81.6%, and that intrapericardial instillation was an effective treatment strategy for malignant pericardial effusion<sup>[17]</sup>.

The most serious complications of pericardiocentesis are laceration and perforation of the myocardium and the coronary vessels. Safe execution of pericardiocentesis was achieved by performing echocardiography under fluoroscopic guidance. Recent large echocardiographic series have shown that the incidence of major compli-



cations after echocardiography was 1.3%-1.6%. In fluoroscopy-guided percutaneous pericardiocentesis, cardiac perforations occurred in 0.9% cases, serious arrhythmias in 0.6%, arterial bleeding in 1.1%, pneumothorax in 0.6%, infection in 0.3%, and a major vagal reaction in 0.3%<sup>[8,18]</sup>. In our study, pericardiocentesis was performed echocardiographically under fluoroscopy guidance, and no complications developed. We believe that echocardiography with fluoroscopy-guided pericardiocentesis appears to be a safer alternative for pericardiocentesis.

The most appropriate type of pericardial drainage is subject to debate. In principle, less aggressive procedures are preferred, but at the same time, the procedures must be able to prevent recurrence of effusion accumulation. Simple needle pericardiocentesis can often resolve tamponade initially, but the probability of relapse is very high.

Recurrence, which is observed in 40%-70% of patients with large malignant pericardial effusion, may be prevented by intrapericardial instillation of sclerotic or cytotoxic agents, immunomodulators, systemic antitumor treatment, radiation therapy, percutaneous balloon pericardiectomy, or surgical methods<sup>[19,20]</sup>. Surgical drainage (or pericardiectomy, its major equivalent) is excessively required for many patients. The best option is to perform pericardiocentesis using the Seldinger technique, i.e. inserting a pigtail drainage catheter that can be retained in the pericardium until the drainage is complete<sup>[17]</sup>. If effusion recurs after the removal of the pigtail catheter, a sclerosing agent (tetracycline or bleomycin) can be instilled into the pericardial sac, or subxiphoid balloon pericardiectomy can be performed<sup>[17]</sup>.

With regard to the cytotoxic agents that can be used for intrapericardial instillation, Maisch *et al.*<sup>[20]</sup> studied the effectiveness of tetracycline, 5-FU, and CDDP in 20 patients with recurrent malignant pericardial effusion, and observed favorable outcomes (no fluid re-accumulation) only after CDDP instillation.

With regard to the side effects and complications of CDDP instillation, Maisch *et al.*<sup>[19]</sup> reported that myocardial ischemia occurred in 1 of 42 patients studied, and there were no other complications. Fiorentino *et al.*<sup>[21]</sup> reported that mild nausea occurred in all patients, but hematologic and renal toxicity and local or infectious complications did not occur in any patients in their study. In our study, 2 patients (28.6%) developed nausea. However, no significant side-effects were observed in the study of Tondini *et al.*<sup>[22]</sup> CDDP instillation did not cause hypotension and retrosternal pain, as is observed after the instillation of some other agents<sup>[19]</sup>; hence, it is thought to be a reasonable cytotoxic agent for intrapericardial administration. After the intrapericardial instillation of CDDP, 3 of the 7 patients in our study underwent systemic chemotherapy (5-FU + CDDP). The overall mean survival time after intrapericardial instillation of CDDP was reported to be  $2.8 \pm 1.3$  mo by Maisch *et al.*<sup>[19]</sup> and it was  $120 \pm 71$  d (range, 68-268 d) in our study. Maisch *et al.*<sup>[19]</sup> performed intrapericardial instillation of CDDP for patients with neoplastic pericardial effusion without esophageal cancer, but our patients had esophageal cancer. Moreover, the overall mean survival

time in our study was longer than that of the study by Maisch *et al.*<sup>[19]</sup>.

We conclude that pericardiocentesis with intrapericardial instillation of CDDP is effective for the treatment of malignant pericardial effusion resulting from esophageal cancer. In our study, the number of patients with pericardial constriction was very small for conducting statistical evaluation, but it is thought that pericardiocentesis with intrapericardial instillation of CDDP is a safe and feasible treatment in cases of medical-surgical emergency. Moreover, additional systemic chemotherapy after pericardiocentesis with intrapericardial instillation of CDDP may prolong the survival time of patient.

## COMMENTS

### Background

Pericardial effusion in patients with esophageal carcinoma is most commonly associated with radiation and/or chemotherapy, and rarely with esophago-pericardial fistula. Here, the authors report the outcome of intrapericardial instillation of cisplatin (CDDP) for the treatment of malignant pericardial effusion and tamponade resulting from esophageal carcinoma.

### Research frontiers

After the intrapericardial instillation of CDDP, 3 of the 7 patients in this study underwent systemic chemotherapy (5-fluorouracil + CDDP). The overall mean survival time after intrapericardial instillation of CDDP was  $120 \pm 71$  d (range, 68-268 d) in this study. And the overall mean survival time in this study was longer.

### Innovations and breakthroughs

The authors conclude that pericardiocentesis with intrapericardial instillation of CDDP is effective for the treatment of malignant pericardial effusion resulting from esophageal cancer.

### Applications

In this study, the number of patients with pericardial constriction was too small to conduct statistical evaluation, but it is thought that pericardiocentesis with intrapericardial instillation of CDDP is a safe and feasible treatment in cases of medical-surgical emergency. Moreover, additional systemic chemotherapy after pericardiocentesis with intrapericardial instillation of CDDP may prolong the survival time of patient.

### Peer review

This is a well written manuscript describing a series of well organized experiments. It is about malignant pericardial effusion and tamponade resulting from esophageal carcinoma. Not many cases of this disease have been reported and this is an original case series.

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## Surgery for gastrointestinal malignant melanoma: Experience from surgical training center

Thawatchai Akaraviputh, Satida Arunakul, Varut Lohsiriwat, Cherdasak Iramaneerat, Atthaphorn Trakarnsanga

Thawatchai Akaraviputh, Satida Arunakul, Varut Lohsiriwat, Cherdasak Iramaneerat, Atthaphorn Trakarnsanga, Division of General Surgery, Department of Surgery, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand  
Author contributions: Akaraviputh T originated the idea and drafted the manuscript; Arunakul S collected the data and conducted statistical analyses; Lohsiriwat V wrote part of the manuscript; Iramaneerat C critically reviewed and edited the manuscript; Trakarnsanga A gave comment and wrote part of the manuscript.

Correspondence to: Dr. Thawatchai Akaraviputh, MD, Endo-Laparoscopic Surgery Unit, Division of General Surgery, Department of Surgery, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand. [sitak@mahidol.ac.th](mailto:sitak@mahidol.ac.th)

Telephone: +66-2-4198005-6 Fax: +66-2-4121370

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### Abstract

**AIM:** To characterize clinical features, surgery, outcome, and survival of malignant melanoma (MM) of the gastrointestinal (GI) tract in a surgical training center in Bangkok, Thailand.

**METHODS:** A retrospective review was performed for all patients with MM of the GI tract treated at our institution between 1997 and 2007.

**RESULTS:** Fourteen patients had GI involvement either in a metastatic form or as a primary melanoma. Thirteen patients with sufficient data were reviewed. The median age of the patients was 66 years (range: 32-87 years). Ten patients were female and three were male. Seven patients had primary melanomas of the anal canal, stomach and the sigmoid colon (5, 1 and 1 cases, respectively). Seven patients underwent curative resections: three abdominoperineal resections, two wide local excisions, one total gastrectomy and

one sigmoidectomy. Six patients had distant metastatic lesions at the time of diagnosis, which made curative resection an inappropriate choice. Patients who underwent curative resection exhibited a longer mean survival time (29.7 mo, range: 10-96 mo) than did patients in the palliative group (4.8 mo,  $P = 0.0006$ ).

**CONCLUSION:** GI MM had an unfavorable prognosis, except in patients who underwent curative resection (53.8% of cases), who had a mean survival of 29.7 mo.

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**Key words:** Melanoma; Gastrointestinal tract; Neoplasm metastasis

**Peer reviewers:** Nadia Peparini, MD, PhD, Department of General Surgery "Francesco Durante", La Sapienza University, Viale del Policlinico, 155, Rome, 00161, Italy; Dr. Oliver Mann, MD, Senior Attending Physician and Deputy Director, Department of General, Visceral and Thoracic Surgery, University of Hamburg, Martini Str. 52, D-20246 Hamburg, Germany; Dr. Yuk Him Tam, Department of Surgery, Prince of Wales Hospital, Shatin, Hong Kong, China

Akaraviputh T, Arunakul S, Lohsiriwat V, Iramaneerat C, Trakarnsanga A. Surgery for gastrointestinal malignant melanoma: Experience from surgical training center. *World J Gastroenterol* 2010; 16(6): 745-748 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v16/i6/745.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i6.745>

### INTRODUCTION

Malignant melanoma (MM) of the gastrointestinal (GI) tract is a rare condition, especially in Eastern countries. However, its incidence is rising with unclear reason<sup>[1]</sup>. It may be either primary or metastatic. GI metastasis of MM is frequently found during autopsy (50%-60% of cases), but a small proportion of melanoma patients are

diagnosed with GI metastasis while living (2%-5% of patients)<sup>[2,3]</sup>. The most common sites of the metastasis are the stomach and small intestine. Meanwhile, primary MM can arise in any GI mucosal site, but is most common in the anorectal region and esophagus.

The prognosis of GI MM is very poor with a 5-year survival of < 10%<sup>[4]</sup>. Surgery is still the mainstay of treatment. Recent studies have reported a trend toward less radical resection because there is no significant advantage of aggressive surgery over limited surgery in terms of local disease control, recurrence, and survival time<sup>[4,5]</sup>.

In Thailand, MM is less common than in western countries and the data are limited. This study, therefore, was conducted to evaluate clinical features, surgical options, and outcome including recurrence and survival of MM of the GI tract in a surgical training center in Bangkok, Thailand.

## MATERIALS AND METHODS

A retrospective review was conducted on patients diagnosed with MM of the GI tract who were admitted to Siriraj Hospital between 1997 and 2007. Patients were identified from the hospital computer database using an ICD-10 system. Patients' charts were reviewed retrospectively for patient characteristics, presenting symptoms, physical examination findings, imaging results, operative records, presence of complications, recurrence, follow-up time, survival time, and cause of death. Diagnosis was confirmed in all patients by histological study and immunochemistry for S-100 protein or HMB45 monoclonal antibody. Survival time was defined as the number of months from the time of diagnosis of GI MM to the time of death or the last follow-up evaluation. The long-term follow-up data were collected by direct contact with patients or their relatives. A Kaplan-Meier method was used for statistical analysis of survival outcome. This study was approved by Siriraj ethics committee, Mahidol University (EC1 2550/307).

## RESULTS

Between 1997 and 2007, there were 14 patients diagnosed with MM of the GI tract in Siriraj Hospital. One patient was excluded from this study due to insufficient data in the medical records; thus, only 13 cases were included in this study. Ten patients were female and three were male. The median age of the patients at presentation was 66 years (range: 32-87 years). Seven patients had a primary GI MM (anorectal,  $n = 5$ ; sigmoid colon,  $n = 1$ ; and stomach,  $n = 1$ ), whereas the others (three patients, 23.1%) had metastatic MM of the GI tract. The primary melanoma sites of these three patients were ocular, thumb, and ovary. There were three patients who had advanced GI MM of unknown primary origin.

The most common presenting symptom was abdominal pain (5 patients, 38.5%), followed by intra-ab-

Table 1 Characteristic of patients with MM of the GI tract

	Curative group	Palliative group	All
<i>n</i>	7	6	13
Median age (range) (yr)	66 (32-87)	57 (42-78)	66 (32-87)
Gender (M/F)	2/5	1/5	3/10
Presenting symptoms <i>n</i> (%)			
Abdominal pain	1	4	5 (38.5)
Intra-abdominal mass	4	0	4 (30.8)
Obstructive jaundice	0	1	2 (15.4)
Small bowel obstruction	0	1	1 (7.7)
Bowel habit change	1	0	1 (7.7)
Tenesmus	1	0	1 (7.7)
Investigation <i>n</i> (%)			
Abdominal CT	5	3	8 (61.5)
Endoscopy	1	1	2 (15.1)
Upper GI study	0	1	1 (7.7)
Barium enema	1	0	1 (7.7)
Origin of GI MM <i>n</i> (%)			
Primary	7	0	7 (53.8)
Secondary	0	3	3 (23.1)
Unknown primary	0	3	3 (23.1)
Site of the tumor <i>n</i> (%)			
Anorectal	5	2	7 (53.8)
Stomach	1	2	3 (23.1)
Jejunum	0	1	1 (7.7)
Pancreas	0	1	1 (7.7)
Sigmoid colon	1	0	1 (7.7)
Survival times (mo)			
Mean	29.7	4.8	17
Range	10-96	4-12	4-96

MM: Malignant melanoma; GI: Gastrointestinal; CT: Computed tomography.

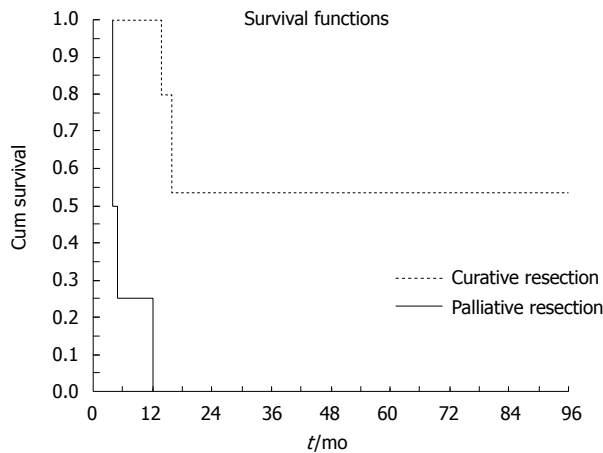
dominal mass in four patients (30.8%). Other presenting symptoms included obstructive jaundice, small bowel obstruction, bowel habit change, and tenesmus. The anorectal region was the most common site of GI MM (7 patients, 53.8%), followed by the stomach (3 patients, 23.1%) (Table 1).

Seven patients (53.8%) underwent curative resection, which consisted of three abdominoperineal resections, two wide local excisions, one total gastrectomy, and one sigmoidectomy. There was no perioperative mortality in this study. Meanwhile, another six patients (46.2%) had distant metastatic lesions at the time of diagnosis. They therefore received only palliative surgical treatment such as colostomy, small bowel resection, and enterointerostomy anastomosis. Patients who underwent curative resection exhibited a longer mean survival time (29.7 mo, range: 10-96 mo) than patients in the palliative group (4.8 mo, range: 4-12 mo) (Figure 1). Survival was significantly increased in patients who underwent curative resection ( $P = 0.0006$ ).

## DISCUSSION

In Thailand, MM is a rare disease. The estimated incidence rate of cutaneous melanoma in Thailand is 0.4 and 0.1 per 100000 in men and women, respectively<sup>[5]</sup>. Incidence of MM of the GI tract is exceedingly rare<sup>[6]</sup>. There have





**Figure 1** Kaplan-Meier curve demonstrating survival of patients with malignant melanoma of the gastrointestinal tract, who underwent curative resection and palliative resection.

been only a few reported cases of documented anorectal MM in Thailand<sup>[7]</sup>. MM of the GI tract can be either primary or metastatic. Primary GI melanoma must be differentiated from metastatic disease by previous history of melanoma and complete physical examination.

Patients often present with bleeding, pain or intestinal obstruction. If the patients present with GI bleeding, an endoscopy with magnification might be the procedure of choice to diagnose MM of the GI tract<sup>[8-10]</sup>. Multiple black, depressed lesions ( $1 \pm 5$  mm in diameter) with a “bull’s eye” appearance are usually viewed in the GI mucosa<sup>[9]</sup>. In the present study, approximately half of the patients presented with pain. A lower number of patients presented with gross GI bleeding and obstruction.

MM of the GI tract remains a fatal disease. Patients often present with advanced disease. In our study, almost half of the patients had metastatic disease at the time of presentation. The prognosis of GI MM is poor. The mean survival time of patients with a local or locoregional disease who underwent curative resection was only 29.7 mo. Several investigators have reported that the overall survival varies from 12 to 18 mo, with a 5-year survival of  $< 10\%$ <sup>[11]</sup>. When systemic metastasis has occurred, mean survival is only 6-8 mo<sup>[12,13]</sup>.

As a result of the poor prognosis of this disease, operative intervention has been discouraged. However, several recent studies have demonstrated better survival outcome in the patients who had complete surgical resection<sup>[14-19]</sup>. Our experiences compare favorably with those of other centers. Our study demonstrated seven patients with GI MM who underwent curative resection with a mean survival comparable to that in other centers. Several adjuvant treatment of GI MM such as chemotherapy, radiotherapy and immunotherapy have been utilized in many countries, but no such treatment was given in our center because of their unclear effectiveness. One recent randomized trial has demonstrated no survival benefit of adjuvant therapy<sup>[20]</sup>.

In conclusion, patients with GI MM had a poor prog-

nosis, especially in nonoperable cases. Surgical resection of the tumor resulted in a longer survival time. In selected patients with local or locally advanced disease, surgery should be performed where possible.

## COMMENTS

### Background

Gastrointestinal (GI) malignant melanoma (MM) is a rare malignancy. As a result of the paucity of cases, the available data about its clinical features, treatment options, and outcomes are very limited, especially in Eastern countries.

### Research frontiers

As a result of the poor prognosis of GI MM, improvement of its treatment options is an area that is in need of research. However, in order to reach that point, some basic understanding of the clinical characteristics and treatment outcomes of current surgical approaches is required. This study provides a picture of clinical experience with this rare disease from one surgical training center in Thailand.

### Innovations and breakthroughs

This study revealed that the nature of MM of the GI tract and its treatment outcomes in Thailand were similar and comparable to those from other centers.

### Applications

With a better understanding of GI MM and its poor prognosis, future research should look at how to improve the treatment outcome, through early diagnosis, selection of patients undergoing surgery, and improvement of surgical techniques.

### Peer review

This is a retrospective study on surgical management of MM of the GI tract. Patients who underwent curative resection exhibited a longer mean survival time than patients in the palliative group. Survival was significantly increased in patients who underwent curative resection.

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## Effects of *in vitro* cultivated *Calculus Bovis* compound on pulmonary lesions in rabbits with schistosomiasis

Tao Li, Zhen Yang, Hong-Jiao Cai, Li-Wei Song, Ke-Yu Lu, Zheng Zhou, Zai-De Wu

Tao Li, Li-Wei Song, Ke-Yu Lu, Zheng Zhou, Department of General Surgery and Oncology, China Mei Tan General Hospital, Beijing 100028, China

Zhen Yang, Hong-Jiao Cai, Zai-De Wu, Department of General Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China

**Author contributions:** Li T designed and performed the research and wrote the paper; Yang Z, Cai HJ and Wu ZD performed the clinical and pathological studies and data acquisition; Song LW, Lu KY and Zhou Z analyzed the data.

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**Correspondence to:** Tao Li, MD, Department of General Surgery and Oncology, China Mei Tan General Hospital, Beijing 100028, China. [litaoforgood2009@tom.com](mailto:litaoforgood2009@tom.com)

Telephone: +86-10-88680627 Fax: +86-10-62560298

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### Abstract

**AIM:** To explore the interventional effects and mechanism of *in vitro* cultivated *Calculus Bovis* compound preparation (ICCBco) on pulmonary lesions in portal hypertensive rabbits with schistosomiasis.

**METHODS:** The experimental group included 20 portal hypertensive rabbits with schistosomiasis treated by ICCBco. The control group included 20 portal hypertensive rabbits with schistosomiasis treated by praziquantel. The morphological changes of the pulmonary tissues were observed under light and electron microscopy. The expression of fibronectin (FN) and laminin (LN) in the lung tissues was analyzed by immunohistochemistry.

**RESULTS:** Under light microscope, the alveolar exudation in the lung tissue was more frequently observed in the control group, while the alveolar space was fairly dry in the lung tissue of ICCBco group. Under electron microscope, more alveolar exudation in the lung tissue, and more

macrophages, alveolar angiotectasis and the blurred three-tier structure of alveolar-capillary barrier could be seen in the control group. In ICCBco group, fibers within the alveolar interspace slightly increased in some lung regions, and the structure of type I epithelium, basement membrane and endodermis was complete, and no obvious exudation from the alveolar space, and novascular congestion could be observed. There was a positive or strong positive expression of FN and LN in the lung tissue of the control group, while there was a negative or weak positive expression of FN and LN in ICCBco group.

**CONCLUSION:** ICCBco can effectively prevent pulmonary complications in portal hypertensive rabbits with schistosomiasis by means of improving lung microcirculation and lowering the content of extracellular matrix.

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**Key words:** *In vitro* cultivated *Calculus Bovis* compound preparation; Schistosomiasis; Portal hypertension; Lung lesion; Fibronectin; Laminin; Pulmonary microcirculation

**Peer reviewer:** Heitor Rosa, Professor, Department of Gastroenterology and Hepatology, Federal University School of Medicine, Rua 126 n.21, Goiania - GO 74093-080, Brazil

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### INTRODUCTION

Portal hypertension is a vascular lesion that initially arises in liver, but is also accompanied with structural and functional changes of blood vessels in extrahepatic portal

system, systemic circulation and pulmonary circulation, which now collectively called portal hypertensive vascular lesions<sup>[1]</sup>. In clinical practice, much attention has been paid to the prevention and treatment of complications such as ascites, esophagogastric variceal bleeding; however the management of pulmonary complications is ignored which affects the prognosis of patients. Hence, drugs used for prevention and treatment of pulmonary complications seems to be very important. *In vitro* cultivated *Calculus Bovis* (ICCB)<sup>[2]</sup> developed by Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, with independent intellectual property rights, is one of the first-class national Chinese herbal medicine certificate of new drugs. ICCB is bilirubin calcium stones from *in vitro* cultivated bovine bile by simulating the formation principle and biochemical processes of gallstone *in vivo* using the modern bio-medical technology. The pharmacy, pharmacology and toxicology of the drug and phase I-IV clinical trials show that ICCB is consistent with natural bezoar in property, structure, composition, content and clinical efficacy, and no obvious toxicity and adverse effects could be observed (SFDA approval number Z20010075). *Calculus Bovis* has effects of clearing heat and toxic materials, promoting blood circulation and reducing swelling, eliminating stasis and facilitating tissue recovery, lowering vascular permeability, clearing softened blood vessels, scavenging free radicals and anti-anoxia in the principle of traditional Chinese medicine. *In vitro* cultivated *Calculus Bovis* compound preparation (ICCBco)<sup>[3-5]</sup> is mainly composed of ICCB, Chinese *Paris Rhizome*, *polygonum cuspidatum*, *appendiculate cremastra pseudobulb*, *frankincense*, and *myrrh*, with functions of clearing away heat and toxic materials, removing blood stasis, reducing swelling, eliminating blood stasis and promoting tissue regeneration. To evaluate the efficacy of ICCBco in the treatment of lung lesions in portal hypertensive rabbits with schistosomiasis as the experimental animal model, we performed a randomized, double-blind, controlled trial to observe the pathological changes and pathological mechanism of fibronectin (FN) and laminin (LN) expressions in the lung tissue of portal hypertensive rabbits with schistosomiasis.

## MATERIALS AND METHODS

### Materials

**Experimental animal:** Forty healthy adult rabbits (male, 2.5 kg in weight) provided by the Medical Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology.

**Medicine:** ICCBco (0.25 g/granule, 60 granules/bottle), Levo-praziquantel (0.1 g/tablet, 10/box), both offered by Tongji Medical College, Huazhong University of Science and Technology.

**Reagents:** Sheep serum, rabbit anti-human FN and LN primary antibody serum (1:100) were purchased from Boster Company, Wuhan. SABC kit was obtained from Zhongshan Company, Beijing.

**Instruments:** OPTON transmission electron microscopy (TEM, Carl Zeiss EM 10 C, Oberkochen, Germany) and optical microscopy (Olympus, Japan) were used.

### Methods

**Animal model establishment:** The hair over the abdomen was shaved off, and the rabbits were infected by cercariae of *oncomelania hupensis* by the sticking and pasting method<sup>[6]</sup>. The solution containing  $200 \pm 5$  cercariae was dripped on the shaved area of each rabbit and covered by a slide for 15 min, which led to acute infection. After 40 d, ICCBco was administered to the rabbits (6 granules/d). After 60 d, levo-praziquantel was perfused to kill parasites with a dosage of 500 mg/d for two consecutive days. The pulmonary fibrosis model was established in 120 d or so, and the experimental animals were killed by necropsy procedure in 4 mo. Pulmonary samples were obtained by autopsy. The experimental animals were divided into two groups: group A (control group), treated with praziquantel ( $n = 20$ ); group B, treated with praziquantel plus ICCBco ( $n = 20$ ).

**Sample collection:** Batches of rabbits were sacrificed by injecting an overdose of anesthesia with 1% Thiopental Sodium (50 mg/kg) *via* the ear vein. A small sample of the left lung tissue about the size of  $2 \text{ cm} \times 1 \text{ cm} \times 1 \text{ cm}$  was harvested after laparotomy, routinely fixed with 10% formaldehyde solution, and embedded in paraffin wax within 12 h for pathological examination. Another sample of the left lung tissue with a size of  $1 \text{ mm} \times 1 \text{ mm} \times 0.5 \text{ mm}$  was immediately put in a vial containing 20 g/L glutaraldehyde, and then sent to the department of ultrastructural pathology for TEM examination in 1 h.

**Staining method:** Samples of lung tissue were fixed in 40 g/L neutral buffered formaldehyde solution, routinely embedded in paraffin wax, and serial sections were made at a thickness of  $5 \mu\text{m}$  for hematoxylin and eosin (HE) staining and observed under optical microscopy.

Samples were fixed in 10% formaldehyde solution, and then treated with 2.5% potassium dichromate mordant prepared by 5% acetic acid for 12-18 h, and immersed in water washing for 10 min. Sections were treated with sodium thiosulfate in order to remove mercury deposition, and then fully washed with water, and stained for 2-5 min in Ehrlich's hematoxylin followed by wash in water. Differentiation was done in acid alcohol and thoroughly washed in running water until the sections turned blue, and then stained in 1% aqueous acid fuchsin solution for 5 min followed by rinsing sections in running water for 30 s or longer until color of collagen disappeared, and rinsed in distilled water. Sections were stained in Aniline Blue/Orange G for 20 min, washed in running water for 2-5 min, dehydrated, differentiated through 95% alcohols, washed in absolute alcohol and then passed into xylene for tissue transparent. At last, sections were mounted with gelatin and observed under optical microscopy.

Flesh sample of the lung tissue were cut into slices about a size of  $1 \text{ cm} \times 1 \text{ cm} \times 1 \text{ cm}$ , and placed in 25 mg/L



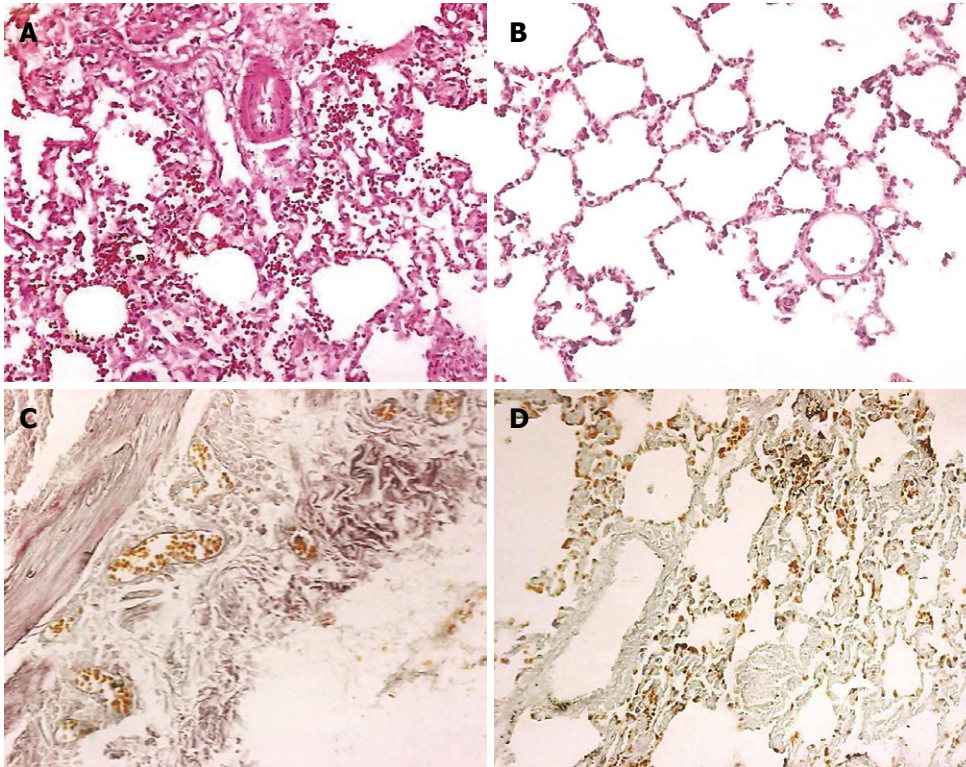


Figure 1 HE staining observation of lung tissues in control group (A) and *in vitro* cultivated *Calculus Bovis* compound preparation (ICCBco) group (B); Mallory trichrome staining in control group (C) and ICCBco group (D).

glutaraldehyde for 2 h as pre-fixation, then washed in pH 7.4 phosphate buffer solution followed by post-fixation in 10 g/L osmium acid under 4°C for 2 h. The slices were dehydrated with progressively increased concentration of alcohol and acetone, embedded in epoxy resin EPON 812. Ultrathin sections were cut and stained with double staining of uranium and lead (acetic acid-uranium-lead citrate) so as to be observed under TEM.

Sections were routinely deparaffinized, treated with 30 mL/L hydrogen peroxide, which was freshly prepared by distilled water, for 10 min at room temperature to inactivate endogenous peroxidases and rinsed three times in distilled water. Normal goat serum blocking solution was added and incubated at room temperature for 30 min in order to reduce non-specific background staining. Excessive liquid was removed without rinse. Sections were incubated in primary antibody (1:100) at 4°C overnight, then washed thrice in PBS (0.1 mol/L), and incubated in biotinylated secondary antibody for 30 min at room temperature, then rinsed thrice in PBS for 5 min. And they were incubated with SABC for 30 min at 37°C followed by rinse four times in PBS for 5 min. Sections were incubated in DAB with DAB reagent kit: One drop of reagent A and reagent B were added in 1 mL distilled water, and placed the well-mixed reagents onto the slices. The reaction was allowed to develop for 3-10 min under the control of microscopy at room temperature, and rinsed in distilled water. Sections were lightly counterstained with hematoxylin. After dehydration, transparent, and mounting process, slides were observed under microscopy. PBS was used as negative control instead of primary antibody and a known positive slice was taken as positive control. Brownish yellow granules in the cytoplasm were considered as positive FN and LN. Selected regions containing

endothelial cells under optical microscopy were input into HPIAS-1000 automatic color image analysis system and the results were shown as the average absorbance.

### Statistical analysis

All data were presented as the mean  $\pm$  SD. Statistical analysis was done using SPSS software version 11.0. Statistical differences between the two groups were analyzed using ANOVA and *t* tests. *P* values less than 0.05 were considered significant.

## RESULTS

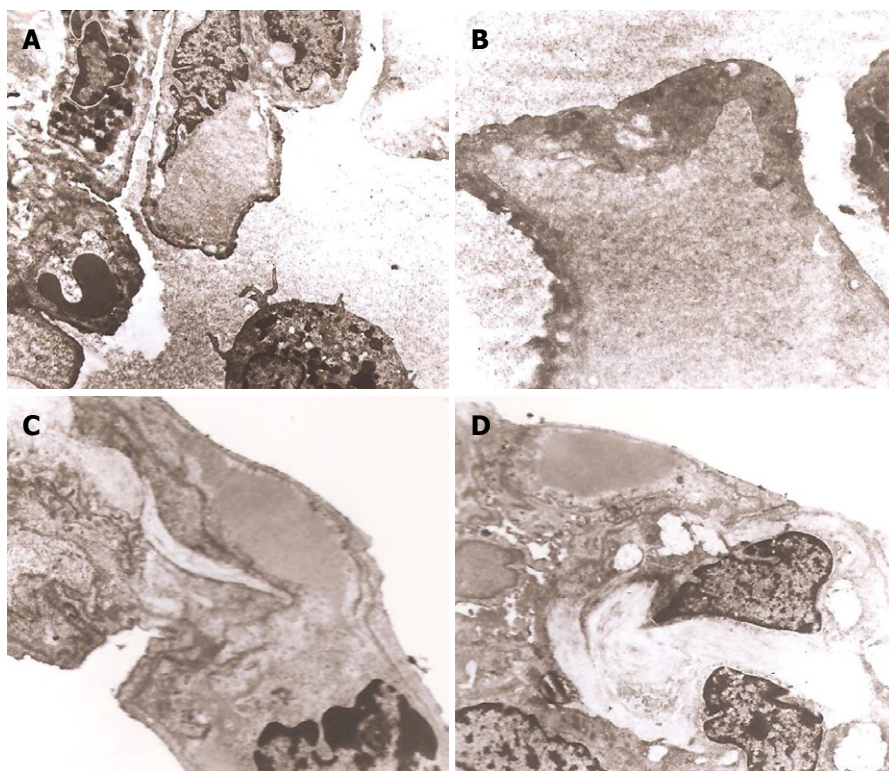
### Morphological results

HE staining results showed that the alveolar exudation in the lung tissue was seen more frequently in group A, while the alveolar space was fairly dry in the lung tissue of group B (Figure 1A and B). Mallory trichrome staining results showed more alveolar exudation and collagen fibers in the lung tissue of group A, while fairly dry alveolar space and less collagen fibers were seen in group B (Figure 1C and D). More alveolar exudation was found in group A, and more macrophages, alveolar angiectasis and the blurred three-tier structure of alveolar-capillary barrier could also be seen under TEM observation (Figure 2A and B). In group B, fibers within the alveolar interspace slightly increased in some lung regions, and the structure of type I epithelium, basement membrane and endodermis was complete, and no obvious exudation in alveolar space and no vascular congestion were observed (Figure 2C and D).

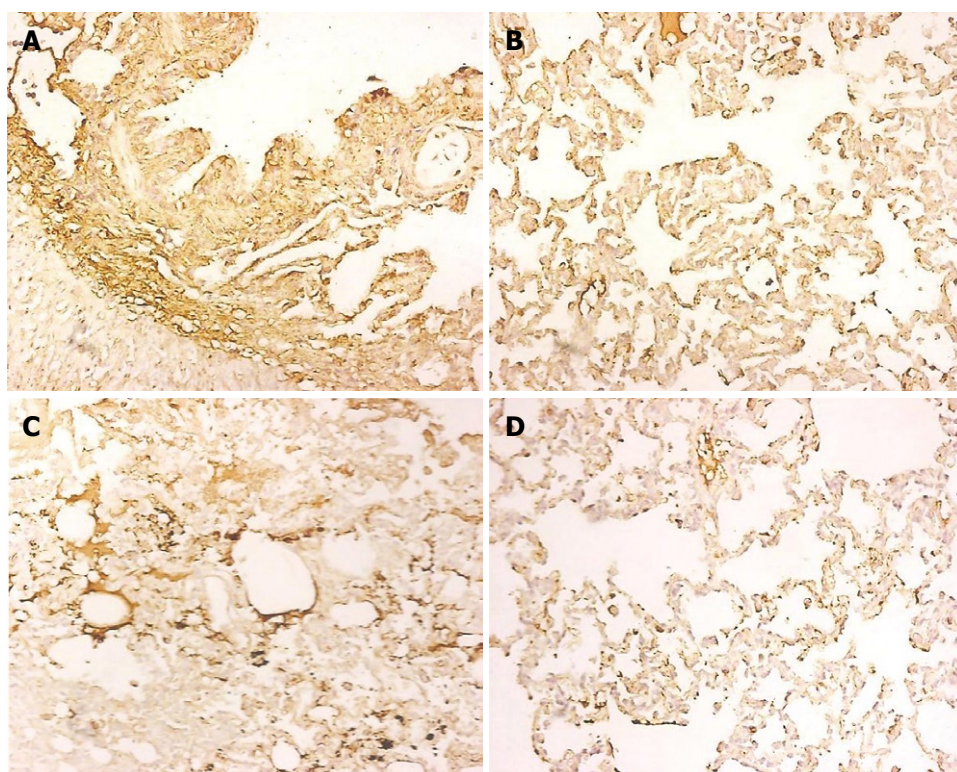
### Immunohistochemical staining results

There was a positive or strong positive expression of FN





**Figure 2** Observation of lung tissues in control group (A, B), and ICCBco group (C, D) under transmission electron microscope.



**Figure 3** Expression of fibronectin (FN) and laminin (LN) of lung tissues in control group (A, C) and ICCBco group (B, D) by histochemical staining.

in the lung tissue of group A, while a negative or weak positive expression of FN in group B (Figure 3A and B). Group A showed a positive or strong positive expression of LN in the lung tissue, while group B showed a negative or weak positive expression of LN (Figure 3C and D). The integrated optical density (IOD) values are shown in Table 1.

## DISCUSSION

Rabbit model of hepatic fibrosis of schistosomiasis was consistent with the natural course and pathological development process of human liver fibrosis; previous studies have confirmed that 4 mo after rabbits were infected with schistosomiasis, the hepatic fibrosis

Table 1 IOD values in lung tissues of both groups (mean  $\pm$  SD)

Group	n	IOD value of FN	IOD value of LN
Control group	20	19.47 $\pm$ 1.21	20.23 $\pm$ 0.87
ICCBco group	20	13.15 $\pm$ 0.94	12.08 $\pm$ 1.26

A marked decrease in the expression of FN and LN in the lung tissue was seen in ICCBco group ( $P < 0.01$ ). IOD: Integrated optical density; FN: Fibronectin; LN: Laminin; ICCBco: *In vitro* cultivated *Calculus Bovis* compound preparation.

formed, indicating it is a mature model of liver fibrosis.

Schistosomiasis can not only cause liver fibrosis and portal hypertension, but also cause tissue lesions. The most dangerous lesion is pulmonary vascular lesion characterized by hypoxemia and decreased oxygen saturation. However, the exact mechanisms of hypoxemia are still controversial, and it is currently believed to be caused by the three factors together - intrapulmonary shunt, ventilation - perfusion imbalance and pulmonary diffusion dysfunction. The important fundamental cause of these pathologic changes is pulmonary vasodilation<sup>[7]</sup>. Our results showed that there was a slight increase in fibers between alveolar gap in some parts of the lung tissue, and the structure of type I epithelium, basement membrane and endodermis was complete, and there was no obvious exudation in alveolar space, and no vascular congestion in the ICCBco group, while more alveolar exudation, more macrophages, alveolar angiotectasis and the blurred three-tier structure of alveolar-capillary barrier were seen in praziquantel control group. This indicates that ICCBco could make a marked improvement in pulmonary ischemia, hypoxia, pulmonary function, blood flow blockade, damage, and connective tissue hyperplasia, which would shed light on pathological basis of clinical manifestations.

Extracellular matrix (ECM) proteins can be categorized into two kinds: collagen and non-collagen protein. ECM not only provides supporting structure and attachment for tissues but also regulates cell adhesion, migration, proliferation, differentiation, and tissue trauma repair and fibrosis. FN is a glycoprotein of large molecular weight with highly active adhesion, mainly derived from macrophage. FN induces chemotactic migration of interstitial cells<sup>[8]</sup>, and promotes fibroblast division and proliferation<sup>[9]</sup>. Studies have found that FN mRNA and protein expression of alveolar macrophages and interstitial fibroblasts were markedly elevated in patients with pulmonary fibrosis<sup>[10]</sup>, FN can transmit messages into cells through adhesion molecules on the surface of fibroblast cells and plays an initial role in the lung fibrosis process. It is currently believed that FN first appears in the early pulmonary fibrosis, and after then other interstitial elements occurs. LN is a large-molecular-weight non-collagen glycoprotein existing in the transparent layer of the basement membrane, which plays an important role in the maintenance of structure and function of alveolar and capillary basement membrane. Basement membrane provides a support for the regeneration of injured epi-

thelial cells, and is the barrier for the entry of molecules and cells into the alveolar cavity<sup>[11,12]</sup>. The normal lung tissue contains very little LN. There is a significant increase of LN in pulmonary fibrosis or liver cirrhosis, which is about 10 times the level of LN in the normal lung and is consistent with collagen content in fibrosis<sup>[13]</sup>. Some scholars found that LN fluorescence in alveolar septa was enhanced in rats with early stage pulmonary fibrosis, later became thicker and deranged, presuming that LN participated in the whole process of experimental pulmonary fibrosis, and might play an important role in the development of fibrosis<sup>[14]</sup>. Previous experiments showed that abnormal accumulation of type I and III collagen, FN and LN in extracellular matrix at the early stage of schistosomiasis-induced liver fibrosis<sup>[15]</sup>. FN and LN are, therefore, better indicators for pulmonary fibrosis. In this study, we found that there was a significant decrease in the expression of FN and LN in ICCBco group compared with praziquantel control group, which suggests ICCBco could effectively inhibit the formation of pulmonary fibrosis. With functions of clearing away heat and toxic materials, removing blood stasis, reducing swelling, eliminating blood stasis and promoting tissue regeneration, it could improve pulmonary microcirculation, inhibit connective tissue proliferation, degrade extracellular matrix, reduce pulmonary damage, hence inhibiting the formation of pulmonary fibrosis.

In summary, ICCBco can effectively prevent pulmonary complications of portal hypertensive rabbits with schistosomiasis. Its function in suppressing pulmonary lesions is achieved by removing heat, toxic materials and blood stasis, reducing swelling, eliminating blood stasis and promoting tissue regeneration to block the activation of alveolar macrophages, improve pulmonary microcirculation, and reduce ECM. The successful development of ICCB and the preliminary study on portal hypertensive pulmonary lesions caused by schistosomiasis suggest that it is of great significance and prospects for further basic and clinical research, development and clinical application of new drugs and preparations to treat portal hypertensive pulmonary lesions induced by schistosomiasis.

## COMMENTS

### Background

Portal hypertension is a vascular lesion that initially arises in liver, but structural and functional changes of blood vessels in extrahepatic portal system, systemic circulation and pulmonary circulation also accompany, which now collectively called portal hypertensive vascular lesions. In clinical practice, much attention has been paid to the prevention and treatment of complications such as ascites, esophagogastric variceal bleeding; however the management of pulmonary complications is ignored which affects the prognosis of patients. Hence, drugs used for prevention and treatment of pulmonary complications seem to be very important.

### Research frontiers

*In vitro* cultivated *Calculus Bovis* compound preparation (ICCBco) is composed of ICCB, Chinese *Paris Rhizome*, *polygnum cuspidatum*, *appendiculate cremastra pseudobulb*, *frankincense*, and *myrrh*, and has the functions of clearing away heat and toxic materials, removing blood stasis, reducing swelling, eliminating blood stasis and promoting tissue regeneration, according to the principle of traditional Chinese medicine. However, the topic has not been unequivocally addressed. This study evaluated the efficacy of ICCBco in the



treatment of lung lesions in portal hypertensive rabbits with schistosomiasis as the experimental animal model.

### Innovations and breakthroughs

The present study explored the pathogenesis of portal hypertension and the prevention and treatment of its pulmonary complications (hepatopulmonary syndrome, pulmonary fibrosis, pulmonary hypertension, pulmonary venous hypertension) from a new perspective of portal hypertensive vascular disease. ICCB is a Class 1 new Chinese medicine developed by Wuhan Tongji Hospital with independent intellectual property rights, is a treasure of traditional Chinese medicine. To investigate its role in the treatment of schistosomiasis-induced pulmonary complications of portal hypertension has far-reaching significance.

### Applications

The successful development of ICCB and the preliminary study on portal hypertensive pulmonary lesions caused by schistosomiasis suggest that it is of great significance and prospects for further basic and clinical research, development and clinical application of new drugs and preparations to treat portal hypertensive pulmonary lesions induced by schistosomiasis.

### Terminology

Composed of ICCB, Chinese *Paris Rhizome*, *polygonum cuspidatum*, *appendiculate cremastra pseudobulb*, *frankincense*, and *myrrh*, ICCBco can effectively prevent pulmonary complications of portal hypertensive rabbits with schistosomiasis. ICCBco could improve pulmonary microcirculation, inhibit connective tissue proliferation, degrade extracellular matrix, reduce pulmonary damage, hence inhibiting the formation of pulmonary fibrosis.

### Peer review

This is a very interesting research but not well planned.

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## Methylation of *Dickkopf-3* as a prognostic factor in cirrhosis-related hepatocellular carcinoma

Bin Yang, Zhi Du, Ying-Tang Gao, Cheng Lou, Shi-Guang Zhang, Tong Bai, Yi-Jun Wang, Wen-Qin Song

Bin Yang, Zhi Du, Ying-Tang Gao, Key Laboratory of Artificial Cells, Third Central Hospital, Tianjin 300170, China  
Zhi Du, Cheng Lou, Yi-Jun Wang, Department of Hepatobiliary Surgery, Third Central Hospital, Tianjin 300170, China  
Tong Bai, Graduate School of Tianjin Medical University, Tianjin 300070, China

Bin Yang, Ying-Tang Gao, Shi-Guang Zhang, Wen-Qin Song, College of Life Science, Nankai University, Tianjin 300071, China

Bin Yang, Postdoctoral Workstation of TEDA, Tianjin 300457, China

**Author contributions:** Du Z and Song WQ designed the research and revised the manuscript; Yang B, Zhang SG and Bai T performed the research; Gao YT provided the analytic tools; Lou C collected the clinical pathological data; Wang YJ collected the tumor tissues; Yang B analyzed the data and wrote the manuscript.

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**Correspondence to:** Zhi Du, Professor, Key Laboratory of Artificial Cells, Third Central Hospital, Tianjin 300170, China. zhi-du@163.com

Telephone: +86-22-84112148 Fax: +86-22-24315132

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### Abstract

**AIM:** To investigate the prevalence and time of *Dickkopf* (*DKK*) family methylation and its clinical significance in hepatocarcinogenesis.

**METHODS:** Methylation of *DKK* family genes was quantitatively analyzed in 115 liver tissue samples, including 50 pairs of primary hepatocellular carcinoma (HCC) and matched noncancerous cirrhotic tissue samples, as well as 15 liver cirrhosis biopsy samples.

**RESULTS:** The methylation level of *DKK3* was significantly higher in HCC tissue samples than in matched noncancerous cirrhotic tissue samples ( $P < 0.0001$ ) or

in liver cirrhosis biopsy samples ( $P = 0.0139$ ). Receiver operator characteristic curve analysis confirmed that the percent of methylated reference (PMR) values of *DKK3* could effectively discriminate HCC tissue samples from noncancerous tissue samples (AUC = 0.8146) or liver cirrhosis biopsy samples (AUC = 0.7093). Kaplan-Meier survival curves revealed that the progression-free survival time of patients with a higher *DKK3* methylation level (PMR > 1%) was significantly shorter than that of those with a lower *DKK3* methylation level (PMR ≤ 1%) ( $P = 0.0255$ ). Multivariate Cox analysis indicated that methylated *DKK3* was significantly and independently related with a shorter survival time (relative risk = 2.527, 95% CI: 1.063-6.008,  $P = 0.036$ ) of HCC patients.

**CONCLUSION:** Methylation of *DKK3* is an important event in early malignant transformation and HCC progression, and therefore might be a prognostic indicator for risk assessment of HCC.

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**Key words:** *Dickkopf*; Hepatocellular carcinoma; Methylation; Biomarker; Prognosis

**Peer reviewer:** Dr. Paolo Del Poggio, Hepatology Unit, Department of Internal Medicine, Treviglio Hospital, Piazza Ospedale 1, Treviglio Bg 24047, Italy

Yang B, Du Z, Gao YT, Lou C, Zhang SG, Bai T, Wang YJ, Song WQ. Methylation of *Dickkopf-3* as a prognostic factor in cirrhosis-related hepatocellular carcinoma. *World J Gastroenterol* 2010; 16(6): 755-763 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v16/i6/755.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i6.755>

### INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world, leading to more than 500 000 deaths every year<sup>[1]</sup>. Most HCC cases (> 80%)

occur in either sub-Saharan Africa or Eastern Asia. HCC cases in China alone account for more than 40% of all cases in the world<sup>[2]</sup>. It was recently reported that the incidence of HCC is increasing worldwide<sup>[3]</sup>. Treatment of HCC is still a great challenge for clinical oncologists because most HCC patients are diagnosed at its advanced stage with distant metastasis<sup>[4]</sup>.

Dysplastic cirrhotic nodules are considered precursors of HCC because of their association with HCC occurrence. Development of HCC is closely associated with cirrhosis and 90% of the tumors are found in chronic hepatitis or cirrhotic patients<sup>[5]</sup>. One reasonable explanation for this close correlation is that necrosis and regeneration of hepatocytes due to chronic liver damage provide the stepwise accumulation of genetic and epigenetic changes necessary for hepatocarcinogenesis<sup>[2]</sup>. Therefore, elucidation of these aberrant alterations involving hepatocellular transformation at the cirrhosis stage is not only crucial to understand the molecular basis of hepatocarcinogenesis but also to provide potentially useful markers for the early diagnosis, risk assessment, treatment, and chemoprevention of HCC.

Aberrant promoter hypermethylation of tumor suppressor genes is a common event during the pathogenesis of human cancers and one of the important epigenetic mechanisms in carcinogenesis. It has been shown that methylation of multiple tumor suppressor genes in HCC may contribute to the pathogenesis of this disease<sup>[5,6]</sup>. Dickkopf (DKK) family is one class of the secreted Wnt antagonists and its functional loss can contribute to activation of the Wnt pathway and result in carcinogenesis through dysregulation of cell proliferation and differentiation<sup>[7]</sup>. It has been recently shown that methylation of *DDK* gene family contributes to carcinogenesis and serves as a potential biomarker for the diagnosis or prognosis of several human malignancies<sup>[8]</sup>. However, few reports are available on the epigenetic silencing of *DDK* gene and its clinical significance in HCC<sup>[9]</sup>.

In the present study, we examined the promoter hypermethylation of human *DDK* family genes in HCC and cirrhosis tissue samples by quantitative methylation-specific polymerase chain reaction (Q-MSP), and evaluated whether quantitative methylation of *DDK* genes can serve as a potentially diagnostic or prognostic biomarker for HCC.

## MATERIALS AND METHODS

### Patients and sample collection

A total of 115 liver tissue samples, including 50 pairs of primary HCC and matched noncancerous cirrhotic liver (NCL) tissue samples, as well as 15 liver cirrhotic (LC) biopsy samples, were analyzed in this study. Tumor tissue samples were collected from patients who underwent surgery in Third Central Hospital of Tianjin between December 2003 and August 2006 and stored at -80°C for further processing. Clinicopathological data were collected from patient records and pathology reports. Written informed consent was obtained from each patient and the study protocol was approved by the

Clinical Research Ethics Committee of Third Central Hospital, Tianjin.

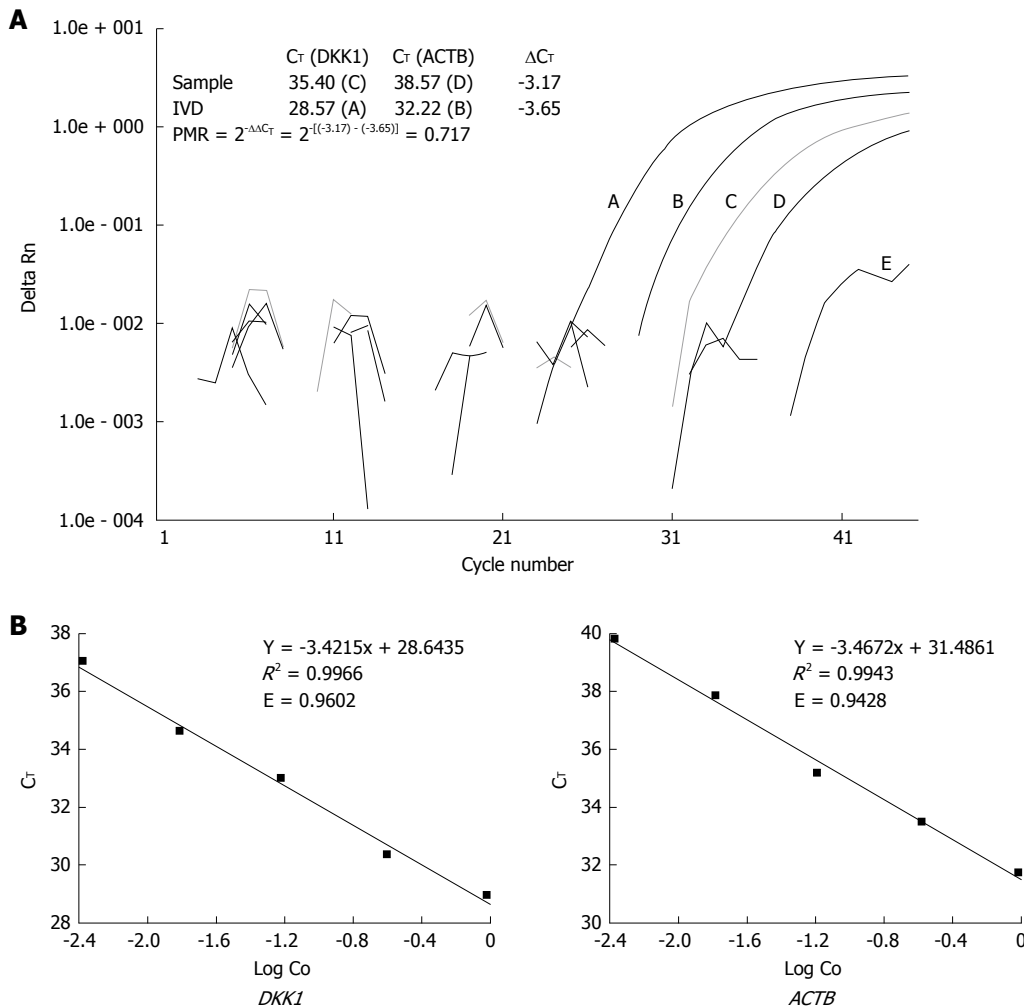
### DNA extraction and bisulfite treatment

Genomic DNA was extracted from tumor tissue samples by digesting with SDS/proteinase K in TE buffer followed by a standard phenol/chloroform extraction. The extracted DNA was subjected to bisulfite treatment as previously described<sup>[10]</sup>. Briefly, 1–2 µg genomic DNA was denatured with 0.3 mol/L NaOH at 37°C for 20 min, and incubated in 3.0 mol/L sodium bisulfite and 10 mmol/L hydroquinone at 55°C for 16 h. The DNA was desalted with a QIAquick gel extraction kit (Qiagen, Valencia, CA) and dissolved in 50 µL of 10 mmol/L TE buffer (pH 8.0). Then, 5.5 µL of 3.0 mol/L NaOH was added and incubated at 37°C for 20 min to desulfonate it. The modified DNA was neutralized with 30 µL of 10 mol/L ammonium acetate, precipitated using 2 volumes of ethanol, and resuspended in 40 µL of 1.0 mmol/L TE buffer (pH 7.6).

### Q-MSP

Fluorogenic quantitative MSP assay was carried out in the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). For each gene of the *DDK* family, a set of primers and a probe covering multiple CpG dinucleotides were designed within the putative CpG islands around the gene promoters. For the endogenous reference gene, *ACTB* (β-actin), the primers and probe were designed to avoid CpG dinucleotides. All primers and probes were designed according to the bisulfite-converted DNA sequences. Genes of interest were amplified in proportional to the degree of CpG methylation, while *ACTB* was amplified independently of its methylation. The sequences of primers and probes are as follows: (1) *DDK1*, 5'-GGGTTCGCGGTATAAAGGTAGTC-3' (sense), 5'-TCCGAAAACCCCTACGATC-3' (antisense), 5'-FAM-TGGCGGTGGCGGCGTAGAGT-BHQ1-3' (Probe); (2) *DDK2*, 5'-GCGAGCGTAGCGTAAGTTCGT-3' (sense), 5'-CACTCACAATTACCCCGAAACG-3' (antisense), 5'-FAM-AGGTATCGTTGCGTTGGTAGCGATTTCG-BHQ1-3' (Probe); (3) *DDK3*, 5'-GGTATCGGCGTTGTTCGTATTTC-3' (sense), 5'-CCACCCCGACTAAACCGAAT-3' (antisense), 5'-FAM-TCGCGGTTTCGTTTATCGCGTC-BHQ1-3' (Probe); and (4) *ACTB*, 5'-TGGTGATGGAGGTTTAGTAAGT-3' (sense), 5'-AACCAATAAAACCTACTCCTCCCTTAA-3' (antisense), 5'-FAM-ACCACCACCAACACACAATAACAAACACA-BHQ1-3' (Probe).

Q-MSP assay was performed in a reaction volume of 20 µL in 96-well plates, and the final reaction mixture was consisted of 1 × real-time PCR master mix (Toyobo Co., Ltd. Shanghai), 200 nmol/L probe, 400 nmol/L primers of each gene, and 2 µL bisulfite-converted DNA templates. PCR was performed at 95°C for 2 min, followed by 45 cycles at 95°C for 15 s, and at 60°C for 45 s. Reactions were done in duplicate and each plate included at least 3 controls with no template (NTC), as well as negative and positive controls on each plate. Leukocyte



**Figure 1** Quantitative methylation analysis using comparative C<sub>T</sub> method. A: Representative quantitative methylation-specific polymerase chain reaction (Q-MSP) amplification plots for *DDK1* and illustration for percent of methylated reference (PMR) calculation with comparative C<sub>T</sub> method; B: Validation experiment for comparative C<sub>T</sub> method, E = 10<sup>(1/Slope)</sup> - 1, where E: PCR efficiency; Slope: Slope of calibration curve. DKK: Dickkopf.

DNA collected from a healthy individual was used as a negative control. The DNA methylated *in vitro* with SssI methyltransferase (New England Biolabs Inc., Beverly, MA) was used as a positive control for all studied genes.

### PMR values

Abbreviation PMR was used to define the percentage of fully methylated molecules at a specific locus as previously described<sup>[11]</sup>. Briefly, the PMR value was calculated by dividing the *GENE: ACTB* ratio in a sample by the *GENE: ACTB* ratio in SssI-treated leukocyte DNA (IVD) and multiplied by 100. Parallel PCR reactions were done for the genes of interest and reference. Given the high efficiency of Q-MSP amplification for both *DDK1* and *ACTB* genes in this study, PMR values were detected with the comparative C<sub>T</sub> method instead of the relative standard curve method, which needs serial dilutions of bisulfite-treated universally methylated DNA to construct a relative standard curve for each gene<sup>[12]</sup>. Relation between the percentages of methylated DNA molecules and C<sub>T</sub> was described as  $PMR = 2^{-\Delta\Delta C_T} \times 100\%$  where  $\Delta\Delta C_T = \Delta C_{T(Gene)} - \Delta C_{T(ACTB)} = [C_{T(Gene)} - C_{T(ACTB)}]_{(Sample)} - [C_{T(Gene)} - C_{T(ACTB)}]_{(IVD)}$ .

The number of cycles at which the fluorescence signal crossed a detection threshold was determined automatically with the ABI prism 7000 detection system, and referred to as C<sub>T</sub>. Representative Q-MSP amplification plots for *DDK1* and corresponding calculation for PMR are illustrated in Figure 1A. For the ΔΔC<sub>T</sub> calculation to be valid, the efficiencies in target and reference amplification should be within 10%. PCR efficiency was calculated and compared according to the following equation recommended in technical manual of Applied Biosystems (Figure 1B).

### RNA preparation and real-time quantitative PCR

RNA was extracted from HCC and matched NCL tissue samples using Trizol (Tiangen, Beijing) according to its manufacturer's instructions. Total mRNA was digested with DNase I (Ambion, Austin, TX) to remove genomic DNA contamination and then subjected to reverse transcription using the reverse transcription system (Promega, Madison, WI). For the reverse-transcriptase PCR of *DDK3*, the sense primer (5'-ATCACCTGGGAGCTAGAGCCTGATG-3') and anti-sense primer (5'-ACC TCTCTGGGCAGCAGGGATCTC-3') were designed. PCR was done on the ABI Prism 7000 sequence detection

system in combination with the SYBR green teal-time PCR master mix (Toyobo Co., Ltd, Shanghai). Melting curve analyses following amplification were performed to assure the product specificity. Relative expression of *DKK3* mRNA was normalized to the housekeeping gene *GAPDH* in the same cDNA using the comparative  $C_t$  method. Primer sequences for *GAPDH* are 5'-CTCAT GACCACAGTCCATGCCATCACTG-3' (sense) and 5'-CATGAGGTCCACCACCCTGTTGCTGTA-3' (anti-sense).

### Receiver operator characteristic (ROC) curve analysis

ROC curves were plotted to assess the PMR values of *DKK* family as diagnosis biomarkers, and their discriminatory capacity was evaluated by calculating the area under the curve (AUC). Generally, a truly useless test has an AUC of 0.5, while a perfect test (one that has zero false positives and zero false negatives) has an AUC of 1.0. For each gene of *DKK* family, the PMR values in HCC tissue samples were considered patient results, while the values in matched NCL tissue samples were considered control results.

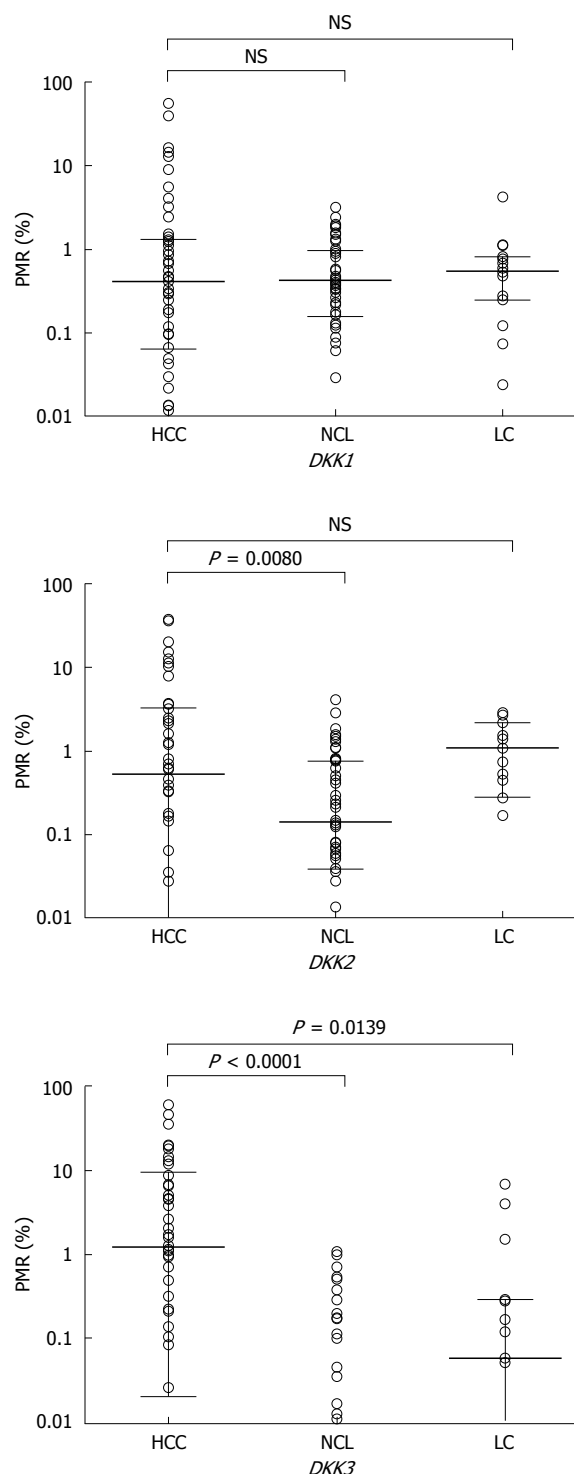
### Statistical analysis

Relation between categorical variables was determined by Pearson  $\chi^2$  test or Fisher's exact test. Difference in median of PMR values between paired HCC and NCL tissue samples was detected by Wilcoxon matched pairs test, difference in HCC tissue and LC biopsy samples was revealed by Mann-Whitney *U* test. Variables associated with overall survival or progression-free survival rate were tested using Kaplan-Meier estimates and compared by log-rank test. Relative risk (RR) of *DKK3* methylation-related death and other clinical variables were estimated from a univariate Cox proportional hazards model. Multivariate Cox models were also constructed to estimate the RR for *DKK3* methylation with adjustments for potential confounding risk factors. All statistical tests were two-sided and  $P < 0.05$  was considered statistically significant. Statistical analyses were performed with GraphPad Prism V5.0 (GraphPad Software, San Diego, CA) and SPSS V11.0 software for Windows (SPSS Inc., Chicago, IL), respectively.

## RESULTS

### Quantitative analysis of *DKK* gene methylation in HCC and cirrhotic liver tissue samples

The methylation levels of *DKK* family in 3 kinds of tumor tissue sample were quantified by Q-MSP. The distribution of PMR values is illustrated in Figure 2. The methylation levels of *DKK* family in the 50 paired HCC tissue samples and NCL tissue samples were compared. Wilcoxon matched pairs test demonstrated that the methylation levels of *DKK2* and *DKK3* were significantly higher in HCC tissue samples than in corresponding NCL tissue samples ( $P = 0.0080$ ,  $P < 0.0001$ ), whereas no significant difference was found in the methylation level of *DKK1*. The difference in median PMR value was further compared between the 50



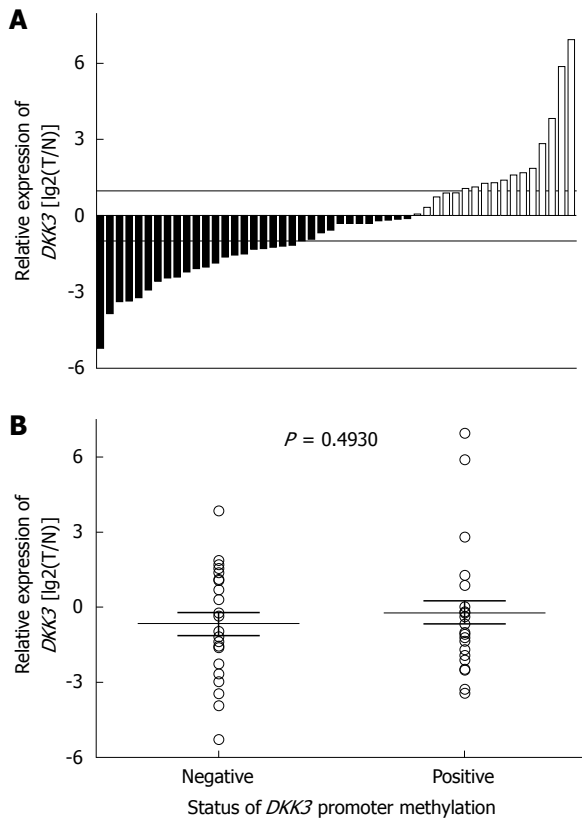
**Figure 2** Methylation levels of *DKK* family genes in hepatocellular carcinoma (HCC) and corresponding noncancerous cirrhotic liver (NCL) tissue samples and liver cirrhosis (LC) biopsy samples. Horizontal bar denotes the median PMR value, and range indicates 25%-75% quartile.

HCC and 15 LC tissue samples by Mann-Whitney test and a significant difference was only found in the median PMR value for *DKK3* gene ( $P = 0.0139$ ).

### Expression of *DKK3* mRNA in HCC tissue samples

The expression of *DKK3* mRNA in primary HCC tissue samples was detected by real-time PCR. Fifty pairs of HCC





**Figure 3** Expression of *DKK3* mRNA in HCC (A) and NCL (B) tissue samples. Horizontal lines represent the median FC value, and range indicates 25%-75% quartile.

tissue samples and corresponding NCL tissue samples were analyzed. The expression level of *DKK3* mRNA was lower in tumor tissue samples than in its adjacent tissue samples (Figure 3A). However, the median of *DKK3* RNA expression was not statistically different between the methylated and unmethylated *DKK3* genes ( $P = 0.4930$ ) (Figure 3B).

#### ROC curve analysis of PMR values in HCC and liver cirrhosis tissue samples

To assess whether quantitative methylation assay of *DKK* family can serve as a diagnosis tool to discriminate malignant from non-malignant liver tissue samples, ROC curves were plotted with PMR values as test results. The overall discriminatory ability of the test was evaluated by calculating the AUC (Figure 4). Two ROC curves were plotted for each gene. The PMR values in HCC tissue samples and the values in two types of liver cirrhosis tissue samples (NCL and LC) were considered patient results and control results, respectively. ROC curve analysis revealed that the AUC value for the PMR of *DKK3* was relatively higher in the two kinds of tumor tissue samples (0.8146 for HCC *vs* NCL tissue samples and 0.7093 for HCC *vs* LC tissue samples, respectively).

#### *DKK* gene methylation and clinicopathological correlation

To investigate the correlation between *DKK* family methy-

**Table 1** *DKK* methylation frequencies in HCC and NCL tissue samples

	Positive (PMR > 1%)	Negative (PMR ≤ 1%)	P
<i>DKK1</i>			
HCC	14	36	0.6484
NCL	12	38	
<i>DKK2</i>			
HCC	21	29	0.0076
NCL	8	42	
<i>DKK3</i>			
HCC	27	23	< 0.0001
NCL	2	48	

*DKK*: Dickkopf; HCC: Hepatocellular carcinoma; PMR: Percent of methylated reference; NCL: Noncancerous cirrhotic liver.

lation and clinicopathological variables, the continuous PMR values were converted into discrete binary data, and the patients were also divided into two subgroups. To exclude the low *DKK* methylation level in a mere minority of cells, which may have little effect on gene activity in tumor tissue samples, PMR (1%) was selected as a criterion for the methylation of *DKK*. That is, the *DKK* methylation was classified into positive (PMR > 1%) and negative (PMR ≤ 1%) groups.

The methylation patterns of *DKK* genes in paired HCC and NCL tissue samples are summarized in Table 1. Consistent with the quantitative analysis above, significantly different methylation patterns of *DKK3* were found in HCC and NCL tissue samples ( $P < 0.0001$ ). The methylation frequency of *DKK2* in HCC (64%, 32 of 50) and NCL (60%, 30 of 50) tissue samples was similar, while the methylation level of *DKK2* was higher in HCC tissue samples (42%, 21 of 50) than in NCL tissue samples (16%, 8 of 50) ( $P = 0.0076$ ). No significant difference of *DKK1* methylation was found in HCC and NCL tissue samples.

Whether methylation of *DKK* family is related with certain clinicopathological variables was further determined. Statistical analysis showed that the methylation frequency of *DKK1* or *DKK2* was not related with the clinicopathological variables (data not shown). The frequency of *DKK3* methylation was higher in multicentric HCC (Table 2).

#### Overall and progression-free survival analysis

To analyze the overall and progression-free survival rate associated with *DKK3* methylation levels, patients were divided into methylation positive and negative groups according to their PMR values. The overall survival rate was not significantly different between the two groups, while the progression-free survival rate of patients with a high *DKK3* methylation level was significantly lower than that of those with a low methylation level ( $P = 0.0255$ ) (Figure 5). Univariate Cox proportional hazards model showed that the portal vein invasion and high *DKK3* methylation level were related with an increased risk of disease progression when the tumor size was larger. Multivariate analysis model further showed that these three factors were the independently prognostic indicators for HCC (Table 3).

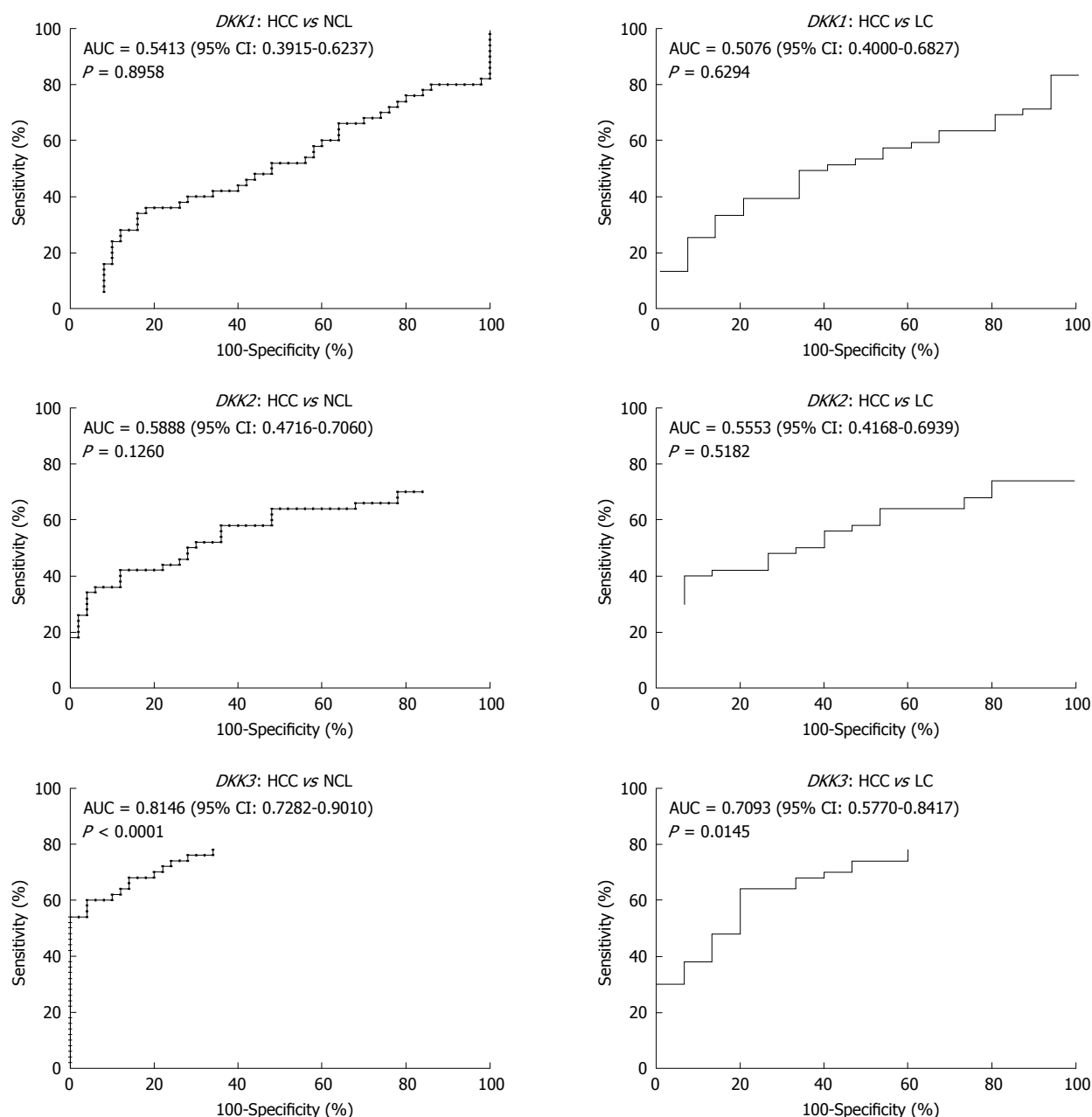


Figure 4 ROC curves for *DKK* genes used to evaluate the ability of methylation levels to distinguish tumor tissue samples from cirrhotic tissue samples.

## DISCUSSION

Development of HCC is a multistep process associated with genetic and epigenetic alterations. Methylation of multiple tumor suppressor genes is frequently observed in the development of cancer and may occur at different stages of HCC. However, not all these epigenetic alterations are directly involved in hepatocarcinogenesis. Aberrant methylation observed in HCC may be a consequence of the normal aging process, persistent viral infection, and chronic inflammation. Nishida *et al.*<sup>[13]</sup> demonstrated that methylation of tumor suppressor genes in HCC is frequent but occurs in a gene-specific and disease-specific manner. Therefore, it is important to determine the prevalence and time of promoter hypermethylation in hepatocarcinogenesis, especially at the

stage of hepatocellular transformation from a cirrhotic background. *DKK1* acts as a powerful inhibitor of the Wnt signaling pathway, and epigenetic inactivation of *DKK1* has been observed in various cancers<sup>[14-16]</sup>. In our study, a high frequency of *DKK1* methylation was also observed in liver tissue samples (Table 1), whereas quantitative methylation analysis revealed that there was no statistically different *DKK1* methylation in HCC and liver cirrhosis tissue samples, including tumor-adjacent cirrhosis and cirrhotic biopsy samples (Figure 2), suggesting that methylation of *DKK1* may be involved in early hepatocarcinogenesis but not directly contributes to neoplastic transformation from the liver cirrhotic background. Methylation of *DKK2* has been observed in some kinds of tumor, but there is little direct evidence that epigenetic inactivation of *DKK2* contributes to tumor development<sup>[14-16]</sup>. In our

**Table 2** Correlation between *DDK3* methylation and clinicopathological characteristics

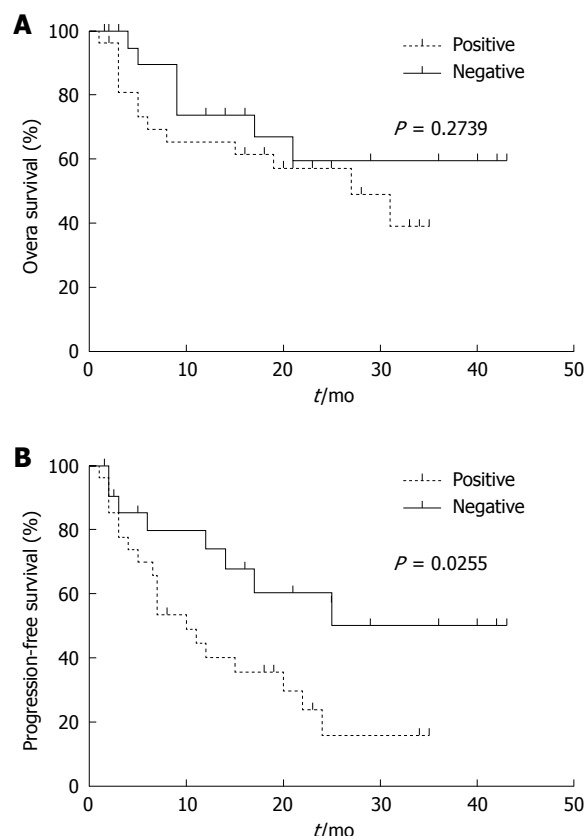
	Positive (PMR > 1%)	Negative (PMR ≤ 1%)	P
Total	27	23	
Sex			
Male (n = 43)	23	20	1.000
Female (n = 7)	4	3	
Age (yr)			
≤ 55 (n = 28)	14	14	0.5773
> 55 (n = 22)	13	9	
Virus status			
Positive (n = 44) <sup>1</sup>	22	22	1.000
Negative (n = 6)	3	3	
AFP (ng/mL)			
≤ 400 (n = 30)	17	13	1.000
> 400 (n = 20)	11	9	
Tumor size (cm)			
≤ 5.0 (n = 19)	9	10	0.5630
> 5.0 (n = 31)	18	13	
Number of tumor			
Single (n = 31)	13	18	0.0417
Multiple (n = 19)	14	5	
Portal vein invasion			
Positive (n = 17)	9	8	1.000
Negative (n = 33)	18	15	

<sup>1</sup>Including 42 cases of hepatitis B virus and 2 cases of hepatitis C virus carriers. AFP:  $\alpha$ -fetoprotein.

study, although *DDK2* methylation occurred in tumor and corresponding cirrhosis tissue samples with a similar frequency (Table 1), the methylation level of *DDK2* was obviously higher in tumor tissue samples than in its adjacent cirrhosis tissue samples (Figure 2), indicating that methylation of *DDK2* may accumulate with the development of HCC. Interestingly, the *DDK2* methylation level was higher in cirrhotic biopsy samples than in HCC tissue samples with no statistical significance, possibly due to the aberrant methylation in fibroblasts and stromal cells of cirrhotic biopsies<sup>[17]</sup>.

In contrast to *DDK1* and *DDK2*, *DDK3* is methylated in a tumor-specific manner during hepatocarcinogenesis. In our study, *DDK3* methylation occurred more frequently in HCC tissue samples than in cirrhosis tissue samples and the *DDK3* methylation level was also dramatically higher in HCC tissue samples than in cirrhosis tissue samples, indicating that *DDK3* promoter methylation may be an important event in the hepatocellular transformation from a cirrhotic background. Hsieh *et al.*<sup>[18]</sup> found that *DDK3* expression level is lower in human hepatoma tissue samples than in noncancerous liver tissue samples. It is thus reasonable to postulate that methylation and subsequently epigenetic inactivation of *DDK3* gene may play an important role in hepatocarcinogenesis.

Although distinct methylation patterns of *DDK3* were observed in HCC tissue samples and its adjacent liver cirrhosis tissue samples, no significant difference in mRNA expression was observed between the methylated and unmethylated groups. Similar results showing a lack of clear inverse correlation between the methylation and gene expression data have also been observed<sup>[19]</sup>. HCC

**Figure 5** Overall (A) and progression-free (B) survival analysis of patients with different *DDK3* methylation levels.

tissue is very heterogeneous and our tissue samples were not microdissected to remove contaminated normal cells. The presence of a substantial amount of normal tissue in specimens prevents an exact assessment of the gene inactivation effects of CpG island hypermethylation. Gene expression in normal stromal and epithelial cells can mask a lack of expression in a subset of cells with CpG island hypermethylation<sup>[19]</sup>. Therefore, analysis of aberrant DNA hypermethylation is advantageous over gene expression analysis in that it has a greater sensitivity in the presence of contaminated normal cells<sup>[19]</sup>.

The potential values of *DDK* gene methylation were further assessed in our study for clinical diagnosis purpose. The distinct methylation patterns of *DDK* gene (methylation frequencies or levels) in benign and malignant tissues are the prerequisite to determine a certain gene methylation as an effective molecular biomarker. Therefore, in addition to comparison of methylation frequencies in tumor and non-tumor groups, we further evaluated the discriminatory ability of quantitative methylation levels (PMR values) to distinguish HCC tissue from liver cirrhosis tissue using ROC analysis. Our results showed that the PMR values of *DDK3* could discriminate HCC from liver cirrhosis with high sensitivity and specificity (Figure 4), suggesting that *DDK3* methylation in combination with other diagnostic tools, may be a promising epigenetic biomarker for early detection of HCC.

It has been reported that aberrant *DDK3* methylation is a major event in early and late liver malignant trans-

Table 3 Cox regression model of progression-free survival

	Univariate analysis			Multivariate analysis <sup>1</sup>		
	RR	95% CI	P	RR	95% CI	P
Tumor size (cm)						
> 5.0	3.653	1.085-12.304	0.036	3.345	1.312-8.526	0.011
≤ 5.0	1			1		
Portal vein invasion						
Positive	2.657	1.023-6.900	0.045	3.188	1.294-7.852	0.012
Negative	1			1		
<i>DDK3</i> methylation						
Positive (PMR > 1%)	2.370	0.965-5.823	0.060	2.527	1.063-6.008	0.036
Negative (PMR ≤ 1%)	1			1		
AFP (ng/mL)						
> 400	1.829	0.759-4.409	0.179			-
≤ 400	1					
Age (yr)						
> 55	1.552	0.569-4.234	0.391			-
≤ 55	1					
Number of tumor						
Multiple	0.922	0.280-3.036	0.893			-
Single	1					

<sup>1</sup>Only the variables with *P* values less than 0.05 are included in the equation. RR: Relative risk.

formation and may constitute a critical target for risk assessment, treatment, and chemoprevention of HCC<sup>[20]</sup>. Therefore, another major question addressed in the present study concerns the prognostic value of *DDK3* methylation in human HCC. In the present study, progression-free survival analysis showed that patients with *DDK3* methylation tended to have relapse or metastasis shortly after resection (Figure 5). Multivariate Cox regression analysis further confirmed that *DDK3* methylation was an independent prognostic indicator (Table 3). Interestingly, the number of tumors (single or multiple) was the only clinicopathological variable associated with *DDK3* methylation in this study. HCC is prone to multicentric occurrence, and some other tumor suppressor genes are specifically methylated in multicentric HCC and can act as clonal markers<sup>[21]</sup>. Epigenetic inactivation of genes associated with multicentric occurrence may play an important role in the relapse or progression of HCC, thus patients with a high *DDK3* methylation level can represent a subset of poor prognosis after surgical resection. Our results showed that a high *DDK3* methylation level may serve as a potential prognostic factor for HCC. However, since the number of patients in the present study was relatively small, the prognostic significance of *DDK3* methylation levels needs to be further investigated in a larger cohort of patients with a longer follow-up period.

In conclusion, methylation of *DDK3* is an important event during early malignant transformation and progression of HCC, thus representing a prognostic indicator for risk assessment of HCC.

## COMMENTS

### Background

Aberrant promoter hypermethylation of tumor suppressor genes is very common in human cancers. It not only presents one of the important mechanisms in carcinogenesis, but also serves as a type of promising biomarkers for the diagnosis or prognosis of cancer patients.

### Research frontiers

Dickkopf (*DKK*) family is one class of the secreted Wnt antagonists. Its functional loss can contribute to activation of the Wnt pathway and result in carcinogenesis. Inactivation of Wnt antagonist genes and its epigenetic mechanism have been recently characterized in many cancers. However, few reports are available on the epigenetic silencing of *DKK* gene and its clinical significance in hepatocellular carcinoma (HCC).

### Innovations and breakthroughs

Using quantitative methylation-specific polymerase chain reaction (Q-MSP) technology, the authors investigated the prevalence and time of *DKK* family methylation in adequate tumor tissue and biopsy samples. The results of this study demonstrate that methylation of *DDK3* is an important event during early malignant transformation and progression of HCC, thus representing a prognostic indicator for risk assessment of HCC.

### Applications

*DDK3* methylation was identified as a specific epigenetic event involving hepatocellular transformation at cirrhosis stage, which not only provides a clue to the molecular basis of hepatocarcinogenesis, but also offers a potentially useful marker for the early diagnosis or prognosis of HCC.

### Terminology

Percent of methylated reference (PMR) is calculated by dividing the *GENE/ACTB* ratio in a sample by the *GENE/ACTB* ratio in SssI-treated DNA and multiplied by 100. The normalization performed to obtain PMR can simplify the cross-gene comparison, since the data range 0-100.

### Peer review

This is a very well-written paper trying to find anomalies in genes and their functions that may contribute to the prognostication and differential diagnosis of HCC with dysplastic nodules.

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## Therapy and prognostic features of primary clear cell carcinoma of the liver

Sheng-Pu Ji, Qiang Li, Hui Dong

Sheng-Pu Ji, Qiang Li, Hui Dong, Department of Hepatobiliary Surgery, Cancer Hospital of Tianjin Medical University, Tianjin 300060, China

Author contributions: Ji SP performed the research and wrote the paper; Li Q analyzed and interpreted the data; Dong H contributed the new analytic tools.

Correspondence to: Qiang Li, PhD, Professor, Department of Hepatobiliary Surgery, Cancer Hospital of Tianjin Medical University, Huanhu Western Road, Hexi District, Tianjin 300060, China. [jishengpu\\_2007@qq.com](mailto:jishengpu_2007@qq.com)

Telephone: +86-22-23340123-3051 Fax: +86-22-23359984

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### Abstract

**AIM:** To clarify the therapeutic strategies and prognosis factors of primary clear cell carcinoma of the liver (PCCCL).

**METHODS:** The clinical pathological data of 64 patients with PCCCL treated with hepatectomy in our hospital from January 2000 to January 2006 were analyzed retrospectively. The patients were divided into two groups to make treatment analysis: curative resection only ( $n = 40$ ); and curative resection and postoperative chemotherapy with calcium folinate and tegafur ( $n = 24$ ). Meanwhile, the PCCCL patients were subdivided into two subgroups on the basis of the proportion of clear cells in the tumor for pathological analysis. There were 36 cases in subgroup A for which the proportion of clear cells was more than 70%, and 28 cases in subgroup B for which the proportion was less or equal to 70%, comparing analysis of median survival time of the counterpart groups. Univariate and multivariate analyses were performed to examine factors that affected clinical prognosis, recurrence and metastasis.

**RESULTS:** Median survival period of the curative surgery group was 38 mo, while the counterpart was 41 mo. Median survival period for group A was 41 mo,

while group B was 19 mo. The Kaplan-Meier method showed that capsule formation, preoperative liver function, hepatitis C virus infection, large vascular invasion and multiple tumor occurrences were related to disease-free survival. Cox regression analysis showed that the clear cell ratio, capsule formation, preoperative liver function and large vascular invasion were independent risk factors for overall survival.

**CONCLUSION:** Postoperative chemotherapy has no obvious effect on survival of patients with PCCCL. Clear cell ratio, capsule formation, preoperative liver function, and vascular invasion were independent risk factors for prognosis.

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**Key words:** Clear cell carcinoma; Hepatectomy; Prognosis; Treatment; Risk factor

**Peer reviewers:** Martin K Schilling, MD, FRCS, Professor of Surgery, Chairman, Department of General, Visceral, Vascular and Pediatric Surgery, University of Saarland, Kirrbergerstrasse, Homburg, D-66424, Germany; Takashi Kobayashi, MD, PhD, Department of Surgery, Showa General Hospital, 2-450 Tenjincho, Kodaira, Tokyo 187-8510, Japan

Ji SP, Li Q, Dong H. Therapy and prognostic features of primary clear cell carcinoma of the liver. *World J Gastroenterol* 2010; 16(6): 764-769 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v16/i6/764.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i6.764>

### INTRODUCTION

Hepatocellular carcinoma (HCC) is a major cause of cancer mortality worldwide and is increasing in incidence; driven largely by the growing hepatitis B and hepatitis C epidemics<sup>[1,2]</sup>, which comprise approximately 75% of all HCC and liver cirrhosis (70%-80% of all cases)<sup>[3,4]</sup>. Surgical resection has long been the only curative treatment;

conventional chemotherapy and radiotherapy are ineffective for HCC<sup>[5]</sup>. Despite recent advances in diagnostic and therapeutic modalities, prognosis is usually poor, particularly in patients with coexisting liver cirrhosis. Survival rates are 3%-5% in cancer registries for the United States and developing countries. In total, 55% of cases (and deaths) are in China alone<sup>[6]</sup>. Many investigations have suggested that tumor size, number of nodules, vascular invasion, tumor encapsulation, blood transfusion, high  $\alpha$ -fetoprotein (AFP) level, and resection margin status are the main prognostic factors associated with the postoperative recurrence of HCC<sup>[7]</sup>.

Primary clear cell carcinoma of liver (PCCCL) is a particular histological type of HCC, PCCCL is not frequent and has been reported to account for 7.5%-12.5% of all liver cancer cases<sup>[8]</sup>. Microscopically, all cases of PCCCL show moderate to marked cytoplasmic accumulation of glycogen and/or macro- and microvascular intracytoplasmic fat droplets that dissolve during hematoxylin-eosin (HE) staining, which leaves behind a clear cytoplasm. Generally, the tumor cells are mainly in the mid-range degree of differentiation, and low-grade malignancy. PCCCL usually has capsule formation and is localized. Surgical resection is the most promising therapeutic method for PCCCL. The outcome for patients with PCCCL is better than for those with common type counterparts, and survival improves with an increasing proportion of clear cells<sup>[9,10]</sup>. Treatment and prognosis of PCCCL are reported rarely in the literature. Also, treatment and clinical prognostic features are not fully clarified.

## MATERIALS AND METHODS

### Subjects

The participants of this study were 64 patients [40 male, 24 female (ratio 1.67:1), aged 23-73 years, mean  $55.16 \pm 10.56$  years], who received curative hepatic resection for PCCCL at the Tianjin Medical University Cancer Hospital between January 2000 and January 2006. We used the diagnostic criteria generally accepted by pathologists in China to diagnose PCCCL as follows: (1) only when it contained > 50% clear cells; (2) exception for metastatic clear cell carcinoma from other organs; and (3) diagnosis by more than two pathologists. A total of 64 patients were eligible for this study.

### Treatment method

All 64 patients underwent surgical tumor resection. Anatomical resection included hemi-hepatectomy, segmentectomy and sub-segmentectomy, based on Child-Pugh classification. Resection margin was 1 cm beyond the tumor, and surgical margins were negative when examined by the pathologists. Clear cell ratio, large vascular invasion (large blood vessels including the portal vein, hepatic vein and/or first level branch), and lymph node metastasis were confirmed by pathology.

Patients with PCCCL who had undergone their first curative hepatic resection at the Cancer Hospital of Tianjin Medical University were eligible for postoperative

adjuvant chemotherapy if they met the following entry criteria: (1) absence of detectable residual or recurrent tumors at 1 mo after curative resection; (2) age < 70 years; (3) liver function belonging to Child A or B class; (4) absence of severe cardiac complications; and (5) general health satisfactory for toleration of the contemplated chemotherapy. The exclusion criteria were the presence of clinically confirmed extrahepatic metastasis, macroscopic evidence of tumor thrombus in the inferior vena cava or the main portal vein, other previous or synchronous malignant disorders, and postoperative dysfunction of any organ. Finally 24 patients were considered as suitable candidates for our studies.

Postoperative chemotherapy of 24 cases consisted of calcium folinate and tegafur. Calcium folinate was administered at a starting dose of 200 mg/m<sup>2</sup>, as a continuous intravenous infusion over 2 h on days 1-5. Tegafur was administered at a starting dose of 850 mg/m<sup>2</sup> given intravenously over 3 h on days 1-5. Adequate intravenous hydration and antiemetic therapy were routinely administered. Chemotherapy courses were repeated every 21 d, provided patients recovered from all toxic effects. Based on the predetermined criteria of toxicity grades, the doses of chemotherapy drugs were increased or decreased by 25%. Criteria for dose reduction included development of grade 3 non-hematological toxicity or grade 4 hematological toxicity. Complete blood, differential, and platelet counts were evaluated at least once weekly and more frequently when patients were myelosuppressed during the rest period. Serum creatinine, blood urea nitrogen, electrolyte, and magnesium levels were monitored regularly during each course.

### Survival analysis methods

The prognostic factors were examined in cumulative and disease-free survival, using the following variables: age (older or younger than 50 years); sex (male *vs* female); serum hepatitis B virus (HBV) surface antigen (HBsAg) (negative *vs* positive); serum hepatitis C virus (HCV) antibody (HCVAb) (negative *vs* positive); proportion of clear cell more than *vs* less than or equal to 70%; tumor size (greater than *vs* less than or equal to 5.0 cm); liver cirrhosis (negative *vs* positive); serum levels of AFP (greater than *vs* less than or equal to 200 ng/mL); operative procedures (anatomical *vs* non-anatomical resection); lymph node metastases (negative *vs* positive); vessel invasion (negative *vs* positive); Child-Pugh classification (Grade A *vs* Grade B or C); capsule formation (negative *vs* positive); number of nodules (solitary *vs* multiple); therapeutic strategies (curative resection *vs* curative resection and postoperative chemotherapy); TNM staging (I, II *vs* III, IV).

### Follow-up

All patients were followed up to January 2009, or up to the time of death; all patients were followed up for > 3 years. Patients were examined regularly with measurement of the serum AFP level, hepatic ultrasonography and chest radiography every month after surgical resection to check

metastasis and recurrence. Six months later, we examined serum AFP level, hepatic ultrasonography and chest radiography every 3 mo. When recurrence was suspected, further evaluations were made by abdominal, chest and brain enhanced computed tomography (CT), if necessary, by ultrasound-guided biopsy or positive electron tomography/CT examination to confirm the diagnosis. Patients who died of another disease were lost to follow-up.

### Statistical analysis

Differences in the means were assessed with the  $\chi^2$  test. The cumulative survival and the life table and Kaplan-Meier method, calculated recurrence-free survival rates and the difference between the two groups was analyzed by the log-rank test. The survival curve was described using the Kaplan-Meier method. Cox regression (proportional hazard model) was adopted for the multivariate analysis of prognostic factors. Statistical software package SPSS 13.0 (SPSS Inc., Chicago, IL, USA) was employed for all of the analyses. *P* values less than 0.05 were considered statistically significant. SPSS 13.0 was employed for all of the analyses.

## RESULTS

In 36 cases (56.25%), the proportion of clear cells was > 70%; 46 cases (71.88%) were positive for HBsAg, 10 cases (14%) were positive for HCVAb, and six cases (9.32%) were negative for both HBsAg and HCVAb. Fifty-six patients had liver cirrhosis (87.50%). In 50 patients, tumor diameter was > 5.0 cm (78.13%). In 26 patients, serum AFP level was > 200 ng/mL (40.63%). Forty-four patients had a solitary tumor (68.75%); 30 had large vessel invasion (46.86%); 12 had lymph node metastasis (18.75%); 24 received postoperative chemotherapy with calcium folinate and tegafur (37.50%); and 40 received curative hepatic resection without chemotherapy (62.50%). The liver function was evaluated using Child-Pugh classification. Forty patients had grade A liver function (62.50%), 21 (32.81%) had grade B, and three (4.69%) had grade C. Forty-six patients had tumor capsule formation (71.88%). Pathological stage of PCCCL was evaluated using TNM staging. Three patients had stage I, nine had stage II, 40 patients had stage III, and two had stage IV.

### Postoperative follow-up

Seventeen patients suffered intrahepatic recurrence during follow-up; extra-hepatic metastasis occurred in 13 cases, and six patients suffered metastasis and recurrence. One patient in the curative surgical group died of perioperative complications, which resulted in a perioperative mortality of 1.56% (1/64). One patient died from traffic accidents, and two from the resection and postoperative chemotherapy group missed postoperative follow-up. After excluding the perioperative deaths and the missing patients, the postoperative cumulative and disease-free survival rates at 1, 3 and 5 years were 78.13%, 49.88% and 37.50% (mean  $\pm$  SD, 41.56  $\pm$  3.72 mo; median,

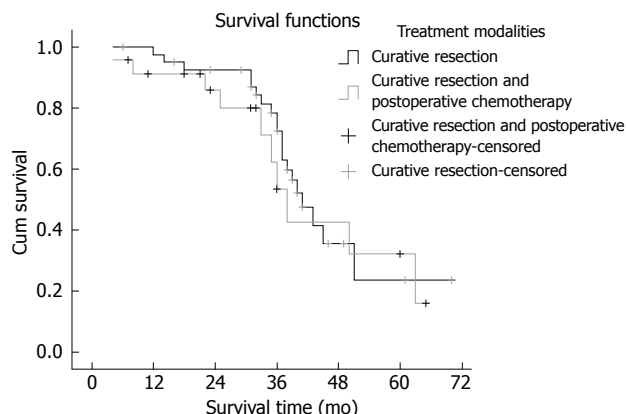


Figure 1 Comparison of survival rates of different treatment modalities.

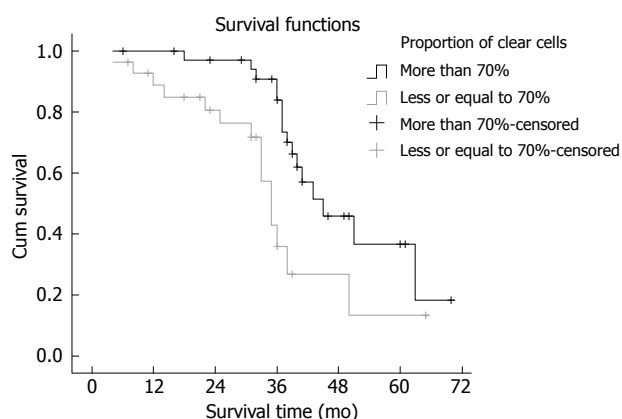


Figure 2 Comparison of survival of patients with different percentage of clear cells.

39.6 mo) and 71.82%, 40.63% and 25.00% (mean  $\pm$  SD, 34.56  $\pm$  3.93 mo; median, 33.0 mo), respectively. Median survival period in the curative surgical group was 38 mo, and 41 mo in its counterpart. There was no statistical significance in the survival time between the two groups ( $\chi^2 = 0.196$ , *P* = 0.658). The survival curves are shown in Figure 1. Median survival period of the group with > 70% clear cells was 41 mo, and 29 mo in its counterpart. The proportion of clear cells had an obvious effect on the median survival period of patients ( $\chi^2 = 7.432$ , *P* = 0.006). The survival curves are shown in Figure 2. The prognosis of the patients with a higher proportion of clear cells was better than that in patients with a lower proportion of clear cells.

### Prognostic analyses

**Univariate and multivariate analyses:** For the univariate analysis, age, sex, serum HBsAg, serum HCVAb, tumor diameter, vascular invasion, capsule formation, background of liver cirrhosis, serum AFP level, Child-Pugh classification, vascular invasion, proportion of clear cells, and lymph node metastases were included. We found that the parameters of vascular invasion, capsule formation, background of liver cirrhosis, number of nodules, proportion of clear cells, and Child-Pugh classification were statistically significant in cumulative survival (Table 1).



**Table 1** Univariate analysis of clinicopathological variables associated with the prognosis of PCCCL

Parameters	Cases	Median survival time (95% CI) (mo)	$\chi^2$ -value	P-value
AFP range (ng/mL)				
≥ 200	26	31 (27.302-34.698)	1.413	0.285
< 200	38	49 (42.211-55.789)		
Liver cirrhosis (+)				
Positive	56	31 (28.013-33.987)	6.032	0.014
Negative	8	43 (38.303-47.697)		
Capsule formation				
Positive	46	61 (53.106-68.894)	10.241	0.001
Negative	18	19 (16.690-21.310)		
Number of nodules				
Single	44	61 (53.014-68.986)	4.028	0.045
Multiple	20	11 (9.675-12.325)		
Child-Pugh classification				
A	35	60 (51.023-68.977)	11.330	0.003
B or C	21	25 (23.302-28.698)		
Vascular invasion				
Positive	30	27 (23.346-30.654)	11.755	0.001
Negative	34	41 (35.437-46.563)		
lymph node metastases				
Positive	12	31 (26.012-35.988)	0.023	0.880
Negative	52	40 (37.342-44.658)		
TNM staging				
I, II	12	43 (39.211-46.789)	16.192	0.001
III, IV	52	27 (25.371-28.629)		
Proportion of clear cells				
≥ 70%	36	41 (35.535-46.465)	7.342	0.006
< 70%	28	19 (16.964-21.036)		

PCCCL: Primary clear cell carcinoma of the liver.

**Table 2** Multivariate analysis of clinicopathological variables associated with the prognosis of PCCCL

Parameters	Regression coefficient	Wald value	P value	RR (95% CI)
Proportion of clear cells	1.409	6.898	0.009	4.090 (1.430-11.702)
Capsule formation	-1.364	5.172	0.023	0.256 (0.079-0.828)
Vascular invasion	1.686	9.923	0.002	5.395 (1.890-15.398)
Child-Pugh classification	1.917	4.119	0.042	6.798 (3.253-17.334)

Then, the parameters of significances were all contained in the Cox regression analysis. Using the Cox regression analysis, four clinic pathological variables were shown to have potential of predicting overall or disease-free survival of PCCCL patients, including rate of capsule formation, vascular invasion, Child-Pugh classification, and proportion of clear cells (Table 2).

**Cumulative recurrence-free survival rates and disease-free prognosis:** The life table and Kaplan-Meier method, calculated the cumulative recurrence-free survival rates and the difference between two groups were analyzed by the log-rank test. The 1-, 3- and 5-year disease-free survival rates were 71.82%, 40.63% and 25.00% (mean  $\pm$  SD, 34.56  $\pm$  3.93 mo; median, 33.0 mo). Kaplan-Meier univariate analysis

showed that the disease-free prognosis of the patients with capsule formation, better liver function, negative HCVAb, no vascular invasion, and solitary tumor were better than the patients with no capsule formation, poor liver function, positive HCVAb, vascular invasion and multiple tumors. Patients with no capsule formation, poor liver function, positive HCVAb, vascular invasion and multiple tumors were prone to metastasis and/or recurrence.

## DISCUSSION

PCCCL is a particular and relatively rare histological type of HCC. Microscopically, it is similar to the clear cell cancers (kidney, ovarian or adrenal), which makes it difficult to differentiate from the metastatic clear cell cancers of the liver. Murakata *et al*<sup>[11]</sup> have recommended hepatocyte antibody as a screening immunostain in working up a clear cell tumor in the liver when diagnostic histological criteria of HCC are absent. In this setting, it distinguishes PCCCL from other clear cell malignancies with a sensitivity of 90% and specificity of 100%. Some other studies have indicated *in situ* hybridization for albumin mRNA as a useful method to distinguish PCCCL from other clear cell tumors metastasizing to the liver<sup>[12]</sup>. In the present study, we made the diagnosis using features that point toward the diagnosis of HCC. This study integrated the patient's pathological features, biopsy, and clinical manifestations, imaging studies, endoscope bile stasis and postoperative long-term follow-up to make a clear diagnosis<sup>[4,13]</sup>. There was no misdiagnosis in our study. Some authors consider < 30% of clear cells within the tumor as sufficient<sup>[9]</sup>, whereas others diagnose PCCCL when the tumor contains > 30% clear cells, however, tumors with clear cells ranging from 90% to 100% are extremely rare<sup>[14]</sup>. We used the diagnostic criteria generally accepted by pathologists in China to diagnose PCCCL, that is, only when it contained > 50% clear cells<sup>[10]</sup>. In our further studies, we formed a group according to whether the clear cell count was 70% of all cells. We found that the group with > 70% clear cells had significantly longer survival ( $\chi^2 = 7.432$ ,  $P = 0.006$ ). This shows that the prognosis was related to the proportion of clear cells. The greater the number of clear cells, the better the prognosis.

Surgical resection is an effective way to achieve favorable outcomes and long-term survival of patients with PCCCL. Lao *et al*<sup>[15]</sup> have reported 1- and 3-year survival rates of 76.5% (13/18) and 47.1% (8/18), in all 13 surgical resection patients; the longest survival was 97 mo, and surgical resection was an effective treatment to achieve long-term survival. Compared with HCC, PCCCL has a slower development process, good differentiation, lower grade malignancy, and easier capsule formation, therefore, the tumor is more limited and prone to resection. Surgical resection is the most important means of achieving long-term survival. If there is recurrence after resection, tumor re-resection is possible, but if it cannot be removed, development is slower than for HCC. In the present study, there were 24 patients in the surgical resection and chemotherapy

group; the median survival period was 38.2 mo, and the median survival of the curative surgical resection group was 39.1 mo. The difference between these two groups was not significant ( $\chi^2 = 0.196$ ,  $P = 0.658$ ), which indicated that postoperative adjuvant chemotherapy with calcium folinate and tegafur was not sensitive to PCCCL and had no obvious effect on the survival time of patients. Other postoperative chemotherapy regimens for PCCCL were not investigated in this study. The prognosis of patients with postoperative chemotherapy requires further study.

Pecorella *et al*<sup>[16]</sup> have reported that a 35-year-old patient who was treated with liver transplantation survived for 17 mo, which was lower than the median survival in our study. Emile *et al*<sup>[17]</sup> have shown that prognosis was better in a large series of transplanted Caucasian patients with PCCCL than in those with other liver malignancies. In the present study, the prognosis of patients with surgical resection was better than for HCC, which may be related to better tumor differentiation, capsule formation, less vascular invasion and lymph node metastasis, and high resectability rate. The prognosis of patients with PCCCL is still controversial. Many studies have reported PCCCL has better prognosis than other HCCs<sup>[8,18]</sup>. Lai *et al*<sup>[9]</sup> have reported that the outcome for patients with PCCCL is better than those with common-type cancers, and survival improves with an increasing proportion of clear cells. Conversely, other investigators have found that the prognosis of patients with PCCCL is similar to that of their common-type counterparts and perhaps even worse<sup>[19,20]</sup>. Yang *et al*<sup>[14]</sup> have reported that the 3- and 5-year survival rate was 54.5% and 33.3%, respectively, which was slightly lower than the rate for non-PCCCL patients (including HCC). However, all these data failed to disclose any statistical significance, or were not statistically analyzed according to the number of cases. Our study confirmed the former results in a series of postoperative patients, and showed significantly higher 1-, 3- and 5-year survival rates in PCCCL patients. The Kaplan-Meier method showed that capsule formation, preoperative liver function, HCV infection, large vascular invasion and multiple tumor occurrences were related to disease-free survival. The prognosis of patients in the PCCCL group was related to clear cell ratio, preoperative liver function, liver cirrhosis, HCV infection, capsule formation, large vascular invasion and multiple tumor occurrences. In this study, lymph node metastasis did not significantly affect survival, which may have been related to the comparatively small number of cases in this study, therefore, we need to increase the number of sample cases for further study. Cox multivariate analysis showed that clear cell ratio, capsule formation, preoperative liver function and large vascular invasion were independent risk factors for survival. In this study, capsule formation of PCCCL was different from the clinical characteristics of HCC. Capsule formation may limit tumor growth and spread and is conducive to tumor resection and treatment. Lower malignancy and better differentiation

of clear cells may have contributed to the improved prognosis. The higher the proportion of clear cells, the better was the prognosis. Preoperative Child-Pugh classification was an independent risk factor for survival. High HCV prevalence led to poor liver function and shorter survival.

In summary, postoperative chemotherapy with calcium folinate and tegafur had no obvious effect on survival time of patients with PCCCL. Patients with a high clear cell ratio had improved prognosis. Capsule formation, poor preoperative liver function, HCV infection, large vascular invasion, and multiple tumor occurrence were risk factors for metastasis and postoperative recurrence of PCCCL. Patients with capsule formation, no large vascular invasion, high clear cell ratio, and better liver function had improved prognosis.

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## COMMENTS

### Background

Primary clear cell carcinoma of the liver (PCCCL) is a type of primary hepatocellular carcinoma (HCC), which is characterized pathologically by diffuse clear cells of the tumor, and a clear cytoplasm that does not stain with hematoxylin-eosin. At present, treatment and prognosis of PCCCL have not been reported widely in the literature. Its treatment and clinical prognostic features have not been fully clarified.

### Research frontiers

PCCCL is a particular histological type of HCC; PCCCL is not frequent and has been reported to account for 7.5%-12.5% of all liver cancer cases. Treatment and clinical prognostic features have not been fully clarified. The research hot topics are how to treat PCCCL, and its independent prognostic risk factors. Surgical resection is an effective way to achieve favorable outcomes and long-term survival of patients with PCCCL.

### Innovations and breakthroughs

Previously, there have been more case studies of PCCCL, and large sample studies have been rare. The present study found that postoperative chemotherapy with calcium folinate and tegafur had no obvious effect on patient survival. The study found that the higher the proportion of clear cells, the better the prognosis. The authors also found that the clear cell ratio, capsule formation, preoperative liver function, and vascular invasion were independent prognostic risk factors, which had not been reported previously.

### Applications

The study results suggest that postoperative chemotherapy with calcium folinate and tegafur has no obvious effect on patient survival. Surgical resection is an effective way to achieve favorable outcomes and long-term survival of patients with PCCCL.

### Terminology

Hepatectomy is a treatment approach that involves the surgical removal of part or all of the liver for therapeutic purposes. Postoperative chemotherapy is the use of certain drugs to further treat cancer after surgery according to certain symptoms and physical signs.

### Peer review

This is a good retrospective study which analyzed therapeutic strategies for patients with PCCCL, who were undergoing liver resection. The authors operated on 64 patients with this infrequent type of HCC within the past 6 years. The major oncological and surgical issues are discussed in the introduction and discussion.

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## Treatment of hepatitis B virus-associated glomerulonephritis: A meta-analysis

Yu Zhang, Jian-Hua Zhou, Xiao-Ling Yin, Feng-Yu Wang

Yu Zhang, Jian-Hua Zhou, Xiao-Ling Yin, Feng-Yu Wang, Department of Pediatrics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China

Author contributions: Zhang Y designed the research, collected and analyzed the data and wrote the manuscript; Zhou JH designed the research and revised the manuscript; Yin XL and Wang FY participated in the data collection.

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Correspondence to: Jian-Hua Zhou, Professor, Department of Pediatrics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China. [jhzhou@tjh.tjmu.edu.cn](mailto:jhzhou@tjh.tjmu.edu.cn)

Telephone: +86-27-83663256 Fax: +86-27-83663256

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### Abstract

**AIM:** To evaluate the efficacy of antiviral or corticosteroid treatment on hepatitis B virus-associated glomerulonephritis (HBV-GN).

**METHODS:** Six and five trials were used respectively to evaluate the efficacy of either antiviral or corticosteroid treatment on HBV-GN. Pediatric patients were pooled separately to assess their response to the above treatment modalities. The primary and secondary outcomes were remission of proteinuria and clearance of Hepatitis B e-antigen (HBeAg), respectively. A fixed or random effect model was established to collect the data.

**RESULTS:** The remission rate of proteinuria (RR = 1.69, 95% CI: 1.08-2.65) and the clearance rate of HBeAg (RR = 6.44, 95% CI: 3.11-13.35) were significantly higher in antiviral treatment group than in control group. The proteinuria remission was significantly associated with HBeAg clearance ( $P = 0.002$ ). However, the difference in proteinuria remission rate was not statistically significant between corticosteroid treatment group and control

group (RR = 1.45, 95% CI: 0.68-3.11). Antiviral therapy could significantly promote the HBeAg clearance in pediatric patients, but neither antiviral nor corticosteroid therapy could significantly decrease proteinuria in pediatric patients compared to controls.

**CONCLUSION:** Antiviral but not corticosteroid treatment can decrease proteinuria and promote HBeAg clearance in HBV-GN patients.

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**Key words:** Hepatitis B virus-associated glomerulonephritis; Drug therapy; Meta-analysis

**Peer reviewer:** Eva Herrmann, Professor, Department of Internal Medicine, Biomathematics Saarland University, Faculty of Medicine, Kirrberger Str., 66421 Homburg/Saar, Germany

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### INTRODUCTION

Hepatitis B virus-associated glomerulonephritis (HBV-GN) remains one of the most common secondary glomerular diseases in Chinese children, although its incidence seems to decrease nowadays after the popularization of HBV vaccination<sup>[1,2]</sup>. Most HBV-GN patients present with nephrotic syndrome and some show mild to moderate proteinuria with hematuria<sup>[3]</sup>. Although spontaneous remission has been reported in many pediatric patients<sup>[3]</sup>, some still develop progressive renal failure<sup>[4-6]</sup>. Therefore, it is very important to attenuate proteinuria and slow down renal disease progression in HBV-GN patients.

HBV-GN is treated with either antiviral drugs including interferon, lamivudine, and entecavir or with corticosteroids



and even immunosuppressive agents like mycophenolate mofetil, leflunomide<sup>[7,8]</sup>. It has been shown that antiviral therapy can promote the clearance of HBV and improve the coexisting renal disease<sup>[3]</sup>, but the efficacy of interferon on HBV-GN has not been confirmed<sup>[9,10]</sup>. Moreover, interferon therapy is not as successful for HBV-GN in children as for HBV-GN in adults<sup>[3]</sup>. Thus, the efficacy of antiviral therapy on HBV-GN remains to have been established, especially in pediatric patients. Corticosteroids are also used in treatment of some patients with nephrotic syndrome. However, it is argued that corticosteroid and immunosuppressive agents are unfavorable for HBV-GN since they inhibit the immune system and activate latent HBV, leading to active replication of HBV and deterioration of renal lesions<sup>[3,11]</sup>. So the efficacy of these treatment modalities on HBV-GN is still uncertain. Up to date, we are not sure if patients with HBV-GN can be treated with antiviral drugs alone and if nephrotic patients can be treated with corticosteroids.

Unfortunately, the data available in studies on HBV-GN treatment are limited and often provide inconsistent results, which can be explained by many factors like variable sample size, racial differences, disease variation as well as interference of other treatment. These inconsistencies can be solved by meta-analysis. In a meta-analysis<sup>[12]</sup> of antiviral therapy for HBV-GN published in 2006, 2 of the 6 trials included were non-controlled studies, other treatments like corticosteroids and pediatric patients were not analyzed. Thus, we performed a meta-analysis including just controlled trials to evaluate the effects of antiviral drugs and corticosteroids on HBV-GN both in adults and in children.

## MATERIALS AND METHODS

### Literature search

All eligible articles in English and Chinese published prior to November 2008 were searched from PubMed, EMBASE, Cochrane Library and CNKI. The terms, including hepatitis B virus (or hepatitis B), nephropathy, nephrotic syndrome and therapy, interferon, lamivudine, corticosteroid, prednisolone, *etc.*, were crossed. Furthermore, bibliographies of retrieved articles, proceedings of major recent meetings on nephrology and hepatology and related dissertations in English or Chinese were manually searched.

### Criteria for inclusion

Controlled clinical trials, cohort studies, and case-control studies were searched for this systematic review. The diagnosis of HBV-GN was established based on renal pathology. The primary and secondary outcomes were remission of proteinuria and clearance of Hepatitis B e-antigen (HBeAg), respectively. Only dissertations, conference papers and full-text papers published in peer-reviewed journals concerning the treatment of HBV-GN were included in the study. The decision was made based on the quality of studies rather than on their results.

### Criteria for exclusion

Publications were excluded if they were non-controlled

studies or on treatment of HBV-GN with Chinese herbal drugs. For serial reports of the same patients, only those who provided the most comprehensive information were included.

### Definition of treatment effect

The assessed outcomes included clinical and virologic responses. Clinical responses were divided into complete remission and partial remission, which were respectively defined as disappearance of proteinuria ( $< 0.3$  g/d) and reduction in urine protein excretion. Virologic response was defined as clearance of HBeAg from serum.

### Data extraction and quality assessment

Two reviewers independently selected the studies, and extracted data and outcomes according to the inclusion criteria. In case of disagreement between the two reviewers, a third reviewer was introduced to discuss with the two reviewers and extracted the data when all the three reviewers reached a consensus.

### Statistical analysis

Meta-analysis was performed using fixed-effect or random-effect methods, depending on the absence or presence of significant heterogeneity. Statistical heterogeneity between trials was evaluated by the Cochran  $\chi^2$  test and significance was considered when  $P < 0.10$ . In the absence of statistically significant heterogeneity, the Mantel-Haenszel method in the fixed-effect model was used for meta-analysis. Otherwise, the DerSimonian and Laird method<sup>[13]</sup> in the random-effect model was selected. The relative risk (RR) with 95% confidence interval (CI) was used to assess the treatment efficacy. The combined result was an average RR and 95% CI weighted according to the standard error of the RR of the trial.  $P < 0.05$  was considered statistically significant. We used funnel plots to assess the publication bias, and tested for funnel plot asymmetry using Egger's test<sup>[14]</sup> and Begg's test<sup>[15]</sup>. All analyses were performed with STATA version 9.0 (Stata Corp, College Station, Tx) and Review Manager version 4.2 (RevMan, Cochrane Collaboration, Oxford, England).

## RESULTS

### Description of included trials in the meta-analysis

Of the 998 studies we identified in the search, 55 and 943 articles were published in English and Chinese, respectively. After a review of the titles and abstracts or full texts, 989 articles were excluded and 9 articles<sup>[16-24]</sup> (8 in English and 1 in Chinese) were included based on the pre-specified criteria. One of them was randomized controlled trial (RCT)<sup>[16]</sup>, others were cohort studies. Among the 9 articles, 5 (55.6%) were from China, corresponding to the high incidence of HBV-GN in China and the low incidence in Europe and North American. The characteristics of 9 clinical trials included are shown in Table 1, and the details of intervention methods like dose and duration of drugs, main outcomes, and follow-up time in each study are provided in Tables 2 and 3.

Table 1 Characteristics of 9 included studies

Study	Country or region	Patients		Study design
		Gender	Age (yr)	
Lin <sup>[16]</sup> , 1995	Taiwan, China	29M, 11F	6.2 ± 2.4	RCT (3 score)
Bhimma <i>et al</i> <sup>[17]</sup> , 2002	South Africa	34M, 5F	8.7, 9.2	Cohort study
Lai <i>et al</i> <sup>[18]</sup> , 1991	Hong Kong, China	14M, 2F	27.2 ± 6.2	Cohort study
Tang <i>et al</i> <sup>[19]</sup> , 2005	Hong Kong, China	14M, 8F	48.3 ± 12.8, 43.1 ± 22.8	Cohort study
Panomsak <i>et al</i> <sup>[20]</sup> , 2006	Thailand	14M, 10F	39.8	Cohort study
Yang <i>et al</i> <sup>[21]</sup> , 2003	Wenzhou, China	28M, 5F	8.01 ± 1.23	Cohort study
Lai <i>et al</i> <sup>[22]</sup> , 1990	Hong Kong, China	10M, 5F	22.8 ± 14.4, 17.2 ± 8.2	Cohort study
Ozdamar <i>et al</i> <sup>[23]</sup> , 2003	Turkey	11M, 3F	10	Cohort study
Peña <i>et al</i> <sup>[24]</sup> , 2001	Spain	11M, 1F	4.52 ± 2.34	Cohort study

RCT: Randomized controlled trial.

Table 2 Design of 6 clinical trials on efficacy of antiviral therapy for HBV-GN

Author	Group	Case (n)	Intervention	Dropped-out (n)	Outcome			Follow-up
					CR	VR	Renal insufficiency (n)	
Lin <sup>[16]</sup> , 1995	Control	20	The same supportive treatment as treatment group	0	7 complete remission, 10 partial remission	0 HBeAg clearance	UA	24 mo
	Treatment	20	rIFN $\alpha$ , 5 mU (weight < 20 kg), 8 mU (weight $\geq$ 20 kg), 3 t/w for 12 mo	0	20 complete remission	16 HBeAg clearance	UA	
Bhimma <i>et al</i> <sup>[17]</sup> , 2002	Control	20	Anti-hypertension and diuretics if needed	0	0 complete remission, 5 partial remission	1 HBeAg clearance	0	40 wk
	Treatment	24	rIFN $\alpha$ -2b, 10 mU/m <sup>2</sup> , 3 t/w for 16 wk	5	10 complete remission, 4 partial remission	10 HBeAg clearance, 4 reverters, 5 failures	2	
Lai <i>et al</i> <sup>[18]</sup> , 1991	Control	11	Diuretic agents or dipyridamole or none	0	0 complete remission, 8 partial remission	0 HBeAg clearance	4	60 mo
	Treatment	5	2 wk of prednisolone 40 mg/d followed by 12 wk of rIFN $\alpha$ -2b 3 mU, 3 t/w	0	1 complete remission, 4 partial remission	1 HBeAg seroconversion	1	
Tang <i>et al</i> <sup>[19]</sup> , 2005	Control	12	ACEI or ARB	0	2 complete remission, 2 partial remission	1 HBeAg clearance, 2 HBeAg seroconversion	5 ESRD	49.2 ± 16.5 mo
	Treatment	10	3TC, 100 mg/d, 49.2 ± 16.5 mo, plus ACEI or ARB	0	7 complete remission, 3 partial remission	8 HBV-DNA clearance (5 HBeAg clearance)	0	
Panomsak <i>et al</i> <sup>[20]</sup> , 2006	Control	10	ACEI, fish oil, or neither	3	2 complete remission	0 HBeAg clearance	2 ESRD	5-120 mo
	Treatment	7	1 month of prednisolone followed by 3TC in 6 case and IFN $\alpha$ in one case	0	2 complete remission, 5 partial remission	1 HBeAg seroconversion	0	
Yang <i>et al</i> <sup>[21]</sup> , 2003	Control	14	The supportive or symptomatic treatment	0	9 complete remission, 2 partial remission	3 HBeAg seroconversion	0	3.8 ± 2.4 yr
	Treatment	6	rIFN $\alpha$ , 1-3 mU, 3 t/w for 3-6 mo	0	3 complete remission, 2 partial remission	3 HBeAg seroconversion	0	

HBV-GN: Hepatitis B virus-associated glomerulonephritis; CR: Clinical response; VR: Virologic response; UA: Unavailable; 3TC: Lamivudine; rIFN $\alpha$ : Recombinant  $\alpha$ -interferon; HBeAg: Hepatitis B e-antigen; ACEI: Angiotension converting enzyme inhibitors; ARB: Angiotensin II receptor blocker; ESRD: End-stage renal disease; t/w: Times per week.

### Therapeutic evaluation: Antiviral therapy

The efficacy of antiviral therapy on HBV-GN was assessed using 6 trials<sup>[16-21]</sup>, including 1 RCT<sup>[16]</sup> and 5 cohort studies<sup>[17-21]</sup>. The total number of patients was 159 (72 in treatment group with 5 dropped out, 87 in control group with 3 dropped out). Among the 159 patients, 133 presented with nephrotic syndrome and 134 with membranous nephropathy. The mean follow-up time was five months to ten years, significantly different between trials.

**Clinical response in antiviral treatment group and control group:** The  $\chi^2$  test of heterogeneity was highly

significant ( $P = 0.0001$ ). Accordingly, a random-effect model was used. The remission rate of proteinuria was significantly higher in antiviral treatment group (91.0%) than in control group (56.0%) with a combined RR of 1.69 (95% CI: 1.08-2.65, Figure 1A). The result of sensitivity analysis remained unchanged even if lamivudine treatment studies were excluded (RR = 1.50, 95% CI: 0.99-2.26, Figure 1B), indicating that the result is stable.

Furthermore, three trials<sup>[16,17,21]</sup> on pediatric patients were analyzed. The  $\chi^2$  test of heterogeneity was also highly significant ( $P = 0.007$ ), so a random-effect model was selected. As shown in Figure 1C, the remission rate

Table 3 Design of 5 clinical trials on efficacy of corticosteroid therapy for HBV-GN

Author	Group	Case (n)	Intervention	Dropped-out (n)	Outcome		Follow-up
					CR	Renal insufficiency (n)	
Lai <i>et al</i> <sup>[22]</sup> , 1990	Control	7	Diuretic agents	0	2 complete remission	UA	14-37 mo
	Treatment	8	Prednisolone 60 mg/d (adult), 40 mg/m <sup>2</sup> per day (< 15 yr), for 6 mo	0	3 complete remission, 4 partial remission, 1 relapse	UA	
Ozdamar <i>et al</i> <sup>[23]</sup> , 2003	Control	4	None	0	4 complete remission	UA	5-120 mo
	Treatment	8	Prednisolone, 2 mg/kg per day	2	1 complete remission, 4 partial remission, 1 death due to sepsis	UA	
Panomsak <i>et al</i> <sup>[20]</sup> , 2006	Control	10	ACEI, fish oil, or neither	3	2 complete remission	2 ESRD	5-120 mo
	Treatment	6	Prednisolone, 2 mg/kg per day	1	3 complete remission, 2 partial remission	0	
Yang <i>et al</i> <sup>[21]</sup> , 2003	Control	14	The same supportive and symptomatic treatment as treatment group	0	9 complete remission, 2 partial remission	0	3.8 ± 2.4 yr
	Treatment	8	Prednisolone, 1.5-2 mg/kg per day, for 3 mo	0	4 complete remission, 2 partial remission	0	
Peña <i>et al</i> <sup>[24]</sup> , 2001	Control	4	Symptomatic treatment	0	4 complete remission	UA	9.95 ± 5.88 yr
	Treatment	7	Prednisone, 1.5-2 mg/kg per day, a minimum of 4 wk (1 case prednisone + CTX)	0	7 steroid-resistant during therapy, but all complete remission at the end of follow-up	UA	

CTX: Cytoxan.

of proteinuria in pediatric patients was slightly higher in treatment group (86.7%) than in control group (61.1%) with a combined RR of 1.40 (95% CI: 0.80-2.47), but the difference was not statistically significant ( $P = 0.24$ ).

**Virologic response in antiviral treatment group and control group:** The  $\chi^2$  test of heterogeneity was not significant ( $P = 0.13$ ), therefore a fixed-effect model was selected. The clearance rate of HBeAg was significantly higher in antiviral treatment group (59.7%) than in control group (8.33%) with a RR of 6.44 (95% CI: 3.11-13.35, Figure 2A).

In addition, 3 trials<sup>[16,17,21]</sup> on pediatric patients were separately analyzed for virologic response. The  $\chi^2$  test of heterogeneity was significant ( $P = 0.05$ ), therefore a random-effect model was used. The clearance rate of HBeAg was significantly higher in antiviral treatment group (73.3%) than in control group (7.4%) with a RR of 10.71 (95% CI: 3.74-30.63, Figure 2B).

**Consistency analysis of clinical and virologic responses:** Kappa analysis showed that proteinuria remission was significantly related with HBeAg clearance after antiviral therapy (kappa = 0.285,  $P = 0.002$ ).

#### Effect of antiviral therapy on protection of renal function

The renal function of patients was observed in 5 of the 6 trials during the follow-up (Table 2). Renal insufficiency was found in only 3 of 47 (6.38%) patients in the antiviral treatment group and in 11 of 64 (17.2%) patients in the control group, respectively.

#### Therapeutic evaluation: Corticosteroid treatment

The efficacy of corticosteroid treatment on HBV-GN was assessed in 5 out of 9 articles<sup>[20-24]</sup>. Two of them were included in meta-analysis of antiviral therapy efficacy. Of the 23 patients in Panomsak's study<sup>[20]</sup>, 7, 6 and 10 were

treated with antiviral drugs, prednisolone and symptomatic treatment, respectively. Of the 28 patients in Yang's study<sup>[21]</sup>, 6, 8 and 14 were treated with antiviral drugs, prednisolone and symptomatic treatment, respectively. All the 5 trials were cohort studies. The clinical response at the end of follow-up is shown in Figure 3A. Of the 76 patients analyzed, 37 were in corticosteroid treatment group with 3 dropped out, 39 were in control group with 3 dropped out. Among them, 52 presented with nephrotic syndrome and 56 with membranous nephropathy. The  $\chi^2$  test of heterogeneity was highly significant ( $P = 0.001$ ), so a random-effect model was used. The combined RR was 1.45 with a 95% CI of 0.68-3.11 ( $P = 0.34$ ), indicating that there is no significant difference in proteinuria remission rate between corticosteroid treatment group and control group. However, this result should be carefully interpreted since a limited number of clinical trials can affect the conclusion of meta-analysis. Besides, it was difficult to assess the protective effect of corticosteroid treatment on renal function since only 2 of 5 clinical trials described the renal function during the followed-up.

Moreover, 3 trials<sup>[21,23,24]</sup> on pediatric patients were separately pooled to analyze the efficacy of corticosteroid treatment. The  $\chi^2$  test of heterogeneity was not significant ( $P = 0.61$ ), so a fixed-effect model was used. The combined RR was 0.91 with a 95% CI of 0.65-1.27 ( $P = 0.58$ ), indicating that the difference in the remission rate of proteinuria was also not significant between corticosteroid treatment group and control group (Figure 3B).

#### Publication bias

Publication bias may exist when no significant findings remain unpublished, thus artificially inflating the apparent magnitude of an effect. Egger and Begg tests showed that the risk of having missed trials was acceptably low, since the  $P$  values for the clinical and virologic responses to antiviral therapy and the clinical response to corticosteroid

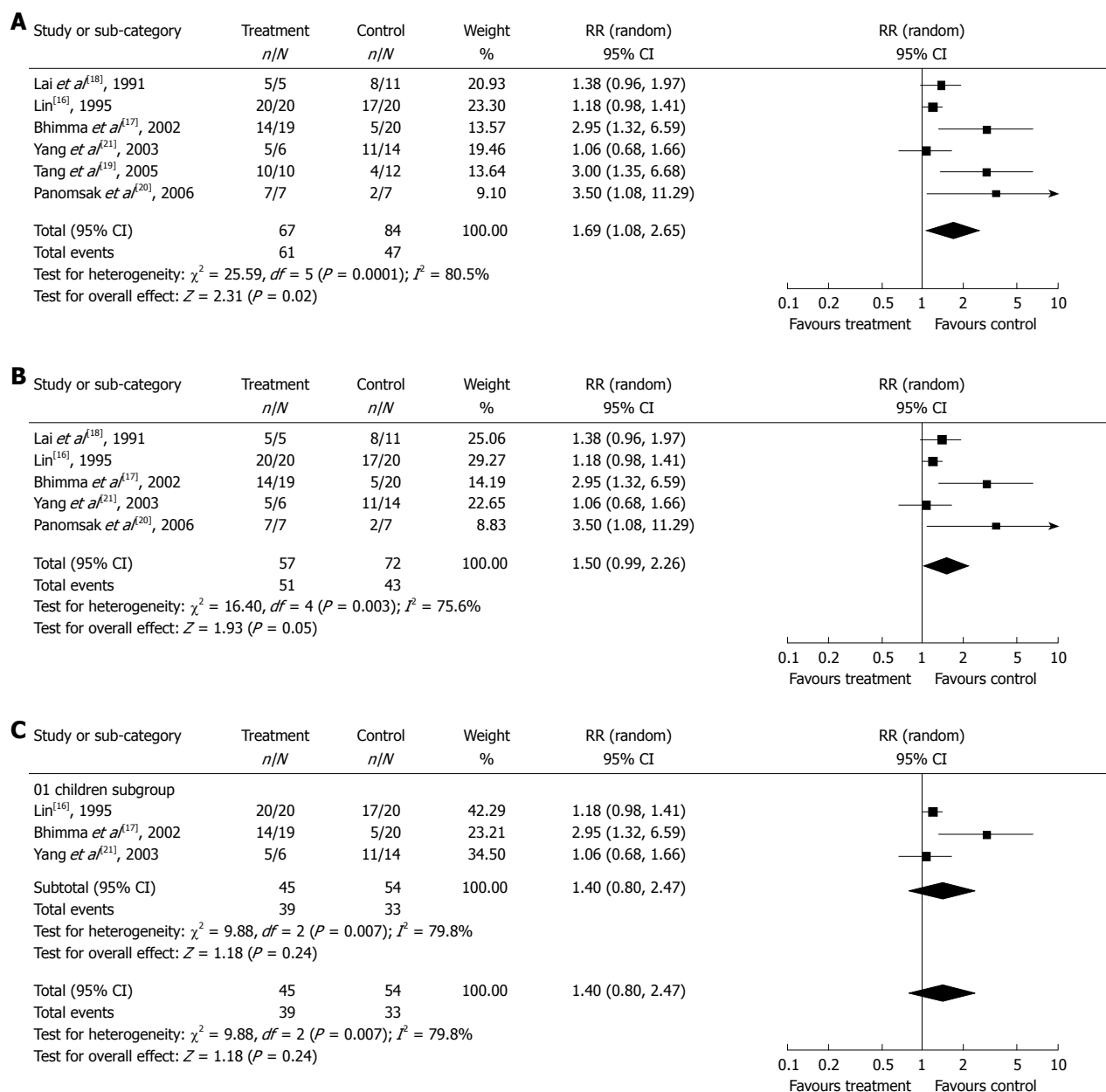


Figure 1 Proteinuria remission rate in antiviral treatment group and control group (A, B) and in pediatric patients (C).

treatment were greater than 0.05. The funnel plots of study results against precision are shown in Figure 4.

### Adverse events

Since adverse events were reported inconsistently in across studies and the relevant information in these studies was incomplete, we did not evaluate their incidence and severity of adverse events of these drugs. Some adverse events such as influenza-like illness, anemia, leucopenia, *etc.*, were reported in patients treated with IFN. Almost all patients showed good tolerance to long-term administration of lamivudine, although some patients complained of headache, dizziness, local myalgia, paresthesia, *etc.*

## DISCUSSION

Most HBV-GN patients presented with nephrotic

syndrome, many of them, especially pediatric patients showed a spontaneous remission trend, so whether the patients should be treated with antiviral drugs or with immunosuppressive agents remains to be elucidated. Antiviral therapy has been recommended in many studies for HBV-GN since it can effectively inhibit HBV replication and attenuate proteinuria<sup>[9,25-33]</sup>. Our results demonstrated that antiviral therapy could significantly improve the remission rate of proteinuria, the clearance rate of HBeAg, and renal progression. Moreover, Kappa analysis showed that proteinuria remission is significantly related with HBeAg clearance after antiviral therapy. Only 5 patients were dropped out in antiviral treatment group due to economical reasons. Almost all patients were tolerable to antiviral drugs. Our results are consistent with Fabrizi's study<sup>[12]</sup>. Since each trial used different kinds, dosages and treatment courses of antiviral drugs, the



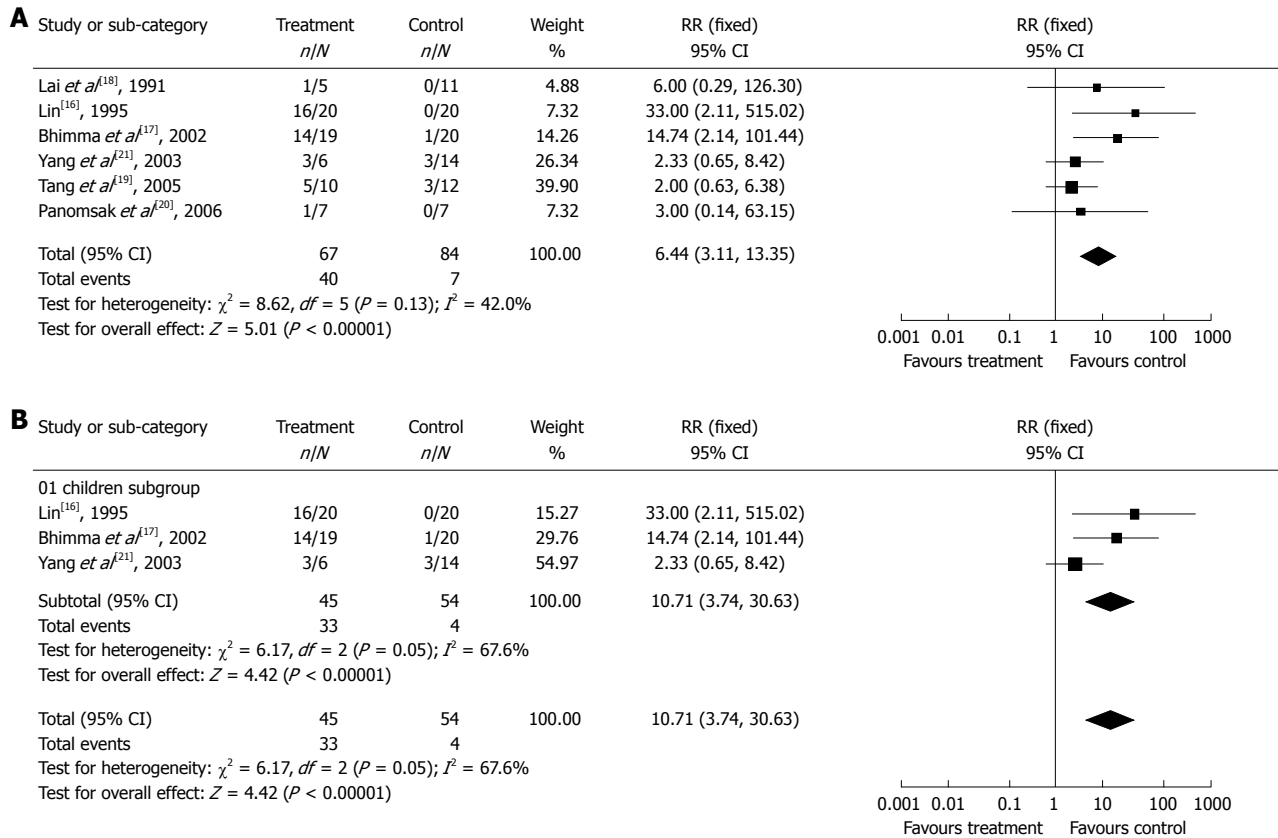


Figure 2 Clearance rate of HBsAg in antiviral treatment group and control group (A) and in pediatric patients (B).

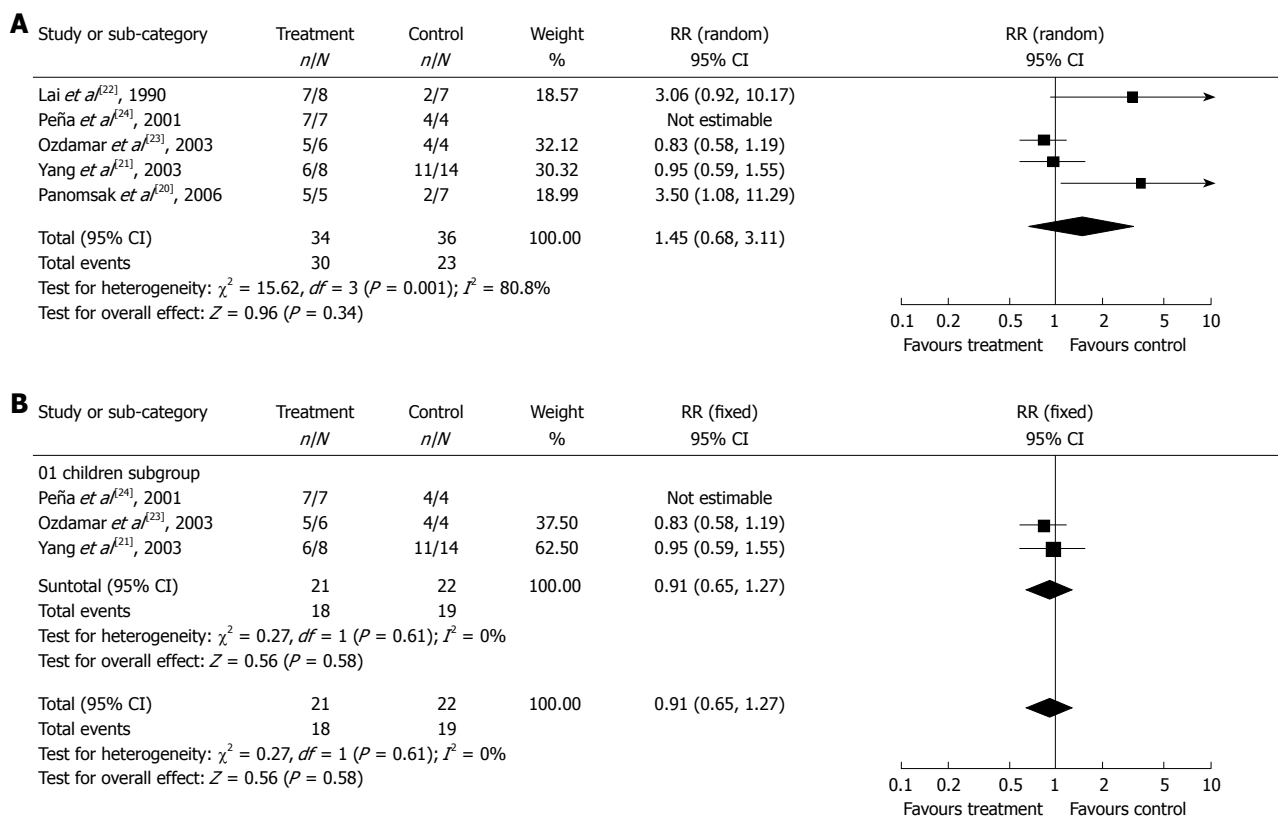
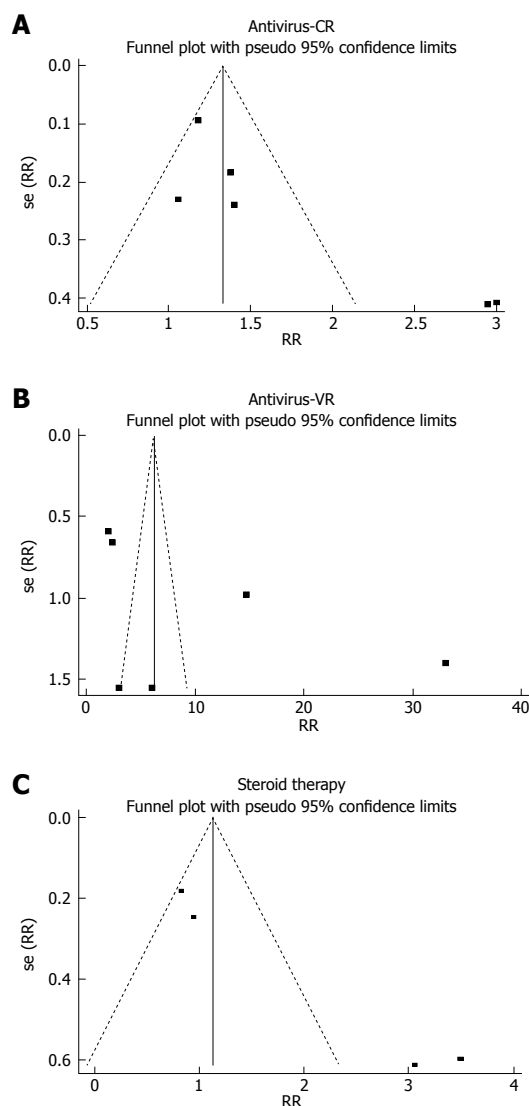


Figure 3 Proteinuria remission rate in corticosteroid treatment group and control group (A) and in pediatric patients (B).



**Figure 4** Funnel plots for 6 articles in meta-analysis of clinical response to antiviral therapy (A), 6 articles in meta-analysis of virologic response to antiviral therapy (B), and 5 articles in meta-analysis of clinical response to corticosteroid treatment (C).

meta-analysis proved the efficacy of antiviral treatment but did not necessarily mean that an exact treatment protocol should be recommended. The meta-analysis of pediatric patients showed that antiviral therapy could significantly increase the clearance rate of HBeAg, but not remarkably improve proteinuria, which is not consistent with our above findings possibly due to the limited sample size. Large-scale randomized controlled trials on pediatric patients are needed to clarify if antiviral therapy can induce remission of proteinuria.

Corticosteroid is the first-line drug for idiopathic nephrotic syndrome, but it may activate potent HBV infection leading to deterioration of liver and renal lesion<sup>[22,34-36]</sup>. Our meta-analysis showed that corticosteroid treatment could not significantly improve proteinuria. The effect of corticosteroids on proteinuria remission was not better than nonspecific symptomatic treatment, but its potent risk could not be neglected. Therefore, based on the results of this meta-analysis, corticosteroid

should not be recommended for HBV-GN patients solely, especially for those with a high viral load and abnormal liver functions. Theoretically, corticosteroid in combination with antiviral drugs is certainly superior over corticosteroid alone, but no trials are available. So corticosteroid may only be used cautiously on the basis of antiviral therapy with viral load closely monitored.

As with all meta-analyses, our study had certain limitations of publication bias<sup>[37]</sup>. The number of high-quality clinical trials and enrolled patients was limited in this study. Moreover, the time of treatment was not long enough to evaluate its effects on chronic HBV-GN.

In conclusion, the efficacy and safety of antiviral therapy (including IFN and lamivudine) on HBV-GN are good. Antiviral therapy is effective on remission of proteinuria, and HBeAg clearance, delaying renal function deterioration. However, corticosteroids cannot ameliorate HBV-GN.

## COMMENTS

### Background

Hepatitis B virus-associated glomerulonephritis (HBV-GN) is one of the common secondary glomerular diseases in China. Although spontaneous remission can occur in many pediatric patients, some still develop progressive renal failure. Therefore, it is very important to attenuate proteinuria and delay renal disease progression.

### Research frontiers

So far HBV-GN has been treated like hepatitis B with antiviral drugs including interferon, lamivudine, entecavir or like primary nephrotic syndrome with corticosteroids and even immunosuppressive agents such as mycophenolate mofetil, leflunomide, etc. However, it is still uncertain up to now about the efficacy of these treatment modalities.

### Innovations and breakthroughs

The data available in previous studies on HBV-GN treatment are limited and often provide inconsistent results. So far only one meta-analysis of antiviral therapy for HBV-GN was published in 2006, but 2 of the 6 trials included were non-controlled studies. The meta-analysis including controlled studies is the first to evaluate the effects of antiviral drugs and corticosteroids on HBV-GN. Moreover, pediatric patients were separately assessed.

### Applications

The results of this study suggest that antiviral but not corticosteroid treatment can decrease proteinuria and promote Hepatitis B e-antigen clearance in HBV-associated glomerulonephritis patients. It may help doctors to optimally treat HBV-GN patients.

### Terminology

Hepatitis B virus-associated glomerulonephritis is an immune-mediated secondary glomerular disease characterized by deposits of hepatitis B viral antigens, immunoglobulins and C3 in the glomerular capillary wall and mesangium. Nephrotic syndrome, proteinuria and/or hematuria are the most common renal manifestations.

### Peer review

The present manuscript describes a meta-analysis for the evaluation of clinical and virologic responses to antiviral and corticosteroid treatment of hepatitis B-associated nephritis. Overall, the methods are appropriate and the results are believable.

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## A case of gouty arthritis following percutaneous radiofrequency ablation for hepatocellular carcinoma

Dae Hee Choi, Hyo-Suk Lee

Dae Hee Choi, Department of Internal Medicine, Kangwon National University College of Medicine, Chuncheon 200-714, South Korea

Hyo-Suk Lee, Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine, Seoul 110-744, South Korea

Author contributions: Choi DH collected the data and drafted the paper; Lee HS reviewed the data and edited the paper.

Correspondence to: Hyo-Suk Lee, MD, Professor, Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine, 28 Yungun-dong, Chongno-gu, Seoul 110-744, South Korea. [hsleemd@snu.ac.kr](mailto:hsleemd@snu.ac.kr)  
 Telephone: +82-2-7457557 Fax: +82-2-7436701

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### Abstract

Percutaneous radiofrequency thermal ablation (RFA) is considered an effective technique for providing local control in the majority of Hepatocellular carcinoma (HCC) patients. Although RFA is generally well tolerated, recent studies have reported complications associated with RFA. We describe a case of acute gouty arthritis in a 71-year-old man with chronic renal failure who was treated with RFA for a HCC lesion and who had hepatitis B-associated cirrhosis and mild renal insufficiency. Regular surveillance of the patient detected a 3.5 cm HCC lesion. Because the patient had declined surgery, RFA was chosen for therapy. On the third post-procedural day, the laboratory results showed increases in his uric acid and potassium levels, which were compatible with a tumor lysis syndrome. On the 6th post-procedural day, the patient complained of new right knee pain. Subsequent joint aspiration revealed monosodium urate monohydrate crystals. We made the diagnosis of acute gouty arthritis arising from tumor lysis and liver infarction caused by HCC ablation, which was aggravated by acute renal insufficiency. After adequate hydration and administration of oral colchicines, the patient's right knee pain subsided and

the uric acid serum level returned to normal. This is the first described case of acute gouty arthritis after RFA for a HCC lesion in a patient with underlying chronic renal insufficiency. To avoid hyperuricemia and an acute attack of gout after RFA therapy for HCC, early identification of patients at risk is warranted, such as those with a large tumor, rapid tumor growth, and renal insufficiency, and preventative measures should be considered.

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**Key words:** Radiofrequency thermal ablation; Hepatocellular carcinoma; Gout; Tumor lysis syndrome; Complications

**Peer reviewer:** Kenji Miki, MD, Department of Surgery, Showa General Hospital, 2-450 Tenjin-cho, Kodaira, Tokyo 187-8510, Japan

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### INTRODUCTION

Although the optimal treatment for hepatocellular carcinoma (HCC) is surgical resection, there are only a small number of patients who meet resectability criteria for HCC<sup>[1]</sup>. Percutaneous radiofrequency thermal ablation (RFA) is one of the emerging therapeutic modalities used for the minimally invasive treatment in the management of liver malignancies, particularly in patients who cannot undergo surgery<sup>[2,3]</sup>. It has been reported that the morbidity and mortality rates are low, and there are few complications associated with RFA<sup>[4,5]</sup>. Although RFA is relatively well-tolerated, severe and potentially fatal complications, such as liver failure, colon perforation and portal vein



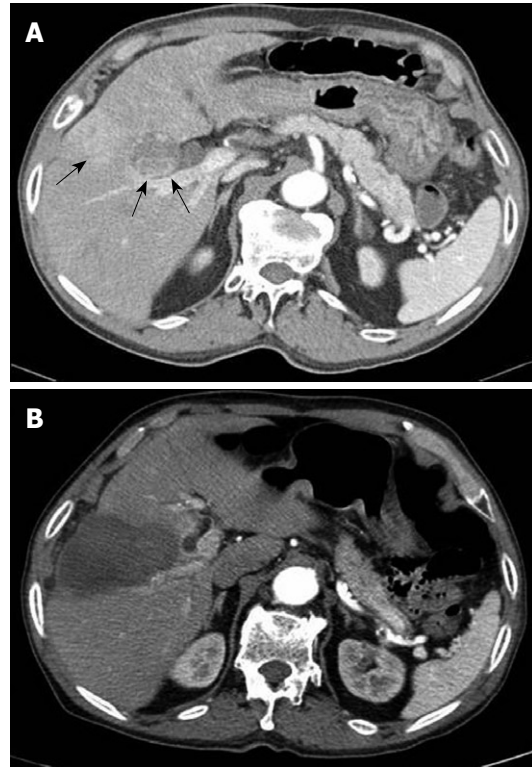
thrombosis can arise<sup>[6,7]</sup>. In addition, there are non-fatal serious complications, such as liver abscess, pleural effusion, skin burns, hypoxemia during treatment, pneumothorax, subcapsular hematoma, acute renal insufficiency, hemoperitoneum, needle tract seeding, and self-limited post-ablation syndrome<sup>[8]</sup>. However, acute gouty arthritis attacks following RFA for HCC have not been reported. We describe a case of gouty arthritis in a 71-year-old man who was treated with RFA for a large HCC in segment IV of the liver adjacent to the gallbladder bed.

## CASE REPORT

A 71-year-old male with hepatitis B virus-associated cirrhosis had undergone a previous percutaneous ethanol injection for a small HCC. Regular surveillance detected a 3.5 cm HCC lesion at segment IV of the liver adjacent to the gallbladder, posterior to the lesion where a previous percutaneous ethanol injection had been performed (Figure 1A). The baseline laboratory tests showed thrombocytopenia (platelet count,  $101 \times 10^3/\text{mm}^3$ ; normal range,  $150\text{--}440 \times 10^3/\text{mm}^3$ ), an elevated blood urea nitrogen level (44 mg/dL; normal range, 8–20 mg/dL), an increased creatinine level (1.8 mg/dL; normal range, 0.4–1.0 mg/dL), a normal uric acid level (5.5 mg/dL; normal range, 2.6–8.0 mg/dL), and a normal potassium level (3.7 mmol/L; normal range, 3.6–5.0 mmol/dL). The liver function tests revealed a borderline low albumin level (3.2 g/dL; normal range, 3.2–4.9 g/dL), a normal total bilirubin level (1.1 mg/dL; normal range, 0.3–1.2 mg/dL) and a normal prothrombin time (international normalized ratio, 0.93). The other laboratory tests, including the total calcium, phosphorus, lactate dehydrogenase (LDH), and aspartate transaminase levels, were within normal limits. Among several curative treatment options available, RFA was chosen for further therapy because the patient had declined surgery. Written informed consent was obtained from the patient before RFA.

RFA was performed under sonographic guidance using a 3.5 MHz convex probe (Sequoia, Siemens Medical Solutions). The treatment was performed under local anesthesia using 100 µg of fentanyl citrate (Myengmun) to control pain. The vital signs were monitored continuously during the procedure. Once proper positioning of the electrode in the tumor area had been confirmed by sonography, the electrodes were connected to a 500 KHz monopolar radiofrequency generator (CC-1, Valleylab) capable of producing 200 W. Four internally cooled 17-gauge electrodes (Cool-Tip, Valleylab) with 3.0 cm exposed tips delivered radiofrequency energy to the tumors. During withdrawal of the electrode, the entire electrode track was heated briefly to a temperature of 80°C by application of radiofrequency. The procedure lasted 23 min with complete ablation of the HCC without apparent complications, such as injury to the gallbladder, on the immediate follow-up computed tomography scan (Figure 1B).

The morning after the procedure, the patient complained of abdominal distension. A simple abdominal radiograph revealed no evidence of bowel perforation,

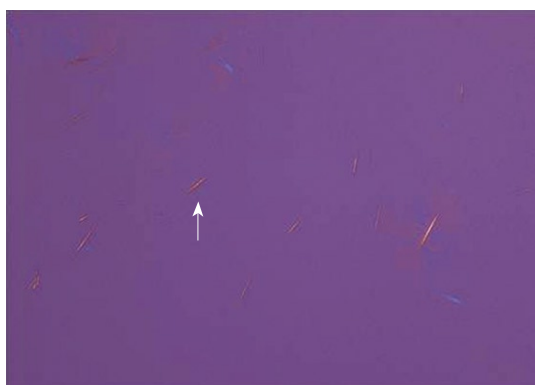


**Figure 1** Computed tomography (CT) scan of the patient. A: CT scan in an arterial phase demonstrates a 3.5 cm hepatocellular carcinoma lesion (arrows) at segment IV of the liver adjacent to the gallbladder, posterior to the lesion where a previous percutaneous ethanol injection has been performed; B: CT scan obtained after radiofrequency thermal ablation reveals complete ablation for the hepatocellular carcinoma without apparent complications such as a gallbladder injury on the immediate follow-up.

although the large intestine was distended (Figure 2). The laboratory results were normal, except an elevation of the potassium level (5.0 mmol/L) and a sustained elevated creatinine level (1.7 mg/dL). On the third post-procedural day, although the patient's symptoms had not changed, repeat laboratory results showed that his uric acid level had increased to 8.7 mg/dL, and the potassium level was maintained at 5.1 mmol/L, which were compatible with a tumor lysis syndrome. The urine output was adequate, and the acid-base gas analysis revealed no disturbances. The patient was hydrated with crystalloids and subjected to close observation. On the 6th post-procedural day, the patient complained of new right knee pain. The physical examination showed swelling and tenderness on the medial side of the right knee that was warm to touch. Joint aspiration revealed a yellow serous fluid that was confirmed, by polarizing microscopy, to be monosodium urate monohydrate crystals (Figure 3). On the same day, the laboratory data revealed that the serum uric acid and creatinine levels increased to 9.8 mg/dL and 3.2 mg/dL, respectively, which were both higher than the baseline levels before the RFA procedure. We made the diagnosis of acute gouty arthritis arising not only from tumor lysis caused by HCC ablation, but also from liver infarction adjacent to the tumor by broad ablation, which was aggravated by acute renal insufficiency in chronic renal failure. We treated the patient with an adequate intravenous



**Figure 2** A simple abdominal radiograph reveals no evidence of bowel perforation, except a distension of the large intestine.



**Figure 3** Strongly negative birefringent, needle-shaped monosodium urate crystals (arrow) in synovial fluid from a patient under compensated polarized light.

crystalloid infusion and oral colchicine (0.6 mg/d). As a result, the patient's right knee pain subsided. On the 11th post-procedural day, the uric acid level was within normal limits (7.2 mg/dL) and the levels of other electrolytes and creatinine had returned to baseline values.

## DISCUSSION

Tumor lysis syndrome has been reported for many different types of poorly differentiated lymphomas, such as high grade non-Hodgkin's lymphoma, Burkitt's lymphoma, and acute lymphocytic leukemia<sup>[9]</sup>. Because this syndrome is mostly frequently related to the cytotoxic treatment of poorly differentiated lymphomas or combination chemotherapy, it has rarely been reported for solid organ tumors, including lung, breast, and advanced gastric cancers<sup>[10-12]</sup>. However, several recent reports have demonstrated that tumor lysis syndrome may occur after various treatments of HCC, including RFA and transarterial chemoembolization (TACE)<sup>[13-15]</sup>. Shiba *et al*<sup>[14]</sup> reported a case of TACE-induced tumor lysis syndrome resulting from necrosis of a large HCC. Moreover, they suggested that factors predisposing a patient to tumor lysis include large tumor size, tumors with high sensitivity to treatment, renal insufficiency, dehydration, and hyperuricemia before treatment<sup>[14]</sup>.

Tumor lysis syndrome causes hyperuricemia, hyperkalemia, hyperphosphatemia and hypocalcemia, and may lead to acute renal failure or metabolic acidosis resulting

from deposition of uric acid in the kidney, and calcium phosphate complex in renal tubules<sup>[16]</sup>. In our patient, although metabolic acidosis did not develop, tumor lysis syndrome, including hyperuricemia, hyperkalemia, and acute renal failure did develop. Therefore, we believe that acute tumor lysis syndrome occurred in our patient as a result of tumor necrosis following RFA.

Lehner *et al*<sup>[15]</sup> reported a death caused by acute tumor lysis syndrome after ablation of a 3.2 cm HCC. They indicated that patients with pre-existing azotemia, along with elevated baseline LDH which exists in most cases of cirrhosis, and elevated uric acid level are at increased risk of tumor lysis syndrome<sup>[15]</sup>. Therefore, we suggest that the predisposing factors in our case included underlying cirrhosis with pre-existing azotemia. Moreover, because we observed that the ablated field was large compared to the target lesion, we believe hyperuricemia could be caused by liver infarction induced by ablation.

We describe, for the first time, a case of acute gouty arthritis as a possible complication following thermal ablation for HCC. A definite diagnosis of gout was made by identification of monosodium urate crystals within phagocytes in the synovial fluid using compensated polarized microscopy<sup>[17]</sup>. It is well known that numerous circumstances, such as surgery, dietary overindulgence, and ingestion of drugs affecting serum uric acid concentrations are associated with acute attacks of gouty arthritis<sup>[18]</sup>. The likely explanation for the development of acute gouty arthritis in this elderly man was the abrupt increase in the serum uric acid level from tumor cell necrosis after HCC ablation. Therefore, in this case, the acute gouty attack on the 6th post-procedural day was likely caused not only by chronic renal insufficiency, but also by hyperuricemia following tumor cell lysis and liver infarction.

After the diagnosis of acute gouty arthritis was established, the patient received colchicine immediately. Although colchicine is not a urate-lowering agent, adequate hydration and colchicine administration resulted in lowering of the serum level of uric acid to the baseline value and resolution of the patient's right knee pain 2 d after drug administration.

To date, RFA is considered an effective procedure for providing local control in HCC patients. RFA is associated with the possibility of effecting long-term, disease-free survival in selected patients compared to other local techniques, such as cryoablation and percutaneous ethanol injection<sup>[19,20]</sup>. Although RFA is a generally well-tolerated and relatively safe localized-regional therapy, complications can develop<sup>[4-8]</sup>. A recent report showed that RFA was associated with a 4% rate of major complications, a 4.8% rate of minor complications, and a negligible risk of death<sup>[4,5]</sup>. As shown in this case, acute gouty arthritis is not a life-threatening condition, but it affects the quality of life of the patient. Therefore, during therapy, patients who are at risk should be monitored closely for this possible complication. To avoid hyperuricemia and acute gouty arthritis after RFA therapy for HCC, early identification of patients at risk is warranted, such as those with a large tumor, rapid tumor growth, and renal insufficiency, and

preventative measures should be considered.

In conclusion, this is the first described case of acute gouty arthritis after RFA for a HCC lesion in a patient with underlying chronic renal insufficiency. This complication should be considered in patients at risk. Appropriate screening, management, and treatment may reduce the complications associated with RFA.

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## Multiple chronic non-specific ulcer of small intestine characterized by anemia and hypoalbuminemia

Yan Chen, Wang-Qian Ma, Jia-Min Chen, Jian-Ting Cai

Yan Chen, Wang-Qian Ma, Jia-Min Chen, Jian-Ting Cai,  
 Department of Gastroenterology, Second Affiliated Hospital  
 of Medical College, Zhejiang University, Hangzhou 310009,  
 Zhejiang Province, China

**Author contributions:** Ma WQ and Cai JT contributed equally  
 to this work, provided the data; Chen JM performed the capsule  
 endoscopy; Chen Y wrote the paper.

**Correspondence to:** Dr. Wang-Qian Ma, Department of  
 Gastroenterology, Second Affiliated Hospital of Medical  
 College, Zhejiang University, Hangzhou 310009, Zhejiang  
 Province, China. [chenyan72\\_72@hotmail.com](mailto:chenyan72_72@hotmail.com)

Telephone: +86-571-87783936 Fax: +86-571-88833653

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### Abstract

A female patient with anemia and hypoalbuminemia was admitted to our hospital due to an over 20-year history of recurrent dizziness, fatigue and ankle edema. She was diagnosed as multiple chronic non-specific ulcer of the small intestine characterized by non-specific histology and persistent gastrointestinal bleeding.

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**Key words:** Small intestinal ulcer; Hypoalbuminemia; Anemia; Gastrointestinal bleeding; Capsule endoscopy

**Peer reviewer:** Dr. Dinesh Vyas, Department of Minimally and Endoscopic Surgery, St John Mercy Hospital, 851 E Fifth Street, Washington, MO 63090, United States

Chen Y, Ma WQ, Chen JM, Cai JT. Multiple chronic non-specific ulcer of small intestine characterized by anemia and hypoalbuminemia. *World J Gastroenterol* 2010; 16(6): 782-784  
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### INTRODUCTION

With the wide use of capsule endoscopy, small intestinal ulcer associated with chronic bleeding is commonly seen in clinical practice. However, its diagnosis and treatment are complicated. After the causes of ulcer such as Crohn's disease, Behcet's disease and tuberculosis are excluded, most cases are diagnosed as "idiopathic chronic ulcerative enteritis"<sup>[1]</sup>. Since 1960, cases of non-specific ulcer of small intestine have been reported<sup>[2-4]</sup>. Most of them were caused by non-steroidal anti-inflammatory drugs (NSAID) or potassium tablets. Matsumoto *et al*<sup>[5,6]</sup> have reported a multiple chronic non-specific ulcer of small intestine (CNSU), which is not related to NSAID. We, here, report a case of multiple chronic non-specific ulcer of the small intestine characterized by non-specific histology and persistent gastrointestinal (GI) bleeding.

### CASE REPORT

A 42-year-old female was admitted to our hospital due to an over 20-year history of recurrent dizziness, fatigue and ankle edema. Over the past 20 years, she had dizziness, fatigue and abdominal distension without any identifiable causes. At the same time, she developed ankle edema which resolved spontaneously after rest. Laboratory test at a local hospital showed that she had severe iron deficiency anemia [hemoglobin (HB) 27 g/L] and hypoalbuminemia (albumin 11 g/L). Her symptoms could always be relieved temporarily after intravenous infusion of albumin and packed red blood cells. However, over the past 4 mo, she had repeated episodes of chest tightness and palpitation after activities. She also complained of intermittent abdominal pain after meal but had no fever, nausea or vomit. Her bowel movements were normal except for occasionally mild diarrhea. She denied any decreased appetite, nausea and vomiting, hematemesis, hematochezia, melena, tenesmus and stools with mucus or pus.



She had no known history of hepatitis or chronic renal disease or smoking or alcohol, and exposure to contaminated water. She denied any use of NSAID. Her parents were healthy and her little brother was dead at the age of 10 years due to unknown cause. Her son and husband were healthy.

Physical examination demonstrated that her temperature was 36.8°C, blood pressure was 92/60 mmHg, respiration was 18/min, and heart rate was 92 bpm. The patient was alert and fully oriented but appeared pale, cold and clammy. She had no jaundice, liver palm or spider angioma, eruption or purpura on the skin. No superficial lymph nodes could be palpated. Her trachea was slightly shifted to the left with no jugular venous distention. Her right chest wall movement and tactile fremitus over the right lung base were decreased with absent breath sounds and dullness to percussion. Her heart had a grade 2/6 diastolic murmur at the apex. An abdominal bulge and positive shifting dullness were found with active bowel sound. No abdominal wall vein dilatation, tenderness or rebound tenderness were found. No mass, liver and spleen were palpable. Digital rectal examination was negative. She had moderate pitting edema of the lower extremities.

Her white blood cells were  $8.0 \times 10^{12}/L$ , HB was 59 g/L, mean cell volume was 65 fL, mean corpuscular hemoglobin was 18.1 pg, and platelets were  $445 \times 10^9/L$ . Her TB/CB was 0.15/0.07 mg/mL, A/G was 1.3/2.0, alanine aminotransferase was 25 U/L, aspartate aminotransferase was 20 U/L, reticulocytes was 2.9%, erythrocyte sedimentation rate was 28 mm/h. Bone marrow aspiration showed proliferation of erythrocyte lineage and mature red blood cells with an increased central pallor. Iron staining revealed 2% intracellular iron but no extracellular iron. No proteinuria or haematuria was detected at a urinalysis. Renal function was normal. Rheumatoid factors and full antinuclear autoantibodies were negative. Serological tests were negative for hepatitis B virus, hepatitis C virus and human immunodeficiency virus. Her serum  $K^+$  and  $Na^+$  were 3.15 mEq/L and 133 mEq/L, respectively. Abdominal ultrasonography showed the presence of moderate ascites and a right pleural effusion. Fecal occult blood test (FOBT) was positive while stool culture was negative. Both upper GI endoscopy and colonoscopy were negative. Capsule endoscopy showed multiple, sharply demarcated ulcers limited in the ileum with a circular shape. The margins of ulcers were clear and the intervening mucosa was normal. Stenosis could also be seen in the ileum.

The patient was treated with intravenous infusion of albumin and packed red blood cells as well as intramuscular injection of iron. After 4 wk of treatment, the dizziness and abdominal distension were gradually improved and lower extremity edema also receded. Hb increased temporarily to 90 g/L and abdominal ultrasound showed only a small amount of ascites and pleural effusion. However, FOBT remained positive and abdominal pain did not relieve. Oral prednisone was given for 1 wk but did not relieve the pain. A laparotomy was proposed

but she refused. She was discharged and scheduled for outpatient follow-up.

## DISCUSSION

GI bleeding-induced anemia is the most typical presenting symptom of patients suffering from CNSU and low serum protein concentration is also seen<sup>[5]</sup>. Our patient had pronounced anemia and hypoalbuminemia. Her initial manifestation was pronounced anemia followed by ascites and pleural effusions. The diagnostic criteria for CNSU were established as previously described<sup>[6]</sup>, including persistent anemia for more than 1 year, small intestinal ulcers, absence of clinical evidence suggestive of mycobacterial infection, absence of clinical evidence suggestive of Crohn's disease, and lack of any dermatologic, ophthalmologic or genital symptom suggestive of Behcet's disease. The ulcers in our patient were different from those of Crohn's disease characterized by longitudinal ulcers and cobblestone appearance. There was also no evidence of complication suggestive of Crohn's disease because no perforation and fistulisation were found although the patient had a very long course of disease. Behcet's disease could also be excluded since there was no clinical evidence showing dermatologic, ophthalmologic or genital symptom in our patient.

Capsule endoscopy confirmed the diagnosis of CNSU in our patient. Matsumoto *et al.*<sup>[5]</sup> reported that ulcers in CNSU patients are predominantly found in the ileum, which are circular or irregular in shape. The margins of ulcers are always clear and the intervening mucosa appears normal. Capsule endoscopy showed typical circular ulcers limited in the ileum of our patient, which is consistent with the reported findings<sup>[5]</sup>.

The clinical and endoscopic features of CNSU are similar to those of NSAID-induced enteropathy. Matsumoto *et al.*<sup>[6]</sup> compared the enteroscopic findings in CNSU and NSAID-induced enteropathy, and found that both are characterized by concentric stenosis and ulcers with non-specific histology while the lesions of small intestine are different in respect to their site and stage. CNSU patients have active, sharply demarcated ulcers limited in ileum while few ulcers are found in NSAID-induced enteropathy. Since our patient had no history of NSAID use, the possibility of NSAID-induced enteropathy could be excluded.

CNSU is different from another idiopathic small intestinal multiple ulcer disease described as cryptogenic multifocal ulcerous stenosing enteritis (CMUSE)<sup>[7]</sup>, which is an independent, rare and poorly understood disease characterized by non-specific small intestinal ulceration and stenosis which responds to corticosteroid therapy<sup>[7]</sup>. Perlemuter *et al.*<sup>[7]</sup> described that CMUSE syndrome is characterized by chronic diarrhea, bouts of intestinal obstruction, and ulcerative stenosis of the small intestine. A very important feature of CMUSE is that patients respond dramatically to corticosteroid therapy<sup>[7]</sup>. However, the therapeutic effect of corticosteroid in our patient was not good. Another dif-

ference is that anemia and hypoalbuminemia are not often seen in CMUSE patients. Our patient had a long history of pronounced anemia and hypoalbuminemia prior to the development of abdominal pain, suggesting that stenosis may not develop rapidly in CNSU.

Capsule endoscopy is the best diagnostic tool for obscure GI bleeding. Our patient was admitted because of her chronic GI bleeding with unknown origin. Since upper GI endoscopy and colonoscopy showed negative results, capsule endoscopy showed multiple circular ulcers. The affected site was limited in the ileum, thus providing the most important evidence for the diagnosis of our patient.

In conclusion, the pathophysiology of CNSU remains poorly understood. CNSU is a disease responsible for obscure GI bleeding arising from the small intestine. Capsule endoscopy contributes to the diagnosis of this peculiar form of small intestine disease.

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## Meta-analysis of capsule endoscopy in patients diagnosed or suspected with esophageal varices

Daniel Ahn, Praveen Guturu

Daniel Ahn, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75390, United States

Praveen Guturu, Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX 77550, United States

**Author contributions:** Guturu P designed and wrote the letter; Ahn D extracted analyzed the data.

**Correspondence to:** Praveen Guturu, MD, Department of Internal Medicine, University of Texas Medical Branch, 301 University Boulevard, Route 0570, Galveston, TX 77555, United States. [prguturu@utmb.edu](mailto:prguturu@utmb.edu)

Telephone: +1-409-7721011 Fax: +1-409-7725462

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### Abstract

The PillCam ESO (Given Imaging, Israel) or esophageal capsule endoscopy (ECE) is a novel technique used in the diagnostic evaluation of esophagus. Many studies have been performed to compare the accuracy of ECE against the current gold standard esophago-gastro-duodenoscopy and a meta-analysis recently published by Lu *et al* suggests that ECE may have an acceptable sensitivity and specificity in detecting esophageal varices. We would like to discuss the importance and implication of publication bias in this meta-analysis.

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**Key words:** Capsule endoscopy; Screening varices

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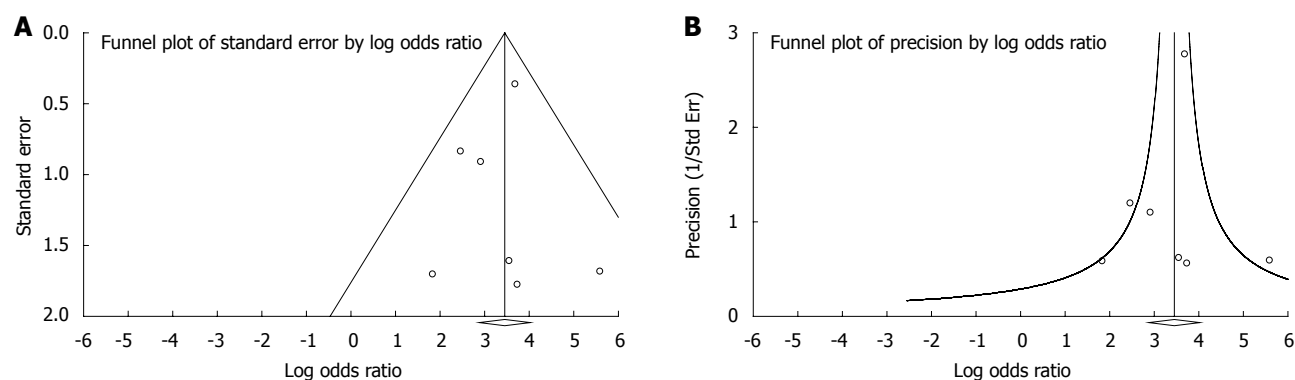
Ahn D, Guturu P. Meta-analysis of capsule endoscopy in patients diagnosed or suspected with esophageal varices. *World J Gastroenterol* 2010; 16(6): 785-786 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i6/785.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i6.785>

### TO THE EDITOR

In March 2009 issue of *World Journal of Gastroenterology*, Lu *et al*<sup>[1]</sup> published their interesting findings regarding the accuracy of esophageal capsule endoscopy (ECE) in detecting esophageal varices and its utility in screening and surveillance of esophageal varices. We would like to add that it is vital to comment on the presence or absence of any bias when reporting a meta-analysis as this will allow the readers to assess the strengths and weaknesses of the recommendation made. A recently published statement on preferred reporting items for systematic reviews and meta-analyses (PRISMA)<sup>[2]</sup>, an evolution of the original quality of reporting of meta-analyses (QUOROM) guidelines, suggests that publication bias should be assessed while reporting meta-analyses and systematic reviews. Since publication bias was not reported in the above meta-analysis, we analyzed the data for the presence or absence of publication bias.

We assessed the publication bias using funnel plot. Funnel plots were plotted using log odds ratio *vs* standard error (Figure 1A) and log odds ratio *vs* precision (Figure 1B), both showed no evidence of publication bias. We complemented the funnel plots with Eggers test<sup>[3]</sup> and rank correlation analysis<sup>[4]</sup> and both also showed no evidence of publication bias ( $P > 0.05$ ).

We hope that this information about the absence of publication bias in this meta-analysis will add more value to the conclusion reported in the above meta-analysis.



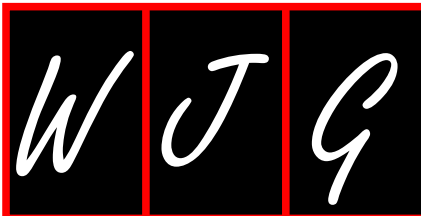
**Figure 1** Funnel plot. A: Log odds ratio vs standard error; B: Log odds ratio vs precision (Precision = 1/standard error).

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**Philip Abraham, Dr., Professor**, Consultant Gastroenterologist & Hepatologist, P. D. Hinduja National Hospital & Medical Research Centre, Veer Savarkar Marg, Mahim, Mumbai 400 016, India

**Taku Aoki, MD**, Division of Hepato-Biliary-Pancreatic and Transplantation Surgery, Department of Surgery, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan

**Marc Basson, MD, PhD, MBA**, Department of Surgery, Michigan State University, 1200 East Michigan Avenue, Suite #655, Lansing, MI 48912, United States

**Christa Buechler, PhD**, Internal Medicine I, Regensburg University Medical Center, Franz Josef Strauss Allee 11, 93042 Regensburg, Germany

**Itaru Endo, MD, PhD, Professor and Chairman**, Department of Gastroenterological Surgery, Yokohama City University, Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama, 2360004, Japan

**Omar Vergara-Fernandez, MD**, Departments of Surgery, National Institute for Medical Sciences and Nutrition Salvador Zubirán, Vasco de Quiroga No. 15, Col. Seccion XVI. Deleg. Tlalpan, CP 14000, México

**Nikolaus Gassler, Professor**, Institute of Pathology, University Hospital RWTH Aachen, Pauwelsstrasse 30, 52074 Aachen, Germany

**Werner Hohenberger, Professor**, Department of Surgery, Hospital Strasse 12, D-91054 Erlangen, Germany

**Kevin Cheng-Wen Hsiao, MD, Assistant Professor**, Colon and Rectal Surgery, Tri-Service General Hospital, No. 325, Sec. 2, Cheng-Kung Rd, Nei-Hu District, 114 Taipei, Taiwan, China

**Masahiro Iizuka, MD, PhD, Director**, Akita Health Care Center, Akita Red Cross Hospital, 3-4-23, Nakadori, Akita, 010-0001, Japan

**Beata Jolanta Jabłońska, MD, PhD**, Department of Digestive Tract Surgery, University Hospital of Medical University of Silesia, Medyków 14 St. 40-752 Katowice, Poland

**Sherif M Karam, Dr., Professor**, Department of Anatomy, Faculty of Medicine and Health Sciences, United Arab Emirates University, PO Box 17666, Al-Ain, United Arab Emirates

**Patricia F Lalor, PhD, Dr.**, Liver Research Laboratory, Room 537 Institute of Biomedical Research, Division of Medical Science, University of Birmingham, Birmingham B15 2TT, United Kingdom

**Vance Matthews, Dr., PhD, BS**, Cellular and Molecular Metabolism Laboratory, Baker University of Texas Medical Branch, IDI, PO Box 6492, St Kilda Road Central, VIC 8008, Melbourne, Australia

**Sri P Misra, Professor**, Gastroenterology, Moti Lal Nehru Medical College, Allahabad 211001, India

**Emiko Mizoguchi, MD, PhD**, Department of Medicine, Gastrointestinal Unit, GRJ 702, Massachusetts General Hospital, Boston, MA 02114, United States

**Naofumi Mukaida, MD, PhD, Chairperson and Professor**, Division of Molecular Bioregulation, Cancer Research Institute, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-0934, Japan

**Akihito Nagahara, Associate Professor**, Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1 Hongo Bunkyo-ku, Tokyo 113-8421, Japan

**Mihaela Petrova, MD, PhD, Dr.**, Clinic of Gastroenterology, Medical Institute, Ministry of Interior, Sofia 1606, Bulgaria

**Andreas G Schreyer, Dr., Professor**, Department of Radiology, University Hospital Regensburg, Franz-Josef-Strauss-Allee 11, Regensburg 93053, Germany

**Rafiq A Sheikh, MBBS, MD, MRCP, FACP, FACG**, Department of Gastroenterology, Kaiser Permanente Medical Center, 6600 Bruceville Road, Sacramento, CA 95823, United States

**Martin Storr, MD, PhD, Associate Professor**, Department of Medicine, Gastroenterology, University of Calgary, 3330 Hospital Dr NW, T2N 2N1, Calgary, Canada

**Shoichiro Sumi, MD, PhD, Associate Professor**, Department of Organ Reconstruction, Institute for Frontier Medical Sciences, Kyoto University, Sakyo-ku, Kyoto, 606-8507, Japan



## Meetings

### Events Calendar 2010

January 25-26  
Tamilnadu, India  
International Conference on Medical  
Negligence and Litigation in Medical  
Practice

January 25-29  
Waikoloa, HI, United States  
Selected Topics in Internal Medicine

January 26-27  
Dubai, United Arab Emirates  
2nd Middle East Gastroenterology  
Conference

January 28-30  
Hong Kong, China  
The 1st International Congress on  
Abdominal Obesity

February 11-13  
Fort Lauderdale, FL, United States  
21th Annual International Colorectal  
Disease Symposium

February 26-28  
Carolina, United States  
First Symposium of GI Oncology at  
The Caribbean

March 04-06  
Bethesda, MD, United States  
8th International Symposium on  
Targeted Anticancer Therapies

March 05-07  
Peshawar, Pakistan  
26th Pakistan Society of  
Gastroenterology & Endoscopy  
Meeting

March 09-12  
Brussels, Belgium  
30th International Symposium on  
Intensive Care and Emergency  
Medicine

March 12-14  
Bhubaneswar, India  
18th Annual Meeting of Indian  
National Association for Study of  
the Liver

March 23-26  
Cairo, Egypt  
14th Pan Arab Conference on  
Diabetes PACD14

March 25-28  
Beijing, China  
The 20th Conference of the Asian

Pacific Association for the Study of  
the Liver

March 27-28  
San Diego, California, United States  
25th Annual New Treatments in  
Chronic Liver Disease

April 07-09  
Dubai, United Arab Emirates  
The 6th Emirates Gastroenterology  
and Hepatology Conference, EGHG  
2010

April 14-17  
Landover, Maryland, United States  
12th World Congress of Endoscopic  
Surgery

April 14-18  
Vienna, Austria  
The International Liver Congress™  
2010

April 28-May 01  
Dubrovnik, Croatia  
3rd Central European Congress  
of surgery and the 5th Croatian  
Congress of Surgery

May 01-05  
New Orleans, LA, United States  
Digestive Disease Week Annual  
Meeting

May 06-08  
Munich, Germany  
The Power of Programming:  
International Conference on  
Developmental Origins of Health  
and Disease

May 15-19  
Minneapolis, MN, United States  
American Society of Colon and  
Rectal Surgeons Annual Meeting

June 04-06  
Chicago, IL, United States  
American Society of Clinical  
Oncologists Annual Meeting

June 09-12  
Singapore, Singapore  
13th International Conference on  
Emergency Medicine

June 14  
Kosice, Slovakia  
Gastro-intestinal Models in  
the Research of Probiotics and  
Prebiotics-Scientific Symposium

June 16-19  
Hong Kong, China  
ILTS: International Liver  
Transplantation Society ILTS Annual  
International Congress

June 20-23  
Mannheim, Germany  
16th World Congress for  
Bronchoesophagology-WCBE

June 25-29  
Orlando, FL, United States  
70th ADA Diabetes Scientific  
Sessions

August 28-31  
Boston, Massachusetts, United States  
10th OESO World Congress on  
Diseases of the Oesophagus 2010

September 10-12  
Montreal, Canada  
International Liver Association's  
Fourth Annual Conference

September 11-12  
La Jolla, CA, United States  
New Advances in Inflammatory  
Bowel Disease

September 12-15  
Boston, MA, United States  
ICAAC: Interscience Conference  
on Antimicrobial Agents and  
Chemotherapy Annual Meeting

September 16-18  
Prague, Czech Republic  
Prague Hepatology Meeting 2010

September 23-26  
Prague, Czech Republic  
The 1st World Congress on  
Controversies in Gastroenterology &  
Liver Diseases

October 07-09  
Belgrade, Serbia  
The 7th Biannual International  
Symposium of Society of  
Coloproctology

October 15-20  
San Antonio, TX, United States  
ACG 2010: American College of  
Gastroenterology Annual Scientific  
Meeting

October 23-27  
Barcelona, Spain  
18th United European  
Gastroenterology Week

October 29-November 02  
Boston, Massachusetts, United States  
The Liver Meeting® 2010--AASLD's  
61st Annual Meeting

November 13-14  
San Francisco, CA, United States  
Case-Based Approach to the  
Management of Inflammatory Bowel  
Disease

December 02-04  
San Francisco, CA, United States  
The Medical Management of HIV/  
AIDS



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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 Xiaohua 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

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 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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- 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. *HRSA 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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- 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. Wiczorek 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

#### 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.

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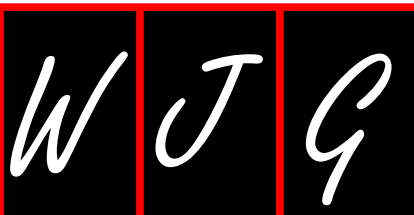
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**EDITING**

Editorial Board of *World Journal of Gastroenterology*, Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China  
Telephone: +86-10-5908-0039  
Fax: +86-10-8538-1893  
E-mail: [wjg@wjgnet.com](mailto:wjg@wjgnet.com)  
<http://www.wjgnet.com>

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Telephone: +86-10-8538-1892  
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**SUBSCRIPTION**

Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China  
Telephone: +86-10-8538-1892  
Fax: +86-10-8538-1893  
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Telephone: +86-10-5908-0039  
Fax: +86-10-8538-1893  
E-mail: [wjg@wjgnet.com](mailto:wjg@wjgnet.com)  
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## Ontogeny, growth and development of the small intestine: Understanding pediatric gastroenterology

Laurie A Drozdowski, Tom Clandinin, Alan BR Thomson

Laurie A Drozdowski, Medical Affairs, Mead Johnson Nutritionals, Ottawa, Ontario, K1S 5N4, Canada

Tom Clandinin, Department of Agricultural, Food and Nutritional Science, University of Alberta, Alberta, T6G 2B7, Canada  
 Alan BR Thomson, Division of General Internal Medicine, Department of Medicine, University of Alberta, Alberta, T6G 2B7, Canada

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**Correspondence to:** Dr. Alan BR Thomson, Division of General Internal Medicine, Department of Medicine, University of Alberta, Alberta, T6G 2B7, Canada. [alan.thomson@ualberta.ca](mailto:alan.thomson@ualberta.ca)  
 Telephone: +1-780-4928154 Fax: +1-780-4927964

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### Abstract

Throughout our lifetime, the intestine changes. Some alterations in its form and function may be genetically determined, and some are the result of adaptation to diet, temperature, or stress. The critical period programming of the intestine can be modified, such as from subtle differences in the types and ratios of n3:m6 fatty acids in the diet of the pregnant mother, or in the diet of the weanlings. This early forced adaptation may persist in later life, such as the unwanted increased intestinal absorption of sugars, fatty acids and cholesterol. Thus, the ontogeny, early growth and development of the intestine is important for the adult gastroenterologist to appreciate, because of the potential for these early life events to affect the responsiveness of the intestine to physiological or pathological challenges in later life.

**Key words:** Intestinal development; Ontogeny; Pediatrics

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### INTRODUCTION

The molecular mechanisms of fetal development of the intestine have been explored using transgenic and knock-out mice, with the suggestion of the importance of Wnt, bone morphogenetic protein (BMP), PTEN/PI3K and Notch signaling<sup>[1]</sup>. After midgestation, the stratified cuboidal intestinal epithelium, derived from endoderm, begins to form villi as a result of epithelial-mesenchymal interactions<sup>[2]</sup>. Wnt and Indian hedgehog signaling interact to stimulate proliferation and to act as a morphogen, in turn acting on BMP signals in the mesenchyme to influence morphogenesis during the development of the intestine<sup>[3]</sup>. The cellular differentiation along the crypt-villus axis is maintained by Wnt pathway target genes<sup>[4,5]</sup>. Unphosphorylated active PTEN in turn controls the activation of the lipid kinase PI3 kinase pathway, where the PDK1 and especially the PKB (Akt) ser1Thr kinases act as the main effector kinases of this proliferative pathway<sup>[6]</sup>.

These complex processes are integrated to produce functionally important alterations during late intrauterine and early postnatal life of intestinal morphology and function, to prepare the infant for early feeding on high-fat milk, and then weaning onto lower fat-but higher carbohydrate-containing solid foods. Understanding these ontogenic events helps to understand the early age-dependent approach to nutritional disease state.

## INTESTINAL MORPHOLOGY

At the time of birth, the human small intestine is morphologically and biochemically more mature than that of other mammals. Of interest though, since rodents are born at a more immature stage than humans, at least some of the brush border membrane (BBM) enzymatic maturation that occurs prenatally in humans only occurs after birth in rodents. This makes the rodent a useful model to better understand the process of intestinal maturation that occurs in premature infants.

The maturity of the small intestine is reflective of the length of the gestational period, with the developments of the human small intestine being largely completed in utero by the end of the first trimester<sup>[7]</sup>. Despite temporal differences in the ontogeny of the small intestine between species, the processes involved in the development of the small intestine remain similar. Thus, the human intestine goes through each of the stages that occur in rodents, so that animal studies may be used to better understand the development of the human intestine.

Development of the small intestine is comprised of three stages: (1) morphogenesis and cell proliferation, (2) cell differentiation, and (3) functional maturation<sup>[8]</sup>. Gastrulation is the process by which the primitive gut tube is formed. This consists of the endoderm, the precursor to the epithelial lining of the gastrointestinal (GI) tract, surrounded by mesenchyme. In humans, this process begins at three weeks gestation<sup>[7]</sup>.

In the embryo, the GI system is one of the first to polarize by forming an entry and exit to the systems along the anterior and posterior axis. The *bax* genes are nuclear transcription factors that activate genes that encode secretory proteins. The *bax* genes play an important role in the formation of distinct regions of the brain and skeleton<sup>[7]</sup>. Through epithelial-mesenchyme interactions, these proteins may also be involved in determining anterior-posterior patterning in the fetal gut. Similarly, Sonic hedgehog and Indian hedgehog pathways mediate epithelial-mesenchymal interactions at early stages of gut formation<sup>[2]</sup>.

Next, there is a transition into columnar epithelium, with the development of polarized enterocytes, and the formation of the BBM and basolateral membrane (BLM) of the enterocyte. The formation of nascent villi and microvilli occurs simultaneously, with cellular proliferation detectable along the villi. In humans, formation of the villus is initiated at 9-10 wk gestation, and proceeds in a cranial-caudal direction<sup>[7]</sup>. Villus and microvillus formation account for the approximate 100 000-fold increase in the intestinal surface area observed from the early first trimester period to birth<sup>[9]</sup>.

The development of intestinal crypts then follows in humans, but in rodents, crypts do not develop until after birth<sup>[10]</sup>. The human fetus and the neonatal rat have transient villus-like structures in the proximal colon with properties similar to enterocytes, including the expression of BBM enzymes and transporters<sup>[11-13]</sup>. In later life, when premalignant changes occur in the colon in the

form of development of colonic adenomatous polyps, the villous structure may recur. Interestingly, CaCO<sub>2</sub> cells derived from human colon cancer cells develop villi and villous functions, and are a good cell culture model for the assessment of, for example, intestinal absorption and metabolism.

The cells of the intestinal mucosa (the antagonists, enteroendocrine cells, Paneth and goblet cells) are compartmentalized within the crypt-villus unit. All four of the differentiated cell types of the intestinal mucosa are derived from one or more multipotent stem cells located in each intestinal crypt<sup>[14]</sup>. As cells move out of the crypt and up the villus or deeper into the crypts, "...differentiation occurs as progeny of the transit cell population migrate in vertically coherent bands..."<sup>[15]</sup>. Fibroblast growth factor receptor 3 (FGFR-3) is highly expressed in the undifferentiated crypt epithelial cells in the developing intestine, and FGFR-3 signaling through  $\beta$ -catenin/Tcf-4-dependent and independent pathways may regulate crypt epithelial stem cell expansion and crypt morphogenesis by the process of crypt bifurcation or fission<sup>[15]</sup>. Other growth factors such as Wnt(s) and FGF2 may cross talk with the  $\beta$ -catenin signaling pathway<sup>[16]</sup>.

Cellular proliferation occurs in the crypts, differentiated cells populate the villi, and the dynamic balance between proliferation and differentiation is balanced by apoptosis of the senescent cells. Hepatocyte nuclear factor 4  $\alpha$  (HNF4 $\alpha$ ) belongs to the family of nuclear receptor transcription factors found in the liver, pancreas, kidney, and intestinal tract<sup>[17,18]</sup>. HNF4 $\alpha$  may instruct "...cells to become specific to the intestinal epithelium..."<sup>[19]</sup>, as well as upregulating genes during epithelial cell differentiation such as Apo A-IV, intestinal alkaline phosphatase, liver and intestinal fatty acid binding proteins<sup>[20-23]</sup>.

Bile acids regulate their own synthesis<sup>[24]</sup>. The luminal concentration of bile acids and the bile acid pool are low in the preterm and term infant, and rise as the animal ages<sup>[25,26]</sup>. These initially low values are associated with malabsorption of lipids<sup>[27]</sup>. The size of the bile acid pool increases with the activity of cholesterol 7 $\alpha$ -hydroxylase (Cyp7a1) and oxysterol 7 $\alpha$ -hydroxylase (Cyp7b1) by mechanisms that are independent of the farnesyl X receptor (FXR), and the short heterodimeric pathway (SHP)<sup>[24]</sup>.

Increased bile acid absorption by the ileal apical sodium-dependent bile acid cotransporter (ASBT) also contributes to the expansion of the bile acid pool.

In mouse models of necrotizing enterocolitis (NEC)<sup>[28]</sup>, the proinflammatory transcription factor NF- $\kappa$ B mediates this intestinal injury as the result of platelet activating factor (PAF) converting p105 into p50. The p50 further upregulates proinflammatory cytokines which lead to a systemic inflammatory response and acute bowel injury<sup>[29]</sup>.

Peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) is a nuclear receptor which associates with retinoid X receptor to "...suppress proliferation and promote differentiation of intestinal epithelial cells..." and to decrease the size of the proliferative zone of the intestinal crypts<sup>[30-32]</sup>. The thiazolidinedione drugs are PPAR $\gamma$  ago-

nists which reduce cholera toxin mediated chloride secretion through the reduced expression of the apical CFTR channels, KCNQ1  $K^+$  channels as well as  $Na^+-K^+-2Cl^-$  cotransporter-1 proteins in the BLM<sup>[33]</sup>.

In addition to the enterocytes, four other small intestinal mucosal cell types develop: goblet cells, enteroendocrine cells, Paneth cells, and M cells. M cells are associated with Peyer's patches, and are detected by 17 wk of gestation<sup>[34]</sup>. In the human intestine, all epithelial cell types known to occur in the adult are present by the end of the first trimester<sup>[34]</sup>. The intestinal epithelium is able to maintain the differentiation programs of each lineage, depending on the location of the cells along the crypt-villous and proximal-distal gradients<sup>[35,36]</sup>.

The regulation of GI development is complex, and involves a host of growth and transcription factors. Receptors for epidermal growth factor (EGF), transforming growth factor  $\beta$  (TGF $\beta$ ), insulin-like growth factor II (IGF-II), hepatocyte growth factor (HGF), and GLP-2 are present in fetal human intestine<sup>[37,38]</sup>. Human fetal cortisone levels in the blood increase late in gestation<sup>[39]</sup>. Corticosterone (a glucocorticoid similar to cortisol) is thought to be the main factor involved in rat small intestinal maturation<sup>[40,41]</sup>.

Studies investigating the development of human fetal small intestine xenografted to SCID mice demonstrate that the transplanted intestine normally undergoes differentiation in the absence of luminal and hormonal factors<sup>[42,43]</sup>. This finding, in conjunction with the observation that villus formation in rodents is autonomous<sup>[44]</sup>, suggests that intestinal development may be "hard-wired", i.e. is regulated largely by intrinsic factors, with extrinsic factors playing only a secondary role. Indeed, several transcription factors including *N-myc*, HNF3 $\beta$  and *Cdx-2* have been identified as potential intrinsic factors implicated in GI development. *N-myc* gene knock-out animals demonstrate defects in GI development<sup>[45]</sup>. Homologous null mutants of HNF3 $\beta$  are lethal, as many structures, including the gut tube do not develop normally<sup>[46,47]</sup>. *Cdx-2* expression is detected at the time of morphogenesis in mouse intestine, and is a known regulator of the expression of the small intestinal BBM enzymes sucrase-isomaltase (SI)<sup>[48]</sup>.

The exogenous expression of *Cdx-2* in a rat intestinal cell line induces the differentiation of goblet and absorptive cells from crypt cells. This suggests a possible role of *Cdx-2* in the ontogeny of the GI tract. Several other signaling pathways (including the Notch, Wnt/ $\beta$ -catenin and BMP pathways) are also thought to play a role in patterning the gut during development, and in regulating epithelial differentiation through epithelial-mesenchymal interactions<sup>[49,50]</sup>. What then is the importance of the extracellular matrix (ECM)?

Indeed, in addition to regulation by transcriptional factors, intestinal development may be controlled through interactions with components of the ECM. Developmental changes in E-cadherins and integrins have been described<sup>[56,51,52]</sup>, suggesting that the ECM

may influence the ontogeny of epithelial cells. Cultures of human fetal enterocytes demonstrate enhanced differentiation, when they are grown on components of the ECM<sup>[53]</sup>. This suggests that a permissive rather than an instructive role may be attributed to the ECM in GI development. Indeed, when major components of the ECM have been deleted, in mice, they show no changes in GI morphogenesis, indicating that these components are not essential for GI development in this model<sup>[54]</sup>.

## FUNCTIONAL DEVELOPMENT

The functional development of the BBM enzyme activity has been well characterized<sup>[55-58]</sup>.

BBM SI is first detected in the human fetus in the first trimester, but is not seen until weaning in the rat<sup>[58]</sup>. Both rat and human fetal small intestine demonstrate detectable BBM lactase-phlorizin hydrolase (LPH) activity, but LPH expression before and after birth varies depending on the species<sup>[59]</sup>. In humans, BBM enzyme activity has been correlated to morphogenesis, with the development of enzyme activity being associated with the formation of enterocytes<sup>[60]</sup>. Proximal-to-distal gradients of enzyme activity along the length of the small intestine are established early in gestation. In addition, crypt-villous gradients are evident, with LPH activity being highest at the villous tip, and SI activity maximal in the mid-villous region<sup>[61]</sup>.

### LPH

The earliest ingested nutrient in mammals is, of course, milk. The major carbohydrate in milk is disaccharide lactose. Lactose is cleaved by BBM LPH into glucose and galactose. LPH is therefore a crucial enzyme for neonates who are solely dependent on their mother's milk for nourishment.

Human LPH is first detected in the proximal small intestine at 8-9 wk of gestation, but later extends along the length of the small intestine<sup>[60]</sup>. In contrast, rat LPH is very low until 24 wk gestation, when its activity begins to increase. A rise in LPH activity in rodents occurs only late in the third trimester. In the human fetal jejunum, LPH activity correlated with the abundance of its mRNA<sup>[62]</sup>, consistent with the proposal that LPH activity is regulated transcriptionally<sup>[63-65]</sup>. Nuclear transcription factors that have been shown to interact with the LPH promoter element CE-LPH1 include CDX-2<sup>[66]</sup>, HOXC11<sup>[67]</sup>, GATA6<sup>[68]</sup>, and HNF1<sup>[67]</sup>.

Once weaning has occurred in nearly all species of mammals, both LPH activity and mRNA abundance decline<sup>[59,69,70]</sup>. Even in humans, the vast majority of the world's population experiences a decline in LPH activity sometime during childhood or adolescence. These lower values of LPH activity are 5%-10% of those values seen in early childhood<sup>[71]</sup>. In contrast, in geographical regions such as Western Europe and North America, where for thousands of years dairy cattle were raised as a continuing source of milk, LPH activity persists throughout adulthood<sup>[59,69,70]</sup>, unless an adverse process affects the small



intestine. This is known as secondary lactose deficiency (i.e. the decline in LPH activity is secondary to a disease). The decline in LPH activity in early life in characteristic locations is primary, i.e. genetically determined. Thus, if an adult with northern European ancestry presents with new onset milk intolerance, lactose deficiency is suspected, and an underlying condition such as celiac disease or inflammatory bowel disease is looked for.

In humans, the correlation between mRNA abundance and activity of LPH suggest that transcriptional and post-transcriptional mechanisms are involved in the development of hypolactasia<sup>[72]</sup>. Post-translational mechanisms may also be involved in the decline in LPH activity, through the modulation of functional protein along the villus. Glycosylation of the protein results in the 225 kDa form, however, the mature BBM LPH enzyme represents a cleavage product of this glycosylated precursor<sup>[59,73,74]</sup>. The initial cleavage occurs intracellularly in a post-Golgi compartment<sup>[75]</sup>. This yields a protein which lacks LPH activity. Once LPH is inserted in enterocyte BBM, LPH is once again cleaved, but this time by extracellular trypsin, and this yields the mature and active 145 kDa form of the LPH and it is cleaved<sup>[76]</sup>.

## SI

SI is a bifunctional enterocyte BBM disaccharidase with sucrase, isomaltase and maltase activity. Sucrase hydrolyzes sucrose into glucose and fructose. In humans, SI is first detected at 9-10 wk gestation, and gradually increases until just prior to birth, when a marked increase in SI occurs. After birth, there is a rapid decline in SI levels to values comparable to those found in early gestation. Sucrase is not normally a part of the infant's diet, so it is not clear why SI activity is so high in the human fetus.

Fetal human SI protein is in the proSI form from 15-30 wk gestation, but after 30 wk most of the protein consists of sucrase and isomaltase subunits<sup>[77]</sup>. Enterokinase activity, which activates proteases that cleave proSI, appears at 26 wk, and coincides with the appearance of the sucrase and isomaltase subunits.

SI is transiently expressed in the colon of both humans and rodents<sup>[11,13,60,78]</sup> in association with the appearance of small intestinal-like morphology. The observation that SI is expressed in colorectal cancer cells suggests that the factors that normally repress SI expression in the colon may be lost in cancer cells.

In mice, low levels of SI mRNA abundance are detectable in the small intestine<sup>[78]</sup>. However, rat studies show that there is no BBM SI activity from birth until weaning<sup>[58]</sup>. Thus, even between different types of rodents there are variations in BBM SI development. At weaning, a dramatic increase in SI activity occurs, with adult rat levels being rapidly established. Expression of SI mRNA and protein is first detected in cells located at the crypt-villous junction, suggesting that the enterocytes containing SI are programmed in crypts. As these enterocytes migrate up the villus, the entire villus ultimately becomes populated with cells expressing SI. SI expression first appears in the proximal small intestine, and then proceeds distally to the

ileum. This is a genetically programmed event that is not significantly affected by the animals' diet<sup>[70,79]</sup>. Premature SI induction can be induced by precocious stress, glucocorticosteroids, insulin, or thyroxine<sup>[70,79,80]</sup>.

There is a correlation between fetal SI activity and mRNA abundance, suggesting control at the level of either mRNA transcription or stability<sup>[81]</sup>. A number of regulatory elements (including SIF1, SIF2, and SIF3) have been identified within the promoter region of the SI gene, and are important for transcriptional induction. CDX-2 binds to the SIF1 element and transactivates the SI gene promoter<sup>[48]</sup>. CSX-2 appears to be the major regulator of SI transcription. A number of other potential transcription factors have been identified, such as HOXC11, which like CDX-2, bind to the SIF1 element of the SI promoter<sup>[67]</sup>. HNF1 $\alpha$  interacts with the SIF3 element and to a lesser extent SIF2, to activate SI transcription<sup>[82]</sup>. GATA zinc-finger transcription factors interact with a region of the SI promoter upstream of the SIF1 element.

Glycosylation of the SI protein occurs in the endoplasmic reticulum (ER) and in the Golgi apparatus, yielding a 245 kDa protein<sup>[83]</sup>. Once the protein is inserted into the BBM, the post-translational processing is by the cleavage of the molecule into two subunits which occurs *via* trypsin digestion in the intestinal lumen<sup>[83]</sup>. The SI subunits remain associated by hydrostatic bonds. Defects in post-translational processing are thought to be responsible for inherited SI deficiency in humans<sup>[84]</sup>.

## Glucose transport

The ontogeny of intestinal nutrient transport is largely dependent on the species that is studied. In all mammals, sugar transporter protein does not appear until the intestine differentiates and forms crypts, villi and microvilli. The time at which this process occurs differs between species (please see above), and may be affected by the length of the gestational period. Differentiation of the mucosa alone, however, is not solely responsible for triggering the appearance of transporters, as many of them do not appear until after birth, or even after weaning.

Much of the research on the ontogeny of intestinal transport comes from rodent studies. Rodents are considered to be altricial, meaning that they are born "premature" as compared to humans. Indeed, many of the postnatal changes in the intestine seen in rats occurs parentally in humans, making neonatal rodents an ideal model for premature infants<sup>[85]</sup>. The pig is also a useful model of ontogeny, due to the similarities between the pig and the human small intestine<sup>[86]</sup>.

The intestinal transport of nutrients, such as glucose, is first detected in the fetal small intestine of mammals, including humans<sup>[87]</sup>. Both placental nutrients<sup>[88,89]</sup> as well as the swallowing of amniotic fluid<sup>[90]</sup> contribute to fetal nutrient acquisition. In fact, the volumes of amniotic fluid which are swallowed in humans *in utero* at term are estimated to be approximately 500 mL/d<sup>[91]</sup>. Taste buds are detected early in gestation<sup>[92]</sup>, and early experiments have shown that human fetal swallowing increases trans-amniotic saccharin infusion, and decreases following the

infusion of noxious substance<sup>[93]</sup>. Injection of galactose into the amniotic fluid of fetal rabbits increases intestinal mucosal weights, as well as the uptake of glucose<sup>[94]</sup>. Thus, even fetal rabbits are able to up-regulate intestinal transport capacity in response to nutrients. The importance of fetal swallowing in the development of the GI tract is also highlighted by experiments in which fetal sheep underwent esophageal ligation to prevent amniotic fluid from reaching the small intestine<sup>[95]</sup>. A decrease in small intestinal villous height, intestinal weight and body weight resulted.

Prenatal intestinal transporters are critical for the development of the fetus, as an estimated 10%-15% of fetal protein requirements in rhesus monkeys are met through nutrients that are present in the amniotic fluid<sup>[96]</sup>. The presence of growth factors released from the GI tract may also be important, as gastric infusion of epidermal growth factor (EGF) reversed the weight loss seen following esophageal ligation<sup>[90]</sup>.

Once the epithelium lining the small intestine differentiates into columnar cells at 9-10 wk of gestation, transport BBM proteins including SGLT1 are expressed<sup>[97]</sup>. Significant levels of SGLT1 mRNA are also detected in fetal tissue, suggesting that carrier-mediated transport of glucose may be occurring<sup>[98]</sup>. Dramatic increases in the site density of SGLT1 are observed in fetal pigs between 74% of term and birth<sup>[98]</sup>. Between 17 and 30 wk gestation in humans, the duodenal-ileal gradient of glucose absorption is established<sup>[99]</sup>. In rats, glucose transport and SGLT1 protein and mRNA increase at weaning to levels higher than those seen in suckling or in adult animals<sup>[100,101]</sup>. Curiously, phloridzin does not block glucose transport by SGLT1 in suckling and mature animals to the same extent that it does in weanlings. This may suggest the presence of an age-specific alternative mechanism of glucose transport, or an age-related difference in the phloridzin binding site on SGLT1.

Kinetic analysis of glucose uptake rates in BBM vesicles from human fetal tissue suggests the presence of two transport systems. In addition to SGLT1, a low affinity, high capacity system is detected in the proximal small intestine<sup>[102,103]</sup>. This may represent GLUT2, which has been described in the BBM of adult rats exposed to high luminal sugar concentrations<sup>[104]</sup>.

Human and rat fetal small intestine also express GLUT1 (as do erythrocytes and brain tissue), which appears earlier than GLUT2, and decreases gradually during fetal life<sup>[105,106]</sup>. Although the mechanism of this developmental regulation is unknown, GLUT1 may be involved in early cell growth proliferation. Intestinal BLM GLUT2 mRNA is expressed at high levels at birth<sup>[101]</sup>, and GLUT2 transports glucose and fructose. In fact, GLUT2 mRNA is detected in fetal rats as early as day 16 following conception, even before intestinal villi are formed<sup>[106]</sup>. GLUT2 mRNA increases after weaning, and subsequently decreases to adult levels<sup>[101]</sup>. GLUT2 in the developing intestine is regulated by luminal glucose and fructose<sup>[107]</sup>. Luminal perfusion of 20 d old rat pups' intestine with fructose or glucose (100 mmol/L) increases

GLUT2 mRNA. This enhancing effect of luminal glucose or fructose was blocked by the transcription inhibitor actinomycin D, but was not affected by the protein synthesis inhibitor cycloheximide. GLUT2 mRNA was also increased in bypassed intestinal loops, suggesting that systemic factors are involved in its regulation. Interestingly, GLUT2 mRNA abundance was even higher in the bypassed loop than in the section that was perfused, suggesting a possible compensatory mechanism due to perceived starvation.

Sugar uptake increases with the gestational age of the animal, and typically peaks immediately after birth, when the intestine takes over the burden of nutrient acquisition from the placenta. Studies done on pigs using the everted sleeve method demonstrate that the maximal transport rate ( $V_{max}$ ) for D-glucose was highest immediately after birth, with a subsequent decrease in the value of  $V_{max}$  associated with the onset of suckling<sup>[86]</sup>. In contrast, in newborn pigs the onset of suckling appears to stimulate increases in BLM GLUT2 density<sup>[108]</sup>. It is not known if GLUT2 activity protein or mRNA can be modified by sugars in the intestinal tract of humans.

At birth, all enterocytes appear to have the capability to transport nutrients. As a result, uptake occurs in enterocytes from all along the villus, rather than just from the upper third, such as occurs in older rats<sup>[109]</sup>. This may contribute to the higher rate of sugar uptake. Soon after birth, the gradient of increasing transport as one moves from the crypt to the villus is established<sup>[109]</sup>. This may be responsible for the reduced uptake capacity of the intestine observed postnatally. The "dilution" of fetal enterocytes with new immature cells that do not express transporters may be responsible for this effect. Indeed, the subsequent age-related decline in transport observed in chickens was attributed to reductions in the site density of SGLT1<sup>[110]</sup>.

Developmental changes in the intestinal transport of nutrients may also be non-specific (for example, changes in mucosal surface area, proliferation and migration of enterocytes, or changes in intestinal permeability). Indeed, the subsequent age-related decline in transport observed in chickens was attributed to reductions in the site density of SGLT1<sup>[110]</sup>.

Studies on human premature neonates have used the urinary excretion of D-xylose and 3-O-methyl-glucose as measures as passive and active carrier-mediated monosaccharide absorption of these sugars, respectively, when compared to those born before 28 wk gestation<sup>[111]</sup>. The replacement of rat fetal enterocytes along the villus requires up to 2 wk, as compared to the 24-48 h required for the replacement of adult enterocytes. Non-specific changes are responsible for ontogenic alterations in mucosal weight, surface area and transport capacity. Postnatal development of enterocytes results in increases in the surface area of microvilli and the BLM<sup>[112,113]</sup>. Reduced turnover rates result in longer lifetimes of enterocytes, resulting in slower replacement of cells.

Reductions in BBM fluidity occur in post-weaning rabbits, in association with increases in the cholesterol-

to-phospholipid ratio in the BBM<sup>[114,115]</sup>. In general, reductions in fluidity result in reductions in permeability. Human neonates show decreases in intestinal permeability within the first 30 d of life, as assessed by lactulose/mannitol urinary excretion<sup>[116]</sup>.

### Fructose transport

Although SGLT1 and GLUT2 are expressed in enterocytes both in the fetus and at birth, the expression of BBM GLUT5 is only detected in post-weaning rats<sup>[101,117-119]</sup>. This contrasts with what is seen in pigs<sup>[86]</sup> and lambs<sup>[120]</sup>. In rats, GLUT5 protein and mRNA abundance parallel fructose transport, and therefore remain low throughout the suckling phase. GLUT5 protein and mRNA also remain low throughout weaning in rats, with higher levels detected in the post-weaning phase when fructose may first appear in the rat<sup>[101,118,119]</sup>. This increase in GLUT5 mRNA and protein coincides with the rise in fructose uptake seen at this period. Although there is a temporal association between the introduction of dietary fructose and the appearance of GLUT5, the expression of the transporter is “hard wired” and occurs at this time even in the absence of dietary stimuli<sup>[121]</sup>. However, the precocious introduction of fructose into the diet of 22 d old rat pups stimulates fructose transport and GLUT5 mRNA expression<sup>[121]</sup>. Jiang *et al.*<sup>[122]</sup> showed that luminal perfusions of high concentrations (100 mmol/L) of fructose resulted in increases in GLUT5 mRNA and activity. This developmental reprogramming of fructose transport required *de novo* mRNA and protein synthesis, as both actinomycin D and cycloheximide (inhibitors of transcription and translation, respectively) abolished the effect.

In humans, the introduction of solid foods and fruit juice containing fructose at earlier stages of infancy, coupled with the increased use of fructose as a sweetener in dietary products, has resulted in increased exposure to fructose during infancy. Fructose has been implicated as the major cause of “toddler’s diarrhea”, largely because it is a late onset transporter that increases postnatally in human infants<sup>[123]</sup>. The infant intestine may not be equipped to absorb high amounts of fructose, resulting in fructose malabsorption. Fructose may then enter the colon, and the high osmolarity may cause osmotic diarrhea. Even in adults, the incidence of fructose intolerance may be increasing: in a recent study malabsorption may be over 70% in persons with persistent, unexplained, non-specific GI symptoms<sup>[124]</sup>. The dose of fructose used in this study was approximately equivalent to the amount of fructose found in two cans of pop.

### Amino acid transport

Amino acid (AA) transporters appear prenatally in the intestines of chickens, rats, rabbits, and humans, and these transporters increase dramatically in the first days after birth<sup>[125]</sup>. In rats, rabbits, and pigs, the highest rates of BBM AA and peptide uptake are seen at birth, with decreases during suckling and post-weaning<sup>[126-128]</sup>. In rats, BBM AA transporters are expressed prenatally at

the same time or shortly after SGLT1<sup>[129]</sup>. In 17-20 wk gestation human fetal small intestine, all of the AA transport systems studied (neutral, acidic, basic, and imino) were found to be functional, with a proximal-distal gradient established shortly after crypt-villus formation<sup>[130]</sup>. In human fetuses, AA transport occurs at 14 wk gestation, with glucose transport at 18 wk, and fatty acid absorption at 24 wk gestation<sup>[131]</sup>.

AA transporters, including NBAT (which transports cationic and neutral AA) and EAAC1 (which transports glutamate), are expressed in suckling rats<sup>[132]</sup>. The intestinal absorption of peptides occurs *via* PepT1, which is distributed throughout the small intestine. The distribution of these transporters in suckling rats parallels that seen in adult animals. NBAT mRNA is highest in the proximal small intestine, while EAAC1 mRNA was highest in the more distal regions. While a marked crypt-villous gradient was found for PEPT1 and NBAT, EAAC1 immunoreactivity is confined to the lower third of the villus and to the crypts. Thus, the EAAC1 transporter of glutamate is the first AA transporter with decreased expression during epithelial cell differentiation<sup>[132]</sup>.

There are developmental changes in AA and peptide transport. The ontogeny of AA transport is complicated due to the major species differences, and by the large number of AA transport systems, and the fact that some AA are essential or non-essential, depending on the age of the animal. Also protein requirements change throughout the lifespan of an animal, necessitating variations in either intake or uptake of protein or AA. For example, intestinal proline uptake per mg of tissue is maximal at birth in rats, decreases at the end of the suckling phase, and decreases further in older animals. This decline matches both the dietary protein levels and protein requirements<sup>[119]</sup>. In addition, the decline in uptake of essential AA is greater than that for non-essential AA<sup>[129]</sup>. This may be because young animals have a disproportionate need for essential AA in early life, due to their rapid growth at this age.

There are also different patterns of uptake for individual AA. For example, in cats, the basic AA transport declines more steeply than does neutral AA transport. In humans, lysine and phenylalanine transport appears later than does the transport of alanine, leucine, taurine and valine<sup>[133]</sup>. In rats, uptake declines at a similar rate with age from proline, methionine and lysine; however the decline in leucine uptake occurs twice as quickly<sup>[129]</sup>. Finally, in rats as transition occurs at weaning when the uptake of glucose, fructose, and lysine increase, this is coupled with decreases in proline and leucine uptake<sup>[119]</sup>. It is unknown why there are such complicated patterns of changes in AA uptake in early life.

### Macromolecule transport

The uptake of macromolecules across the intestinal epithelial barrier is an important route by which immunoglobulins, growth factors and antigens are absorbed. This route of entry is especially important in neonates, who rely on it to obtain important immune factors from



maternal colostrum or milk. Most species (including rats, mice, and humans) are born hypoglobulinemic, and absorb IgG from maternal milk through proximal small intestine absorption<sup>[134,135]</sup>.

The transport of macromolecules across the BBM may occur by receptor-mediated or non-specific transcytosis. The transport of macromolecules is facilitated by the presence of protease inhibitors in the maternal colostrum. Rodent studies demonstrate specific intestinal receptors that bind to the Fc portion of IgG<sup>[136]</sup>. These receptors are transcriptionally regulated, and are present in highest amounts in the duodenum<sup>[137]</sup>. In humans, IgG is transferred from the placenta to the fetus in the third trimester of pregnancy, with receptors for IgG being detected in the intestine of both the fetus and the neonate. Macromolecular movement across the BBM persists after birth, and then gradually decreases<sup>[136]</sup>. The initially high permeability of the intestine declines after birth, leading to a process commonly referred to as “gut closure”. The time at which gut closure occurs and macromolecular transport ceases varies between species, with a rapid decrease in transport observed in pigs within the first few postnatal days, and a similar decrease seen around 21 d after birth in rats and rabbits<sup>[138]</sup>. In humans, the exact time that gut closure occurs is unknown, but intrinsic features as well as growth factors, hormones and breast milk may play a role in regulating this process. The decline in permeability may also be related to changes in the thickness and viscosity of the mucus gel layer which coats the BBM. The intervillus mucus gel is increased in weaned as compared to suckling rats. This would potentially increase the effective resistance of the unstirred water layer, and thereby contribute to the decrease in the uptake of macromolecules.

In human infants, uptake of macromolecules or lactalbumin declines with advancing postconceptual and postnatal age<sup>[139]</sup>. The ability of the neonatal intestine to adapt to the presence or absence of luminal stimuli is apparent from studies demonstrating a delay in spontaneous closure of the intestine if breastfeeding is postponed beyond the first 30 h of life<sup>[140]</sup>.

### Pancreatic enzymes

The higher fecal fat losses in preterm infants compared with term infants is thought to be attributable to lower pancreatic and intestinal lipase activities. Despite the presence of lipase in breast milk and of lipases in the newborn tongue and stomach, micellar absorption of lipids appears later when pancreatic lipase and bile acid concentrations increase<sup>[141]</sup>.

The human exocrine pancreas is functionally immature at birth, with substantial development occurring after birth. Proteolytic enzymes are detected early in the human fetus (20–25 wk gestation), with each enzyme developing in a unique manner. Trypsin increases during fetal life, to reach 90% of childhood levels at term<sup>[142]</sup>. In contrast, at birth chymotrypsin and carboxypeptidase B levels are less than 60% and 25% of childhood levels, respectively. Despite the differences in the temporal de-

velopment of proteolytic enzymes, protein digestion in the preterm neonate is adequate, and may be supported by the early development of gastric pepsin and mucosal peptidases<sup>[143]</sup>.

Pancreatic amylase activity is negligible in the human fetus, and is not detectable until one month after birth<sup>[144]</sup>. Salivary amylase is detected at 20 wk gestation, and while levels are low at birth, they increase to adult levels by the third month following birth<sup>[145]</sup>. Amylase is also present in human milk, and may aid in the digestion of starch contained in weaning foods<sup>[145]</sup>. The reduced levels of amylase activity may reflect the low levels of starch in the neonatal diet. At weaning, an increase in amylase activity is detected<sup>[144]</sup>. While this may be influenced by the appearance of starch in the infant's diet, animal studies demonstrate a persistent increase in amylase activity in rats subjected to prolonged nursing<sup>[146]</sup>. This suggests the presence of an inherent genetic program for the expression of amylase activity, not necessarily related to the starch content of the diet.

Pancreatic lipase activity in humans is detectable at 32 wk gestation. It remains low at birth, and increases 10 wk after birth<sup>[147]</sup>. Lingual and gastric lipases, however, are detected at 26 wk gestation<sup>[148]</sup>. At birth these lipases are able to hydrolyze 60%–70% of ingested fat, even in the absence of pancreatic lipase<sup>[149]</sup>. Lipase and esterase activity is present in human milk, and contributes to the increased fat absorption observed in breast-fed infants<sup>[150]</sup>. Low lipolytic activity may be the rate-limiting step in the development of efficient fat absorption<sup>[143,151,152]</sup>. Authors propose that it is the ability to take up long chain fatty acids (LCFA) from the lumen that is the rate-limiting step<sup>[153]</sup>.

The ontogeny of the intestine is “hard wired”, and occurs even in the absence of luminal and hormonal factors<sup>[41,42]</sup>. Still, a number of studies have demonstrated that variations in maternal diets, as well as weaning diets, can influence the ontogeny of the intestine<sup>[154–156]</sup>. “Critical period programming” is a phenomenon by which a biological mechanism is irreversibly turned on or off once during a lifetime in response to prevailing conditions at a critical stage<sup>[157]</sup>. This concept, which has also been referred to as “metabolic programming” or “imprinting”<sup>[158,159]</sup>, has been used to explain associations between prenatal/neonatal environment events, alterations in growth and development, and later pathophysiology<sup>[160,161]</sup>. Early exposure to a diet high in fructose during the suckling-weaning transition may contribute to modest dyslipidemia later in life<sup>[162]</sup>. Intestinal sugar uptake is also prone to critical period programming<sup>[163]</sup>.

The role of dietary lipids in the programming of intestinal nutrient transport has been studied<sup>[154–156,164,165]</sup>. Thomson *et al.*<sup>[164]</sup> demonstrated that feeding eight-week old rabbits a low cholesterol diet for two weeks reduced intestinal glucose uptake, and that this effect persisted for at least ten weeks after the animals returned to eating a normal diet. The response to diet depended on the duration of feeding, the age of the animals, and whether or not there was previous exposure to the diet. In this



study, effects on sugar transport were not explained by changes in food intake, body weight or intestinal weight. Furthermore, persistent changes were seen in the active transport of glucose, galactose, leucine and bile acids, while changes in the passive uptake of lipids were reversible.

When the ratio of polyunsaturated to saturated fatty acids in the diet of weanling rats is altered, diets enriched in saturated fatty acids increase hexose uptake, and these alterations were fast, progressive and irreversible<sup>[154]</sup>. Feeding the same diets to pregnant and lactating rats resulted in similar increases in sugar uptake in their weanling offspring<sup>[166]</sup>. Curiously, these changes were not seen in the suckling offspring, suggesting that the mechanisms responsible for adaptation may not be fully developed in these animals. Perin *et al.*<sup>[165]</sup> confirmed that the weanling intestine was capable of adaptation, by continuing to feed the offspring of pregnant dams the same diet for three weeks post-weaning. Persistent alterations in sugar uptake were seen in response to variations in dietary lipids, once again emphasizing the importance of early exposure in the programming of intestinal nutrient transport. In addition to the differences between suckling and weanling offspring, the pattern of adaptation also appeared to differ between the jejunum and ileum.

Further studies went on to characterize the effect of diets enriched with arachidonic acid, docohexanoic acid and diets with different ratios of n6 to n3 fatty acids on intestinal nutrient transport<sup>[156]</sup>. As in the previous study by Perin *et al.*<sup>[166]</sup>, these maternal diets critically influenced the ontogeny of the intestine, with many of the changes in transport being irreversible. Furthermore, responsiveness to later dietary challenges depended on early-life feeding experiences, once again emphasizing the importance of early dietary exposure to the development and later adaptability of intestinal transport of nutrients.

### Lipid and bile acid transport

Studies using human fetal jejunal explants (14–20 wk gestation) maintained in serum-free organ culture demonstrated increases in chylomicron, VLDL and HDL, paralleled by increases in triglycerides and cholesterol esters. This demonstrates the ability of the fetal intestine to absorb fat in conjunction with ontogenic increases in lipid and lipoprotein synthesis<sup>[167]</sup>. Apolipoprotein B synthesis is developmentally regulated: fetal intestine synthesizes only apoB-100 at 11 wk, but both apo-48 and apo-B100 are synthesized at 16 wk, with apoB-46 being predominant in the mature intestine.

Pinocytosis of lipid globules is important after birth. The immature rat intestine is also able to absorb fatty acids and cholesterol<sup>[168]</sup>. Triglycerides (TG) are digested by gastric and lingual lipases into fatty acids and 2-monoacylglycerols, and their uptake is higher in the immature intestine than in adults<sup>[168,169]</sup>. Fatty acid binding proteins on the BBM are present in adults<sup>[170]</sup>. Lipid uptake is thought to be passive in sucklings (Meddings and Theisen, 1989). Once taken up into the enterocytes,

lipids are resynthesized into TG, phospholipids (PL) and cholesterol esters (CE)<sup>[171]</sup>.

Bile acids include solubilizing lipids in the intestinal lumen, which facilitate their diffusion through the intestinal unstirred water layer external to the BBM. Intestinal bile acid uptake is an important step in the enterohepatic circulation of bile acids (recently reviewed in Kullak-Ublick *et al.*<sup>[172]</sup>), and this uptake is therefore important in the overall process of lipid absorption. Bile acid transporters are curiously absent during suckling when fat intake is high and when bile acid secretion and recycling would be expected to be maximal. It is speculated that the malabsorption may allow bile acids to enter the colon and affect the development of the enteric flora. It is likely that passive absorption of bile acids during the suckling period may be the mechanism by which bile acids are recirculated<sup>[172]</sup>.

Sodium-dependent bile acid transporters in the BBM or cytosol are detected in the rat at weaning<sup>[173,174]</sup>. Abrupt increases in bile acid transport at weaning occur in rat and human ileum, and are due to parallel increases in the steady state mRNA abundance and transporter number<sup>[175,176]</sup>.

## CONCLUSION

Thus, the ontogeny, early growth and development of the intestine is important for the adult gastroenterologist to appreciate, because of the potential for these early life events to affect the responsiveness of the intestine to physiological or pathological challenges in later life.

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## Is the control of dietary cholesterol intake sufficiently effective to ameliorate nonalcoholic fatty liver disease?

Munechika Enjoji, Makoto Nakamuta

Munechika Enjoji, Department of Clinical Pharmacology, Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan

Makoto Nakamuta, Department of Gastroenterology and Clinical Research Center, Kyushu Medical Center, National Hospital Organization, 1-8-1 Jigyohama, Chuo-ku, Fukuoka 810-8563, Japan

**Author contributions:** Enjoji M drafted the editorial; both authors wrote the final version.

**Correspondence to:** Munechika Enjoji, MD, PhD, Department of Clinical Pharmacology, Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan. [enjoji@adm.fukuoka-u.ac.jp](mailto:enjoji@adm.fukuoka-u.ac.jp)

Telephone: +81-92-8716631 Fax: +81-92-8630389

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**Peer reviewers:** Dr. Mihaela Petrova, MD, PhD, Clinic of Gastroenterology, Medical Institute, Ministry of Interior, Sofia 1606, Bulgaria; Akihito Tsubota, Assistant Professor, Institute of Clinical Medicine and Research, Jikei University School of Medicine, 163-1 Kashiwa-shita, Kashiwa, Chiba 277-8567, Japan

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### Abstract

In our examination of the distribution of abdominal fat, dietary intake and biochemical data in patients with nonalcoholic fatty liver disease (NAFLD), non-obese NAFLD patients without insulin resistance presented a characteristic pattern of dietary intake. Dietary cholesterol intake was superabundant in non-obese patients compared with obese patients, although total energy and carbohydrate intake was not excessive. Namely, excess cholesterol intake appears to be one of the main factors associated with NAFLD development and liver injury. Therefore, the control of dietary cholesterol intake may lead to an improvement in NAFLD, and the NPC1L1 inhibitor ezetimibe might be a promising treatment for NAFLD. We review one pathogenic aspect of lipid metabolism dysregulation in NAFLD and survey new strategies for NAFLD treatment based on the modification of cholesterol metabolism.

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**Key words:** Cholesterol; Ezetimibe; Nonalcoholic fatty

### INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD), which encompasses a broad spectrum of liver disorders ranging from simple hepatic steatosis to steatohepatitis and cirrhosis, is currently the most common cause of chronic liver disease and abnormal liver function tests in Western countries. The development of hepatic steatosis is considered to be associated with an excess intake of calories, visceral obesity and insulin resistance, which result in an increased release of free fatty acids from adipocytes and increased rates of fatty acid synthesis in the liver<sup>[1,2]</sup>. However, the mechanisms involved in the pathogenesis of NAFLD in humans have not been thoroughly investigated. Because of the associated triglyceride accumulation in hepatocytes, NAFLD has been mainly investigated as a lipogenic disorder. Indeed, fatty acid overload because of the acceleration of *de novo* synthesis and cellular uptake results in mitochondrial dysfunction, oxidative stress and impaired VLDL formation, which lead to disease progression<sup>[1,2]</sup>. These changes related to lipid metabolism were positively linked to transcriptomic and metabolomic profiles in rats with NAFLD induced by a high fat diet<sup>[3]</sup>. In addition, from our analyses of the expression profile of fatty acid metabolism-associated genes in

biopsy samples from NAFLD liver, a similar expression pattern was seen, which indicated that the expression of sterol regulatory element-binding protein-1c (SREBP-1c), a positive regulator of fatty acid synthesis, was still upregulated and the expression of AMP-activated protein kinase, a negative regulator of fatty acid synthesis, was down-regulated despite the increased uptake of free fatty acids and intracellular accumulation of fatty acids and triglycerides<sup>[4,9]</sup>. These results suggest a breakdown of the feedback regulation from the increased level of intracellular fatty acids. Recently, it has been considered that cholesterol metabolism has a significant role in the pathogenesis of NAFLD. In the examination of cholesterol metabolism-associated genes, despite cholesterol overload in hepatocytes, *de novo* synthesis of cholesterol is still activated in the NAFLD liver, meaning that cholesterol metabolism is dysregulated in NAFLD<sup>[10]</sup>. This review focuses on the intrahepatic cholesterol dysregulation in NAFLD and potential emerging therapies for NAFLD.

To understand the nature of NAFLD, a nutritional approach provided helpful information. Among NAFLD patients, a large percentage of patients have obesity with insulin resistance, however, many non-obese individuals are also included<sup>[11,12]</sup>. Considering visceral fat and insulin resistance, which are evident in obese patients, the distribution of abdominal fat, dietary intake and biochemical data were compared between obese (BMI > 25 kg/m<sup>2</sup>) and non-obese patients (BMI < 25 kg/m<sup>2</sup>) to identify potential nutritional factors that affect NAFLD<sup>[13,14]</sup>. Visceral fat and dietary intake of total energy and carbohydrates were at overtly higher levels in the obese group as a matter of course. In contrast, in non-obese patients, dietary cholesterol was significantly higher and dietary polyunsaturated fatty acids (PUFA) were significantly lower than those in obese patients. Mean concentrations of serum total cholesterol, LDL-cholesterol and triglycerides were near the upper limit of the normal range, and serum levels of adipocytokines were not in the abnormal range in either group.

Namely, superabundant dietary cholesterol and decreased dietary PUFA intake may contribute to NAFLD development without the presence of obesity or insulin resistance. These findings are supported by some animal models fed a high-cholesterol diet, which show hepatic steatosis without obesity<sup>[15-17]</sup>. However, these animals had obvious hypercholesterolemia in contrast to NAFLD patients. This might be because the dietary cholesterol levels are considerably higher (0.2%-1.25%) in animal models than in our examined NAFLD patients. Furthermore, in these patients, hypercholesterolemia might be masked by the overwork of hepatocytes, resulting in cholesterol overload in tissues. Cholesterol supply and fatty acid synthesis are associated on a stream of the liver X receptor  $\alpha$  (LXR $\alpha$ )-SREBP-1c pathway. In hepatocytes, LXR $\alpha$  is a key regulator of cholesterol and fatty acid metabolism, and its endogenous agonistic ligands are oxysterols, which are metabolites

of cholesterol. Surplus cholesterol produces increased levels of oxysterols, resulting in activation of the LXR $\alpha$ -SREBP-1c pathway and enhancement of fatty acid synthesis. Furthermore, upregulation of LXR $\alpha$  expression was more noticeable in non-obese than in obese NAFLD patients<sup>[8]</sup>. Also, in the study of PUFA, patients with NAFLD were found to have lower levels of hepatic n-3 and n-6 PUFA, and n-3 PUFA dietary intake had therapeutic effects on fatty liver in patients with NAFLD<sup>[18-20]</sup>. n-3 PUFAs, such as eicosapentaenoic acid, which function as suppressors of SREBP-1c, are considered to reduce hepatic levels of triglycerides. However, clinically, a drug containing eicosapentaenoic acid does not have a high enough efficacy in many cases to overcome NAFLD (our own data).

Until now, investigations of therapeutic interventions have largely focused on agents that modify oxidative stress and insulin sensitivity, but clearly, an effective therapy for NAFLD has not been proven. If excess cholesterol plays a key role in the onset and progression of NAFLD, the control of dietary cholesterol intake should be a beneficial treatment strategy. Niemann-Pick C1 like 1 (NPC1L1), found in the proximal jejunum and canalicular aspect of hepatocytes, is essential for the absorption/reabsorption of cholesterol from the intestines and liver. Accordingly, the NPC1L1 inhibitor ezetimibe is expected to decrease intracellular cholesterol levels and to down-regulate/inactivate the LXR $\alpha$ -SREBP-1c pathway, and may be a suitable candidate for NAFLD treatment. In animal models, knocking out NPC1L1 or treatment with a NPC1L1 inhibitor provides resistance against steatosis<sup>[21,22]</sup>. Clinically, we encountered and reported a patient with NAFLD in whom ezetimibe clearly provided an improvement against liver injury and steatosis<sup>[23]</sup>. In a clinical study, to reduce cholesterol load, ezetimibe was administered (10 mg/d, orally) to non-obese NAFLD patients ( $n = 12$ ) without any other treatments and any lifestyle modifications (unpublished data). In fact, ezetimibe was effective for liver injury because significant improvements were seen in serum aminotransferase levels, with 75% of subjects normalizing their transaminases. Six months after the treatment, alanine aminotransferase levels decreased by nearly 60% on average. However, a steatotic appearance remained as determined by liver echotexture in many of the patients (9/12) after 12 mo of treatment, indicating that a significant attenuation of fat content was not necessarily found. Of note, suppression of dietary cholesterol absorption may be a feasible option to successfully treat NAFLD, particularly in non-obese patients.

Considering the above findings, cholesterol-modifying treatments are favorable for NAFLD patients, and ezetimibe is expected to show a prompt clinical effect on laboratory findings for at least non-obese patients. Hence, the following should be examined and determined: (1) Are HMG-CoA reductase (HMGR) inhibitors (statins), which suppress *de novo* cholesterol synthesis, effective for NAFLD as well as ezetimibe? In recent reports, some



affirm but some deny the effect<sup>[24-26]</sup>. However, statins, with the exception of pravastatin, have generally shown promising results with improved serum aminotransferase levels. Combination therapy with an HMGCR inhibitor plus ezetimibe might be more effective than monotherapy, although several cases of hepatic injury as an adverse effect have been reported in patients with pre-existing chronic liver disease<sup>[27,28]</sup>; (2) Is the control of cholesterol levels also effective in obese NAFLD patients with insulin resistance? Because dietary cholesterol intake was also significantly higher in obese patients than in normal individuals<sup>[13]</sup>, ezetimibe is possibly effective for obese patients. However, in obese patients, it is difficult to remove the impact of other factors such as lifestyle modifications and other baseline agents; therefore, a study in obese patients requires circumspection; (3) Does the control of cholesterol levels improve steatosis in long-term observations? In the studies of NAFLD treatment by statins, a consistent opinion has not been drawn on the matter of the improving effect in hepatic steatosis<sup>[24,25]</sup>; (4) Further studies are required to determine whether cholesterol modifications are effective for both types of NAFLD, simple steatosis and steatohepatitis; and (5) Does the control of cholesterol levels show an additive therapeutic effect with any other treatments such as antioxidants, hepatoprotective agents or insulin sensitizers?

## CONCLUSION

According to our nutritional examinations, increased cholesterol intake may be one of the main causes of an increase in the prevalence of NAFLD. Therefore, as a potential treatment, cholesterol-lowering agents look promising. Indeed, several recent studies endorse the clinical indication of statin therapy for NAFLD. Ezetimibe has recently been viewed as an alternative to statin therapy in patients with hypercholesterolemia. Ezetimibe targets the cholesterol absorption/reabsorption step, and accordingly ezetimibe may be a suitable treatment for NAFLD. Larger trials are needed to confirm whether ezetimibe or statins are really efficacious as monotherapeutic agents and, to maximize clinical benefits while minimizing side effects, further trials may be required to investigate the best combination partners for the treatment of NAFLD.

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Luca Stocchi, MD, Series Editor

## Current indications and role of surgery in the management of sigmoid diverticulitis

Luca Stocchi

Luca Stocchi, Department of Colorectal Surgery, Digestive Disease Institute, Cleveland Clinic, Cleveland, OH 44195, United States

Author contributions: Stocchi L performed the research on current literature, analyzed the data and wrote the paper.

Correspondence to: Luca Stocchi, MD, Department of Colorectal Surgery, Digestive Disease Institute, Cleveland Clinic, 9500 Euclid Avenue, Desk A30, Cleveland, OH 44195, United States. [stocchi@ccf.org](mailto:stocchi@ccf.org)

Telephone: +1-216-4456378 Fax: +1-216-4458627

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### Abstract

Sigmoid diverticulitis is a common disease which carries both a significant morbidity and a societal economic burden. This review article analyzes the current data regarding management of sigmoid diverticulitis in its variable clinical presentations. Wide-spectrum antibiotics are the standard of care for uncomplicated diverticulitis. Recently published data indicate that sigmoid diverticulitis does not mandate surgical management after the second episode of uncomplicated disease as previously recommended. Rather, a more individualized approach, taking into account frequency, severity of the attacks and their impact on quality of life, should guide the indication for surgery. On the other hand, complicated diverticular disease still requires surgical treatment in patients with acceptable comorbidity risk and remains a life-threatening condition in the case of free peritoneal perforation. Laparoscopic surgery is increasingly accepted as the surgical approach of choice for most presentations of the disease and has also been proposed in the treatment of generalized peritonitis. There is not sufficient evidence supporting any changes in the approach to management in younger patients. Conversely, the available evidence suggests that surgery should be indicated after one attack of

uncomplicated disease in immunocompromised individuals. Uncommon clinical presentations of sigmoid diverticulitis and their possible association with inflammatory bowel disease are also discussed.

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**Key words:** Sigmoid diverticulitis; Diverticulitis management; Diverticulitis surgery; Acute diverticulitis; Complicated diverticulitis; Perforated diverticulitis; Laparoscopic colectomy

**Peer reviewer:** Mr. Chris Briggs, Department of Hepatobiliary and Pancreatic Surgery, K Floor, Royal Hallamshire Hospital, Glossop Road, Sheffield, S10 2JF, United Kingdom

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### INTRODUCTION

Sigmoid diverticulitis is a common disease of the Western World and results in a significant number of hospital admissions<sup>[1]</sup> with considerable societal costs due to loss of productivity. The prevalence of diverticula in the sigmoid increases proportionally with aging and only rarely results in the inflammation referred to as sigmoid diverticulitis. Sigmoid diverticula may cause significant bleeding which is generally unrelated to diverticular inflammation and is generally referred to as diverticular bleeding or bleeding diverticulosis. Bleeding caused by diverticula will therefore not be included in this review article. The spectrum of sigmoid diverticulitis ranges from a single episode of mild sigmoid inflammation amenable to outpatient treatment to a life-threatening generalized peritonitis caused by acute diverticular perforation which

requires urgent surgical intervention.

The aim of this review article is to analyze the clinical presentation, treatment modalities for the various forms of sigmoid diverticulitis, the indications for elective and urgent surgery and the postoperative and functional outcomes reported in the literature.

## RISK FACTORS AND PREVENTIVE STRATEGIES

There are few studies which present evidence of a causal relationship with preventable factors. The data obtained from a prospective cohort of 47 228 male health professionals who were free from diverticular disease in 1986 has been fundamental in providing evidence-based outcomes. Obesity is significantly associated with an increased incidence of both diverticular bleeding and diverticulitis, which have often been considered together in the studies from this large dataset. The relative risk of diverticulitis was found to be between 1.5 and 2, depending on whether body mass index (BMI), waist circumference or waist to hip ratio were considered<sup>[2]</sup>. Correspondingly, physical activity, particularly if vigorous, is associated with decreased incidence of sigmoid diverticulitis and diverticular bleeding<sup>[3]</sup>. A diet with an increased fiber intake, particularly cellulose, is also significantly associated with a decreased risk of diverticular disease<sup>[4]</sup>. On the other hand, the presumed correlation between incidence of sigmoid diverticulitis and the consumption of nut, corn and popcorn has not been confirmed when analyzing this large prospective cohort of men<sup>[5]</sup>. With respect to the use of medications, the regular and consistent use of nonsteroidal antiinflammatory drugs and acetaminophen is associated with symptoms of severe diverticular disease, particularly bleeding<sup>[6]</sup>.

## CLINICAL PRESENTATION AND DIAGNOSIS

Sigmoid diverticulitis generally presents with abdominal pain, typically located in the left lower quadrant and associated with a variable degree of peritoneal irritation, which can range from none to generalized peritonitis. Localized peritoneal reaction with guarding and rebound tenderness may be noted. Fever and elevation of the white blood cell count can aid in the diagnosis when present. A redundant sigmoid colon may reach the right lower quadrant, and sigmoid diverticulitis under these circumstances may resemble acute appendicitis. In cases of complicated diverticulitis a stricture may lead to obstructive symptoms with nausea and vomiting as the most noticeable symptoms. On the other hand, a history of recurrent urinary tract infection, dysuria with or without urgency, pneumaturia and fecaluria can suggest a colovesical fistula. When a patient reports passing stools per vagina, insertion of a vaginal speculum can reveal a fistulous opening at the vaginal apex, thus confirming a colovaginal fistula. A previous history of hysterectomy

is a valuable clinical clue to the correct diagnosis as colovaginal and colovesical fistulas are rare in females with their uterus in place, as the uterus becomes a screen interposed between the inflamed colon and the bladder and vagina. Less commonly, sigmoid diverticulitis can involve other surrounding structures and cause coloenteric, colouterine or colocutaneous fistulas.

A full colonoscopy should be typically avoided during an episode of acute diverticulitis because of an increased risk of perforation. In select cases and experienced hands, a gentle flexible sigmoidoscopy can provide additional information and help rule out alternative diagnoses such as cancer, inflammatory bowel disease, or ischemic colitis. Computed tomography (CT) is the most commonly used imaging modality to determine the diagnosis of sigmoid diverticulitis. In this respect, CT has supplanted barium enema and gastrografin enema in the routine evaluation of the sigmoid colon<sup>[7]</sup>. It can also help establish a differential diagnosis with other conditions which might exhibit similar symptoms such as gynecologic or urinary tract disorders. Irritable bowel syndrome and diverticulitis may present with similar symptoms and physical findings. It is therefore important to confirm the diagnosis of sigmoid diverticulitis by imaging before recommending surgery.

## CLASSIFICATIONS OF SIGMOID DIVERTICULITIS AND IMPLICATIONS FOR MANAGEMENT

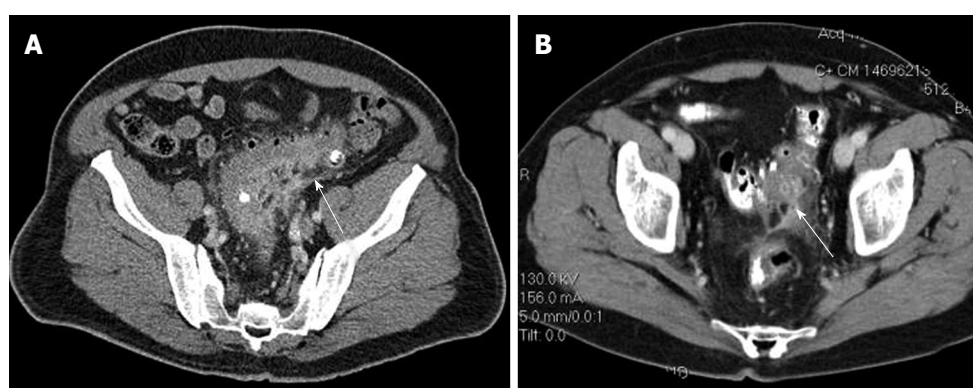
It is appropriate to classify sigmoid diverticulitis into different categories as the morbidity and mortality of this condition are greatly variable. Traditionally, the Hinchey classification has been used to subdivide sigmoid diverticulitis into subgroups based on the degree and extent of the abdominal and pelvic disease identified at the time of surgery and associated with perforated diverticular disease of the colon<sup>[8]</sup>. Of note, Hinchey credited Hughes for the development of an earlier, similar classification in 1963<sup>[9]</sup>. The Hinchey classification, developed before the advent of routine CT imaging, remains the most widely used classification and a few updated modifications have therefore been proposed in recent years (Table 1). In fact, the original Hinchey classification might not be the most practical classification to help in the contemporary management of at least some cases of diverticular disease. For example, the Hinchey classification separates a pericolic abscess (Hinchey 1) from a distant abscess (Hinchey 2). However, larger pericolic abscesses and similarly sized distant abscesses might carry similar morbidity and require similar management. In these cases, more important factors in the clinical management of this complication of diverticular disease might instead be the abscess size, location in the pelvis or mesocolon and also the ability to percutaneously drain the abscess regardless of its vicinity to the sigmoid, and therefore maximize the feasibility of a subsequent one-stage operation. In this respect, some proposed modifications of the Hinchey classification spe-



**Table 1** Hinchey classification and its modifications

	Original Hinchey classification	Sher <sup>[10]</sup> , Kohler modification <sup>[11]</sup>	Wasvary modification <sup>[33]</sup>	Kaiser modification <sup>[71]</sup>
Stage I	Pericolic abscess confined by the mesentery of the colon	Pericolic abscess	I a phlegmon I b pericolic abscess	I a confined pericolic inflammation-phlegmon I b confined pericolic abscess
Stage II	Pelvic abscess resulting from a local perforation of a pericolic abscess	II A distant abscess amenable to percutaneous drainage II B complex abscess associated with/without fistula	Pelvic abscess	Pelvic, distant intrabdominal or retroperitoneal abscess
Stage III	Generalized peritonitis resulting from rupture of pericolic/pelvic abscess into the general peritoneal cavity	Generalized purulent peritonitis	Purulent peritonitis	Generalized purulent peritonitis
Stage IV	Fecal peritonitis results from the free perforation of a diverticulum	Fecal peritonitis	Fecal peritonitis	Fecal peritonitis

<sup>1</sup>This modification also includes stage 0, defined as mild clinical diverticulitis.



**Figure 1** Diverticulitis. A: Uncomplicated sigmoid diverticulitis with colonic thickening and straining at CT (arrow), also referred to as “mild” CT diverticulitis. Two diverticula contain contrast medium without evidence of extravasation outside the sigmoid; B: “Severe” CT diverticulitis with extravasation of contrast and small amount of extraluminal air (arrow). This patient was initially managed non-operatively and eventually required surgery for recurrent disease.

**Table 2** Ambrosetti classification<sup>[12]</sup>

Moderate diverticulitis	Severe diverticulitis
Localized sigmoid wall thickening (> 5 mm) Inflammation of pericolic fat	Same as mild diverticulitis plus one of the following: Abscess Extraluminal air Extraluminal contrast

cifically include the ability to percutaneously drain the abscess<sup>[10,11]</sup>. Furthermore, the Hinchey classification was developed based on the description of surgical findings and was not specifically designed to evaluate cases of sigmoid diverticulitis treated with antibiotics only. More recently, CT scanning has become the imaging modality of choice to diagnose sigmoid diverticulitis and has been proposed as being the imaging modality providing the most important and valuable indication as to the likelihood that medical treatment with antibiotics will fail. In this regard, Ambrosetti *et al*<sup>[12]</sup> have proposed a CT-based classification of sigmoid diverticulitis subdivided into “moderate disease” or “mild disease” in the case of localized sigmoid wall thickening (greater than 5 mm) and inflammation of the pericolic fat (Figure 1A). On the other hand, the term

“severe disease” is used instead in the case of abscess, extraluminal air or extraluminal contrast extravasation (Figure 1B and Table 2).

## UNCOMPLICATED DIVERTICULITIS

When the inflammatory process is limited to the sigmoid it is generally treated with antibiotics. If symptoms are not severe and the patient is otherwise healthy and compliant with medical treatment, wide spectrum antibiotic treatment can be administered orally on an outpatient basis and the patient followed with serial office visits. On the other hand, if the patient is systemically ill, elderly or has significant comorbidities, a hospital admission and treatment with intravenous antibiotics are warranted. Even when hospital admission is necessary, the appropriateness of an initially conservative approach with antibiotic management has been confirmed<sup>[13-17]</sup>. Most patients with uncomplicated sigmoid diverticulitis respond to medical treatment and generally experience significant decreases in their abdominal pain, temperature and white blood cell count within the first 48 h after initiation of antibiotic treatment<sup>[17,18]</sup>.

In a minority of patients non-operative treatment fails, and symptoms either persist or worsen. In these cases,

urgent or semi-urgent surgery may become necessary during the same hospital stay. Among the remaining patients who successfully recover from their first episode of sigmoid diverticulitis, only a few eventually require elective sigmoid resection for recurrent disease and even more rarely are urgent operations necessary.

Following recovery from a new onset attack of uncomplicated diverticulitis the patient should undergo colonoscopy, or alternatively a barium enema, to rule out alternative diagnoses such as ischemic colitis, inflammatory bowel disease or, most importantly, a carcinoma.

## INDICATIONS FOR ELECTIVE SURGERY

The indications for elective operation for sigmoid diverticulitis are evolving. For several years the traditional teaching has been that elective sigmoidectomy was warranted after 2 attacks of uncomplicated diverticulitis. This recommendation was based on the assumptions that after 2 attacks there was not only a very high probability of recurrent attacks of uncomplicated diverticulitis but also an increased risk of complicated diverticulitis including free perforation causing diffuse peritonitis. From this viewpoint surgery would therefore prevent the risk of complicated diverticulitis with its inherently increased morbidity and mortality. Recent studies have questioned this hypothesis<sup>[19]</sup> and suggest instead that most patients who have complicated diverticulitis experience this clinical presentation as their first manifestation of diverticular disease<sup>[20,21]</sup>. Other studies based on decision analysis models have indicated that the preferred timing of elective surgery to optimize life expectancy should be after the third<sup>[22]</sup> or fourth<sup>[23]</sup> attack of uncomplicated diverticulitis. This changed view on the indications for elective surgery has reduced the overall number of surgical procedures performed for diverticulitis. In a study of 685 390 hospital discharges for sigmoid diverticulitis, based on the Nationwide Inpatient Sample during the period 1991-2005, the ratio of hospital discharges for diverticulitis increased from 5.1 to 7.6 cases per 1000 inpatients. However, the proportion of patients who underwent surgery for uncomplicated diverticulitis declined from 17.9% to 13.7% ( $P < 0.001$ ). In spite of these shifts, the percentage of patients with free perforation from diverticular disease remained stable throughout the study period at 1.5%<sup>[24]</sup>. With the limitation of a retrospective study based on administrative data, this study with a large number of patients also confirms that a less aggressive strategy for elective surgery did not result in any worrisome increase in the rate of presentation with diffuse peritonitis from diverticular perforation. Contemporary proponents of surgery after 2 attacks argue that earlier surgery favorably impacts patient symptoms<sup>[25]</sup> and that an increased number of diverticulitis attacks proportionally increases the conversion rates at the time of elective laparoscopic sigmoidectomy<sup>[26]</sup>.

Overall, the recent data from the literature defining the natural history of uncomplicated diverticulitis has contributed to reducing the emphasis on the rule of

surgery after the second attack. As a result of this shift, the most recent version of the Practice Parameters for Diverticulitis from the American Society of Colon and Rectal Surgery states that “the number of attacks of uncomplicated diverticulitis is not necessarily an overriding factor in defining the appropriateness of surgery”<sup>[27]</sup>.

## SURGICAL TREATMENT

The tenets of surgical treatment of diverticulitis are resection of the entire sigmoid and anastomosis between a soft and pliable area of descending colon and the upper rectum. The latter is generally recognized by the confluence of the teniae, which frequently occurs at the level of the sacral promontory. Failure to completely remove the sigmoid is associated with increased recurrence rates<sup>[28,29]</sup>. Some surgeons have emphasized preservation of the inferior mesenteric artery which might minimize the risk of anastomotic leakage<sup>[30]</sup>, sexual dysfunction from intraoperative nerve injury<sup>[31]</sup>, and optimize functional results<sup>[32]</sup>. Mobilization of the splenic flexure should be left to the discretion of the operating surgeon and is generally not necessary in the case of redundant left colon. The involvement of the tissue surrounding the sigmoid colon by the inflammatory process is variable. Often it is possible to identify the ureters intraoperatively and the required pelvic dissection can be limited to the upper rectum. However, there may be cases of complicated diverticulitis in which the extent and degree of inflammatory changes warrant the use of ureteral stents and/or the creation of a colorectal anastomosis in the more distal rectum. In such cases a difficult, prolonged dissection with significant blood loss may also justify the creation of a proximal diverting stoma. With respect to the required extent of resection, it is not necessary to remove the entire colonic segment bearing diverticula, which may actually be impossible in some cases due to the extent and density of diverticula throughout the colon. However, care should be taken to prevent inclusion of any diverticula into a stapled colorectal anastomosis. These principles are generally accepted and should apply equally to open or laparoscopic surgery. On the other hand, the timing of surgery in relation to the last diverticulitis attack has been the subject of controversy. The traditional practice entails a waiting period of 4-6 wk after a diverticulitis attack before performing an elective operation. Alternatively, some surgeons have suggested that early intervention for complicated diverticular disease may avoid the prolonged hospitalization and possibly multiple hospital admissions related to the traditional stepwise approach with initial antibiotic management and delayed elective surgery<sup>[33]</sup>. It has also been suggested that early surgery might obviate the creation of a stoma with its associated possible complications<sup>[34]</sup>. In addition, there is some evidence suggesting that an earlier timing of surgery, to within 30 d from the last diverticulitis attack, is not associated with increased morbidity when compared with operations performed between 30 and 60 d, or after 60 d following the last attack<sup>[35]</sup>. However, other investigators have reported less encouraging results. In the

case of laparoscopic surgery early surgical intervention has been associated with an increased conversion rate due to inflammation<sup>[36]</sup>. More importantly, a prospective study evaluating early elective sigmoid resection, carried out after 5-8 d of initial antibiotic treatment, has shown that this approach was associated with increased morbidity when compared with operations carried out 4-6 wk after the initial hospitalization<sup>[37]</sup>. While the data regarding the outcomes of early surgery following hospitalization for sigmoid diverticulitis remains controversial, there does not seem to be sufficiently consistent evidence at the moment to justify any anticipation of elective surgery before the traditional 4-6 wk waiting period.

## INCREASED ROLE OF LAPAROSCOPIC SURGERY

While open surgery continues to be performed, especially in low volume centers and by low volume surgeons<sup>[38]</sup>, laparoscopic surgery is increasingly preferred in the elective treatment of sigmoid diverticulitis. Several single-institutional series have confirmed feasibility and safety of the laparoscopic approach<sup>[39-42]</sup>. Laparoscopic sigmoidectomy is associated with reduced recovery time and return to bowel function, reduced hospital stay, and at least in some cases decreased morbidity<sup>[43-47]</sup> and costs<sup>[45,48]</sup>. Single-institutional series by experienced surgeons have reported conversion rates of as low as 2.8% and a median hospital stay of 4 d<sup>[42]</sup>. Minimally invasive sigmoidectomy can be performed using a straight laparoscopic technique or a laparoscopic hand-assisted technique<sup>[49,50]</sup>. A single-access sigmoidectomy has also been recently described<sup>[51]</sup>. The controversy persists as to whether the hand-assisted technique allows a reduction of operative times and conversion rates while extending the benefits of laparoscopic surgery to more difficult cases.

In general, the benefits of laparoscopic surgery have been confirmed by a large study based on data from the Nationwide Inpatient Sample during the years 1998-2000, which included 709 patients treated laparoscopically *vs* 17735 treated with the open technique. Laparoscopically completed patients had a mean reduction of hospital stay of almost 2 d and also reduction of postoperative morbidity when compared to their open counterparts. An important limitation of this study was that, due to the nature of the administrative database used, converted patients were not analyzed combined with the cases completed laparoscopically, which skews the results in favor of laparoscopic surgery<sup>[52]</sup>. However, a more recent study using the University Health System Consortium Database, in which converted patients were appropriately included in the laparoscopic group, confirmed a reduction in hospital stay, overall postoperative morbidity and total hospital cost in favor of laparoscopic sigmoidectomy for benign diseases<sup>[53]</sup>. In addition, there is further evidence of the benefits of laparoscopic surgery emerging from a prospective randomized trial, which has demonstrated reduction of major complications after laparoscopic surgery when

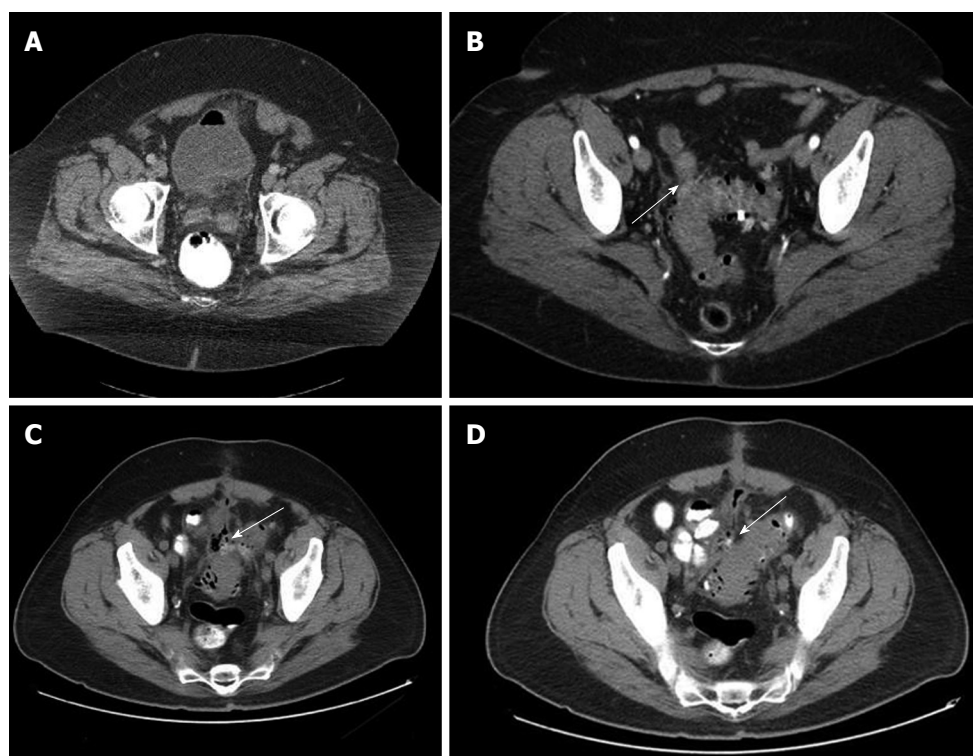
compared with open sigmoidectomy<sup>[54]</sup>. This multicenter, randomized, double-blinded study accrued 104 patients in 5 centers from 2002 to 2006. Double-blinding was carried out by covering the patient abdomen with a large dressing at the time of surgery so that patients, as well as physicians in charge of patients discharge, were unaware of the surgical technique used. Eligible patients were randomized to open *vs* laparoscopic sigmoid resection. Patients were similar with respect to gender, age, BMI, comorbidities, indications for surgery and previous surgical procedures. Conversion rate was 19% and mortality 1%. Laparoscopic surgery resulted in expected recovery benefits including significant reduction of pain based on visual analog scores and systemic analgesia requirements, decreased hospital stay and improved quality of life based on short-term SF-36 questionnaires. In addition, laparoscopic surgery resulted in significant reduction of major complications, defined as a composite inclusive of intrabdominal abscess, anastomotic leakage, pulmonary embolism and myocardial infarction. Major complications combined for a 25% rate after open surgery *vs* 10% after laparoscopic procedures<sup>[54]</sup>.

Based on the data from the last decade, it is reasonable to offer laparoscopic surgery in the surgical management of sigmoid diverticulitis and expect at least the recovery advantages reported after laparoscopic bowel resection.

## RELATIONSHIP BETWEEN SURGICAL VOLUME AND OUTCOMES

A number of studies have investigated possible differences in outcomes related to the experience of the operators. With respect to the use of laparoscopic surgery, there is evidence that the volumes of both individual surgeons and hospitals are directly proportional to the likelihood of performing laparoscopic surgery for diverticular disease. Using National Inpatient Sample Data based on over 55 000 patients, high-volume surgeons were almost 9 times more likely to perform laparoscopic surgery and high-volume hospitals were over 3 times more likely to perform laparoscopic surgery than their low-volume counterparts. These differences remained statistically significant when the data were stratified for age of the patient and timing of surgery; elective *vs* nonelective<sup>[38]</sup>. Volume/outcome studies have also been conducted within the subgroup of patients treated with laparoscopic surgery. In the multicenter, observational, German study from the Laparoscopic Colorectal Surgery Study Group of 1545 patients, the 52 participating institutions were divided into 3 groups according to the number of cases performed; greater than 100, between 30 and 100, and less than 30. While the percentage of patients with complicated diverticulitis was significantly increased in high-volume institutions (21% *vs* 8% in low-volume centers), operating times in these same institutions were shorter by approximately 30 min. Intraoperative complications, conversion rates and postoperative morbidity and mortality were numerically lowest in the high-volume centers, but these differences were not





**Figure 2** **Fistula.** A: Colovesical fistula as indicated by the presence of air in the bladder. This patient had symptoms and other CT findings consistent with sigmoid diverticulitis; B: Sigmoid diverticulitis and colovaginal fistula. This patient had undergone previous hysterectomy and complained of feculent discharge from her vagina. CT scan indicated inflamed sigmoid with adherent small bowel loop (arrow). The small bowel loop could be successfully separated from the sigmoid at the time of laparoscopic sigmoidectomy. There was no evidence of coloenteric fistula; Sigmoid diverticulitis with colocolic fistula (arrows) (C and D) (courtesy of Dr. Ravi Pokala Kiran, Department of Colorectal Surgery, Digestive Disease Institute, Cleveland Clinic, Cleveland, Ohio, USA).

statistically significant<sup>[55]</sup>. The results from this study seem to indicate that experienced surgeons in high-volume centers might be more facile at treating more complex cases with laparoscopic surgery. However, even low-volume centers can still achieve comparable postoperative outcomes and should therefore not be discouraged from performing laparoscopic surgery.

## RESULTS OF SURGERY FOR DIVERTICULITIS

Contemporary surgical treatment of diverticulitis following the principles described above is considered curative with a less than 5% recurrence rate<sup>[29,56]</sup>. A suspicion of recurrent sigmoid diverticulitis following surgical resection should be confirmed by CT scan of the abdomen and pelvis after which antibiotic treatment should be initiated, as for a case of primary uncomplicated sigmoid diverticulitis. It is important to preoperatively discuss with the patient that the risk exists that surgery might not lead to resolution of the patient's complaints. When this is the case, an anastomotic stenosis should be ruled out as a possible source of the problem which can often be successfully treated<sup>[57]</sup>. However, persistent or recurrent symptoms can be more difficult to elucidate. At least one contemporary series has reported a 25% rate of persistent symptoms after surgery<sup>[58]</sup>, which the authors felt could be only partially explained by an overlap with irritable bowel syndrome. One of the

limitations of the assessment of symptoms and functional results after surgery is that sigmoid diverticulitis can cause a significant impairment of quality of life before surgery, a time at which quality of life is even more rarely assessed. The functional results of surgery should therefore be most accurately assessed when compared to the patient's preoperative status. A recent study has appropriately addressed this issue and reported a prospective evaluation of functional outcomes after laparoscopic sigmoid colectomy. A sample of 46 individuals underwent evaluation of their quality of life using the gastrointestinal quality of life indicator (GIQLI) administered before surgery and then at 3, 6, and 12 mo postoperatively. The quality of life significantly improved for the majority of the overall group, whereas it declined in only 5 patients. Urinary and sexual function were also tested using validated scores and did not change as a result of surgery<sup>[31]</sup>. When appropriately diagnosed by CT scan, sigmoid diverticulitis requiring surgery should be followed by improvement in symptoms and function in a substantial majority of cases.

## COMPLICATED DIVERTICULITIS

There are several complications which may be associated with diverticular inflammation. These include fistula (Figure 2), phlegmon, stricture, abscess and free perforation. At times the definition of complicated disease may depend on the individual clinical judgment, as uncomplicated and complicated diseases are a continuum of in-





**Figure 3 Sigmoid stricture (arrow) causing large bowel obstruction with proximal colonic dilatation.** Clinical and imaging findings at presentation did not allow ruling out sigmoid carcinoma. This patient was treated with initial Hartmann procedure and the pathology report revealed sigmoid diverticulitis. He subsequently underwent Hartmann takedown after 3 mo.

creasingly severe inflammation which can cause a variable degree of stricture, intramural abscess or phlegmon. In the United States, complicated disease at presentation is more common in African-American patients and in individuals who lack medical insurance, based on an analysis from the Nationwide Inpatient Sample<sup>[59]</sup>.

In general, surgery is recommended for complicated diverticulitis after the first episode as the risk of recurrent disease without surgery is very high. However, when age or comorbidities prohibitively increase perioperative risks, it may be appropriate to approach complicated diverticulitis with medical treatment alone<sup>[60]</sup>.

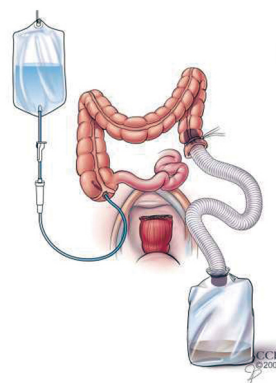
Laparoscopic surgery remains feasible also for complicated diverticulitis<sup>[55,61,62]</sup> including cases with fistulas<sup>[63-66]</sup>. The morbidity after laparoscopic surgery for complicated diverticulitis might exceed that of uncomplicated disease, but this has not been uniformly proven<sup>[42]</sup>.

It remains controversial whether the act of conversion, which is more likely for complicated diverticular disease<sup>[67]</sup>, increases postoperative morbidity or not. It is generally accepted that when a conversion is necessary, an early conversion can minimize major complication so that it causes only minor morbidity<sup>[68]</sup> or does not result in any increased morbidity rate at all<sup>[69]</sup>.

In general, a more selective use of laparoscopic surgery for more straightforward, uncomplicated cases of diverticular disease could minimize conversion rates and therefore capitalize on the advantages derived from the laparoscopic approach. On the other hand, a more liberal use of laparoscopic surgery, including for complicated cases in patients with a previous laparotomy, is likely to result in increased conversion rates. However, this less stringent patient selection could still offer the potential benefits of laparoscopic surgery to an increasing number of individuals requiring surgery for sigmoid diverticulitis without adverse effects on long-term patient outcomes<sup>[68]</sup>.

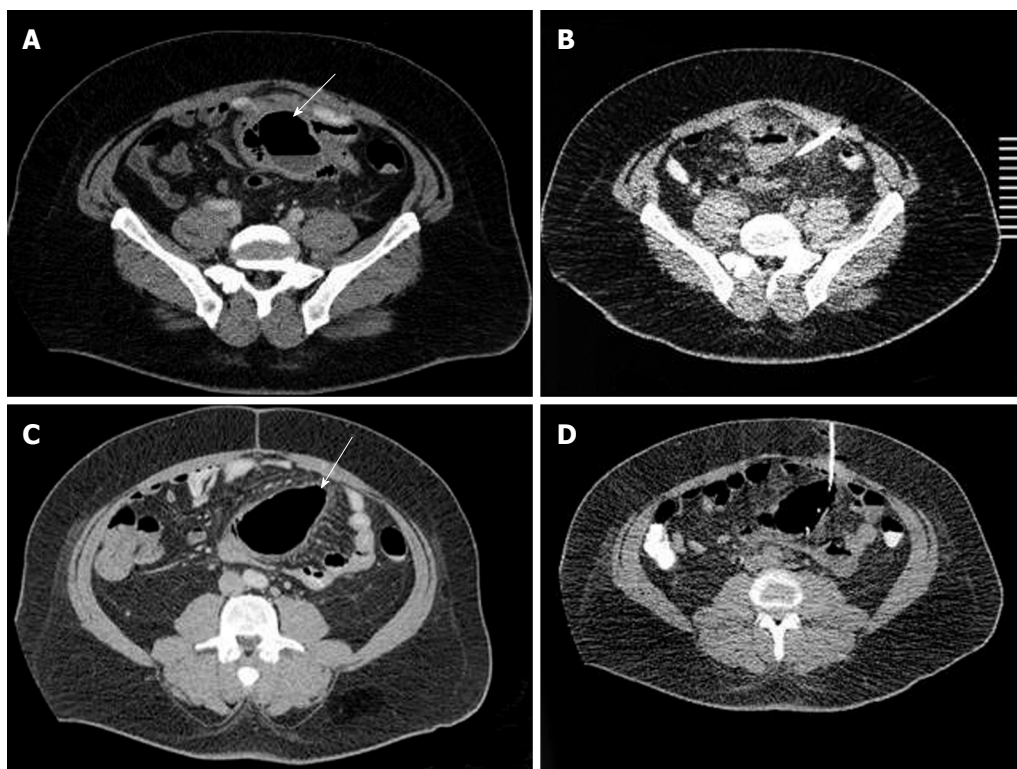
## STRICTURE

Sigmoid diverticulitis can present in the form of a stric-



**Figure 4 On-table intraoperative colonic lavage (see explanation in text).**

ture which may or may not be associated with typical symptoms. In the case of stricture, the indications for surgery may range from colonic obstruction requiring acute surgical intervention to the inability to rule out carcinoma as the cause of stricture (Figure 3). Sigmoid strictures can cause significant dilatation in the proximal colon, which can complicate the creation of a colorectal anastomosis after sigmoid resection. A staged procedure with sigmoidectomy and creation of a colostomy may therefore become necessary. A possible option in the surgical management of severe sigmoid stricture causing significant fecal loading is a resection with on-table colonic lavage and primary anastomosis (Figure 4). This is carried out by inserting a large Foley catheter through an appendicostomy or distal ileal enterotomy secured with a purse-string suture with the tip of the catheter placed into the cecum. This Foley catheter is connected to a bag of warm saline solution which is typically used for irrigation. A large corrugated tube, such as an anesthesia ventilator tube, is then placed in the open end of the descending colon and secured with umbilical tape or large suture to the bowel wall. The distal end of the tubing is placed into a bucket on the floor where the effluent is collected. It is frequently necessary to mobilize both the hepatic and splenic flexures and manually propel solid stools towards the distal end which can significantly increase operative times. A proximal stoma diversion in addition to a colorectal anastomosis may be a prudent adjunct to the operative procedure, with or without intraoperative colonic lavage. Alternatively, a stricture can be treated with placement of endoluminal metallic stents to correct the obstruction, reduce the discrepancy in bowel diameter and allow a subsequent one-stage surgical procedure consisting of sigmoid resection and primary colorectal anastomosis<sup>[70]</sup>. Other options in the management of large bowel obstruction related to diverticular disease are subtotal colectomy and primary ileorectal anastomosis, and, in the most difficult cases, creation of a decompressive colostomy proximal to the strictured sigmoid followed by delayed sigmoid resection. The choice among these various options depends on both the individual patient and the surgeon's level of confidence in performing each of the approaches described above.



**Figure 5** Sigmoid diverticulitis complicated by pericolic abscesses (A and C, arrows) requiring treatment by placement of two separate CT-guided percutaneous drains (B and D). This patient underwent laparoscopic sigmoidectomy with primary colorectal anastomosis and removal of both drains 6 wk after percutaneous drain placement.

## PERIDIVERTICULAR ABSCESS

There is evidence suggesting that clinical presentation of sigmoid diverticulitis as peridiverticular abscess has increased in recent years<sup>[24]</sup>. It is generally acknowledged that elective surgery should be performed after percutaneous drainage of peridiverticular abscess (Figure 5) due to the high risk of recurrent diverticulitis<sup>[13,71]</sup>. In these cases surgery is generally performed 4-6 wk after initial percutaneous drainage. Some surgeons prefer to leave the drain in place until surgery, others remove the drain if the output becomes minimal and a drain contrast study rules out an existing sigmoid fistula. An accepted exception is the use of percutaneous drainage alone to obviate the need of surgery in poor risk patients<sup>[72]</sup>.

The safety and effectiveness of percutaneous drainage in controlling the immediate symptoms of diverticular disease presenting with an abscess have been reported by several authors<sup>[14,73-77]</sup>. A number of variables have been examined as possible factors associated with the success rate of non-operative management.

Firstly, the size of the abscess seems to be an important indicator for success of non-operative management, especially when antibiotics alone are considered as first line treatment. A diameter of approximately 3-4 cm or less is more likely to be associated with successful antibiotic treatment<sup>[14,76,77]</sup>. Based on the ability of antibiotics alone to control smaller abscesses, some authors have suggested that the role of CT-guided drainage of diverticular-related abscesses should be re-evaluated and

percutaneous drainage should be utilized less often<sup>[14]</sup>. Another factor with a possible impact on management is the abscess location. In fact, there is evidence suggesting that an abscess located in the mesocolon might be more responsive to non-operative treatment than a pelvic abscess<sup>[15,71]</sup>. In this regard, in a study analyzing 73 patients initially treated with antibiotics and undergoing CT-guided drainage only in case of failure of medical treatment, 71% of patients with a pelvic abscess ultimately required surgery *vs* 51% after percutaneous drainage of a mesocolic abscess. Based on these results, the authors suggested that sigmoid colectomy should be recommended after drainage of a pelvic abscess but not necessarily after percutaneous drainage of a mesocolic abscess<sup>[74]</sup>. The success of non-operative treatment in at least some patients has prompted other investigators to question the role of routine surgery after successful drainage of pericolic abscess<sup>[20]</sup>.

It remains difficult to critically evaluate the results of the various treatment options available for abdominal and pelvic abscesses related to diverticulitis because of both variability in clinical practices and data reporting. In some institutions percutaneous drainage is the preferred approach whenever technically feasible, which generally requires an abscess diameter of at least 3 cm. On the other hand, in other institutions the initial treatment of diverticular abscesses includes antibiotics alone and only after failure of antibiotic treatment is a percutaneous drainage considered. In addition, the data regarding the effectiveness of percutaneous drainage alone without

subsequent surgery remain limited, because of both small sample sizes and short follow-up. Further studies will be necessary before the standard of care of elective surgery after initial percutaneous drainage is abandoned.

## GENERALIZED PERITONITIS FROM PERFORATED SIGMOID DIVERTICULITIS

A perforation of a sigmoid diverticulum into the free peritoneum is a life-threatening condition requiring immediate surgical intervention. The standard of care in most of these cases is a resection of the colonic segment including the perforation and creation of a proximal colostomy. Several authors refer to this operation as a Hartmann procedure, which by definition involves the resection of the sigmoid, closure of the rectal stump and creation of an end-descending colostomy, and which has also been performed laparoscopically<sup>[78,79]</sup>. Other surgeons have suggested that especially when the patient is severely septic and hemodynamically unstable the initial goal should be an expedited resection limited to the involved segment<sup>[80]</sup>, sometimes referred to as a “perforectomy”, in which at least some of the sigmoid should be left intact until the patient completes his or her recovery from the initial operation. In this case, a completion sigmoid resection would be typically performed at the time of colostomy takedown several months later so that the patient ultimately receives appropriate surgical treatment for sigmoid diverticulitis<sup>[81]</sup>. The morbidity and mortality from Hartmann procedure for free diverticular perforation remain substantial. The aggregate mortality in a total of 1051 patients reported in 54 combined studies conducted between 1966 and 2003 was almost 19% and was associated with a 24% incidence of wound infection and a 10% incidence of stoma complications<sup>[82]</sup>. In spite of advancements in intensive care, imaging and medical treatments, the mortality for this condition has remained stable over time<sup>[83]</sup>. Intestinal continuity can generally be reestablished 3–6 mo after the initial operation<sup>[84]</sup> although it has been reported that between approximately 30% and 70% of patients never have their colostomy closed<sup>[81,85–87]</sup>. In addition, a Hartmann takedown remains a difficult elective procedure<sup>[88]</sup> fraught with significant morbidity<sup>[89]</sup>.

Considering the significant morbidity and mortality associated with a Hartmann procedure and its sequelae, some authors have suggested that in select circumstances it might be possible to resect the perforated segment and primarily reestablish intestinal continuity<sup>[90,91]</sup>, which some surgeons feel can benefit from intraoperative colonic lavage as described above<sup>[92,93]</sup> (Figure 4). This view remains controversial and most surgeons would not recommend a resection and primary colorectal anastomosis for generalized peritonitis from diverticular perforation. However, in select circumstances it is possible to perform a colorectal anastomosis and proximal diverting loop ileostomy. This approach seems to be preferable to a Hartmann resection when the degree of intraoperative contamination and the

underlying patient condition allow this approach. In these cases, the use of a defunctioning stoma in addition to colorectal anastomosis might result in a good compromise between postoperative morbidity, quality of life and probability of permanent stoma<sup>[94]</sup>.

## LAPAROSCOPIC LAVAGE, A NOVEL SURGICAL APPROACH TO GENERALIZED PERITONITIS

The advent of laparoscopic surgery and the increased use of the laparoscopic approach to treat perforated peptic ulcers and appendicitis have led to the development of laparoscopic strategies for the treatment of perforated diverticulitis. In this regard, laparoscopic lavage is a recently proposed treatment option which would potentially save the patient from both a major bowel resection and the creation of a stoma. The initial experiences of laparoscopic lavage have been promising with respect to perioperative mortality and complications<sup>[95]</sup>. In addition, while most proponents of initial laparoscopic lavage have decided in favor of an elective, delayed sigmoidectomy<sup>[96–100]</sup>, a multicenter study from Ireland has reported encouraging results following a policy of lavage followed by continued observation. In fact, Myers *et al*<sup>[101]</sup> noted recurrence of sigmoid diverticulitis in 4 out of 92 treated patients, none of whom required surgery after a median follow-up of 36 mo. These data from different centers suggest that laparoscopic lavage has the potential to become, at least in select cases, the definitive treatment for perforated diverticulitis. However, the data on laparoscopic lavage for diverticular peritonitis remains limited and further investigations into this option are warranted to confirm these initial, promising results.

## YOUNGER PATIENTS: SHOULD THE INDICATIONS FOR SURGERY CHANGE?

The indication for surgery in younger patients, generally defined as those who are 50 years old or younger, has been the subject of controversy. It has been reported that younger patients more frequently require surgery for diverticulitis<sup>[102]</sup> or are prone to recurrent disease<sup>[103]</sup>. Based on the presumed association between younger age and more virulent disease, some surgeons have suggested that elective surgery should be recommended in patients younger than 50 years old after their first attack of uncomplicated diverticulitis<sup>[104,105]</sup>. However, other retrospective series have not confirmed a correlation between younger age and more severe disease<sup>[106–109]</sup>. In addition, prospective data do not support a more aggressive surgical approach for younger patients. In this regard, Guzzo and Hyman<sup>[110]</sup> examined 762 patients admitted to their institution with sigmoid diverticulitis between 1990 and 2001, including 259 individuals younger than 50. The risk of requiring surgery during the first admission was comparable between older and younger patients. In addition,



out of 196 younger patients who were treated medically at the time of their initial admission, only one (0.5%) presented with perforation during a median follow-up of 5.2 years. In another prospective study with a median follow-up of 9.5 years, 118 patients were followed after their initial attack of diverticulitis, 28 of whom were 50 years old or younger. Age and findings at initial CT scan were analyzed as possible predictive factors for risk of poor outcome during the follow-up period, defined as recurrent, persistent or complicated diverticulitis. The probability of poor outcome at 5 years was 54% in younger patients with initially severe CT diverticulitis *vs* 19% for older patients with mild disease, based on CT imaging. At univariate analysis, age was a predictive factor for poor outcome. However, after stratification for severity of disease, age was no longer a significant factor<sup>[111]</sup>. Based on the available contemporary data there does not seem to be sufficient justification to recommend elective surgery after one attack of sigmoid diverticulitis in younger patients and rather the disease should be treated similarly in both younger and older patients depending on its severity and inclination to recurrence.

## IMMUNOSUPPRESSED OR IMMUNOCOMPROMISED PATIENTS

Transplant recipients or patients with chronic diseases affecting the immune system are at increased risk of more aggressive and complicated diverticulitis<sup>[112-114]</sup>, including initial presentation as free peritoneal perforation<sup>[115,116]</sup>. Chronic use of steroids is also associated with increased postoperative mortality after surgery for diverticulitis<sup>[120]</sup>.

Therefore, it is generally recommended that surgery should be offered to this subset of patients after their first documented episode of diverticulitis. The studies supporting this practice are generally retrospective with small sample sizes<sup>[114]</sup>. On the other hand, there is no data presenting evidence against this practice. Therefore it seems reasonable to continue offering surgery after one episode of uncomplicated diverticulitis in immunocompromised patients. In this respect, some surgeons have emphasized that surgery should be carried out after the diverticulitis attack during the same hospital stay and a proximal diversion should be considered<sup>[117]</sup>. Other authors have even suggested that patients with one episode of uncomplicated diverticulitis who are transplant candidates should undergo prophylactic sigmoidectomy before their transplant. The evidence in favor of this practice remains scant, based on earlier studies and generally restricted to renal transplant recipients<sup>[118,119]</sup>. On the other hand, patients awaiting liver, heart and lung transplant are typically in poor health from their primary disease and generally should not be considered for prophylactic sigmoidectomy prior to their transplantation.

With respect to HIV infection and AIDS, there is no substantial data specific for sigmoid diverticulitis<sup>[120]</sup>. In general, the outcome of major abdominal surgery in

HIV-positive individuals without AIDS is not significantly different from the general population. However, when a patient develops diverticulitis in the presence of AIDS or other causes of acute immunosuppression, postoperative infections are more likely. If surgery becomes necessary in these cases, a Hartmann procedure or a primary sigmoid resection with anastomosis and proximal diversion should be therefore preferable.

## EVOLVING CONCEPTS IN DIVERTICULAR DISEASE

Sigmoid diverticulitis may have clinical manifestations which are difficult to accurately characterize. Its symptoms may overlap in some cases with the conditions collectively referred to as irritable bowel syndrome. Our understanding and therapeutic approach for this condition are evolving. From a surgical perspective it is imperative to minimize unnecessary surgery if diverticulitis cannot be documented radiologically, especially with a concurrent clinical history suggestive of irritable bowel syndrome. However, if irritable bowel syndrome can be ruled out, there seem to be a group of patients with chronic left lower quadrant abdominal pain and occasional alteration of bowel habits, but without fever or leukocytosis, who might still benefit from surgery. The condition of this subgroup of patients has been referred to as “smoldering diverticulitis”. Horgan and colleagues identified smoldering diverticulitis in 47 patients, corresponding to approximately 5% out of their denominator of 930 patients undergoing sigmoid resection for diverticulitis. A total of 88% of these patients remained pain-free after at least 12 mo of follow-up following sigmoidectomy and primary anastomosis<sup>[121]</sup>. Atypical sigmoid diverticulitis should be part of the differential diagnosis in the patient with left lower quadrant pain, as surgery is curative in the majority of these cases.

An additional, novel clinical syndrome recently proposed as a separate entity within the realm of diverticular disease is referred to as segmental colitis associated with diverticulosis (SCAD)<sup>[122-124]</sup>. This is a non-specific, localized inflammatory process associated with diverticulosis involving the sigmoid but not the rectum or the proximal colon, generally presenting in middle-aged or elderly patients with rectal bleeding, diarrhea and abdominal pain variably combined. It most commonly affects males. Histology indicates inflammation without granulomas and serology should be negative for anti-neutrophil cytoplasmic antibodies (ANCA) and anti-Saccharomyces cerevisiae antibodies (ASCA). Treatment with 5-aminosalicylate is generally effective in resolving the inflammation both symptomatically and endoscopically<sup>[122]</sup>.

The pathogenesis of SCAD and its relationship with inflammatory bowel disease remain controversial<sup>[122,125]</sup>. Regardless, SCAD is becoming increasingly accepted as a separate entity from the traditional sigmoid diverticulitis and its known complications. While anti-inflammatory agents have been effective in the management of SCAD,



their role in the more common forms of diverticular disease remain unproven.

Another area of investigation concerns the potential causal relationship between sigmoid diverticulitis and colorectal cancer, which has been suggested based on comparisons with patients having diverticulosis without diverticulitis<sup>[126]</sup>. This association has not yet been validated and will therefore require further study. At the moment, sigmoid diverticulitis is not considered a pre-cancerous or high-risk condition for the development of colorectal cancer and the recommended screening modalities do not differ from the guidelines accepted for the average-risk population.

## CONCLUSION

Sigmoid diverticulitis is a condition ranging from mild inflammation of the sigmoid to life-threatening colonic perforation. Antibiotics are generally effective in mild forms of the disease while surgery is indicated in cases of multiple recurrences or complicated disease. Based on recent data, the systematic indication for surgery after 2 attacks should be abandoned in favor of a more individualized approach. Laparoscopic surgery is gaining favor in the surgical treatment of sigmoid diverticulitis. A subset of patients with atypical presentation presents a significant challenge in management; some may benefit from surgery whereas others could benefit from anti-inflammatory agent treatment.

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## Vascular invasion in pancreatic cancer: Imaging modalities, preoperative diagnosis and surgical management

Nicolas C Buchs, Michael Chilcott, Pierre-Alexandre Poletti, Leo H Buhler, Philippe Morel

Nicolas C Buchs, Michael Chilcott, Leo H Buhler, Philippe Morel, Clinic for Visceral and Transplantation Surgery, Department of Surgery, University Hospitals of Geneva, Geneva, 1211, Switzerland

Pierre-Alexandre Poletti, Department of Radiology, University Hospitals of Geneva, Geneva, 1211, Switzerland

Author contributions: Buchs NC, Chilcott M, Poletti PA and Buhler LH wrote the paper; Buchs NC, Buhler LH and Morel P designed the study; Buchs NC, Buhler LH and Morel P performed the research.

Correspondence to: Dr. Nicolas C Buchs, MD, Clinic for Visceral and Transplantation Surgery, Department of Surgery, University Hospitals of Geneva, 24, rue Micheli-du-Crest, 1211 Geneva 14, Switzerland. [nicolas.c.buchs@hcuge.ch](mailto:nicolas.c.buchs@hcuge.ch)

Telephone: +41-22-3727698 Fax: +41-22-3727689

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pancreatic surgery. The aim of this article is to provide a complete review of the different imaging modalities in the detection of vascular invasion in pancreatic cancer.

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**Peer reviewer:** Georgios Papachristou, MD, Assistant Professor of Medicine, Division of Gastroenterology, Hepatology and Nutrition, UPMC Presbyterian, Mezzanine Level, C-Wing, 200 Lothrop Street, Pittsburgh, PA 15213, United States

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### Abstract

Pancreatic cancer is associated with a poor prognosis, and surgical resection remains the only chance for curative therapy. In the absence of metastatic disease, which would preclude resection, assessment of vascular invasion is an important parameter for determining resectability of pancreatic cancer. A frequent error is to misdiagnose an involved major vessel. Obviously, surgical exploration with pathological examination remains the "gold standard" in terms of evaluation of resectability, especially from the point of view of vascular involvement. However, current imaging modalities have improved and allow detection of vascular invasion with more accuracy. A venous resection in pancreatic cancer is a feasible technique and relatively reliable. Nevertheless, a survival benefit is not achieved by curative resection in patients with pancreatic cancer and vascular invasion. Although the discovery of an arterial invasion during the operation might require an aggressive management, discovery before the operation should be considered as a contraindication. Detection of vascular invasion remains one of the most important challenges in

### INTRODUCTION

The incidence of pancreatic cancer has gradually increased over the 20th century and in the early years of this century<sup>[1,2]</sup>. Cancer of the pancreas is the sixth most common cancer and fourth cause of death from cancer (22% of deaths among gastrointestinal cancers)<sup>[1-3]</sup>.

Pancreatic cancer is associated with a poor prognosis, with less than 5% of patients surviving 5 years after the diagnosis<sup>[4]</sup>. Surgical resection remains the only chance for curative therapy in these patients<sup>[5-7]</sup>. Accurate preoperative staging of pancreatic cancer is essential to avoid unnecessary surgery in those with unresectable disease and, at the same time, in order not to deny the opportunity for cure in patients with resectable disease<sup>[5,6,8]</sup>.

Only 16% of patients initially present a disease confined to the pancreas (stage I)<sup>[6,7]</sup>. Thus, of patients seen, 85%-90% have surgically unresectable tumors at the time of diagnosis<sup>[6,7,9-11]</sup>.

There is no evidence-based consensus on the optimal preoperative imaging assessment of patients with suspected pancreatic cancer<sup>[6,8,12]</sup>.

The criteria of unresectability are numerous<sup>[7,13-23]</sup>. However, in the absence of metastatic disease which precludes resection, assessment of vascular invasion is an important parameter for determining resectability for pancreatic cancer<sup>[5]</sup>. A frequent error is to misdiagnose an involved major vessel<sup>[11]</sup>. Vascular invasion is a relatively frequent discovery in pancreatic cancer; found in 21%-64% of patients, depending on the population studied<sup>[7,24]</sup>.

From the point of view of arterial vessels, a tumoral infiltration of a large trunk (celiac axis, superior mesenteric artery, or hepatic artery) must be carefully analyzed because it constitutes a contraindication to surgery<sup>[25-27]</sup>. However, isolated involvement of smaller branches such as the gastro-duodenal artery will not preclude surgical resection<sup>[25]</sup>. The superior mesenteric vessels are the most frequently involved vessels in this cancer, due to their intimate relationship with the head, the uncinate process, and body of the pancreas<sup>[25,28]</sup>.

Limited venous invasion does not represent an absolute contraindication for surgery<sup>[4,26,27,29-31]</sup>. Obviously, surgical exploration with pathological examination remains the "gold standard" in terms of evaluation of resectability, especially from the point of view of vascular involvement. However, current imaging modalities have improved and allow detection of vascular invasion with more accuracy. Detection is the key to the surgeon's preoperative planning, because the posterior and lateral surfaces of the portal and superior mesenteric vein can be evaluated only after the surgical procedure is well advanced<sup>[14]</sup>. Thus, the management of a suspicious tumoral adhesion to a vessel is one of the most important challenges in a Whipple type procedure.

In this review, the current imaging modalities for assessing vascular involvement of pancreatic cancer will be discussed. Subsequently, the management and outcome of vascular invasion in patients with pancreatic cancer will also be reviewed briefly.

## COMPUTED TOMOGRAPHY

Computer tomography (CT) gives information about localization, size and extension of tumor<sup>[8,18]</sup>, while being non-invasive<sup>[32]</sup>. A recent meta-analysis showed CT to be 91% sensitive and 85% specific for tumoral detection<sup>[33]</sup>. Phoa *et al.*<sup>[34]</sup> showed that, with regard to tumor convexity towards a vessel, Grades D (concave contour of the tumor towards vessel) or E (circumferential involvement of vessel) have a risk of invasion of 88%; and a possibility of resection of 7% for the type D and of 0% for the type E<sup>[35]</sup>. Loyer *et al.*<sup>[35]</sup> found that Grades A (fat plane separating the tumor from the vessel) and B (normal pancreatic tissue between tumor and vessel) had a resection rate of 95%, therefore these two grades are factors of better prognosis.

On the other hand, the length of tumor contact with the vessel (if it is greater than 5 mm) is a relatively good

predictive factor for vascular invasion (78% for portal vein and 81% for superior mesenteric vein)<sup>[34]</sup>.

A circumferential contact of more than 180 degrees has been shown to have a good correlation with unresectability<sup>[34,36,37]</sup>. For this criterion, Lu *et al.*<sup>[38]</sup> found a sensitivity of 84%, a specificity of 98%, a positive predictive value (PPV) of 95%, and a negative predictive value (NPV) of 93%, for unresectability. Furthermore, Phoa *et al.*<sup>[34]</sup> reported a sensitivity of 60%, and a specificity of 90%, if tumor convexity Grades D or E were combined with circumferential involvement of > 90 degrees. In addition, a strongly narrowed vessel also has an important risk of being invaded<sup>[34,36]</sup>, but prudence is essential, especially for a vein, due to the mass effect of the tumor without the presence of vascular invasion<sup>[10,39,40]</sup>. In addition, an artery may be completely invaded, with no apparent change in vessel caliber<sup>[36,39]</sup>.

Concerning the irregularity of the vascular wall, Li *et al.*<sup>[36]</sup> reported a sensitivity and a specificity of 45% and 99%, respectively, for tumor detection in arteries, and 63% and 100% in the case of veins.

Regarding the rare superior mesenteric vein teardrop sign, Hough *et al.*<sup>[41]</sup> found a sensitivity of this CT sign of 91% and a specificity of 98%; similar findings were reported in other series<sup>[36]</sup>.

Consequently, Li *et al.*<sup>[36]</sup> reported that the CT criteria for arterial invasion might be: an arterial embedment in tumor, or the combination of tumor involvement of more than one-half of the circumference of the arteries with artery wall irregularity or with artery stenosis (sensitivity of 79%, specificity of 99%). The criteria for venous invasion might be venous occlusion, tumor involvement of more than one-half of the circumference of the veins, vein wall irregularity, vein caliber stenosis, and teardrop superior mesenteric vein sign (sensitivity of 92%, specificity of 100%).

From the point of view of the detection of vascular invasion, many studies have evaluated CT (Table 1). CT has improved much these last years. Technology has developed multi-slice with 4-64 detector rows, allowed thin-sections and dual-phase, with faster time of acquisition, and numerous possibilities of image post-processing (3D reconstructions, multiplanar reconstructions)<sup>[19,29,40,42-45]</sup>.

Fourteen years ago, Yoshimi *et al.*<sup>[46]</sup> reported one of the first cases of 3D vascular reconstruction, allowing the evaluation of portal invasion with a higher accuracy than angiography alone. Currently, pancreatic section thickness of 1 mm is obtained in approximately 20 s, allowing true volume acquisition, with vascular details better than angiography<sup>[28,47,48]</sup> useful when assessing vascular invasion<sup>[44]</sup>. Furthermore, CT angiography allows anatomical study of small pancreatic vessels with a remarkable degree of accuracy<sup>[49,50]</sup>.

Moreover, dilation of the peri-pancreatic veins with no visualization of inferior branches on CT suggests tumor invasion of peri-pancreatic tissue<sup>[50]</sup>.

Several studies have highlighted the importance of

**Table 1** CT performance in the detection of vascular invasion in more than 50 patients with pancreatic cancer

Studies (yr)	n	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Megibow <i>et al</i> <sup>[24]</sup> (1995)	118	47	69	89	28
Raptopoulos <i>et al</i> <sup>[208]</sup> (1997)	82	NA	NA	NA	96
Sugiyama <i>et al</i> <sup>[91]</sup> (1997)	73	65 <sup>1</sup>	77	NA	NA
McCarthy <i>et al</i> <sup>[16]</sup> (1998)	67	NA	NA	55/94 <sup>2</sup>	95/94
Diehl <i>et al</i> <sup>[209]</sup> (1998)	89	86	NA	NA	NA
Böttger <i>et al</i> <sup>[110]</sup> (1998)	255	22.2 <sup>3</sup>	96.4	72.7	74.1
Sugiyama <i>et al</i> <sup>[88]</sup> (1999)	91	64 <sup>4</sup>	79	NA	NA
Nakao <i>et al</i> <sup>[105]</sup> (1999)	55	82.1 <sup>5</sup>	74.1	76.7	80
Pietrabissa <i>et al</i> <sup>[130]</sup> (1999)	50	82	53	NA	NA
Gress <i>et al</i> <sup>[89]</sup> (1999)	151	15	100	100	60
Squillaci <i>et al</i> <sup>[69]</sup> (2003)	50	97	100	100	95
House <i>et al</i> <sup>[210]</sup> (2004)	115	85-87 <sup>6</sup>	95-99	83-93	92-98
Soriano <i>et al</i> <sup>[8]</sup> (2004)	62	67	94	89	80
Li <i>et al</i> <sup>[36]</sup> (2005)	54	92/79 <sup>7</sup>	100/99	NA	NA
Buchs <i>et al</i> <sup>[98]</sup> (2007)	153	54.5 <sup>8</sup>	91.2	66.7	86.1

<sup>1,3,4,5</sup>Only evaluated for portal vein invasion; <sup>2</sup>PPV of 55% for venous invasion and 94% for arterial invasion; NPV of 95% for venous invasion and 94% for arterial invasion; <sup>6</sup>Sensitivity of 85% for the superior mesenteric and portal vein invasion, 86% for the superior mesenteric artery invasion, 87% for the celiac trunk invasion; specificity of 95% for the superior mesenteric vein and portal vein involvement, 97% for the superior mesenteric artery invasion, 99% for celiac trunk involvement; PPV of 90% for the superior mesenteric vein and portal vein invasion, 83% for the superior mesenteric artery involvement, 93% for celiac trunk invasion; NPV of 92% for the superior mesenteric vein and portal vein involvement, 98% for the superior mesenteric artery and celiac trunk invasion; <sup>7</sup>Sensitivity of 92% for venous invasion and 79% for arterial invasion; specificity of 100% for the veins and 99% for the arteries; <sup>8</sup>For multi-slice CT. CT: Computer tomography; PPV: Positive predictive value; NPV: Negative predictive value; NA: Not available.

the moment of image acquisition. With regard to the pancreas, it seems that a portal venous phase (60 s after intravenous administration of iodinated contrast medium) or that a pancreatic phase (40-70 s) provides more information than an arterial phase (18 s) or that of a hepatic phase (70 to 100 s)<sup>[19,29,51-54]</sup>. McNulty *et al*<sup>[51]</sup> reported that an arterial phase can be reserved for patients in whom CT angiography is required.

Lastly, Imbriaco *et al*<sup>[55]</sup> showed that dual-phase helical CT (arterial: 20 s, and pancreatic late: 70 s) was interesting but was comparable with single-phase helical CT (pancreatic early: 50 s).

In conclusion, CT is the assessment of choice in first intention, permitting in one non-invasive examination a TNM staging evaluation.

From the vascular point of view, many criteria exist (especially circumferential involvement of vessel of more than 180 degrees, radiological absence of a fat plane between tumor and vessel, vascular occlusion with collaterals, teardrop sign) which allow accuracy in diagnosing vascular invasion. Development of new radiological techniques (3D reconstructions, multiplanar reconstructions) has improved accuracy of assessment of vascular invasion.

## MAGNETIC RESONANCE IMAGING (MRI)

MRI with cholangiopancreatography gives much information for the evaluation of primary tumor and metastatic dissemination, improved by the use of gadolinium or mangafodipir trisodium<sup>[1,13,47,56-58]</sup>. Currently, the use of

MRI in an “all-in-one” staging method (MRI, coupled with angiography and cholangiopancreatography) is a subject under deliberation<sup>[58-60]</sup>.

MRI criteria for vascular invasion are: (1) occlusion of the vessel, with or without collaterals, (2) tumoral infiltration of peri-vascular fat tissue, (3) circumferential contact of more than 180 degrees between the tumor and the vessel, and (4) mass effect along one side of the vessel for more than 2 cm<sup>[7,56,60,61]</sup>.

As regards the detection of vascular invasion, MRI has an accuracy of approximately 94% for enhanced T1-weighted imaging<sup>[62]</sup>. Romijn *et al*<sup>[58]</sup> found in their study an accuracy of 81% with mangafodipir trisodium (definitely higher than MRI without contrast medium).

Other studies have attempted to analyze the performance of MRI in the detection of vascular invasion. They found a sensitivity of 47%-83%<sup>[24,60]</sup>, a specificity of more than 95%<sup>[7,59]</sup>, a PPV of more than 70%<sup>[7,8]</sup>, and a NPV of 23%-96%<sup>[24,60]</sup>.

Modern MRI technology makes it possible to obtain 3D reconstructions, facilitating the study of the peri-pancreatic vessels<sup>[61,63,64]</sup>. Some series have also demonstrated the adequate time for vascular pancreatic image acquisition: biphasic imaging at 15 and 45 s after arrival of contrast material (gadolinium) in the abdominal aorta<sup>[65]</sup>.

Accuracy of MRI for vascular visualization is quite similar to that of CT<sup>[56,66,67]</sup>. It consequently seems logical to reserve this expensive and time-consuming technology for those patients not able to benefit from CT (allergy to iodine, renal insufficiency, pregnancy) or if CT findings are inconclusive<sup>[68]</sup>.

## ANGIOGRAPHY

Currently, conventional angiography is no longer part of the diagnostic protocol in most centers<sup>[13]</sup>, because this examination does not permit the detection of the tumor itself<sup>[1]</sup>, and can easily be replaced by other less invasive methods which give more information on tumoral extension.

On the other hand, preoperative arteriography may visualize vascular abnormalities (anatomical variations, acquired stenosis), allowing a possible modification of surgical strategy (revascularisation, replacement hepatic artery, embolization of an aneurism)<sup>[17,69,70]</sup>.

With regard to vascular invasion, angiographic criteria are: (1) vascular stenosis or occlusion, with or without collaterals, (2) thrombosis of a vessel, (3) acute angle appearing in the venous wall, and (4) envelopment of the vessel within tumor<sup>[69,71-74]</sup>.

In at least 20% of cases, angiography misses the vascular invasion<sup>[10]</sup>, because it gives only information about the lumen of the vessel<sup>[72]</sup>. Angiography depends upon displacement of vessels and distortion of vascular contours unless clear vessel occlusion is present. Furthermore, the tumor may completely encase and invade the small amount of fat surrounding the vessel, and yet not cause a distortion of the contour of the vascular lumen, which is required for detection on angiography. This feature can be visualized during endoscopic ultrasonography or CT. Thus, angiography requires more extensive vascular involvement in order for it to be detected<sup>[5,74,75]</sup>.

The results reported for detection of vascular invasion by pancreatic cancer using angiography are: a sensitivity between 21%<sup>[5,8]</sup> and more than 80%<sup>[10,76]</sup>, a specificity between 50%<sup>[72]</sup> and 100%<sup>[8,69]</sup>, a PPV more than 60%<sup>[5,72]</sup>, and a NPV between 50%<sup>[72]</sup> and 83%<sup>[10]</sup>. Late angiographic times allow visualization of the portal vein, and possible invasions. In addition, it is possible to inject contrast medium directly into the portal vein by a transhepatic access, for example at the time of intravascular ultrasonography (see below).

In conclusion, studies show that angiography is paradoxically relatively poor in the detection of vascular invasion. On the other hand, it permits the visualization of arterial and venous anomalies, allowing a change in surgical strategy.

## ABDOMINAL ULTRASONOGRAPHY (US)

Abdominal US is often the first line examination for a patient presenting with jaundice and pain<sup>[13]</sup>.

From the vascular point of view, US coupled with Doppler gives a reasonably reliable measure of vascular patency and can improve accuracy in assessing vascular invasion<sup>[13,77,78]</sup>. Its sensitivity ranges between 60%<sup>[79]</sup> and more than 90%<sup>[80]</sup>; its specificity has been reported to be higher than 90%<sup>[79,80]</sup>, the PPV is higher than 90%<sup>[81]</sup>, and the NPV is higher than 75%<sup>[82,83]</sup>. Very recently, authors reported US to be 93% accurate in detecting

portal vein invasion, by using 3D vascular reconstruction technology<sup>[84]</sup>.

Color Doppler sonographic criteria for vascular invasion are: (1) absence of hyperechoic tissue between the tumor and the vessel, (2) more than 2 cm continuity between tumor and vessel, (3) circumferential contact between the tumor and the vessel, (4) circumferential narrowing of vessel lumen, and (5) vascular occlusion or thrombosis<sup>[81-83,85-87]</sup>.

In addition, perioperative US has been reported as 100% sensitive in identifying tumors, and 92% sensitive and specific in detecting portal invasion<sup>[88]</sup>. In 22% of patients with pancreatic neoplasms, US-Doppler makes it possible to modify therapeutic strategy<sup>[86]</sup>.

In conclusion, US coupled with Doppler is a relatively accurate, cheap, and non-ionizing imaging modality for initial screening of patients with suspicion of tumors of the pancreas. However, US has demonstrated weakness in recognition of deeper localizations.

With regard to the detection of vascular invasion, studies have shown that US coupled with Doppler is a reliable method. However, these series evaluated almost exclusively the portal vein and its tributaries. Recent improvement in US imaging, allowing 3D reconstruction, offers new potential for this technology in the assessment of tumoral vascular involvement.

## ENDOSCOPIC ULTRASONOGRAPHY (EUS)

EUS is a relatively new technique, providing direct ultrasonic imaging of the pancreas through the gastrointestinal lumen<sup>[2,13]</sup>. However, the probes are expensive and EUS requires a trained endoscopist<sup>[13,63]</sup>.

EUS has been shown to be accurate in diagnosing and staging pancreatic cancer<sup>[89]</sup>, with the help of fine needle aspiration (FNA), with 96.6% sensitivity, 99.0% specificity, 96.2% NPV, and 99.1% PPV<sup>[90]</sup>.

EUS criteria for vascular invasion are: (1) loss of the hyperechoic vessel wall/tumor interface, (2) direct visualization of tumor within the vessel lumen, (3) vascular encasement or occlusion, (4) non-visualization of a major vessel, in the presence of collaterals, (5) proximity of the tumor (< 3 mm) to the vessel, and (6) irregularity of the vascular wall<sup>[5,8,11,89,91-96]</sup>.

Sugiyama *et al.*<sup>[91]</sup> reported that EUS is more accurate than CT, US, and angiography for the detection of portal invasion; similar findings were shown in other series<sup>[97,98]</sup>. In addition, Brugge *et al.*<sup>[93]</sup> showed that EUS was highly sensitive in the detection of portal and splenic vein invasions.

Arterial invasion is assessed with more difficulty by EUS<sup>[92,98-100]</sup>. Globally, the sensitivity is 50%-100%<sup>[92,95,101,102]</sup>, the specificity 58%-100%<sup>[92,102]</sup>, the PPV 28%-100%<sup>[92,96]</sup>, and the NPV 18%-93%<sup>[89,94]</sup>.

Very recently, Fritscher-Ravens *et al.*<sup>[103]</sup> reported the use of 3D linear EUS in the assessment of vascular involvement with very interesting results compared with classical EUS. Linear 3D EUS enhanced the evaluation



of vascular involvement of pancreatic lesions, especially in chronic pancreatitis.

In conclusion, it is appropriate to incorporate EUS in the preoperative assessment when there is suspicion of pancreatic cancer. From the point of view of the detection of vascular invasion, EUS has shown good accuracy, especially for venous invasion.

## INTRAVASCULAR ULTRASONOGRAPHY (IVUS)

When a tumor appears to be contiguous with the portal vein or with the superior mesenteric vein, the diagnosis of vascular invasion can be difficult. Some limited reports have suggested that IVUS might allow the distinction between a simple compression by mass effect and invasion<sup>[71]</sup>.

Moreover, IVUS makes it possible to detect intra-portal thrombus, sometimes missed by CT<sup>[71]</sup>. IVUS is performed either by a transhepatic access, or by a transmesenteric catheterization (during operative time)<sup>[71,104-108]</sup>. Complications are rare<sup>[72,104-106]</sup>.

IVUS criteria for vascular invasion are: (1) obliteration of the echoic band of the portal vein by the hypoechoic tumor, (2) tumor mass blended with the venous wall, and (3) tumor protrusion into the vascular lumen<sup>[71,72,76,104-106,109]</sup>.

One of the limitations of IVUS is the lack of specificity in the case of pancreatitis<sup>[71,105]</sup>. Moreover, IVUS has a limited penetration, allowing only localised investigations. Another weakness remains the lack of spatial orientation, making the interpretation of the images difficult<sup>[72,106]</sup>.

There are few studies concerning IVUS in detection of vascular invasion in pancreatic cancer. Moreover, they report only portal and superior mesenteric vein results, not evaluating arterial invasion. The results are: sensitivity more than 95%<sup>[71,76]</sup>, specificity more than 90%<sup>[71,76]</sup>, PPV more than 90%<sup>[105]</sup>, and NPV more than 95%<sup>[105]</sup>.

Kaneko *et al.*<sup>[109]</sup> has pioneered the use of IVUS in staging of pancreatic cancer, recently using 3D reconstructions of IVUS with a high degree of accuracy. Tezel *et al.*<sup>[110]</sup> also reported that a contact of more than 18 mm between the tumor and the portal or the superior mesenteric vein was a factor of poor prognosis. The use of IVUS allows stent placement<sup>[111]</sup>, a possibility in the palliative treatment of portal stenosis.

In conclusion, studies show that IVUS is probably superior to CT and portography for the detection of vascular invasion. However, data is available only for the portal vein and for the superior mesenteric vein. To our knowledge, there are no data concerning the utility of IVUS in detecting tumoral arterial invasion.

Because IVUS is expensive and invasive, Nakao *et al.*<sup>[105]</sup> recommend performing this examination only in cases in which the distinction between compression and invasion cannot be made by conventional imaging techniques.

## LAPAROSCOPY AND LAPAROSCOPIC ULTRASONOGRAPHY (LUS)

For almost 30 years<sup>[112]</sup>, laparoscopic examination of the

abdominal cavity has offered an excellent, although invasive, visualization of peritoneum and the liver<sup>[13,47,63,113,114]</sup>.

From the vascular point of view, incision of the gastrohepatic omentum allows a direct access to the underlying vessels<sup>[47,115]</sup>. However, it seems certain that laparoscopy alone cannot detect vascular invasion, in particular mesenteric, without help of perioperative ultrasonography<sup>[116]</sup>.

Currently, routine laparoscopy is not recommended in cases of cancer of the head of the pancreas, because it influences further surgical strategy in only 14%-19% of cases<sup>[116,117]</sup>. On the other hand, a study showed that in the case of cancer of the body or the tail of the pancreas, laparoscopy could avoid up to 50% of the operations, because of metastases not identified during staging<sup>[116]</sup>.

Obviously, laparoscopy can also be used with a palliative aim (double derivations), if the tumor is unresectable<sup>[21,117-120]</sup>. Laparoscopy has its limits: it only allows visualization of the liver surface; impossibility of analyzing the retroperitoneum and its vessels; technical problems due to adhesences<sup>[21,47,63,120,121]</sup>.

LUS was subsequently developed, and this allows detailed study of the liver, the lymphatic area, and the corresponding vessels<sup>[47,121-126]</sup>. Vascular structures can be accurately visualized by LUS in approximately 95% of patients with tumors in the head of the pancreas<sup>[126]</sup>.

LUS criteria for vascular invasion are: (1) loss of the hyperechoic vessel - tumor interface, (2) obliteration or thrombosis of a vessel, (3) a fixed stenosis, (4) vessel encasement by tumor encirclement and rigidity, and (5) presence of invading tumor within the vessel lumen<sup>[122,127-129]</sup>.

There are numerous studies evaluating resectability by LUS, but to our knowledge few have focused on vascular invasion. They have found a sensitivity of more than 50%<sup>[129]</sup>, a specificity of more than 80%<sup>[130]</sup>, a PPV of 93%<sup>[127]</sup>, and a NPV of 73%<sup>[128]</sup>.

Despite these encouraging results, several authors do not recommend systematic use of laparoscopy or LUS. They prefer to recommend this technique for doubtful cases<sup>[21,121,131-133]</sup>.

## POSITRON EMISSION TOMOGRAPHY (PET) AND POSITRON EMISSION TOMOGRAPHY COUPLED WITH COMPUTED TOMOGRAPHY

PET is a non-invasive imaging method, which gives information about cellular metabolic activity.

Currently, 18F-fluorodeoxyglucose (FDG) is injected and taken up preferentially by malignant tumors, and secondary localizations, rather than by healthy tissue<sup>[13,17,18,47,63,134-136]</sup>. The FDG is not metabolized and is trapped inside the cell<sup>[47]</sup>, allowing it to be imaged in contrast to surrounding tissue<sup>[18]</sup>.

PET is accurate in diagnosing small tumors (< 2 cm), as well as peritoneal implants and metastases<sup>[13,47,63,102,135,137-141]</sup>. In addition, PET is able to differentiate

inflammatory pathologies from tumoral ones<sup>[47,135,139,142,143]</sup>. PET differentiates malignant and benign pathologies with a sensitivity of 85%-100% and a specificity of 67%-99%; often higher than that of CT<sup>[135,141,144-146]</sup>.

In addition, false negatives exist in the case of strongly differentiated tumors, small periaampullary tumors or in cases of hyperglycemia<sup>[63,135,146,147]</sup>. In the case of normoglycemic patients, PET has a sensitivity for tumoral detection of 93%-98%<sup>[135,137,146,148,149]</sup>, although in the case of hyperglycemic patients, this falls to 63%, or even less<sup>[135,137,146,149]</sup>, in parallel with the NPV which falls from 96% to 38%<sup>[146]</sup>.

Concerning lymphatic invasion, PET detection has proved poor, probably due to the proximity of regional lymph nodes to the primary tumor<sup>[102,134,135,137,150]</sup>, and the lack of anatomic detail<sup>[13,18,139]</sup>. PET alone is unable to visualize vessels and cannot assess vascular invasion<sup>[63,135,151]</sup>. Thus, the association of PET with CT (PET/CT) seems promising<sup>[139,152]</sup>.

Heinrich *et al.*<sup>[139]</sup> showed recently that PET/CT has a PPV for the differentiation between a benign and a malignant pathology of 91%, whereas its NPV is 64%. PET/CT detects a cancer of the pancreas with a sensitivity of 93%, and is more specific than CT alone (69% *vs* 21%, respectively,  $P = 0.07$ ). However, data are lacking regarding the assessment of vascular involvement. The use of multislice CT coupled with PET, and angio-CT protocols, might allow better visualization of the vessels.

## SURGICAL MANAGEMENT OF VASCULAR INVASION

Frequently, vascular invasion may be assessed only when the operation is already quite advanced (section of the pancreas, digestive transection)<sup>[22,27,153-156]</sup>. Palpation at the time of the Kocher maneuver (maneuver which permits exposure of structures behind duodenum and pancreatic head) is commonly performed to assess the relationship of a pancreatic head tumor to the superior mesenteric artery. However, if the tumor is large, if there is associated pancreatitis, or if the patient is undergoing reoperation, palpation is an inaccurate way to assess this critical tumor-vessel relationship prior to gastric and pancreatic transection<sup>[22]</sup>.

The management of a suspicious tumoral adhesion to a vessel is one of the most important challenges in a Whipple procedure. In such a case, the surgeon is confronted with three options: (1) leave tumor attached to the vessel, resulting in a grossly positive margin of resection; (2) try to separate the tumor from the vessel, with a considerable hemorrhagic risk; and (3) or perform a partial or segmental resection of the portion of invaded vessel with reconstruction<sup>[22]</sup>.

### Arterial invasion

If the invasion of the superior mesenteric or portal vein is not in itself a criterion of unresectability<sup>[4,154,155,157]</sup>, arterial invasion is a more controversial issue. Many authors regard

this invasion as a contraindication to surgery<sup>[27,154,158]</sup>, because of the high morbidity and mortality rates associated with arterial resection and reconstruction<sup>[159]</sup>. Furthermore, arterial invasion usually includes extensive involvement of the mesenteric neural plexus<sup>[160]</sup>, rendering radical resection oncologically unsound because of the frequent finding of positive margins<sup>[154]</sup>.

However, in many cases, the preoperative assessment cannot diagnose such an invasion. The surgeon must then adapt his surgical strategy. Fortner<sup>[161]</sup> recommended the resection of invaded arterial segment, if a reconstruction seemed possible.

From the arterial point of view, celiac or hepatic invasion, discovered during the operation, can be the object of a resection and a reconstruction, either by direct anastomosis, by interposition of a venous graft (for example reverse saphenous or internal jugular vein), or with a prosthesis<sup>[156,161-163]</sup>. An arterial graft (for example the splenic artery) can also be used<sup>[156,163]</sup>. These techniques seemed relatively reliable, with a mortality of 5%, in a recent study<sup>[164]</sup>.

Regarding the modified Appleby's operation (*en-bloc* resection of the celiac trunk with distal pancreatectomy and total gastrectomy) for advanced cancers of body and tail of the pancreas, several Japanese groups propose an extended resection of the celiac trunk, splenic artery, common hepatic artery, and/or superior mesenteric artery, resulting in 5-6 mo of average survival. Hepatic vascularization must be maintained and evaluated during the whole operation, and if necessary, compensated, in order to avoid an acute hepatic insufficiency<sup>[163,165-168]</sup>.

Recently, Gagandeep *et al.*<sup>[169]</sup> reported their experience using celiac axis resection for pancreatic cancer with a prolonged survival, and proposed the consideration of this technique for central and distal pancreatic cancer invading the celiac trunk.

Hirano *et al.*<sup>[170]</sup> reported a high R0 resectability rate (91%) with distal pancreatectomy with *en bloc* celiac axis resection.

When the superior mesenteric artery is invaded, an arterial jejunal branch is isolated. Heparin is injected there, in order to allow the clamping of the superior mesenteric artery with full safety. The artery is then reconstructed either by direct anastomosis, or by anastomosis to the aorta<sup>[161]</sup>.

In the case of an invasion of the hepatic artery, techniques of reconstruction require a venous graft (jugular, reverse saphenous, gonadic veins) or prosthesis, or an arterial graft (splenic, gastro-epiploic, gastroduodenal)<sup>[22,163,171-173]</sup>.

In some cases of cancer of the body of the pancreas, with invasion of the common hepatic artery and celiac trunk, Kondo *et al.*<sup>[174]</sup> tried to embolize the hepatic artery, obtaining a collateral pathway from the superior mesenteric artery. This allowed a distal pancreatectomy with *en bloc* resection of the celiac trunk, without hepatic ischemia.

Other authors have described more traditional techniques of resection-reconstruction, using the gastroduodenal artery<sup>[175]</sup>. Combined resection of the celiac trunk with a distal pancreatectomy has been found to improve the overall prognosis of patients with locally advanced cancer of the body and tail of the pancreas<sup>[176]</sup>.

### Venous invasion

Contrary to arterial involvement, the invasion of the superior mesenteric vein or portal vein is not in itself a criterion of unresectability<sup>[4,154,155,157,177]</sup>.

In uncommon cases, the pancreatic tumor infiltrates the anterior surface of the inferior vena cava. It is possible to excise the invaded part, and to replace it with a synthetic prosthesis. Often, autologous tissues are preferred (jugular, saphenous veins)<sup>[22]</sup>.

When the portal vein is involved, it is legitimate to attempt a resection, especially if the vein is invaded by more than 2 cm, in order to obtain negative margins (Table 2)<sup>[4,178-180]</sup>. Portal invasion is not a predictor of aggressive tumor biology, but rather a reflection of tumor size and location<sup>[153,157,177,179]</sup>. Up to 50% of tumors thought to have vascular invasion intraoperatively have been found subsequently to have only inflammatory adhesions to the portal vein after histologic examination<sup>[157,181,182]</sup>. This finding underlines the difficulty in determining tumoral venous invasion before and during surgery, since peritumoral inflammation may simulate true tumor infiltration<sup>[178]</sup>. Very recently, Fukuda *et al.*<sup>[183]</sup> reported that the depth of portal vein invasion significantly alters survival after curative pancreatic resection combined with portal vein resection. The survival rate was similar for patients with no portal invasion and those with superficial invasion. However, a deeper portal invasion was associated with a poorer survival rate, similar to that of patients undergoing non-curative resection.

The excision is done either by a segmentary resection, or by a tangential resection<sup>[22,184,185]</sup>. The reconstruction requires an end-to-end anastomosis either by direct suture or by using an interposition venous or prosthetic graft<sup>[22,74,154,156,157,161,162,184-189]</sup>. The technical limit of portal vein resection without graft is 4 cm in the hepatic hilus and 7 cm after pancreatic resection<sup>[189]</sup>. For minimal tumor invasion into the portal vein, autologous saphenous vein patch has been described<sup>[27]</sup>. Wide resection of the portal vein may require transection of the splenic vein. To avoid segmental portal hypertension, end-to-side reanastomosis of the splenic vein to the interposition graft is recommended<sup>[184]</sup>.

If the portal clamping lasts longer than 30 min, it is recommended to clamp also the superior mesenteric artery, in order to prevent intestinal congestion<sup>[22,189]</sup>. If the portal clamping lasts longer than 60 min, it is necessary to consider a bypass between the superior mesenteric vein and femoral vein<sup>[189,190]</sup>.

Resection of the portal vein is associated with a higher morbidity rate (bleeding, infections, cardiopulmonary complications), than when this is not performed<sup>[4,185,191]</sup>.

Table 2 Recent results of portal resections in pancreatic cancer

Studies (yr)	n	Mortality (%)	Survival at 1 year (%)	Median survival (mo)
Sindelar <i>et al.</i> <sup>[159]</sup> (1989)	20	20 <sup>1</sup>	50	12
Tashiro <i>et al.</i> <sup>[189]</sup> (1991)	27	8.4	51.9	NA
Ishikawa <i>et al.</i> <sup>[174]</sup> (1992)	35	5.7	NA	9+/-5
Launois <i>et al.</i> <sup>[193]</sup> (1993)	9	0	NA	6.1
Takahashi <i>et al.</i> <sup>[156]</sup> (1994)	79	16.5	17-61.5 <sup>2</sup>	6-14
Allema <i>et al.</i> <sup>[192]</sup> (1994)	20	15	30%	7
Nakao <i>et al.</i> <sup>[211]</sup> (1995)	89	8	5.5-39.6 <sup>3</sup>	NA
Nakao <i>et al.</i> <sup>[190]</sup> (1995)	104	8	NA	NA
Roder <i>et al.</i> <sup>[27]</sup> (1996)	31	0	39	8
Fuhrman <i>et al.</i> <sup>[154]</sup> (1996)	23	4	NA	NA
Harrison <i>et al.</i> <sup>[157]</sup> (1996)	58	5	59	13
Leach <i>et al.</i> <sup>[196]</sup> (1998)	31	0	NA	22
Launois <i>et al.</i> <sup>[188]</sup> (1999)	14	0	23	5
Bachelier <i>et al.</i> <sup>[195]</sup> (2001)	21	3.2	NA	13
van Geenen <i>et al.</i> <sup>[185]</sup> (2001)	34	0	55	14
Shibata <i>et al.</i> <sup>[197]</sup> (2001)	23	4	31	6.8-20.6 <sup>4</sup>
Hartel <i>et al.</i> <sup>[212]</sup> (2002)	68	4	<sup>5</sup>	NA
Aramaki <i>et al.</i> <sup>[194]</sup> (2003)	22	4.5	NA	NA
Nakagohri <i>et al.</i> <sup>[213]</sup> (2003)	33	6	35-81	15
Li <i>et al.</i> <sup>[164]</sup> (2004)	79	5 <sup>6</sup>	NA	NA
Tseng <i>et al.</i> <sup>[206]</sup> (2004)	110	1	85	23.4
Wagner <i>et al.</i> <sup>[4]</sup> (2004)	51	7.7	NA	NA
Shimada <i>et al.</i> <sup>[177]</sup> (2006)	86	1	<sup>7</sup>	14
Carrère <i>et al.</i> <sup>[182]</sup> (2006)	45	4.4	<sup>8</sup>	15
Riediger <i>et al.</i> <sup>[181]</sup> (2006)	53	3.8	<sup>9</sup>	NA
Fukuda <i>et al.</i> <sup>[183]</sup> (2007)	37	2.4	47.7	NA

<sup>1</sup>Included 3 arterial reconstructions. 17 patients benefited from adjunctive radiotherapy; <sup>2</sup>17% survival at 1 year if margins were positive (median survival: 6 mo) and 61.5% if margins were negative (median survival: 14 mo); <sup>3</sup>Survival at 1 year: 39.6% if the vessel was not invaded, 11.3% if the media was invaded, and 5.5% if the intima was invaded; <sup>4</sup>Median survival was 6.8 mo if the intima was invaded, 15.3 mo if the intima was spared, and 20.6 mo if there was no true vascular invasion; <sup>5</sup>5-year survival rate: 23%; <sup>6</sup>This mortality also includes arterial reconstructions (11 patients); <sup>7</sup>5-year survival rate: 12%; <sup>8</sup>3-year survival rate: 22%; <sup>9</sup>5-year survival rate: 17.9%.

In addition, Fuhrman *et al.*<sup>[154]</sup> reported an operative time, an operative blood loss, and perioperative transfusion requirements of greater magnitude in patients who required venous resection. The mortality rate is also higher after portal vein resection but this value is not always significant<sup>[4,188,192,193]</sup>. These findings are not confirmed by other series<sup>[22,27,157,181,182,185,187,191,194-201]</sup>. Numerous authors have reported a mortality rate below 5%, similar to that of standard pancreatoduodenectomy<sup>[27,154,157,164,178,181,182,185,188,194-197]</sup>.

In 62%-85% of cases, the vascular margins are found to be positive<sup>[27,31,185,192]</sup>, explaining a very poor median survival. However, recently, Siriwardana *et al.*<sup>[202]</sup> reported, in a systematic review of synchronous portal-superior mesenteric vein resection during pancreatectomy for cancer, a high rate (67.4%) of nodal involvement during the procedure. For the authors, this implied that by the time a pancreatic tumor involves the portal vein the risk of metastases is high, rendering the possibility of cure by surgery improbable<sup>[202]</sup>.

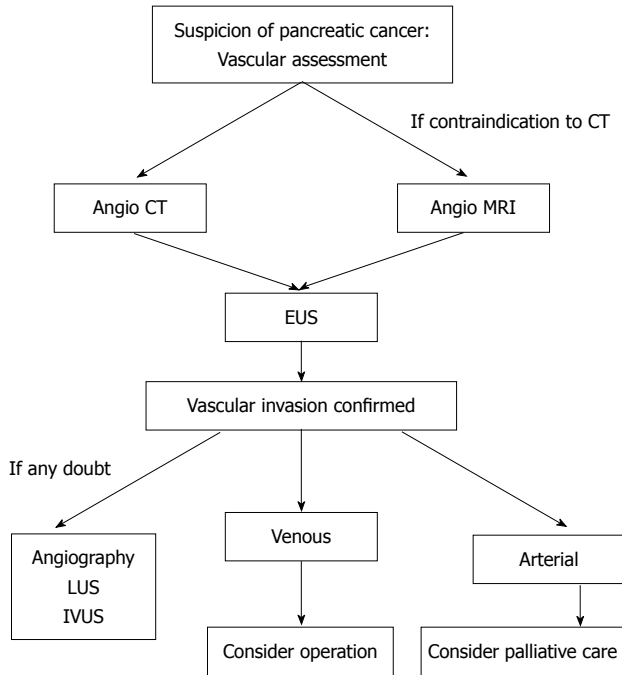
If the tumor invades the superior mesenteric vein, it is not a criterion of unresectability. Various techniques exist to allow complete resection of the tumor,



one of the most important challenges in pancreatic surgery.

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**Figure 1** Proposed algorithm for the management of suspected vascular invasion in pancreatic cancer.

either by tangential excision, or by excision-reconstruction<sup>[153,155,161,162,185,197,203,204]</sup>.

In conclusion, various studies show that venous resection in pancreatic cancer is a feasible technique and relatively reliable, at least with regard to mortality, but (importantly) at the price of a higher morbidity. However, a survival benefit is not achieved by curative resection in patients with pancreatic cancer and vascular invasion<sup>[205,206]</sup>. On the other hand, the discovery of an arterial invasion during the operation might require an aggressive management, using vascular reconstruction. Furthermore, neoadjuvant treatment (combination of 5-fluorouracil/cisplatin chemoradiation) showed only limited impact on survival but appeared to be associated with improved local control<sup>[207]</sup>.

## CONCLUSION

In the absence of metastatic disease, assessment of vascular invasion is a key aspect in the evaluation of resectability for pancreatic cancer. A frequent error is to misdiagnose an involved major vessel. Obviously, surgical exploration with pathological examination remains the “gold standard” in terms of evaluation of resectability, especially from the point of view of vascular involvement. However, current imaging modalities have improved and now allow detection of vascular invasion with more accuracy. Multi-slice CT has become the best imaging modality for this purpose, and the adjunction of PET might be a means to improve results further. EUS is useful, but it remains very operator-dependant. Data are still lacking for the exact role of MRI regarding this issue (Figure 1). Detection of vascular invasion remains



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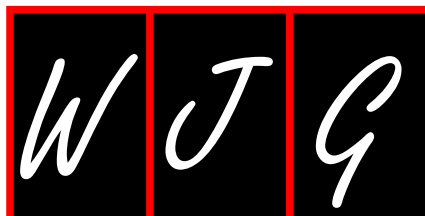
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## Diffusion-weighted MRI in abdominal oncology: Clinical applications

Reiji Sugita, Kei Ito, Naotaka Fujita, Shoki Takahashi

Reiji Sugita, Department of Radiology, Sendai City Medical Center, Sendai 983-0824, Japan

Kei Ito, Naotaka Fujita, Department of Gastroenterology, Sendai City Medical Center, Sendai 983-0824, Japan

Shoki Takahashi, Department of Diagnostic Radiology, Graduate School of Medicine, Tohoku University, Sendai 980-8574, Japan

Author contributions: All authors wrote the manuscript.

Correspondence to: Reiji Sugita, MD, Department of Radiology, Sendai City Medical Center, Sendai 983-0824, Japan. [rsugita@openhp.or.jp](mailto:rsugita@openhp.or.jp)

Telephone: +81-22-2521111 Fax: +81-22-2529431

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### Abstract

Diffusion-weighted magnetic resonance imaging (DWI) provides image contrast that is different from that obtained by conventional magnetic resonance techniques. Although previously, DWI has been used to evaluate various diseases of the central nervous system, several technical advances have expanded the clinical applications of DWI beyond the central nervous system. As a result, many reports have been published on the use of DWI in abdominal diseases. Particularly, abdominal DWI has now being focused on evaluation of patients with abdominal cancer. DWI can be used for pretreatment tumor detection, characterization including predicting tumor response to therapy, monitoring tumor response during therapy, and follow-up study after treatment to detect possible tumor recurrence.

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**Key words:** Diffusion weighted magnetic resonance imaging; Abdominal neoplasms

**Peer reviewer:** Xiao-Peng Zhang, Professor, Department of Radiology, Peking University School of Oncology, Beijing Cancer Hospital & Institute, No. 52 Haidian District, Beijing 100142, China

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### INTRODUCTION

Diffusion-weighted magnetic resonance imaging (DWI) has enabled us to obtain additional information derived from the microscopic motion of water protons, which is not possible using conventional magnetic resonance imaging (MRI). Previously, DWI has been used to evaluate various diseases of the central nervous system. The most established clinical application of DWI for the central nervous system is evaluation of acute stroke<sup>[1]</sup>.

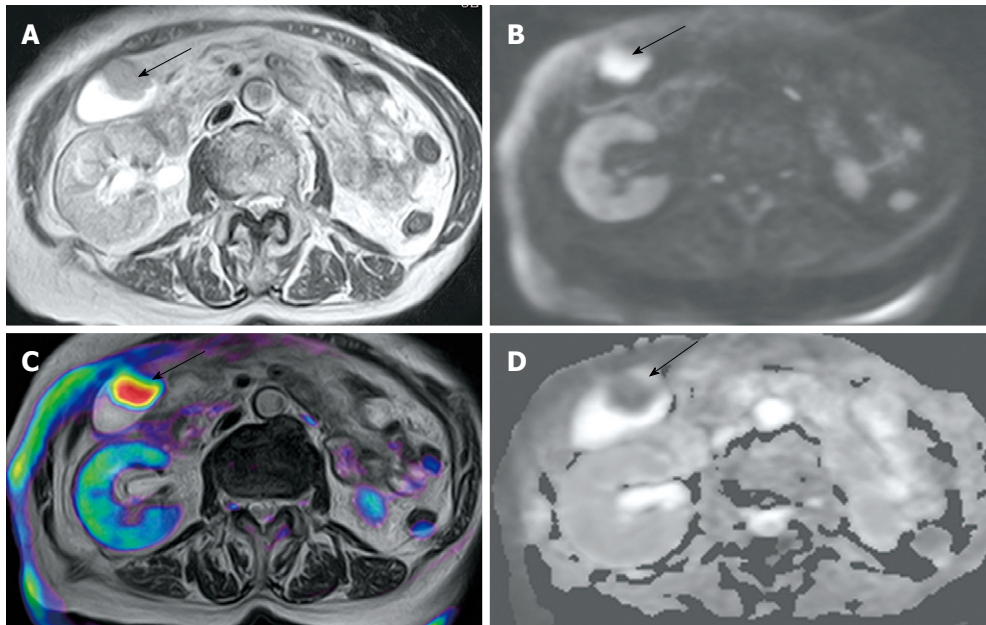
DWI has many advantages. First, it is completely noninvasive, does not require exposure to ionizing radiation or injection of contrast material, and does not cause patient discomfort. Second, because it is derived from a well-established MRI technique, DWI does not require expert technicians with sophisticated technical skills or expensive equipment, such as a cyclotron that is required for positron emission tomography. Another advantage of DWI is that it can be added easily to a routine MRI protocol because it requires only a very short prolongation of examination time<sup>[2]</sup>.

Recently, several technical advances have expanded the clinical applications of DWI beyond the central nervous system, and many studies have been published on the use of DWI in abdominal diseases. Particularly, abdominal DWI has now being focused on evaluating patients with abdominal cancer<sup>[3-9]</sup>. In this article, the application of DWI in abdominal oncology is described.

### HOW TO INTERPRET DWI

DWI can offer qualitative and quantitative information





**Figure 1 MRI of a patient with gallbladder carcinoma.** A: Axial T2-weighted MRI of a patient with gallbladder carcinoma shows a mass (arrow) protruding into the gallbladder lumen; B: Corresponding axial DW image shows high intensity (arrow); C: Corresponding color fusion image of T2-weighted image and DW image shows gallbladder carcinoma (arrow). On color fusion images, the red area corresponds to high signal intensity on DW images and blue correspond to low intensity; D: Corresponding ADC map shows low intensity (arrow).

that can be helpful for tumor assessment (Figure 1). The former assesses visual differences in signal intensity between a tumor and its surrounding normal tissue, and the latter enables calculation of values (apparent diffusion coefficient, ADC) obtained from DWI, such as a computed tomography value.

#### Qualitative assessment in DWI

Visual assessment of relative tissue signal intensity on DWI is being used for tumor detection and characterization<sup>[3]</sup>. Tumors generally tend to block diffusion more than the tissue from which they originate and show relative high signal intensity on DWI (Figure 1B); however, some normal organs, such as the spleen, adrenal gland and seminal vesicle, also show high signal intensity on DWI. Moreover, DWI has a pitfall known as “T2 shine-through”. DWI is obtained by adding a diffusion-weighting gradient (known as an MPG) to T2-weighted images, the basic sequence of conventional MRI. Thus, because DWI shows signal intensity that depends on diffusion and T2 signal intensity, a region with a high T2 signal retains the high signal on DWI, and may be mistaken for restricted diffusion. Therefore, special care must be taken with these pitfalls in diagnosing with DWI. DWI is usually interpreted by superimposing DWI and conventional morphological T2-weighted images because DWI cannot show minute morphological structures (Figure 1C).

#### Quantitative analysis in DWI

Quantitative tumor assessment is possible by calculating ADC after performing DWI with changed parameters (known as *b* values). ADC values in various malignant lesions generally tend to decrease, probably due to increased tissue cellularity or cell density, because the latter correlates with malignancy (Figure 1D). In addition to the cellular membranes, intracellular cytoskeleton, organelles, matrix fibers and soluble macromolecules contribute

to diffusion restrictions in tumors<sup>[10]</sup>; therefore, ADC values are expected to reflect histopathological tissue characteristics. ADC is calculated for each pixel of the image and is displayed as a map. By setting regions of interest within tumors on these maps, ADCs of the tumor can be measured.

## CLINICAL APPLICATIONS OF DWI IN ABDOMINAL ONCOLOGY

### Tumor detection and characterization

Tumors generally tend to show relative high signal intensity on DWI. Using qualitative assessment, Nasu *et al*<sup>[11]</sup> have shown that DWI is superior to superparamagnetic iron oxide (SPIO)-enhanced MRI in detecting liver metastases, which had been the best available examination technique. They have reported that the sensitivity and specificity of DWI was 82% and 94%, respectively. Koh *et al*<sup>[12]</sup> also have reported that the sensitivity and specificity of DWI for detecting liver metastases was 78% and 95%. Thus, qualitative assessment with DWI has superior ability for assessing liver metastasis.

In colorectal tumors, Ichikawa *et al*<sup>[7]</sup> have shown that DWI has high sensitivity and specificity for detecting tumors, and several authors have shown that DWI has high sensitivity and specificity for detecting tumors even in the pancreato-biliary system<sup>[6,9]</sup>.

In quantitative assessment of DWI, ADC measurement has the potential to differentiate benign and malignant liver tumors. In many studies, malignancy has a lower ADC value than benignity. Taouli *et al*<sup>[13]</sup> have shown that metastatic liver tumors have the lowest ADC in malignant and benign focal lesions of the liver, and have revealed a significant difference between benign and malignant lesions. Chan *et al*<sup>[14]</sup> have shown that DWI can be used to distinguish between hepatic abscess and cystic or necrotic malignant liver tumor; ADC of abscess



cavities has a lower value than that of cystic or necrotic malignant liver tumors. Also in abdominal tumors other than in the liver, ADCs of malignant lesions have shown lower values<sup>[9,15,16]</sup>. However, most studies have reported that ADC measurement has no clear threshold to discriminate malignant and benign tumors because of substantial overlapping<sup>[9,13,15-18]</sup>.

### Predicting and monitoring response to therapy

Conventional criteria using morphological images have been used to evaluate antitumor therapy; however, measuring tumor size is often not adequate when tumors are treated with cytotoxic therapy and molecular targeting agents, because changes in tumor size after therapy with these drugs are not expected<sup>[14,19]</sup>; therefore, a new method for evaluating tumor response is required that can precisely reflect the clinical outcome, earlier than conventional imaging modalities.

The ability of DWI to predict therapy outcome has been shown in many clinical studies. Several authors have reported that tumors with low pretreatment ADC values show a better response to various therapies than those with high ADC<sup>[20-24]</sup>. However, studies of areas other than the abdomen have addressed that the relationship between pretreatment ADC and prognosis yield, with different results: patients suffering from a tumor with high pretreatment ADC show better long-term post-treatment prognosis than those with low ADC<sup>[25,26]</sup>.

Many researchers have reported that DWI has the potential for evaluating tumor response during treatment. The results of animal studies have proved that ADC increases can be depicted in those responding to treatment<sup>[27]</sup>. In clinical studies, researchers have reported that an early increase in the ADC value after starting therapy suggests a better treatment outcome<sup>[20,28-32]</sup>.

Monitoring response to therapy by visual assessment of DWI has been reported in brain tumors and bone metastasis, but not in the abdominal region<sup>[21,33]</sup>. Studies on bone metastasis have revealed that the treatment response after therapy could be assessed as a decrease in signal intensity<sup>[33]</sup>. Several authors have shown that tumors demonstrate an increase in ADC after treatment before a change in tumor size occurs, which heralds later diminution of the tumor size<sup>[18,22,34-38]</sup>. Chen *et al.*<sup>[34]</sup> have reported that patients with hepatocellular carcinoma show a significant rise in ADC value when they respond to treatment. Koh *et al.*<sup>[22]</sup> also have reported that patients with colorectal hepatic metastases show an increase in ADC, at least in those who show a partial response to treatment, but not in non-responders. A decrease in ADC during follow-up suggests tumor recurrence<sup>[27]</sup>.

### FUTURE DEVELOPMENT

Several studies have indicated that DWI may be useful for tumor staging, including lymph node and distant metastases<sup>[21,39-42]</sup>. For tumor staging, whole-body imaging is desirable. Takahara *et al.* have shown that whole-body

DWI is promising<sup>[43-45]</sup> using their method to examine the whole body by composite construction of segmented imaging. The images are processed using maximum intensity projection and 3D display<sup>[43-45]</sup>. More clinical research on this technique is needed because their study was preliminary.

The most important issue regarding DWI is non-standardization among MRI manufactures and researchers. Substantial differences in the ADC values of the same normal and diseased organs have been presented<sup>[5]</sup> by researchers using a different imaging technique; therefore, standardization of the imaging protocol is fundamental.

Currently, spatial resolution of DWI is not high enough. In order to compensate for such limited resolution, qualitative assessment might need superimposition of DWI on corresponding T2-weighted images, and quantitative assessment may require meticulous ADC measurements for small lesions. Utilization of high-field MRI may be able to solve the issue of limited spatial resolution.

### CONCLUSION

DWI is a promising imaging technique to evaluate abdominal tumors. This technique can be used for pretreatment tumor detection, characterization including predicting tumor response to therapy, monitoring tumor response during therapy, and follow-up study after treatment to detect possible tumor recurrence. Standardization of the imaging protocol and large clinical trials regarding the usefulness of DWI are needed.

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## Analgesic effects of JCM-16021 on neonatal maternal separation-induced visceral pain in rats

Zhao-Xiang Bian, Man Zhang, Quan-Bin Han, Hong-Xi Xu, Joseph JY Sung

Zhao-Xiang Bian, Man Zhang, School of Chinese Medicine, Hong Kong Baptist University, Hong Kong, China

Quan-Bin Han, Institute of Chinese Medicine, The Chinese University of Hong Kong, Hong Kong, China

Hong-Xi Xu, Chinese Medicine Laboratory, Hong Kong Jockey Club Institute of Chinese Medicine, Hong Kong, China

Joseph JY Sung, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, China

**Author contributions:** Bian ZX made contributions to the study design, data interpretation, and wrote the manuscript; Zhang M performed the majority of experiments and data analysis; Han QB conducted the majority of quality control for the formula; Xu HX and Sung JY made contributions to the study design; Xu HX contributed to the quality control of herbs and products.

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Correspondence to: Zhao-Xiang Bian, PhD, Associate Professor, School of Chinese Medicine, Hong Kong Baptist University, Hong Kong, China. [bzxiang@hkbu.edu.hk](mailto:bzxiang@hkbu.edu.hk)

Telephone: +852-34112905 Fax: +852-34112929

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### Abstract

**AIM:** To investigate the pharmacological effect of JCM-16021, a Chinese herbal formula, and its underlying mechanisms.

**METHODS:** JCM-16021 is composed of seven herbal plant materials. All raw materials of the formula were examined according to the quality control criteria listed in the Chinese Pharmacopeia (2005). In a neonatal maternal separation (NMS) model, male Sprague-Dawley rats were submitted to daily maternal separation from postnatal day 2 to day 14, or no specific handling (NH). Starting from postnatal day 60, rats were administered JCM-16021 (2, 4, 8 g/kg per day) orally twice a day for 28 d. Pain threshold pressure and electromyographic activities of external oblique muscles

in response to colorectal distention recorded with a Power Lab System (AD Instruments International), were tested as pain indices. Changes in serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) concentrations in the colon of rats were analyzed; the enterochromaffin cell numbers and serotonin transporter in the colon of rats were also evaluated with an immunohistochemistry method.

**RESULTS:** NMS treatment significantly reduced pain threshold pressure ( $37.4 \pm 1.4$  mmHg), as compared to that of NH rats ( $57.7 \pm 1.9$  mmHg,  $P < 0.05$ ). After JCM-16021 treatment, the pain threshold pressure significantly increased when compared to that before treatment ( $34.2 \pm 0.9$  mmHg vs  $52.8 \pm 2.3$  mmHg in the high dose group,  $40.2 \pm 1.6$  mmHg vs  $46.5 \pm 1.3$  mmHg in the middle dose group, and  $39.3 \pm 0.7$  mmHg vs  $46.5 \pm 1.6$  mmHg in the low dose group,  $P < 0.05$ ). Also JCM-16021 significantly and dose-dependently decreased electromyographic activity to the graded colorectal distention (CRD), (the mean  $\Delta$ AUC values were:  $0.17 \pm 0.03$ ,  $0.53 \pm 0.15$ ,  $1.06 \pm 0.18$ ,  $1.22 \pm 0.24$  in the high dose group;  $0.23 \pm 0.04$ ,  $0.68 \pm 0.17$ ,  $1.27 \pm 0.26$ ,  $1.8 \pm 0.3$  in the middle dose group; and  $0.29 \pm 0.06$ ,  $0.8 \pm 0.16$ ,  $1.53 \pm 0.24$ ,  $2.1 \pm 0.21$  in the low dose group for the pressures 20, 40, 60, 80 mmHg), as compared to the NMS vehicle group. The mean  $\Delta$ AUC values were:  $0.57 \pm 0.12$ ,  $1.33 \pm 0.18$ ,  $2.57 \pm 0.37$ ,  $3.08 \pm 0.37$  for the pressures 20, 40, 60, 80 mmHg ( $P < 0.05$ ). JCM-16021 treatment significantly reduced the 5-HT concentrations (from high, middle and low dosage groups:  $60.25 \pm 5.98$  ng/100 mg,  $60.32 \pm 4.22$  ng/100 mg,  $73.31 \pm 7.65$  ng/100 mg), as compared to the NMS vehicle groups ( $93.11 \pm 9.85$  ng/100 mg,  $P < 0.05$ ); and increased the 5-HIAA concentrations (after treatment, from high, middle and low dosage groups:  $54.24 \pm 3.27$  ng/100 mg,  $50.34 \pm 1.26$  ng/100 mg,  $51.37 \pm 2.13$  ng/100 mg) when compared to that in the NMS vehicle group ( $51.75 \pm 1.98$  ng/100 mg,  $P < 0.05$ ); but did not change the enterochromaffin cell numbers in the colon of rats. In addition, NMS rats had higher SERT expression ( $n = 10$ ) than NH rats ( $n = 8$ ,



$P < 0.05$ ). JCM-16021 treatment significantly decreased SERT expression when compared to the NMS group ( $P < 0.01-0.001$ ).

**CONCLUSION:** JCM-16021 can attenuate visceral hypersensitivity, and this analgesic effect may be mediated through the serotonin signaling pathway in the colon of rats.

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**Key words:** Analgesia effect; Neonatal maternal separation; Visceral hyperalgesia; Herbal medicine; Serotonin pathway

**Peer reviewer:** Million Mulugeta, DVM, PhD, Professor, Department of Medicine, Division of Digestive Diseases, UCLA, David Geffen School of Medicine, Los Angeles, CA 90073, United States

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## INTRODUCTION

Irritable bowel syndrome (IBS) is characterized by chronic abdominal pain and altered bowel movements such as diarrhea and constipation<sup>[1,2]</sup>. Although conventional therapies (e.g. laxatives, antidepressants, antispasmodics, and bulking agents) are used to relieve the symptoms of IBS, the overall efficacy of these agents is poor<sup>[3,4]</sup>; and these agents have not been proven to be more effective than placebo in providing overall relief of symptoms in randomized, controlled clinical trials. Therefore, increasing numbers of IBS sufferers are seeking help from complementary and alternative medicines. Recently, our research group showed that JCM-16021, an herbal formula composed of seven herbs, can relieve symptoms in IBS patients<sup>[5]</sup>. In this randomized, double-blinded, and placebo-controlled trial, 84 diarrhea-predominant IBS patients received treatment (28 patients in each arm). At the end of the 8 wk treatment, 52% of participants in the JCM-16021 plus Holopon (hyoscine methobromide)-placebo group (Group A), 32% in the Holopon plus herbal-placebo group (Group B), and 42.7% in the double placebo group (Group C) experienced overall symptom improvements. Patients in Group A had the highest percentage improvement (Group A *vs* Group B *vs* Group C: 52% *vs* 32% *vs* 42.7%), but the mechanism of this effect remains unclear.

Serotonin (5-HT), an important neurotransmitter and paracrine signaling molecule, alters visceral perception and motor function by influencing the sympathetic, parasympathetic, and enteric nervous systems<sup>[6]</sup>. The majority of 5-HT is synthesized and stored in entero-

chromaffin (EC) cells in the gastrointestinal tract. Previous studies have shown that the changes in EC cells and the increased 5-HT concentrations in the human colon are associated with the generation of IBS symptoms and in other gastrointestinal functional disorders<sup>[7-10]</sup>. Furthermore, novel serotonergic agents, such as the 5-HT<sub>3</sub> antagonist alosetron and the 5-HT<sub>4</sub> agonist tegaserod, have significant impacts on IBS symptoms through their visceral analgesic properties and diverse effects on motor functions in the lower gastrointestinal tract<sup>[11]</sup>. Therefore, the 5-HT signaling pathway represents a promising target for IBS treatment.

A neonatal maternal separation (NMS)-induced visceral hyperalgesia rat model was previously established<sup>[12]</sup>. Because its characteristics mimic the symptoms of IBS patients, it is often used to study the mechanism of visceral hyperalgesia and to evaluate the pharmacological effects of potential IBS therapies<sup>[13,14]</sup>.

Considering the effects of JCM-16021 in IBS patients and the function of serotonin in visceral hyperalgesia, this study aimed to investigate the analgesic effect of JCM-16021 on NMS-induced visceral hyperalgesia in rats, and its potential underlying mechanism. We hypothesized that JCM-16021 could attenuate visceral pain through the 5-HT signaling pathway in the colon of rats. These results were previously presented as a poster at the 16th United European Gastroenterology Week in October 2008 in Vienna, Austria<sup>[15]</sup>.

## MATERIALS AND METHODS

### Herb materials

JCM-16021 is composed of seven plant materials, which are listed in Table 1. Purchasing, authentication and quality control of all seven herbs were performed based on the requirements of the Chinese Pharmacopoeia<sup>[16]</sup>. The authenticated voucher specimens (the voucher numbers are CMED-0043-02, CMED-0018-17, CMED0024-02, CMED-0044-02, CMED-0180-02, CMED-0179-02, CMED-0118-02) were stored in the Research Laboratory, Hong Kong Jockey Club Institute of Chinese Medicine, Hong Kong, China.

### Reagents

Chloral hydrate was purchased from Fluka. Hematoxylin, 5-HT, and 5-HIAA were purchased from Sigma (Sigma-Aldrich Co., St. Louis, MO, USA). Holopon (hyoscine methobromide, 99%) was purchased from GSK Hong Kong.

### Preparation and quality analysis of JCM-16021

JCM-16021 was prepared in the form of granules as follows: mixed medicinal materials weighing approximately 110 g (equal to the total amount of raw materials in one day's dosage of JCM-16021 formula for IBS patients) were macerated for 30 min, subsequently decocted for 60 min three times, and rinsed 10 times (v/w) with distilled water. The filtrates were combined and dried in a vacuum at 40°C. A water-soluble pale yellow powder, approximately 22 g,

Table 1 Composition of JCM-16021 and related quality analysis results

Composition and samples	Atractylone <sup>1</sup>	Gallic acid <sup>1</sup>	Corilagin <sup>2</sup>	Paeoni-florin <sup>3</sup>	Magnolol <sup>4</sup>	Honokiol <sup>5</sup>	Tetrahydropal-matine <sup>6</sup>	Quercitrin <sup>7</sup>	Heavy metal & pesticide residues
<i>Fructus Terminaliae Chebulae</i> ( <i>Terminalia chebula</i> Retz.) 9%		+	2.18%						Pass
<i>Radix Paeoniae Lactiflorae</i> ( <i>Paeonia lactiflora</i> Pall.) 14%				0.45%					Pass
<i>Cortex Magnoliae Officinalis</i> ( <i>Magnolia officinalis</i> Rehd. et Wils.) 9%					1.76%	0.89%			Pass
<i>Rhizoma Corydalis Yanhusuo</i> ( <i>Corydalis yanhusuo</i> W. T. Wang) 14%							0.10%		Pass
<i>Herba Polygoni Chinensis</i> ( <i>Polygonum chinense</i> L) 18%								0.04%	Pass
<i>Rhizoma Atractylodis</i>	+								Pass
<i>Macrocephalae</i> ( <i>Atractylodes macrocephala</i> Koidz) 18%									Pass
<i>Semen coicis Lachryma-jobi</i> [Coix lacryma-jobi L. var. ma-yuan (Roman.) Stapf] 18%									Pass
Final product	-	+	2.43%	0.70%	ND	ND	0.01%	ND	Pass

This table presents the composition of formula and its ratio of each component in whole formula. <sup>1</sup>TLC test, + means detected, - means not detected; <sup>2</sup>Y = 4309.9x + 11.158, R<sup>2</sup> = 1, 0.155-7.75 µg; <sup>3</sup>Y = 1141.8x + 54.916, R<sup>2</sup> = 0.9999, 0.322-18.343 µg; <sup>4</sup>Y = 7822.7x + 1167.9, R<sup>2</sup> = 0.9994, 0.375-3.75 µg; <sup>5</sup>Y = 7947.5x + 1465.9, R<sup>2</sup> = 0.9982, 0.34-3.4 µg; <sup>6</sup>Y = 5782.6x - 11.063, R<sup>2</sup> = 1, 0.028-1.4 µg; <sup>7</sup>Y = 2612.4x + 1.6674, R<sup>2</sup> = 1, 0.0228-1.14 µg; ND means that the chemical marker was detected but not determined due to peak area being too small.

was obtained. To ensure the quality of the final product, all raw materials were examined according to the quality control criteria listed in the Chinese Pharmacopeia 2005<sup>[16]</sup>. As recommended by the Chinese Pharmacopeia 2005<sup>[16]</sup>, paeoniflorin, magnolol, honokiol, and tetrahydropalmatine were selected as the chemical markers for *Radix Paeoniae Lactiflorae*, *Cortex Magnoliae Officinalis*, and *Rhizoma Corydalis Yanhusuo*, respectively. Corilagin, a constituent of *Fructus Terminaliae Chebulae* was also selected since it is a major component of the final product. Quercitrin was used as the chemical marker of *Herba Polygoni Chinensis*. In addition, gallic acid and atractylone were qualitatively checked in *Fructus Terminaliae Chebulae* and *Rhizoma Atractylodis Macrocephalae*, respectively. Heavy metals and pesticide residues were monitored to ensure safety.

### Animals and neonatal maternal separation

Primiparous timed-pregnant Sprague-Dawley female rats were obtained from the Laboratory Animal Services Centre, The Chinese University of Hong Kong, on gestational day 13-14. Dams were housed individually in macrolon cages and maintained in rooms with temperature kept at 23 ± 2°C and an alternating 12: 12 h light-dark cycle. All of the experimental protocols were carried out with the approval of the Committee on Use of Human-Animal Subjects in Teaching and Research of the Hong Kong Baptist University and according to the Regulations of the Department of Health, Hong Kong, China.

The neonatal maternal separation (NMS) rat model was established based on a previous report<sup>[12]</sup>. Briefly, pups in the NMS group were separated from their mothers and placed into individual cages in another room 180 min daily from postnatal day 2 to day 14, whereas normally-handled

(NH) pups remained undisturbed in their home cage with the dam. All pups were weaned on postnatal day 22, and only male pups were used in the present study to avoid hormonal cycle induced variations. Male rats on postnatal day 60 were used in a series of three experiments.

### Experimental design

This experiment involved three sets of studies: The first series of experiments aimed to evaluate the pharmacological effects of JCM-16021 on visceral pain by assessing changes in pain threshold pressure before and after JCM-16021 treatment. Six groups of rats were used. Group 1 (*n* = 10) with NH rats and Group 2 (*n* = 10) with NMS rats were given distilled water as a control. Groups 3, 4 and 5 (*n* = 10, 9, 9) with NMS rats received JCM-16021 at 8, 4 and 2 g/kg per day, respectively. Group 6 received 0.3 mg/kg per day Holopon (hyoscine methobromide, 99%, GSK Hong Kong) as an active control (*n* = 8). All pain threshold pressure detection tests were conducted in the morning between 9 am and 12 pm.

The second series of experiments aimed to test the analgesic effect of JCM-16021 through assessing electromyographic (EMG) activities of the left external abdominal oblique muscles to colorectal distension (CRD) before and after treatment with JCM-16021. Grouping (*n* = 7-10) was the same as that in the first series, with surgeries performed on treatment day 23, and EMG recording conducted on treatment day 28. The pain threshold test was not performed in this set of rats.

A third series of experiments with five groups (46 rats, *n* = 8-10 each group) of rats aimed to test the effects of JCM-16021 on the concentration of 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), EC cell number and expression of serotonin transporter (SERT). Group

1 with NH rats and Group 2 with NMS rats received distilled water as a control. Groups 3, 4 and 5 with NMS rats received JCM-16021 at dosages of 8, 4, and 2 g/kg per day respectively. When the treatment course finished, the rats were deeply anesthetized with an overdose of midazolam hydrochloride, and 4 cm of the colon (5-6 cm from the anus) was harvested immediately. A piece of the colon was immediately fixed in 4% neutral-buffered paraformaldehyde for immunostaining. The rest was frozen with liquid nitrogen, and stored at -80°C for later analysis of 5-HT/5-HIAA content.

### Drug administration

Starting at postnatal day 60 (body weight around 250 g), rats were daily treated orally with different dosages of JCM-16021 (8, 4 and 2 g/kg per day body weight, in 10 mL/kg distilled water), Holopon at a dosage of 0.3 mg/kg per day body weight, or vehicle (distilled water at 10 mL/kg body weight). The dosage of JCM-16021 was set according to the clinical trial and 4 g/kg per day was determined to be equivalent to the clinical dosage<sup>[5]</sup>.

### Abdominal withdrawal reflex (AWR) test

AWR tests were performed as previously described<sup>[17]</sup>. Briefly, a flexible latex balloon (medical finger glove, 4 cm long, 2.3 cm diameter flaccid) was inserted into the rat colon. Rats were allowed to adapt in a transparent box alone for 30 min after insertion of the colorectal balloon. CRD was then applied in increments of 5 mmHg, maintained for 2 s at each step to observe. The pain threshold pressure was defined as the intensity of CRD that induced a sudden and persistent abdominal muscle contraction in rats with abdomen lift off the platform. The experiments were repeated three times with at least 5 min intervals for recovery. During the test, the observers were blinded to the treatment groups of the rats.

### EMG recording test

To measure rat's visceral sensitivity, the visceral motor response (VMR) to CRD was studied by recording the EMG as previously described with modification<sup>[12,18]</sup>. Briefly, a pair of Teflon-coated stainless wires (Cooner Wire, Chatsworth, CA) was surgically implanted into the left external abdominal oblique muscles on treatment day 23 and EMG recording tests were conducted on treatment day 28. On the test day, animals were subjected to CRD. A flexible latex balloon (medical finger glove, 4 cm long, 2.3 cm diameter flaccid) tied around a urethral catheter (3 mm diameter) was lubricated with liquid paraffin oil and inserted intrarectally in its descending colon with the distal tip 1 cm from the anal verge and secured to the base of the tail under short ether anesthesia. CRD was initiated by Barostat (Distender Series II R, G&J electronics, Canada). The EMG recording signal was amplified and filtered (50-5000 Hz) by Power Lab System (AD Instruments International). Three cycles of graded CRD (20, 40, 60, and 80 mmHg; 20 s duration; 2 min inter-stimulus interval) were applied to each rat. During the five day recovery from surgery, the treatments were

continued. The overall effect of any given reagents was determined by calculating the changes of the area under the curve (AUC) of the raw EMG amplitude response after treatment, based on the formula  $\Delta AUC\% \text{ baseline} = (\text{AUC during CRD} - \text{AUC before CRD}) / \text{AUC before CRD}$ .

### 5-HT and 5-HIAA content assays

5-HT and 5-HIAA concentrations in the colon were analyzed following a procedure with a slight modification<sup>[19]</sup>. Fluorescence of the handled sample was measured at an activation wavelength of 365 nm and an emission wavelength of 470 nm.

### Immunohistochemistry assay for EC cells and SERT

Immunohistochemical detection of EC cells in the colon of rats was performed using a routine streptavidin-biotin peroxidase technique employing Chr-A antibody (1:250, Santa Cruz Biotechnology, Santa Cruz, CA, USA)<sup>[20]</sup> and mouse monoclonal anti-SERT antibody (1:250, Advanced targeting system, AB-N09) as previously described<sup>[19]</sup>. The immunoreaction products were observed under a light NIKON microscope equipped with a NIKON color digital camera system. A NIKON 20 × objective was used to collect images of colon sections. The mean densities of the positive immunoreaction of serotonin transporter receptors and the positive cell numbers of Chr-A in at least 6 serial slides from the colonic sections of each rat were analyzed.

### Statistical analysis

All data are expressed as mean ± SE. The changes in visceral pain threshold pressure were analyzed by comparing the values before and after treatment for each group using a paired *t*-test, and the differences between before and after treatment in a group using a one-way analysis of variance (ANOVA). EMG activity data were analyzed by one-way ANOVA between different groups to determine whether the overall change was significant, in a similar way to the analysis of the changes of concentrations of 5-HT and 5-HIAA, EC cell numbers and SERT in rat colon. *P* < 0.05 was considered statistically significant.

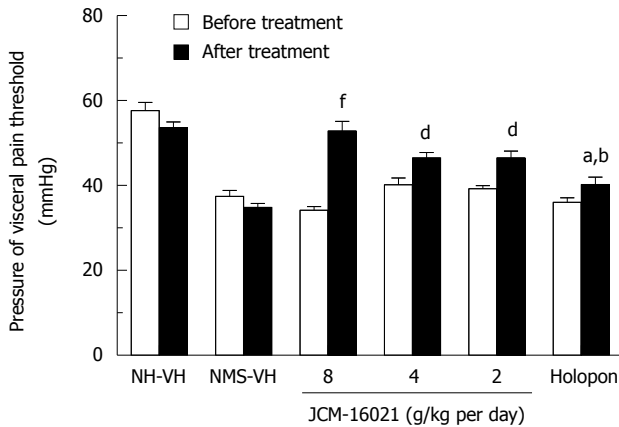
## RESULTS

### Quality control of JCM-16021

To ensure the quality of JCM-16021, eight chemical markers were qualitatively and quantitatively tracked from the raw materials to the final product, and heavy metals and pesticide residues were also examined. The results are listed in Table 1<sup>[5]</sup>.

### NMS induced visceral hyperalgesia

As shown in Figure 1, there is a significant decrease in pain threshold pressure in the NMS vehicle rats before the treatment, when comparing with that of the NH vehicle group before the treatment (*P* < 0.001). The pain threshold pressure values were  $37.4 \pm 4.4$  mmHg



**Figure 1 Pain threshold pressure assessment in different groups.** Data are presented as the mean  $\pm$  SE. Before treatment, the pain threshold of NMS groups was significantly decreased, as compared with that of the NH-VH group ( $^aP < 0.001$ ). After treatment, JCM-16021 and Holopon significantly increased the pain threshold pressure ( $^fP < 0.05$ ,  $^dP < 0.01$ ,  $^bP < 0.001$ ) compared with that before treatment of each group.

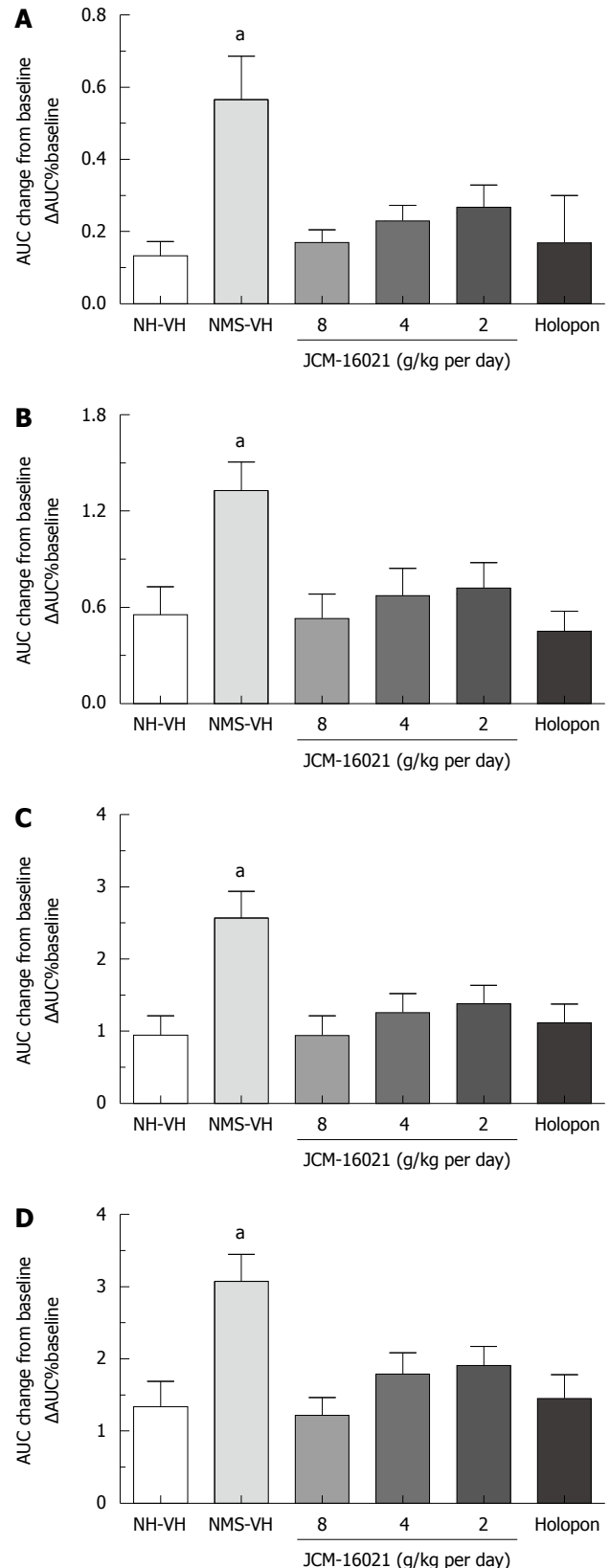
and  $57.7 \pm 5.9$  mmHg in NMS vehicle and NH vehicle groups before treatment, respectively.

In EMG tests, the visceromotor response to CRD, which was reflected as AUC changes over the baseline in the NMS vehicle group after vehicle treatment ( $0.57 \pm 0.12$ ,  $1.33 \pm 0.18$ ,  $2.57 \pm 0.37$ ,  $3.08 \pm 0.37$  under the pressures 20, 40, 60, 80 mmHg) was significantly increased compared to that of the NH vehicle group after vehicle treatment ( $0.11 \pm 0.04$ ,  $0.58 \pm 0.19$ ,  $0.96 \pm 0.3$ ,  $1.39 \pm 0.39$  under the pressures 20, 40, 60, 80 mmHg, Figure 2) ( $P < 0.05$ ). These results indicate that NMS induces allodynia (20 mmHg) and visceral hyperalgesia (40-80 mmHg) in rats.

### Analgesic effect of JCM-16021 in NMS rats

As shown in Figure 1, JCM-16021 can significantly reduce the pain threshold pressure in three dosage groups (from high dose to low dose:  $52.8 \pm 2.3$  mmHg,  $46.5 \pm 1.3$  mmHg, and  $46.5 \pm 1.6$  mmHg) comparing with that of the NMS vehicle group ( $34.8 \pm 0.9$  mmHg,  $P < 0.05$ ). After treatment the pain threshold pressure in three JCM-16021 groups also significantly decreased when compared to that before treatment (from high dose to low dose:  $52.8 \pm 2.3$  mmHg *vs*  $34.2 \pm 0.9$  mmHg,  $46.5 \pm 1.3$  mmHg *vs*  $40.2 \pm 1.6$  mmHg and  $46.5 \pm 1.6$  mmHg *vs*  $39.3 \pm 0.7$  mmHg,  $P < 0.01$ ). Holopon also had a similar analgesic effect to JCM-16021 when comparing the pain threshold pressure values either with that of NMS vehicle group or the value before the Holopon treatment.

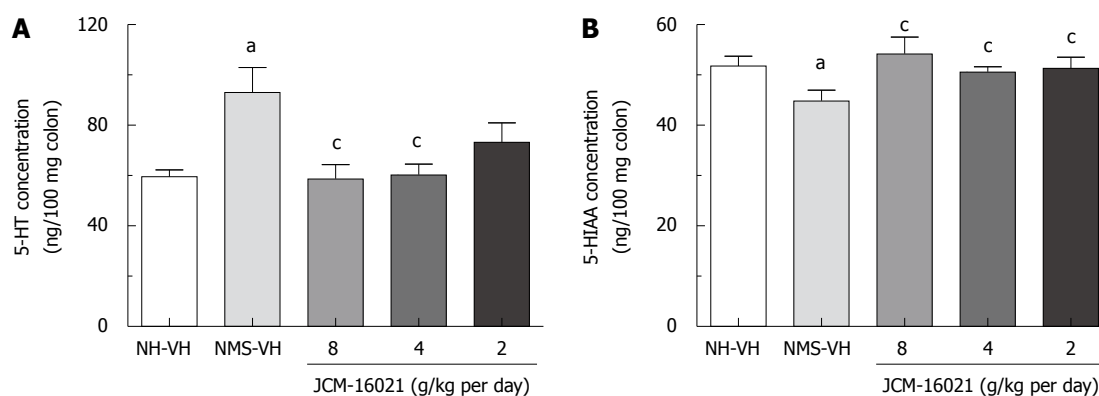
In the EMG test, as shown in Figure 2A-D, the EMG activity to the graded CRD, which was reflected as AUC changes over the baseline, significantly and dose-dependently decreased after JCM-16021 treatment compared to that of the NMS vehicle group ( $P < 0.05$ ). The mean  $\Delta$ AUC significantly fell in the high dose group ( $0.17 \pm 0.03$ ,  $0.53 \pm 0.15$ ,  $1.06 \pm 0.18$ ,  $1.22 \pm 0.24$  for the pressures 20, 40, 60, 80 mmHg), middle dose group ( $0.23 \pm 0.04$ ,  $0.68$



**Figure 2 JCM-16021 effect on the electromyographic activity in response to graded CRD at pressures of 20 mmHg (A), 40 mmHg (B), 60 mmHg (C), 80 mmHg (D).** Data are presented as mean  $\pm$  SE ( $n = 7-10$ ). Significant difference is indicated by  $^aP < 0.05$  when compared with the NMS-VH group.

$\pm 0.17$ ,  $1.27 \pm 0.26$ ,  $1.8 \pm 0.3$  for the pressures 20, 40, 60, 80 mmHg), and low dose group ( $0.29 \pm 0.06$ ,  $0.8 \pm 0.16$ ,





**Figure 3** JCM-16021 effects on the concentrations of 5-HT and 5-HIAA in the colons of rats. A: JCM-16021 significantly decreased 5-HT concentration in neonatal maternal separation (NMS) rats; B: JCM-16021 significantly increased 5-HIAA concentration in NMS rats. Data are presented as mean  $\pm$  SE (ng/100 mg colon tissue,  $n = 8-10$ ). Significant difference is indicated by <sup>a</sup> $P < 0.05$  when compared with the NH control group and by <sup>c</sup> $P < 0.05$  when compared with the NMS control group.

1.53  $\pm$  0.24, 2.1  $\pm$  0.21 for the pressures 20, 40, 60, 80 mmHg), compared to that of the NMS vehicle group. The mean  $\Delta$ AUC values were: 0.57  $\pm$  0.12, 1.33  $\pm$  0.18, 2.57  $\pm$  0.37, 3.08  $\pm$  0.37 for the pressures 20, 40, 60, 80 mmHg,  $P < 0.05$ . Also Holopon significantly reduced the EMG activity compared to the NMS vehicle group ( $P < 0.05$ ).

#### JCM-16021 decreases the 5-HT concentration in the colon of rats

As shown in Figure 3A, 5-HT concentration in NMS vehicle groups ( $n = 9$ , 93.11  $\pm$  9.85 ng/100 mg) was significantly higher than that in NH vehicle groups ( $n = 8$ , 59.53  $\pm$  7.57 ng/100 mg,  $P < 0.01$ ). JCM-16021 treatment at the high and middle dosage, but not the low dosage, significantly decreased the 5-HT concentration in the colon of rats ( $P < 0.01$ ), when compared to the NMS vehicle group. After treatment with JCM-16021, the 5-HT concentrations in high, middle and low dosage groups ( $n = 9, 10, 10$ ) were 60.25  $\pm$  5.98 ng/100 mg, 60.32  $\pm$  4.22 ng/100 mg, 73.31  $\pm$  7.65 ng/100 mg, respectively. Further, although there was no significant difference in 5-HT concentration between the low dosage group and NMS vehicle group, the value was still lower than that of the NMS-VH group (93.11  $\pm$  9.85 ng/100 mg). Clearly, there is a tendency that JCM-16021 treatment could reduce the 5-HT concentration.

#### JCM-16021 significantly increases the 5-HIAA concentration in the colon of rats

NMS treatment significantly decreased 5-HIAA concentration in the colon of rats. 5-HIAA concentration in NMS vehicle groups ( $n = 9$ , 44.86  $\pm$  2.13 ng/100 mg) was significantly higher than that in NH vehicle groups ( $n = 8$ , 51.75  $\pm$  1.98 ng/100 mg,  $P < 0.05$ ). JCM-16021 treatment significantly increased the 5-HIAA concentration in the colon of rats when compared with that of the NMS control group ( $P < 0.05$ ). After treatment with JCM-16021, the 5-HIAA concentrations were 54.24  $\pm$  9.81 ng/100 mg in the high dosage group, 50.61  $\pm$  1.26 ng/100 mg in the middle dosage group, and 51.37  $\pm$  2.13 ng/100 mg in the low dosage group (Figure 3B).

#### JCM-16021 does not change EC cell numbers in the colon of rats

As shown in Figure 4, EC cell number in NMS vehicle groups ( $n = 9$ , 12.97  $\pm$  1.17) was significantly higher than that in NH vehicle groups ( $n = 8$ , 9.70  $\pm$  0.92,  $P < 0.05$ ). JCM-16021 treatment did not significantly change the EC cell number in the three dose groups.

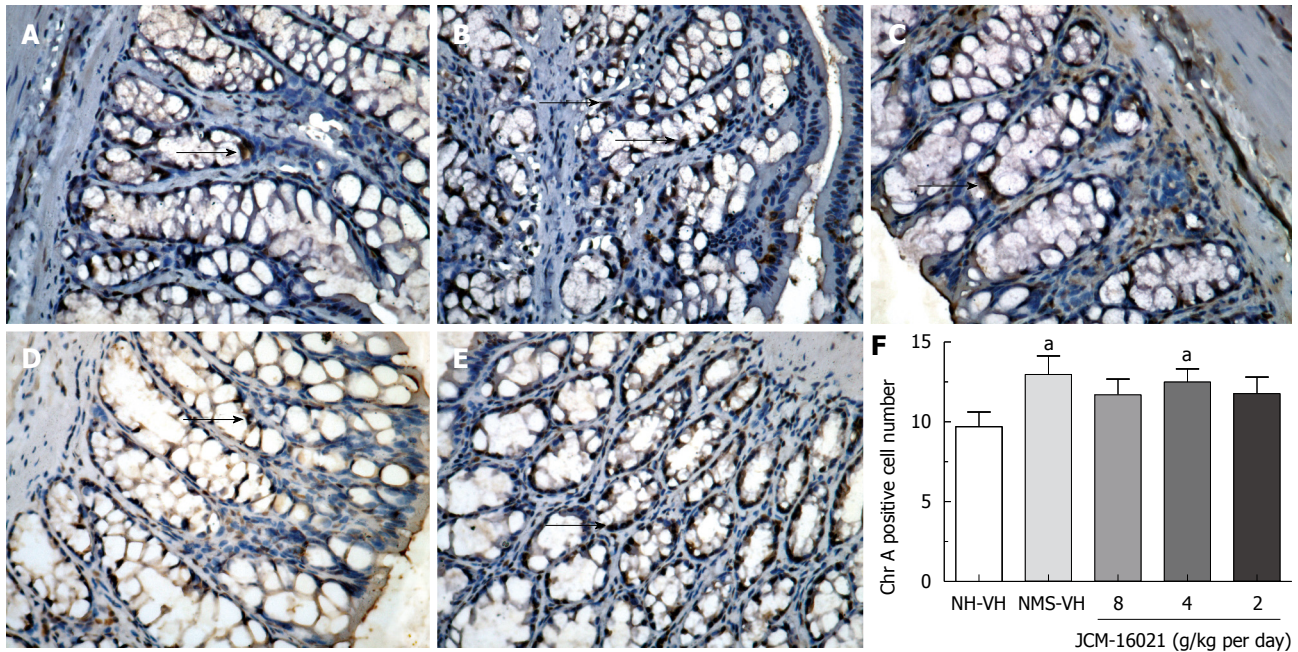
#### JCM-16021 decreased SERT expression in the colon of rats

As shown in Figure 5, NMS rats had higher SERT expression (0.61  $\pm$  0.03,  $n = 10$ ) than NH rats (0.56  $\pm$  0.05,  $n = 8$ ,  $P < 0.05$ ). JCM-16021 treatment at different dosages ( $n = 10$  in each group) significantly decreased SERT expression in the colon. The mean gray indexes among JCM-16021 groups were 0.59  $\pm$  0.03 in the high dose group, 0.54  $\pm$  0.02 in the middle dose group and 0.53  $\pm$  0.03 in the low dose group ( $P < 0.01-0.001$ ).

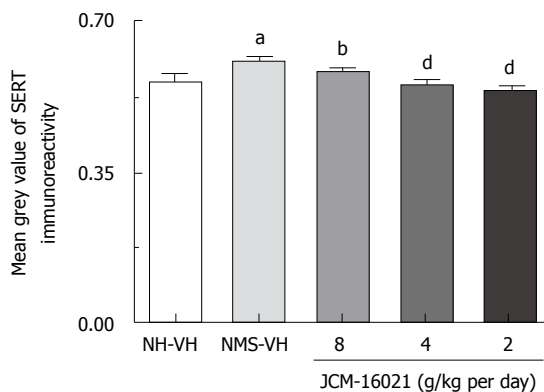
## DISCUSSION

This study demonstrated that JCM-16021 can dose-dependently attenuate the visceromotor response to CRD in NMS rats. Moreover, it decreases 5-HT concentration and increases 5-HIAA concentration in the colon of rats. These findings indicate that JCM-16021 has an analgesic effect on visceral hyperalgesia, and this effect may be mediated through the serotonin signaling pathway in the colon of rats.

Chronic visceral hyperalgesia is an important and characteristic feature of IBS and other functional bowel disorders<sup>[21]</sup>. In order to investigate the mechanism of visceral hyperalgesia, animal models have been developed, such as the early life colon irritation model<sup>[17]</sup>, neonatal maternal separation model<sup>[12]</sup>, and adult repeated stress model in rodents<sup>[22]</sup>. The current study showed that NMS induced a lower pain threshold pressure than that seen in NH rats, and increased EMG activity in response to CRD, thus confirming that NMS induces visceral hyperalgesia in adulthood<sup>[12]</sup>. Further, our results also showed that even in 20 mmHg CRD stimulation, NMS rats still have



**Figure 4** JCM-16021 effect on enterochromaffin cell number (Chr A positive cell number) in the colons of rats. Chr A staining cells (arrows) are present in colon tissues in the normal control group (A), the NMS control group (B), the NMS with high dosage (C), the NMS with middle dosage (D), and the NMS with low dosage of JCM-16021 (E). NMS significantly increased the number of Chr A positive cells in the colons of rats, and JCM-16021 did not change the number of Chr A positive cells (F). Data are presented as mean ± SE ( $n = 8-10$ ). Significant difference is indicated by <sup>a</sup> $P < 0.05$  compared with the normal control group.



**Figure 5** SERT expression in rat colon by immunohistochemistry assay. SERT expression in the colonic tissues of NMS rats was significantly increased over that of NH rats. JCM-16021 treatment decreased SERT expression. Results are expressed as mean ± SE,  $n = 8-10$  in each group. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>d</sup> $P < 0.001$  vs NH.

significant EMG changes compared to NH rats, thus NMS induces not only visceral hyperalgesia, but also allodynia in adulthood. Our results also showed that JCM-16021 increased the pain threshold pressure with a dose-related effect, and dose-dependently reduced the EMG activity to CRD in NMS rats. Therefore, these results indicate that JCM-16021 has an analgesic effect which can attenuate allodynia and visceral pain in NMS rats. Interestingly, the results from the AWR test showed that there is no dose-related response, but the results from the EMG test did show a dose-dependent response. The difference may originate from the objectivity of the two pain indexes; the EMG data, as a quantitative value, is more reliable than that of pain threshold pressure.

As for the mechanism of visceral hyperalgesia, it is believed that the up-regulation of visceral pain perception results, at least in part, from profound and long-lasting changes in the development of the central nervous system, including systems that regulate stress responsiveness<sup>[23,24]</sup>. With regard to the 5-HT effect in visceral hyperalgesia, previous data is not consistent. Our previous study reported that the amount of colonic 5-HT in rats with visceral hyperalgesia induced by mechanical colorectal irritation significantly increased with postnatal day<sup>[19]</sup>. Another study showed that serotonin is actively involved in pathophysiological processes of visceral hyperalgesia because serotonin concentration is significantly decreased in the spinal cord and but not in the colon; and 5-HT significantly increased in the colons of rats after CRD<sup>[13]</sup>. It is well known that 5-HT in the gastrointestinal tract is generally believed to be one of the most important mediators and regulators of bowel sensation and motility<sup>[9,25]</sup>. The current study found that 5-HT concentrations in the colons of the NMS vehicle group were significantly higher than that in the NH vehicle group supporting the concept that 5-HT is an important mediator involved in NMS-induced visceral hyperalgesia.

Our data also showed that JCM-16021 not only significantly reduces the 5-HT concentration but also significantly increases the 5-HIAA concentration. Serotonin, as an important gastrointestinal signaling molecule, is synthesized from the amino acid tryptophan *via* a short metabolic pathway consisting of two enzymes: tryptophan hydroxylase and amino acid decarboxylase. Recent studies have demonstrated that distinct changes in intestinal EC cell number and 5-HT content have significant relationships with symptoms in IBS



patients<sup>[26,27]</sup>. Therefore, the serotonin signaling pathway has been proposed as a therapeutic target to improve the symptoms of IBS<sup>[11]</sup>. Our current study found that NMS not only increases 5-HT concentration but also decreases the levels of its metabolite 5-HIAA in the colon of rats. After JCM-16021 treatment, the 5-HT concentration was decreased while 5-HIAA concentration increased. 5-HIAA is a major product of 5-HT breakdown, which is excreted in the urine. The increase in 5-HIAA indicates that more 5-HT was broken down after JCM-16021 treatment. Therefore, the data indicate that JCM-16021 affected 5-HT action and its metabolism in the colon.

It is well-known that a large proportion of 5-HT in the body is found in the gastrointestinal tract, and is primarily contained within EC cells<sup>[26]</sup>. This study found that EC cell number in NMS control rats is higher than that in NH control rats. It is possible that NMS induces hyperplasia of EC cells, thus NMS rats have higher concentrations of 5-HT in their colons compared to NH rats. Our results also showed that JCM-16021 cannot significantly change EC cell number in the colon of rats. This suggests that the hyperplasia of EC cells may be a permanent change similar to the elevated activation of the cingulate cortex and sensitization of the ascending pathway involving the spinal cord and the thalamo-cortico-amygdala pathway<sup>[24]</sup>. Therefore, EC cell number was not changed with treatment.

SERT is necessary for termination of serotonergic action in the colon. After the release by EC cells, serotonin is taken up again from the mucosa into the nerve fibers<sup>[11]</sup>. Altered SERT expression and function could contribute to the abdominal hypersensitivity and abnormal colonic motility associated with IBS and IBD<sup>[27]</sup>. A previous report showed 5-HT and mucosal SERT are both decreased in ulcerative colitis, diarrhea-predominant IBS and constipation-predominant IBS<sup>[28]</sup>. Our study showed that NMS rats with vehicle have significantly increased SERT expression in the colon with increased concentration of 5-HT, compared with NH rats with vehicle. The increase in SERT expression could be due to an adaptive response to improve disturbed gut function and ameliorate symptoms; thus increased 5-HT could be terminated quickly under different stimulations. After treatment with JCM-16021, SERT expressions were decreased along with 5-HT content. Such decreases could result in the inhibition of SERT function. It is reported that inhibition of SERT function leads to decreased transiency in the gut and lower sensitivity<sup>[27,29,30]</sup>. Our data showed that JCM-16021 reduced SERT expression in the colon of rats, indicating that JCM-16021 inhibits SERT function so as to induce lower sensitivity.

In summary, the present findings provide evidence for the analgesic effect of JCM-16021 on visceral hyperalgesia in rats. This effect may be mediated through changes in the synthesis and metabolism of 5-HT in the colons of rats.

## COMMENTS

### Background

Increasing numbers of irritable bowel syndrome (IBS) sufferers are seeking help

from complementary and alternative medicines because conventional therapies have not been proven to be more effective than placebo in providing overall relief of symptoms in randomized, controlled clinical trials. The 5-HT signaling pathway represents a promising target for IBS treatment. JCM-16021 improved the symptoms of IBS patients but the mechanism is unknown.

### Research frontiers

A neonatal maternal separation (NMS)-induced visceral hyperalgesia rat model is often used to study the mechanism of IBS and to evaluate the pharmacological effects of potential IBS therapies. This study aimed to investigate the analgesic effect of JCM-16021 on NMS-induced visceral hyperalgesia in rats, and its potential underlying mechanism.

### Innovations and breakthroughs

Recent studies have highlighted the role of serotonin (5-HT) in the generation of IBS symptoms and in other gastrointestinal functional disorders. Furthermore, novel serotonergic agents, such as the 5-HT<sub>3</sub> antagonist alosetron and the 5-HT<sub>4</sub> agonist tegaserod, have significant impacts on IBS symptoms through their visceral analgesic properties and diverse effects on motor functions in the lower gastrointestinal tract. This is the first study to report that the analgesic effect of JCM-16021, a Chinese herbal formula, on visceral hyperalgesia in rats may be mediated through changes in the synthesis and metabolism of 5-HT in the colons of rats.

### Applications

This study provides direct evidence for the analgesic effect of JCM-16021 on visceral hyperalgesia in rats. JCM-16021 might become a reliable therapy to relieve the symptoms of IBS patients.

### Peer review

This is an interesting paper showing the analgesic effect of a Chinese Medicine herb, JCM-16021 on maternal separation stress induced visceral hypersensitivity in male rats. Together with their previous data in IBS patients, this additional pre-clinical work suggests that JCM-16021 may be of therapeutic interest for IBS.

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## Celecoxib inhibits *Helicobacter pylori* colonization-related factors

Jing Wang, Wei-Hong Wang, Jiang Li, Fang-Xun Liu

Jing Wang, Wei-Hong Wang, Jiang Li, Fang-Xun Liu, Department of Gastroenterology, Peking University First Hospital, Beijing 100034, China

**Author contributions:** Wang J and Wang WH contributed equally to this work; Wang J performed the most parts of experiment and wrote the manuscript; Wang WH designed the study and wrote the manuscript; Li J and Liu FX offered the technical assistance.

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**Correspondence to:** Wei-Hong Wang, Professor, Department of Gastroenterology, Peking University First Hospital, 8 Xishiku Avenue, Xicheng District, Beijing 100034, China. wangweihong@medmail.com.cn

**Telephone:** +86-10-83572616 **Fax:** +86-10-66518105

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### Abstract

**AIM:** To investigate the effect of celecoxib, a selective COX-2 inhibitor, on *Helicobacter pylori* (*H. pylori*) colonization-related factors and its mechanism.

**METHODS:** After co-incubation with celecoxib, morphology of *H. pylori* strain 26695 was observed under a transmission electron microscope. Flagella motility was assessed by stab agar motility test. Adherence of *H. pylori* to AGS cells was determined by enzyme linked immunosorbent assay. Levels of mRNA expression in flagellar genes (*flaA*, *flaB*), urease genes (*ureA*, *ureB*) and adhesin genes (*babA*, *sabA*, *alpA*, *alpB*, *hpaA*, *hopZ*) were measured by real-time polymerase chain reaction.

**RESULTS:** Separation and non-integrity of bacterial cell wall, rarefaction and asymmetry of cytoplasm, and even lysis of *H. pylori* were observed in the presence of celecoxib. When *H. pylori* strains were incubated in the presence of celecoxib, their flagellar motility and

adherence to AGS cells were inhibited. The expression of *ureA*, *ureB*, *babA*, *sabA*, *alpA*, *alpB*, *hpaA*, *hopZ* was up-regulated while the expression of *flaA*, *flaB* was down-regulated in the presence of celecoxib.

**CONCLUSION:** Celecoxib inhibits flagellar motility and adherence of *H. pylori* to AGS cells, and destructs their normal structure *in vitro*.

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**Key words:** *Helicobacter pylori*; Celecoxib; Colonization; Ultrastructure

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Wang J, Wang WH, Li J, Liu FX. Celecoxib inhibits *Helicobacter pylori* colonization-related factors. *World J Gastroenterol* 2010; 16(7): 846-853 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v16/i7/846.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i7.846>

### INTRODUCTION

About 30% of the population in developed countries and up to 90% of the population in developing countries are chronically infected with *Helicobacter pylori* (*H. pylori*)<sup>[1,2]</sup>. Non-steroidal anti-inflammatory drugs (NSAID) are the most commonly used drugs, on a world-wide scale, which are used by at least 30 million people<sup>[3]</sup>. NSAID and *H. pylori* infection are two major factors for gastric injuries. Subjects taking NSAID are often infected with *H. pylori*. However, whether these two factors exert synergistic or antagonistic actions on gastric mucosa is still controversial<sup>[4,5]</sup>. Data from a meta-analysis review have shown that the risk of peptic

ulcer is approximately 60-fold higher in *H. pylori* positive subjects taking NSAID than in *H. pylori* negative subjects not taking NSAID<sup>[6]</sup>. Since both *H. pylori* and NSAID are responsible for mucosal damage, they can increase the risk of developing uncomplicated and complicated peptic ulcer. However, data from several studies do not always confirm such an assumption<sup>[5]</sup>. A large clinical trial demonstrated that eradication of *H. pylori* delays the healing of gastric ulcers in NSAID users after treatment with omeprazole<sup>[7]</sup>, implying that *H. pylori* may protect individuals against NSAID-induced ulcer, possibly by stimulating mucosal prostaglandins and other protective factors.

Recent studies *in vitro* also suggested that aspirin and celecoxib, a selective COX-2 inhibitor, inhibit the growth of *H. pylori* and decrease the activity of urease and vacuolating cytotoxin in a dose-dependent manner<sup>[8-12]</sup>, indicating that NSAID may antagonize injuries of gastric mucosa caused by *H. pylori* infection. Colonization of *H. pylori* in gastric mucosa is a prerequisite for pathogenicity and needs to have at least 4 basic characteristics: integrate helical shape, motility of flagella, specific binding to adhesin and its receptors, and urease activity that provides an appropriate microenvironment<sup>[13]</sup>. We hypothesize that NSAID and celecoxib may influence the pathogenicity of *H. pylori* in gastric mucosa injury by altering the colonization. Therefore, the aim of the present study was to investigate the effect of celecoxib on *H. pylori* colonization-related factors and its mechanism *in vitro*.

## MATERIALS AND METHODS

### Bacterial culture

*H. pylori* 26695 strain was cultured at 37°C in a microaerobic atmosphere containing 5% O<sub>2</sub>, 85% N<sub>2</sub>, and 10% CO<sub>2</sub> for 48 h on Colombia agar medium supplemented with 8% (v/v) defibrinated goat blood containing 0.02 mmol/L celecoxib or vehicle control (1/1000 DMSO).

### Stab agar motility test

*H. pylori* strains were grown on Colombia agar medium for 48 h and then harvested into a brain heart infusion (37 g/L). After the concentration of bacteria was adjusted to 10<sup>8</sup> CFU/mL, 10 µL was inoculated into a 0.3% agar Brucella broth medium containing 8% defibrinated goat blood using a sterile picker. Five days after incubation under microaerobic condition at 37°C, the halo diameter was measured.

### Ultrastructural analysis

Forty-eight hours after exposure to 0.02 mmol/L celecoxib, *H. pylori* cells were collected and rinsed three times with 0.01 mol/L PBS, fixed in phosphate-buffer solution containing 2.5% glutaraldehyde at 4°C for 2 h. After centrifugation, pellets were embedded in 2% agar, fixed in 1% osmium tetroxide (OsO<sub>4</sub>) at 4°C, and rinsed three times with 0.01 mol/L PBS. After dehydrated

in a series of graded acetone at 4°C, specimens were embedded in Epon 812 (Emicon). The sample was cut into 90 nm-thick sections which were stained with uranyl acetate and lead citrate, and observed under a JEM1230 transmission electron microscope.

### Adhesion of *H. pylori* to AGS cells

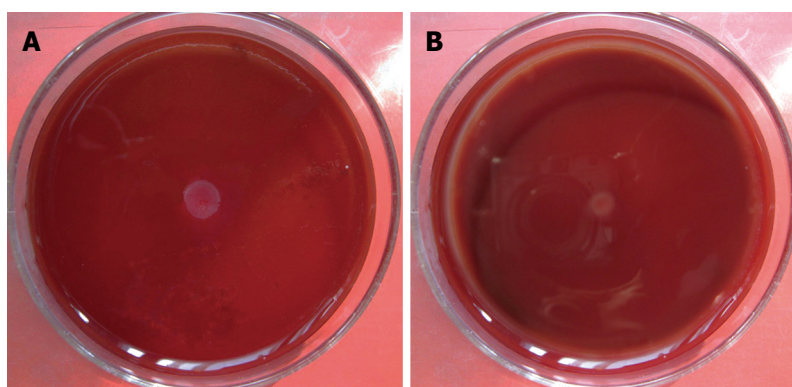
AGS cells (10<sup>4</sup>/well) were seeded in RPMI 1640 medium (Gibco) containing 10% fetal bovine serum in a 96-well plate containing 5% CO<sub>2</sub> at 37°C for 20 h. *H. pylori* (10<sup>7</sup> CFU/well) were pretreated with celecoxib at the concentrations of 0.01, 0.02 and 0.03 mmol/L. The plate was agitated at 60 r/min for 30 min at 37°C. Cultures were fixed with 1% paraformaldehyde. After washed with PBS, *H. pylori* cells were blocked with 5% bovine serum albumin (BSA) for 30 min, and incubated for 24 h with mouse monoclonal anti-*H. pylori* antibody (Santa cruz). After washed three times with PBS, goat anti-mouse IgG-HRP (Santa cruz) was added for 1 h. Binding was visualized by incubating with 100 µL TMB substrate for 30 min. Absorbance was read at 450 nm after 2 mol/L of sulphuric acid was added to terminate the reaction. Adherence of *H. pylori* to AGS cells was calculated according to the formula: [(4 AGS cells with *H. pylori* - 4 AGS cells without *H. pylori*) / (4 positive control - 4 negative control)] × 100. For positive control, only bacteria were added and allowed to adhere to the well. Wells containing neither AGS cells nor *H. pylori* were prepared as a negative control.

### *H. pylori* RNA isolation and reverse transcription

Forty-eight hours after pretreatment with 0.02 mmol/L celecoxib, strains of *H. pylori* were rinsed with Tris-HCl and cleared with 1 mL of TRIzol. After 200 µL of chloroform was added, the sample was vigorously shaken and centrifuged. RNA in aqueous phase was precipitated with 0.5 mL of isopropanol. The pellet was washed with ethanol and dried. The RNA was resuspended in sterile water and quantified by UV absorbance. Total RNA (4 µg) treated with RO1 RNase-free DNase (Promega) to remove DNA was used for reverse transcription reaction. In brief, 1.5 µL of random primers was added, the samples were heated to 70°C for 5 min. Then, 10 µL of 5 × RT buffer, 2.5 µL of dNTPs, and 2 µL of M-MLV were added. cDNA synthesis reaction was performed for 60 min at 37°C and then at 70°C for 10 min. Aliquots of cDNA were stored at -70°C.

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

mRNA levels of flagellar genes (*flaA*, *flaB*), urease genes (*ureA*, *ureB*) and adhesin genes (*babA*, *sabA*, *alpA*, *alpB*, *bpaA*, *hopZ*) were measured by real-time PCR using the ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, Calif). Specific primers and house-keeping gene 16S rRNA were designed with the aid of Primer Express 3.0 software (Applied Biosystem Perkin-Elmer) (Table 1). Real-time PCR was performed in a 25 µL reaction volume containing 2.5 µL



**Figure 1** Stab agar motility tests showing the *H. pylori* motility. A: DMSO control (1/1000); B: Celecoxib (0.02 mmol/L).

**Table 1** Primers and probes used in real-time quantitative PCR

Gene	Primer (5'-3')
<i>flaA</i> -F	ATTGGCGTGTAGCAGAAGTGA
<i>flaA</i> -R	TGACTGGACCGCCACATC
<i>flaB</i> -F	ACATCATGTGTAGCGGTGTGA
<i>flaB</i> -R	GCCCCTAACCGCTCTCAAAT
<i>ureA</i> -F	GCTGGTGCGATTGGCTTTA
<i>ureA</i> -R	GGATAGCGACTTGACATCGT
<i>ureB</i> -F	TCCGTATGGGACAAAACCTCGTA
<i>ureB</i> -R	ACGGCTTTTTTGCCCTTCGT
<i>babA</i> -F	TGCTCAGGGCAAGGGAATAA
<i>babA</i> -R	ATCGTGGTGGTTACGCTTTTG
<i>sabA</i> -F	GGTGTGCTGCAACAGACTCAA
<i>sabA</i> -R	CATAAGCTGTTGCGCCAAATT
<i>alpA</i> -F	GCACGATCGGTAGCCAGACT
<i>alpA</i> -R	ACACATTCCCCGCAATTCAG
<i>alpB</i> -F	ACGCTAAGAAACAGCCCTCAAC
<i>alpB</i> -R	TCATGCGTAACCCACATCA
<i>hpaA</i> -F	GAGCGTGGTGGCTTTGTAGT
<i>hpaA</i> -R	TCGCTAGCTGGATGGTAATTCA
<i>hopZ</i> -F	GCGCCGTACTAGCATGATCA
<i>hopZ</i> -R	GAAATCTTTCGGCGCGTTT
16SrRNA-F	CCGCCTACGCGCTCTTTAC
16SrRNA-R	CTAACGAATAAGCACCGGCTAAC

PCR: Polymerase chain reaction.

of cDNA, 12.5  $\mu$ L of SYBR green real time PCR master mix (Toyobo), 1  $\mu$ L of sense and antisense primers (5 pmol/L), and 9  $\mu$ L of DEPC water. PCR was carried out at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, at 61°C for 1 min. A further melting curve step analyzing the purity of PCR products was performed at 95°C for 15 s, at 61°C for 30 s, and at 96°C for 15 s. A standard curve was plotted using 10-fold serial dilution of each cDNA. mRNA level was expressed as the ratio of detected mRNA to 16S rRNA mRNA [detected mRNA (U/mL)/16S rRNA mRNA (U/mL)  $\times$  100 000]. PCR was carried out in quintuple using samples prepared at the same time.

### Statistical analysis

All experiments were performed at least in triplicate. Data were presented as mean  $\pm$  SD. Statistical analysis between sample and control was conducted by Student's

*t*-test using SPSS 11.0 software.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Effects of celecoxib on *H. pylori* motility

The halo diameter for the growth of *H. pylori* in the presence of celecoxib was  $5.92 \pm 1.20$  mm after 5-d incubation, which was significantly smaller than that ( $8.21 \pm 1.63$  mm) of DMSO control ( $P < 0.05$ , Figure 1), indicating that the motility of *H. pylori* is decreased in the presence of celecoxib.

### Ultrastructural effects of celecoxib on *H. pylori*

Transmission electron microscopy demonstrated that both cytoplasmic and outer membranes of *H. pylori* were intact, the cytoplasm was well-distributed and the electron density was moderate in DMSO control. When incubated with 0.02 mmol/L of celecoxib, V- and U-shaped *H. pylori* were observed. The cell wall of *H. pylori* was attenuated with abscission, or even perforation but no integrity. Separation of the outer membrane from the cytoplasmic membrane (cell wall breakaway) and even cell lysis were observed. Rarefaction and asymmetry were observed in cytoplasm of *H. pylori* and the components of *H. pylori* cells disappeared and distributed abnormally (Figure 2).

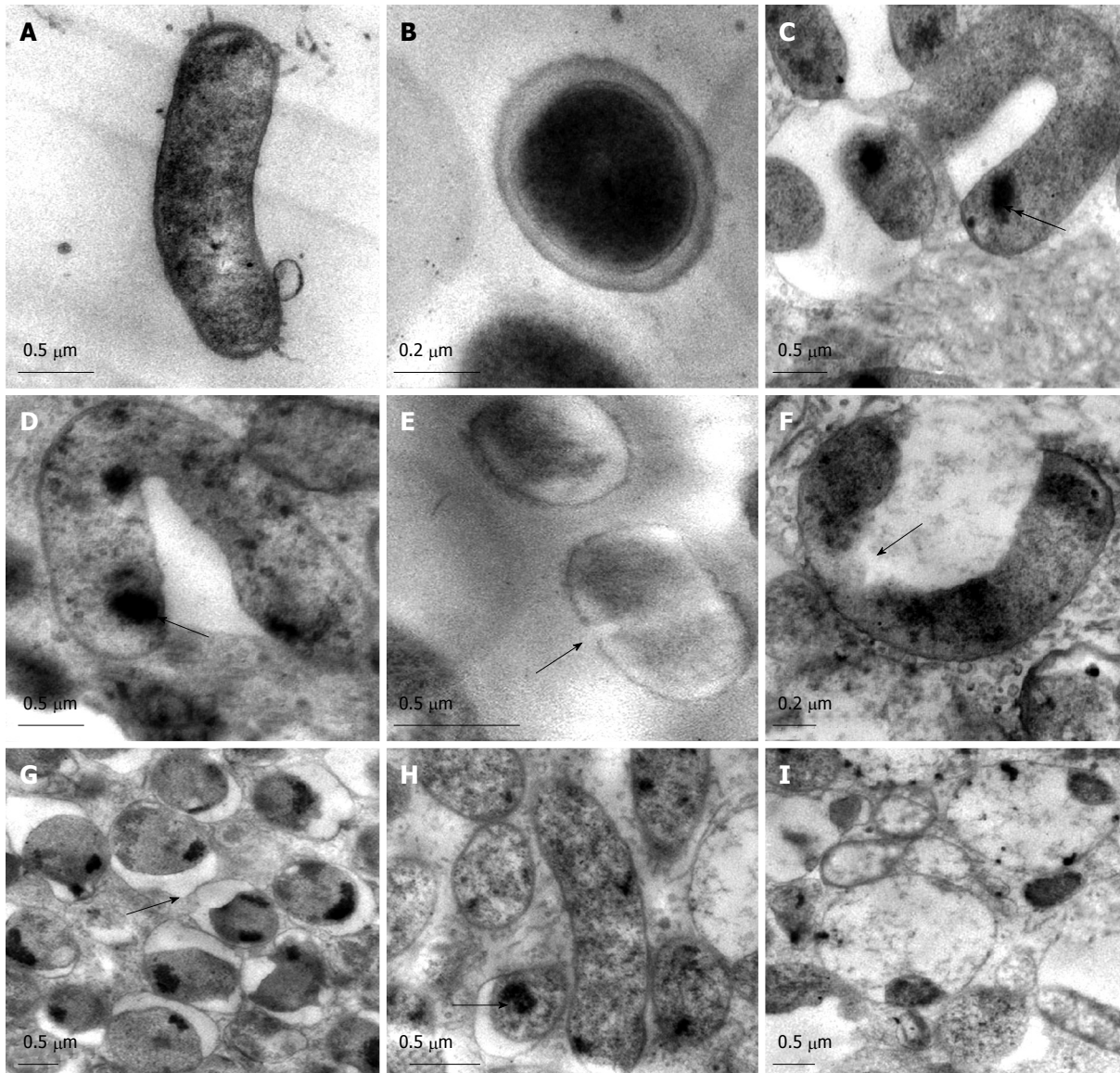
### Effects of celecoxib on *H. pylori* adherence to AGS cells

Compared to the DMSO control (1/1000), celecoxib significantly inhibited the adherence of *H. pylori* to AGS cells in a dose-dependent manner ( $P < 0.05$ ) (Figure 3).

### Effects of celecoxib on *H. pylori* flagellin, urease and adhesin gene expression

The mRNA expression levels in flagellar genes (*flaA*, *flaB*), urease genes (*ureA*, *ureB*) and adhesin genes (*babA*, *sabA*, *alpA*, *alpB*, *hpaA*, *hopZ*) were measured by real-time PCR. After treatment with 0.02 mmol/L celecoxib, the mRNA expression levels in *flaA* and *flaB* were lower than those in DMSO control ( $P < 0.05$ ). However, the mRNA expression levels were higher in urease genes (*ureA*, *ureB*) and adhesin genes (*babA*, *sabA*, *alpA*, *alpB*, *hpaA*, *hopZ*) than in DMSO control ( $P < 0.05$ ). The





**Figure 2 Transmission electron microscopy (TEM).** TEM showing rod-shaped *H. pylori* (A), well-distributed cytoplasm and moderate electron density (B), U-shaped (C, arrow) and V-shaped (D, arrow) *H. pylori*, non-integrity (E, arrow) and abscission (F, arrow) of *H. pylori* cell wall, outer membrane separated from the cytoplasmic membrane (G, arrow), decreased electron density in cytoplasm (H, arrow), and cell lysis (I) after treatment with celecoxib.

**Table 2 mRNA levels in *H. pylori* flagellin, urease and adhesin genes measured by real-time quantitative PCR (mean  $\pm$  SD)**

Gene	Celecoxib (0.02 mmol/L)	DMSO control (1/1000)
<i>flaA</i>	23.08 $\pm$ 1.70 <sup>a</sup>	51.08 $\pm$ 6.91
<i>flaB</i>	16.01 $\pm$ 0.04 <sup>a</sup>	34.80 $\pm$ 7.13
<i>ureA</i>	19.61 $\pm$ 1.78 <sup>a</sup>	7.65 $\pm$ 0.38
<i>ureB</i>	29.59 $\pm$ 5.31 <sup>a</sup>	13.80 $\pm$ 1.63
<i>babA</i>	16.78 $\pm$ 0.91 <sup>a</sup>	12.38 $\pm$ 0.38
<i>sabA</i>	49.00 $\pm$ 4.10 <sup>a</sup>	22.55 $\pm$ 2.26
<i>alpA</i>	15.55 $\pm$ 0.78 <sup>a</sup>	7.34 $\pm$ 0.20
<i>alpB</i>	14.07 $\pm$ 0.23 <sup>a</sup>	8.95 $\pm$ 0.38
<i>hpaA</i>	123.98 $\pm$ 11.82 <sup>a</sup>	57.15 $\pm$ 2.56
<i>hopZ</i>	100.25 $\pm$ 4.37 <sup>a</sup>	45.54 $\pm$ 11.64

<sup>a</sup>*P* < 0.05 vs DMSO control.

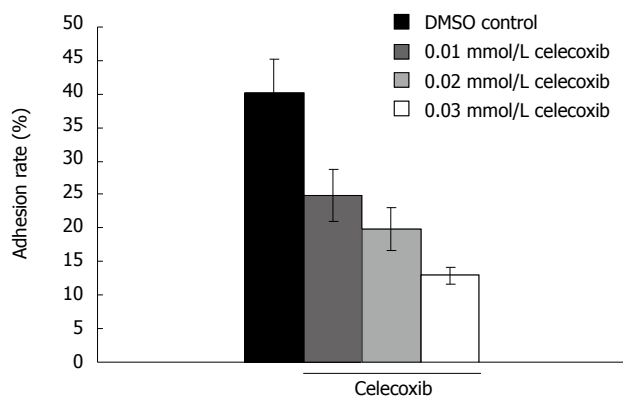
mRNA expression levels in the above genes increased

or decreased 1.5-2.5 folds in the presence of celecoxib (Table 2).

## DISCUSSION

NSAID and *H. pylori* infection are the two main etiological factors for peptic ulcers. However, their role in the pathogenesis of gastric mucosal damage is still controversial<sup>[6]</sup>. It has been demonstrated that eradication of *H. pylori* can decrease the recurrence rate of peptic ulcer and its complications in chronic NSAID users<sup>[14]</sup>, while their co-existence aggravating gastric mucosal damage has not been confirmed<sup>[4,5]</sup>. It was reported that the prostaglandin synthesis level in mucosa is significantly higher in *H. pylori* positive patients than in *H. pylori* negative patients<sup>[15,16]</sup>, demonstrating that colonization of *H. pylori* reduces the inhibitory effect of NSAID





**Figure 3** Adhesion of *H. pylori* to AGS cells after treatment with celecoxib at different concentrations.

on prostaglandin synthesis. *In vitro* studies further revealed that NSAID can inhibit the growth of *H. pylori*, and decrease the activity of urease and vacuolating cytotoxin<sup>[8-12]</sup>, suggesting that NSAID may alter the pathogenicity of *H. pylori* in gastric mucosa injury when the two factors are co-existed in gastric mucosa.

*H. pylori* infection may persist for many years in the host and *H. pylori* colonization-related factors include its spiral shape, flagellar motility, urease and adhesin. Urease neutralizes the pH around *H. pylori* during exposure to the acidic lumen of stomach. The flagella and the spiral shape of *H. pylori* enable *H. pylori* strains to move and penetrate the mucin layer where they come into contact with gastric epithelial cells. Adherence of *H. pylori* to AGS cells is a crucial initial step in colonization<sup>[17,18]</sup>, as non-adhering *H. pylori* strains would be washed away during peristalsis-mediated flushing of stomach. NSAID and celecoxib do not increase the colonization of *H. pylori* in gastric mucosa<sup>[19-23]</sup>. On the contrary, the incidence of *H. pylori* infection in patients taking NSAID is low<sup>[24,25]</sup>, which may be partially explained by the fact that celecoxib can destruct the normal structure of *H. pylori*, and inhibit the motility of flagella, and the adherence of *H. pylori* to AGS cells and the activity of urease<sup>[12]</sup>, which is consistent with the findings in our study.

Adhesin, exposed on the surface of *H. pylori* cells, facilitates interaction with host cellular receptors. The particularly more important adhesins of *H. pylori* are BabA, SabA, AlpA, AlpB, HpaA, HopZ<sup>[26-30]</sup>. Their content and expression under different environmental conditions are variable. In our *in vitro* study, celecoxib inhibited the adherence of *H. pylori* to AGS cells, but increased the mRNA expression levels in *babA*, *sabA*, *alpA*, *alpB*, *hpaA*, *hopZ*. Whether the increased mRNA expression in such genes is accompanied with an increased competent protein or just a compensatory increase in mRNA expression for the inhibition of *H. pylori* growth and adherence activity remains to be further studied. On the other hand, variable expression of cell receptors in a single host and genetic variability of receptor expression in different hosts make the adherence system

very complex. Host receptor expression is up-regulated following *H. pylori* adherence<sup>[31]</sup>. In this study, the impaired adherence of *H. pylori* to AGS cells in the presence of celecoxib down-regulated the host receptor expression. In this condition, although the expression of *H. pylori* adhesins increases, the adherence of *H. pylori* to AGS cells may decrease.

Urease in *H. pylori* accounts for approximately 10% of the total bacterial protein pool<sup>[32]</sup>. Urease hydrolyzes urea and releases ammonia, which neutralizes acid, thus enabling survival and initial colonization. It has been shown that urease activity is essential for the initial bacterial colonization<sup>[33-35]</sup>. Anti-ulcer drug, ecabet, interferes with *H. pylori* colonization by inhibiting urease activity<sup>[36]</sup>. In the present study, celecoxib inhibited the urease activity in a dose-dependent manner, suggesting that it may further influence *H. pylori* colonization.

Urease is composed of two structural subunits, UreA and UreB. Urease gene clusters include *ureA*, *B*, *C*, *D*, *E*, *F*, *G*, *H*, *I*, with *ureA* and *ureB* being the structural genes. *ureC* and *ureD* are located before the structural genes. *ureI*, *ureE*, *ureF*, *urgG* and *ureH* are auxiliary genes. These genes and the structural genes are necessary for urease activity<sup>[37]</sup>. Urease is a metal enzyme possessing nickel and its activity depends on the two Ni<sup>2+</sup> inserted into its 6 active sites. The insertion process is accomplished by proteins encoded by auxiliary genes in a urease gene cluster. At present, a variety of identified proteins can regulate the activity of urease by influencing nickel ions. Besides proteins, different ion concentrations also accommodate urease activity<sup>[38]</sup>. Urease inhibitors can be generally classified into active site-directed (substrate-like) and mechanism-directed inhibitors. Since active site-directed inhibitors bridge the two paramagnetic nickel ions in the active site of urease, the octahedral nickel ions and the amino acid residues in the active site-directed inhibitors are in an orientation similar to those of the urease substrate, the mechanism-directed inhibitors are designed to interfere with the urease's catalysis mechanism leading to enzyme inactivation. In the present study, celecoxib inhibited the urease activity in *H. pylori*, but increased the mRNA expression levels in *ureA* and *ureB*. The mechanism still remains unclear. Further studies are needed to determine whether alterations occur at protein translation or modification level or some other mechanisms are involved.

The motility of *H. pylori* is considered another colonization factor. Less motile strains are less able to colonize or survive in the host than fully motile strains. It has been demonstrated that the degree of the motility of *H. pylori* strains is correlated with the degree of infectivity in gnotobiotic piglets. The most motile strains have a 100% infection rate, while the least motile strains have an infection rate of only 17%<sup>[39]</sup>. Strains without flagella or flagellar mutant strains cannot colonize the gastric mucosa, thus losing their pathogenicity. The flagella consist mainly of the flagellins, FlaA and FlaB.

Both genes coding for these flagellins are necessary for the full motility of *H. pylori*. Elimination of *flaB* yields normal-looking flagella that retain some functions and propel about 60% of the bacteria<sup>[40,41]</sup>. Elimination of *flaA* yields truncated flagella that only slightly move the bacteria. Elimination of both flagellins results in aflagellated immobile bacteria<sup>[41]</sup>. It was reported that NSAID inhibit the movement of *Proteus vulgaris*, *Proteus mirabilis*, *Providencia rettgeri*, *Providencia stuartii* and *Burkholderia cepacia* in a dose- dependent manner<sup>[42]</sup>, and prevent emergence of *Escherichia coli* flagella by inhibiting flagellin synthesis<sup>[43]</sup>. In this study, celecoxib inhibited the motility of *H. pylori* and decreased the mRNA expression in *flaA* and *flaB*.

The relation between the degree of *H. pylori* motility, cytokine response levels and the severity of disease has been extensively studied<sup>[44,45]</sup>. The *H. pylori* motility levels are correlated with IL-8 induction<sup>[44]</sup>. Kurihara<sup>[45]</sup> also found that the degree of *H. pylori* motility is low in strains isolated from remnant gastritis, which is distinct from chronic gastritis, peptic ulceration or gastric cancer, indicating that the type and phase of *H. pylori*-related diseases dictate the selective pressure for maintenance of high *H. pylori* motility levels. Further study is needed to demonstrate whether celecoxib prevents the progress of *H. pylori*-related diseases by inhibiting *H. pylori* motility.

Besides the flagella, the shape of *H. pylori* strains makes them possible to penetrate the mucin layer where they come into contact with the gastric epithelial cells. In the present study, transmission electron microscopy showed that celecoxib could impair the formation of *H. pylori*, break the bacterial outer membrane, and destruct its structure. Since the spiral shape of *H. pylori* is one of the important virulence factors, celecoxib-related morphological changes may have an impact on the progress of *H. pylori*-induced diseases.

Gastric carcinoma is the forth most common cancer and the second leading cause of cancer-related deaths worldwide. The high mortality is largely attributed to the huge number of at-risk individuals. Chemoprevention appears to be the most promising approach in reducing the incidence and mortality of *H. pylori*-related gastric cancer. WHO defined *H. pylori* as a risk factor for gastric carcinoma and classified *H. pylori* strains as group I carcinogen in 1994<sup>[46]</sup>. The prevalence of *H. pylori* infection increases with age<sup>[47]</sup>, and 50% of NSAID users are over 60-year old. NSAID contribute to the chemoprevention of gastric cancer and prevention of lymphatic metastasis by inhibiting angiogenesis and inducing apoptosis of epithelial cells through the COX-dependent and independent pathway. It has been shown that long-term intake of NSAID and aspirin can significantly reduce the incidence of non-cardial gastric cancer in a dose-dependent manner<sup>[48]</sup>. The results of our study further suggest that celecoxib can reduce *H. pylori* colonization, thus attenuating the pathogenesis in gastric mucosa. Although regular use of aspirin can prevent gastric cancer,

it may be disadvantageous for populations with a lower risk of gastric cancer. Those with a high risk of gastric cancer can use celecoxib, a selective COX-2 inhibitor with few gastrointestinal side-effects.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Use of non-steroidal anti-inflammatory drugs (NSAID) and *Helicobacter pylori* (*H. pylori*) infection are the two main etiological factors for gastric injuries. Subjects taking NSAID are often co-infected with *H. pylori*, but the interaction between NSAID taking and infection with *H. pylori* remains unclear. Data from clinical and epidemiological studies are still controversial.

### Research frontiers

The relation between NSAID and *H. pylori* in the pathogenesis of gastric mucosal damage is still controversial. A number of studies have shown that it is not simply additive, synergistic or antagonistic. There may be complex interactions between them which affect the pathogenicity of each other.

### Innovations and breakthroughs

NSAID, as a harmful factor for gastric mucosal barrier, may be expected to increase the colonization of *H. pylori* in gastric mucosa. However, evidence from epidemiological studies indicates a lower prevalence of *H. pylori* infection in patients taking NSAID, which may partially be explained by the fact that celecoxib destructs the normal structure of *H. pylori*, and inhibits the flagellar motility, the adherence of *H. pylori* to AGS cells and the urease activity, as observed in this study.

### Applications

Colonization of *H. pylori* is a crucial initial step in the pathogenesis of *H. pylori* in gastric mucosa. The present study suggested that celecoxib could reduce the colonization of *H. pylori*, thus attenuating the pathogenicity in gastric mucosa.

### Terminology

SYBR green real-time polymerase chain reaction (PCR): a quantitative PCR method for determination of the copy number of PCR templates such as DNA or cDNA in a PCR reaction. SYBR green: A dye that binds to the minor groove of double stranded DNA. When SYBR green dye binds to double stranded DNA, the intensity of fluorescent emissions increases. As more double stranded amplicons are produced, SYBR green dye signals increase.

### Peer review

The study described the effect of celecoxib on *H. pylori*. The study is well-designed. The experimental data are sufficient to support its conclusion.

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## MicroRNA155 is induced in activated CD4<sup>+</sup> T cells of TNBS-induced colitis in mice

Da-Fan Chen, Bang-Dong Gong, Qing Xie, Qi-Wen Ben, Jun Liu, Yao-Zong Yuan

Da-Fan Chen, Jun Liu, Yao-Zong Yuan, Department of Gastroenterology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, 197 Ruijin Second Road, Shanghai 200025, China

Bang-Dong Gong, Qing Xie, Department of Infectious Disease, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, 197 Ruijin Second Road, Shanghai 200025, China  
 Qi-Wen Ben, Department of Gastroenterology, Changhai Hospital of Second Military Medical University, Shanghai 200433, China

**Author contributions:** Yuan YZ and Xie Q designed the research; Chen DF and Gong BD performed the majority of experiments; Ben QW and Liu J provided the new reagents and analytic tools and were also involved in editing the manuscript; Yuan YZ, Xie Q and Chen DF analyzed the data; Yuan YZ and Chen DF wrote the paper.

**Correspondence to:** Yao-Zong Yuan, Professor, Department of Gastroenterology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, 197 Ruijin Second Road, Shanghai 200025, China. [yyz28@medmail.com.cn](mailto:yyz28@medmail.com.cn)

Telephone: +86-21-64370045 Fax: +86-21-64150773

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### Abstract

**AIM:** To investigate the expression of microRNA155 (miRNA155) in trinitrobenzene sulphonic acid (TNBS)-induced colitis and the relationship between miRNA155 and tumor necrosis factor (TNF) expressions.

**METHODS:** In TNBS colitis mice, miRNA155 and TNF mRNA expressions were measured in colons and CD4<sup>+</sup> T cells of draining lymph nodes (LNs). CD4<sup>+</sup> T cells were cultured *in vitro* with or without anti-CD3/CD28 antibody, and the expressions of miRNA155 and TNF mRNA in cells and TNF concentration in culture media were examined.

**RESULTS:** miRNA155 and TNF mRNA expressions in colons and in cells of LNs were significantly increased

in TNBS colitis compared with controls. In TNBS colitis, miRNA155 and TNF mRNA expressions in CD4<sup>+</sup> T cells of LNs and TNF concentration in CD4<sup>+</sup> T cells culture media increased compared with controls. When cultured with anti-CD3/CD28 antibody, miRNA155 and TNF mRNA expressions in CD4<sup>+</sup> T cells and TNF concentration in the CD4<sup>+</sup> T cells culture media were significantly higher than those cultured without anti-CD3/CD28 antibody. Following analysis using the Pearson's correlation coefficient, miRNA155 expression had a significant positive correlation with either TNF mRNA expression in CD4<sup>+</sup> T cells ( $r = 0.860$ ,  $P < 0.05$ ) or TNF concentration in CD4<sup>+</sup> T cells culture media ( $r = 0.892$ ,  $P < 0.05$ ).

**CONCLUSION:** miRNA155 is induced in colons and activated CD4<sup>+</sup> T cells in TNBS colitis, and the levels of miRNA155 and TNF expressions have a significant positive correlation.

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**Key words:** Colitis; Crohn's disease; Lymph nodes; microRNA; Tumor necrosis factor

**Peer reviewers:** Pingchang Yang, MD, PhD, Department of Pathology & Molecular Medicine, McMaster University, BBI-T3330, 50 Charlton Ave East, Hamilton, L8N 4A6, Canada; Mitsunori Yamakawa, Professor, Department of Pathological Diagnostics, Yamagata University, Faculty of Medicine, 2-2-2 Iida-Nishi, Yamagata 990-9585, Japan

Chen DF, Gong BD, Xie Q, Ben QW, Liu J, Yuan YZ. MicroRNA155 is induced in activated CD4<sup>+</sup> T cells of TNBS-induced colitis in mice. *World J Gastroenterol* 2010; 16(7): 854-861 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i7/854.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i7.854>

### INTRODUCTION

Crohn's disease (CD) is a chronic relapsing inflammatory

disorder of the gastrointestinal tract. CD is thought to be a multifactorial, polygenic disease, however, the exact pathogenesis of CD is still unclear<sup>[1-3]</sup>. Some studies have suggested that CD is mediated by T helper type 1 (Th1) cells producing interferon- $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor (TNF) and interleukin (IL)-12<sup>[4-8]</sup>. TNF, which is a proinflammatory cytokine secreted by monocytes/macrophages, T cells, B cells, NK cells and mast cells, is necessary for both the initiation and persistence of the Th1 response and contributes to intestinal inflammation in CD patients<sup>[9-11]</sup>. Today, many animal models of CD are available, and each model has reflected one aspect of human CD. Hapten-induced colitis, in which trinitrobenzene sulfonic acid (TNBS) is delivered intrarectally to rodents, displays Th1 activity of local CD4<sup>+</sup> T cells and is considered to closely resemble CD<sup>[12-14]</sup>.

MicroRNAs (miRNAs) are a group of small non-coding RNAs which posttranscriptionally regulate gene expression<sup>[15,16]</sup>. More than 700 miRNAs have been identified in mammals and are involved in a wide variety of biological processes<sup>[15,17]</sup>. They are transcribed as primary transcripts by RNA polymerase II, cleaved into a precursor miRNA by the Drosha nuclease, and exported from the nucleus by exportin 5. In the cytoplasm, the precursor miRNAs are further processed by the Dicer nuclease and are incorporated into the RNA-induced silencing complexes. The miRNAs guide the RNA-induced silencing complexes binding to the mRNAs 3'-untranslated regions (UTRs), resulting in either the mRNAs degradation or translational inhibition<sup>[18-22]</sup>.

Although the exact functions of most miRNAs have yet to be elucidated, many studies have suggested that miRNAs have been implicated in many aspects of innate and acquired immunity, such as differentiation, survival and functions of immune cells, and the intracellular signaling pathways<sup>[23-25]</sup>. miRNA155, which was reported to be involved in the production of TNF and regulation of immunity<sup>[26,27]</sup>, is processed from an exon of the noncoding RNA known as bic<sup>[27,28]</sup>. In this study, we mainly evaluated the expression of miRNA155 in TNBS colitis, and speculated on the relationship between miRNA155 and TNF expressions.

## MATERIALS AND METHODS

### Mice

Female 6- to 8-wk-old BALB/C mice weighing 18-22 g were obtained from Shanghai Experimental Animals Centre of Chinese Academy of Sciences. The mice were maintained under specific pathogen free conditions in a room at 23  $\pm$  2°C with a 12 h light-dark cycle, and had free access to food and water during the study. They were allowed to acclimate to these conditions for at least seven days before inclusion in an experiment. All procedures were approved by the Investigation and Ethics Committee of Shanghai Jiaotong University School of Medicine.

### Establishment of TNBS-induced colitis

Colitis was induced in BALB/C mice as described previously with some modification<sup>[13,29]</sup>. For sensitization, a 2 cm  $\times$  2 cm field of the abdominal skin was shaved and 150  $\mu$ L of 2.5% TNBS (Sigma Chemical Co., St. Louis, MO, USA) in 50% ethanol was applied. Five days after sensitization, mice were anesthetized slightly with an intraperitoneal injection of xylazine (10 mg/kg) and ketamine (50 mg/kg), and then intrarectally administered 100  $\mu$ L solution of 1% TNBS dissolved in 45% ethanol *via* a 3.5-French catheter equipped with a 1 mL syringe. The tip of the catheter was inserted 4 cm proximal to the anal verge. Mice were held in a vertical position for 1 min after the intrarectal injection. Control mice were given 100  $\mu$ L 45% ethanol solution without TNBS using the same technique.

### Clinical observations and histologic assessments of colitis

Daily body weight, stool consistency, and occult blood (measured by the guaiac reaction, hemoccult) were assessed. Three days after intrarectal injection, mice were killed by cervical dislocation after being anesthetized with diethyl ether and entire colons were removed from the cecum to the anus, and flushed with saline. Colon specimens located 2 cm above the anal verge were achieved. One section of the specimen was fixed overnight in 4% paraformaldehyde and embedded in paraffin, and then sections stained with hematoxylin and eosin were examined. The other sections of the colon were immediately frozen in liquid nitrogen after dissection and used for quantification of miRNA155, IL-1 $\beta$ , IL-6, TNF and IFN- $\gamma$  mRNA.

### Cell preparation

Three days after intrarectal injection, colon draining lymph nodes (LNs) were aseptically removed. Single-cell suspensions were prepared by pressing LNs through a 40  $\mu$ m cell strainer using the plunger of a 1 mL syringe. CD4<sup>+</sup> T cells were isolated from the cell suspensions with magnetic beads labeled with anti-CD4 (L3T4) monoclonal antibodies (Miltenyi Biotec Inc, Bergisch Gladbach, Germany). Cells were incubated in media (RPMI 1640 supplemented with 100 U/mL penicillin/streptomycin, 2 mmol/L L-glutamine, 50 mol/L 2-mercaptoethanol, and 10% fetal calf serum) at 8  $\times$  10<sup>4</sup> cells in 150  $\mu$ L media per well in 96-well plates for 48 h in the absence or presence of dynabeads CD3/CD28 T cells activator (Invitrogen, Carlsbad, CA, USA) at a concentration of 2  $\mu$ L/well.

### Enzyme-linked immunosorbent assay (ELISA)

After incubation for 48 h, the supernatants of the culture media were harvested and assayed for TNF concentration by ELISA using an ELISA kit (R&D Systems, Minneapolis, MN, USA).

Table 1 Primers used for RT or PCR of mRNA or miRNA

Gene name		Primer sequences (5'-3')
IL-1 $\beta$	Sense	GCAACTGTTCTGAACTCAACT
	Antisense	ATCTTTTGGGGTCCGTCAACT
IL-6	Sense	CCACTTCACAAGTCGGAGGCTTA
	Antisense	GCAAGTGCATCATCGTTGTCATAC
IFN- $\gamma$	Sense	TCAAGTGGCATAGATGTGGAAGAA
	Antisense	TGGCTCTGCAGGATTTTCATG
TNF	Sense	CCACCACGCTCTTCTGTCTAC
	Antisense	TGGGCTACAGGCTTGTCACT
$\beta$ -actin	Sense	CTAGGCACCAAGGGTGTGAT
	Antisense	TGCCAGATCTTCTCCATGTC
miRNA155	Stem-loop primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGACTGGATACGACACCCCT
	Sense	GCCCGCTTAATGCTAATTGTGAT
	Antisense	GTGCAGGGTCCGAGGT
U6	Sense	CTCGCTTCGGCAGCACA
	Antisense	AACGCTTCACGAATTTGCGT

RT: Reverse transcription; PCR: Polymerase chain reaction.

### Quantitative real-time polymerase chain reaction (qPCR) analysis of mRNA detection

Total RNA from cells and colon samples were extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentrations were determined with a spectrophotometer (Eppendorf, Hamburg, Germany). 0.2-0.5  $\mu$ g of total RNA was reverse transcribed, and RNA expression levels were quantified by sybergreen-based qPCR using a sequence detection system (Prism 7500; Applied Biosystems Inc., Foster City, USA).  $\beta$ -actin served as the endogenous control. Gene-specific primers for the reported genes are indicated in Table 1. To evaluate the relative expression of each target gene, the comparative threshold (Ct) cycle method was used according to the manufacturer's manual. The threshold cycle (Ct) for each gene was determined as the cycle number at which the reaction crossed an arbitrarily placed threshold, and the relative amount of each mRNA to  $\beta$ -actin was described using the formula  $2^{-\Delta Ct}$  where  $\Delta Ct = (Ct_{mRNA} - Ct_{\beta-actin})$ .

### qPCR analysis of miRNA detection

Total RNA from cells and colon samples were isolated using the TRIzol reagent. Real time quantitative analyses for miRNAs were performed using stem-loop RT-PCR<sup>[30,31]</sup>. 0.2-0.5  $\mu$ g of total RNA was reverse transcribed to cDNA using a target-specific stem-loop primer indicated in Table 1. qPCR was performed on a sequence detection system (Prism 7500; Applied Biosystems Inc., Foster City, USA). In brief, cDNA in water was added to 5  $\mu$ L of the 2  $\times$  SYBR green master mix (Applied Biosystems Inc., Foster City, USA), 400 nmol/L of gene-specific primer and water to 10  $\mu$ L. The reactions were amplified at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. U6 small nuclear RNA (U6) served as the endogenous control. At the end of the qPCR, the thermal denaturation protocol was run to determine the number of products that were

present in the reaction. The relative amount of miRNA to U6 was calculated using the Ct cycle method. The relative amount of each miRNA to U6 was described using the formula  $2^{-\Delta Ct}$  where  $\Delta Ct = (Ct_{miRNA} - Ct_{U6})$ <sup>[30,31]</sup>.

### Statistical analysis

Each group contained 5-8 mice, and results were expressed as the mean  $\pm$  SD. A comparison between the two groups was made using the Student's *t*-test. The relationship between the two targets was tested with Pearson's correlation coefficient. Differences were considered significant at  $P < 0.05$ .

## RESULTS

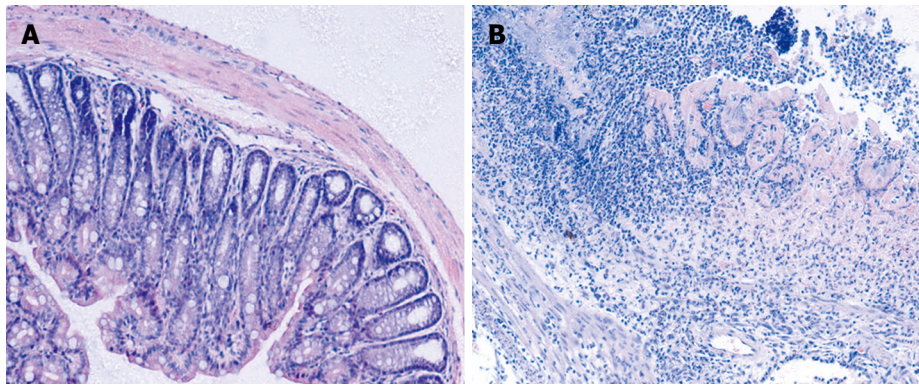
### Successful establishment of experimental colitis

Administration of TNBS to presensitized mice resulted in a severe illness characterized by bloody diarrhea, rectal prolapse accompanied by sustained weight loss. At day 3-4, the disease reached a peak. Histologic examination of the colons showed severe depletion of mucin-producing goblet and epithelial cells, large areas of ulceration, a marked increase in the thickness of the muscular layer, and transmural inflammation involving all colon wall layers with infiltration of lymphocytes, macrophages and neutrophils extending from the mucosa into the muscular and serosal layers (Figure 1).

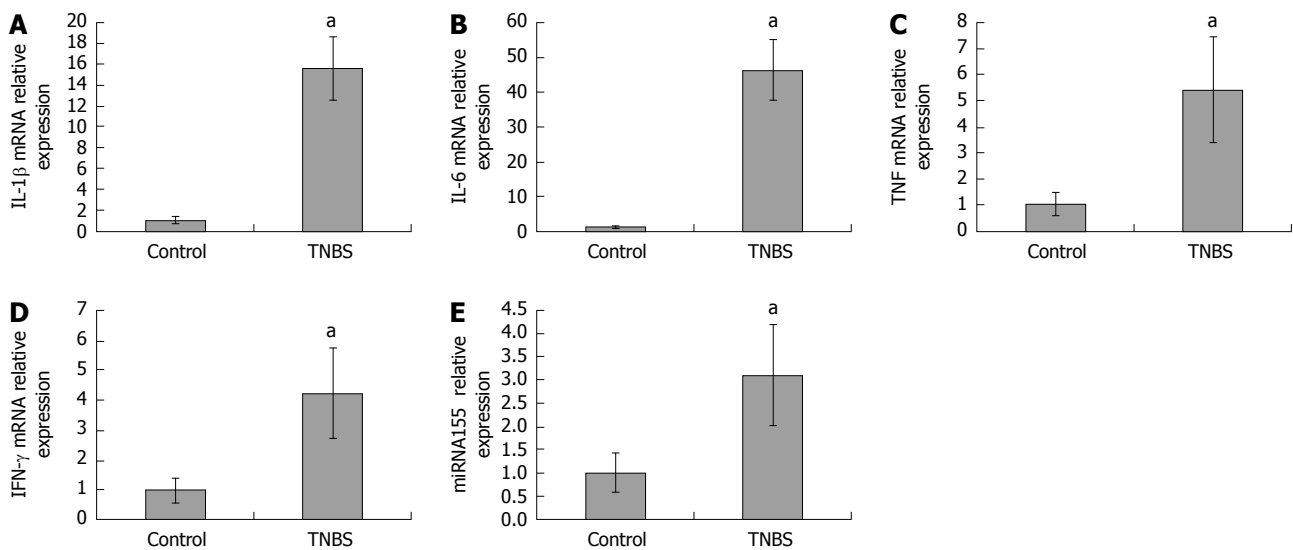
### Increased expressions of miRNA155, IL-1 $\beta$ , IL-6, TNF, and IFN- $\gamma$ mRNA in the colons of TNBS-induced colitis mice

To investigate miRNA155 and cytokine expressions in colons, we assessed miRNA155, IL-1 $\beta$ , IL-6, TNF and IFN- $\gamma$  mRNA in colon homogenates by qPCR. We found that miRNA155 expression in colon homogenates was significantly increased in TNBS-induced colitis, which was 3.10-fold higher than in control mice. IL-1 $\beta$ , IL-6, TNF, and IFN- $\gamma$  mRNA expressions in colon homogenates were also significantly increased in TNBS-induced colitis,





**Figure 1** Representative microphotographs of the colon sections stained with hematoxylin and eosin (original magnification,  $\times 100$ ). A: Control colon; B: Colon was obtained on day 3 after intrarectal TNBS.



**Figure 2** IL-1 $\beta$  (A), IL-6 (B), TNF (C), IFN- $\gamma$  (D) mRNA and miRNA155 (E) expressions in colon homogenates of control and TNBS colitis groups. qPCR-derived IL-1 $\beta$ , IL-6, TNF, IFN- $\gamma$ , and miRNA155 expressions in colon homogenates were significantly increased in TNBS-induced colitis. <sup>a</sup> $P < 0.05$  vs control.

and were 15.58-, 46.34-, 5.43-, and 4.23-fold higher than in control mice, respectively (Figure 2).

#### **miRNA155 and TNF mRNA expressions in the CD4<sup>+</sup> T cells of colon draining LNs and TNF concentration in CD4<sup>+</sup> T cells culture media**

As CD4<sup>+</sup> T cells play a central role in Th1 response, we evaluated the levels of miRNA155 and TNF mRNA expressions in LNs and CD4<sup>+</sup> T cells from LNs. In TNBS colitis, miRNA155 in draining LNs or CD4<sup>+</sup> T cells from LNs increased and were 3.74- and 3.07-fold higher than in controls, respectively (Figure 3). TNF mRNA in draining LNs or CD4<sup>+</sup> T cells from LNs of TNBS colitis were 3.34- and 2.06-fold higher than in controls (Figure 3). In the CD4<sup>+</sup> T cells culture media, the concentration of TNF protein increased in TNBS colitis and was 4.19-fold higher than in controls ( $128.04 \pm 38.71$  pg/mL *vs*  $30.55 \pm 8.37$  pg/mL,  $P < 0.05$ , Figure 4).

#### **Anti-CD3/CD28 antibody promoted the miRNA155 and TNF mRNA expressions in the CD4<sup>+</sup> T cells and the TNF concentration in the supernatants of CD4<sup>+</sup> T cells culture media**

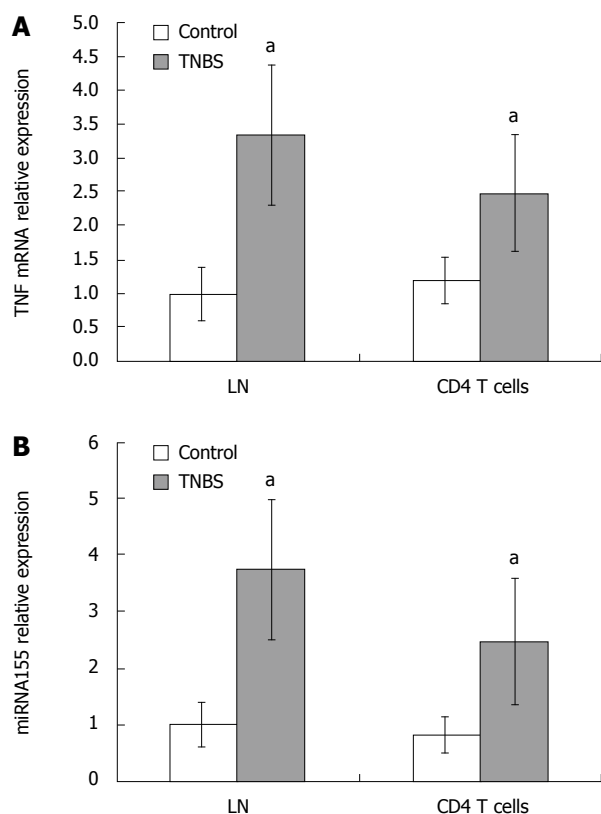
To study whether the T cell receptor (TCR) and the

costimulatory receptor were involved in miRNA155 expression, and to study the relationship between miRNA155 and TNF production, we used anti-CD3/CD28 antibody to stimulate CD4<sup>+</sup> T cells. When cultured with anti-CD3/CD28 antibody, the CD4<sup>+</sup> T cells became larger and displayed an activated appearance. In control and TNBS colitis, the miRNA155 expressions in CD4<sup>+</sup> T cells cultured with anti-CD3/CD28 antibody were 4.72- and 3.61-fold higher than cells cultured without anti-CD3/CD28 antibody, and the TNF mRNA expression in CD4<sup>+</sup> T cells cultured with anti-CD3/CD28 antibody were 3.42- and 3.03-fold higher than cells cultured without anti-CD3/CD28 antibody, respectively (Figure 4). The TNF concentration in the supernatants of culture media which contained anti-CD3/CD28 antibody increased both in control and TNBS colitis mice, and were 4.28- and 6.87-fold higher than in media cultured without anti-CD3/CD28 antibody ( $135.66 \pm 32.11$  pg/mL *vs*  $30.55 \pm 8.37$  pg/mL,  $P < 0.05$ ;  $850.94 \pm 219.49$  pg/mL *vs*  $128.04 \pm 38.71$  pg/mL,  $P < 0.05$ , Figure 4).

#### **Relationship between miRNA155 and TNF gene expressions**

Since TNF plays an important role in the pathogenesis





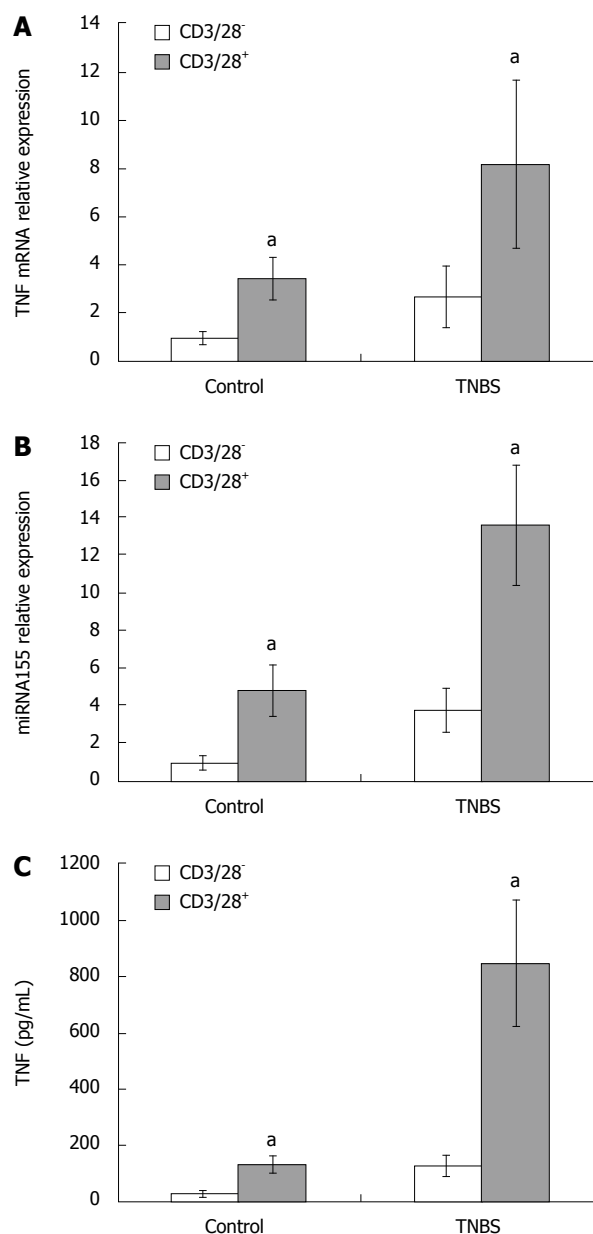
**Figure 3** TNF mRNA (A) and miRNA155 (B) expressions in LNs and CD4<sup>+</sup> T cells in control and TNBS colitis groups. TNF mRNA and miRNA155 expressions in LNs and CD4<sup>+</sup> T cells were significantly increased in TNBS-induced colitis. <sup>a</sup> $P < 0.05$  vs control.

of CD, we evaluated the potential correlation between miRNA155 and TNF gene expressions. Our data indicated that there was a significant positive correlation between miRNA155 and TNF mRNA expressions in CD4<sup>+</sup> T cells ( $r = 0.860$ ,  $P < 0.05$ , Figure 5), and miRNA155 expression in CD4<sup>+</sup> T cells and TNF protein concentration in CD4<sup>+</sup> T cells culture media ( $r = 0.892$ ,  $P < 0.05$ , Figure 5).

## DISCUSSION

As the exact etiology of CD is still unclear, TNBS-induced colitis was used to study many important aspects of the pathogenesis in CD. TNBS colitis is thought to resemble CD because of the mucosal inflammation mediated by excessive IFN- $\gamma$ , TNF and other proinflammatory cytokine production<sup>[12-14]</sup>. In agreement with previous results, our data also showed that TNBS colitis is a Th1 model with elevated IFN- $\gamma$  and TNF expressions in colon. In this study, we found that miRNA155 was increased in colons and in CD4<sup>+</sup> T cells of LNs in TNBS colitis and the levels of miRNA155 and TNF expressions had a significant positive correlation.

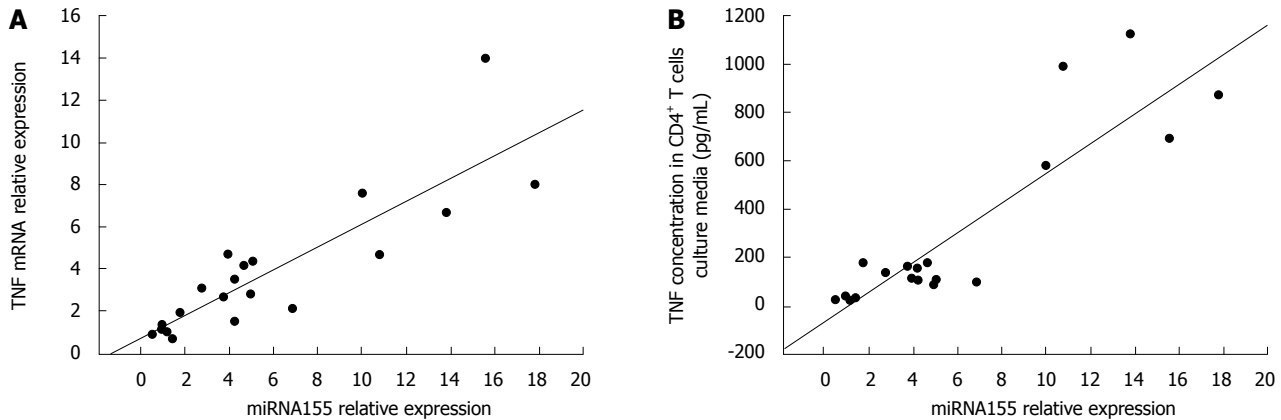
MiRNAs are a group of small noncoding RNAs which are thought to posttranscriptionally regulate gene expressions. Dysregulation of miRNAs has been associated with several autoimmune diseases<sup>[32,33]</sup>. miRNA155 is processed from an exon of the noncoding RNA. Some



**Figure 4** TNF mRNA (A) and miRNA155 (B) expressions in CD4<sup>+</sup> T cells cultured with or without anti-CD3/CD28 antibody, and the TNF protein expression (C) in the supernatants of CD4<sup>+</sup> T cells culture media with or without anti-CD3/CD28 antibody. TNF mRNA and miRNA155 expressions in CD4<sup>+</sup> T cells cultured with anti-CD3/CD28 antibody, and the TNF concentration in the supernatants of culture media containing anti-CD3/CD28 antibody increased in comparison to those in cells or media cultured without anti-CD3/CD28 antibody. <sup>a</sup> $P < 0.05$  vs control.

studies have shown that miRNA155 is required for normal innate and acquired immunity<sup>[26,27,34,35]</sup>.

In macrophages, miRNA155 was reported to enhance the production of TNF, but may target transcripts encoding for several proteins, such as I $\kappa$ B $\epsilon$  kinase and Fas-associated death domain protein whose ultimate function results in the activation of the lipopolysaccharide (LPS)/TNF pathway<sup>[26]</sup>. Therefore, miRNA155 may exert both positive and negative effects on the activation of innate immunity. In acquired immunity, miRNA155 was reported to affect lymphoid cell development<sup>[27,34,36]</sup>. In



**Figure 5** Relationships between miRNA155 expression in CD4<sup>+</sup> T cells and TNF mRNA expression in CD4<sup>+</sup> T cells (A) or TNF concentration in CD4<sup>+</sup> T cells culture media (B). Data analyzed by Pearson's correlation coefficient.

miRNA155<sup>-/-</sup> mice, CD4<sup>+</sup> T cells are intrinsically biased toward Th2 differentiation. miRNA155 modulates the level of a transcription factor c-Maf in T cells and is likely to induce the attenuation of Th2 cell responses *in vivo*<sup>[34]</sup>. With regard to B cells, several studies have proved that B cells require miRNA155 for normal production of isotype-switched, high-affinity antibodies and for a memory response<sup>[27,36]</sup>. However, the expression of miRNA155 and its relationship with CD remains unclear. In this paper, we reported that miRNA155 expression is increased in the colon and its draining LNs in TNBS colitis. This result hints that miRNA155 may have a role in the pathogenesis of TNBS colitis. As CD4<sup>+</sup> T cells play a central role in Th1 response, we determined the level of miRNA155 expression in the CD4<sup>+</sup> T cells from LNs in TNBS colitis and found that miRNA155 was increased in CD4<sup>+</sup> T cells in TNBS colitis.

Considering TNF plays an important role in the pathogenesis of CD, we evaluated the relationship between miRNA155 and TNF expression in CD4<sup>+</sup> T cells. Our data indicated that the gene expression of miRNA155 in CD4<sup>+</sup> T cells of draining LNs in TNBS colitis has a significant positive correlation with either TNF mRNA in CD4<sup>+</sup> T cells or the concentration of TNF protein in the culture media. Some reports have shown that miRNA155 may be involved in TNF production<sup>[26,27]</sup>. Eu-miR-155 transgenic mice which specifically overexpress miRNA155 in B cells produced more TNF when challenged with LPS, and were hypersensitive to LPS/D-galactosamine-induced septic shock<sup>[26]</sup>. In addition, miRNA155<sup>-/-</sup> B cells produce less TNF when activated *in vitro* by BCR cross-linking<sup>[27]</sup>. Scientists have found that the miRNA155 effects on TNF production are at both the transcriptional and posttranscriptional level<sup>[26,27]</sup>. More TNF transcripts were observed in wild-type mice compared with miRNA155<sup>-/-</sup> mice<sup>[27]</sup>. At the posttranscriptional level, miRNA155 may target the 3'-UTR of TNF transcripts to increase the stability of transcripts and enhance translation<sup>[26]</sup>. Our results showed that the TNF protein concentration in the CD4<sup>+</sup> T cells culture media increased more than the TNF mRNA in CD4<sup>+</sup> T cells, which is in agreement with the

proposition that miRNA155 regulated TNF production at both the transcriptional and posttranscriptional levels. These findings suggested that miRNA155 may prompt the production of TNF in some types of immune cells. From our results, miRNA155 increased and had a positive relationship with TNF expression in CD4<sup>+</sup> T cells, therefore we suppose that miRNA155 may influence TNF expression in CD4<sup>+</sup> T cells and is possibly involved in the pathogenesis of TNBS colitis.

The exact mechanism of miRNA155 expression regulation in CD4<sup>+</sup> T cells remains unclear. To study the role of TCR in miRNA155 expression and the relationship between miRNA155 expression and the activation of CD4<sup>+</sup> T cells, we used anti-CD3/CD28 antibody to stimulate CD4<sup>+</sup> T cells, and found that the miRNA155 expression level was elevated in CD4<sup>+</sup> T cells when cultured with the antibody. Stimulation of the TCR/CD3 complex and costimulatory receptor CD28 in CD4<sup>+</sup> T cells by anti-CD3/CD28 antibody can lead to activation of multiple transcription factors, including NF-AT and NF-κB, which ultimately control transcription of cytokines and T-cell proliferation<sup>[37]</sup>. miRNA155 level was reported to have a link with the NF-κB pathway. NF-κB activity is required for the change in miRNA155 levels following TNF stimulation in macrophages, however, this relationship remains elusive<sup>[26]</sup>. The precise mechanism of the regulation of miRNA155 expression may be a new issue for future research.

In conclusion, miRNA155 expression was found to increase in colons and activated CD4<sup>+</sup> T cells in TNBS colitis. A significant positive correlation was observed between miRNA155 expression in CD4<sup>+</sup> T cells and the expression of TNF mRNA in CD4<sup>+</sup> T cells and the TNF protein concentration in CD4<sup>+</sup> T cells culture media. miRNA155 may be involved in the activation and TNF production of CD4<sup>+</sup> T cells in TNBS colitis.

## COMMENTS

### Background

MicroRNAs (miRNAs) are a group of small noncoding RNAs which post-

transcriptionally regulate gene expression. miRNA155 is reported to be involved in the production of TNF and the regulation of immunity. Tumor necrosis factor (TNF) plays an important role in the pathogenesis of Crohn's disease (CD). However, the expression of miRNA155 and its relationship with TNF production in CD remains unclear.

### Research frontiers

The exact etiology of CD is still unclear. MiRNA research may represent a new way to explore the pathogenesis of CD. The expressions and functions of most miRNAs in CD remain a mystery.

### Innovations and breakthroughs

The study found that miRNA155 is increased in colons and draining lymph nodes. In CD4<sup>+</sup> T cells which play a central role in Th1 response, miRNA155 expression is higher in TNBS colitis than in controls. As CD4<sup>+</sup> T cells were activated by anti-CD3/CD28 antibody, the miRNA155 expression was higher than that when cultured without anti-CD3/CD28 antibody. In addition, the levels of miRNA155 and TNF expressions had a significant positive correlation.

### Applications

The results of this study may enhance our understanding of the pathogenesis of CD. By investigating the exact function of miRNA155 in the pathogenesis of CD, the findings may contribute to improvements in future drug therapies.

### Terminology

MiRNA: MicroRNAs (miRNAs) are a group of small noncoding RNAs which post-transcriptionally regulate gene expressions. More than 700 miRNAs have been identified in mammals and are involved in a wide variety of biological processes. Stem-loop RT-PCR: RNA is reverse transcribed to cDNA using a gene-specific stem-loop RT primer, and then the RT products are quantified using conventional real time PCR.

### Peer review

This paper reports a set of relatively new data about the pathogenic mechanism of IBD that may be referred by those investigators working on IBD.

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## Colorectal cancer prognosis twenty years later

Luis Bujanda, Cristina Sarasqueta, Elisabeth Hijona, Lander Hijona, Angel Cosme, Ines Gil, Jose Luis Elorza, Jose I Asensio, Santiago Larburu, José M Enríquez-Navascués, Rodrigo Jover, Francesc Balaguer, Xavier Llor, Xavier Bessa, Montserrat Andreu, Artemio Paya, Antoni Castells, Gastrointestinal Oncology Group of the Spanish Gastroenterological Association

Luis Bujanda, Elisabeth Hijona, Lander Hijona, Angel Cosme, Ines Gil, Department of Gastroenterology, Donostia Hospital, University of Basque Country, Centro de Investigación Biomédica en Enfermedades Hepáticas y Digestivas (CIBEREHD), 20014 San Sebastián, Spain

Cristina Sarasqueta, Department of Epidemiology, Donostia Hospital, CIBERESP, 20014 San Sebastián, Spain

Jose Luis Elorza, Jose I Asensio, Santiago Larburu, José M Enríquez-Navascués, Department of Surgery, Donostia Hospital, 20014 San Sebastián, Spain

Rodrigo Jover, Antoni Castells, Department of Gastroenterology, General Hospital, 03010 Alicante, Spain

Francesc Balaguer, Artemio Paya, Department of Gastroenterology, Clinic Hospital, CIBEREHD, IDIBAPS, 08036 Barcelona, Spain

Xavier Llor, Digestive Diseases and Nutrition Secion, University of Illinois, Chicago, IL 60607, United States

Xavier Bessa, Montserrat Andreu, Department of Gastroenterology, Del Mar Hospital, 08003 Barcelona, Spain

Gastrointestinal Oncology Group of the Spanish Gastroenterological Association, Principal investigators in the EPICOLON Group are listed in the ACKNOWLEDGMENTS

**Author contributions:** Bujanda L and Hijona L participated in the experimental design and writing of the manuscript; Sarasqueta C performed data analysis and interpretation of results; Hijona L, Cosme A, Gil I, Elorza JL, Asensio JI, Larburu S, Enríquez-Navascués JM, Jover R, Balaguer F, Llor X, Bessa X, Andreu M, Paya A and Castells A collected and supplied the clinical data of the patients; Study organization and investigators from the Gastrointestinal Oncology Group of the Spanish Gastroenterological Association participated in the study. All authors have critically reviewed the manuscript and approved the final version. Supported by CIBEREHD is funded by the Instituto de Salud Carlos III

**Correspondence to:** Luis Bujanda, Professor, Department of Gastroenterology, CIBEREHD, University of Country Basque, Donostia Hospital, Avda. Sancho El Sabio, 17-2ºD, 20010 San Sebastián, Spain. [medik@telefonica.net](mailto:medik@telefonica.net)

Telephone: +34-943007173 Fax: +34-943471922

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### Abstract

**AIM:** To evaluate changes in colorectal cancer (CRC) survival over the last 20 years.

**METHODS:** We compared two groups of consecutive CRC patients that were prospectively recruited: Group I included 1990 patients diagnosed between 1980 and 1994. Group II included 871 patients diagnosed in 2001.

**RESULTS:** The average follow up time was 21 mo (1-229) for Group I and 50 mo (1-73.4) for Group II. Overall median survival was significantly longer in Group II than in Group I (73 mo vs 25 mo,  $P < 0.001$ ) and the difference was significant for all tumor stages. Post surgical mortality was 8% for Group I and 2% for Group II ( $P < 0.001$ ). Only 17% of Group I patients received chemotherapy compared with 50% of Group II patients ( $P < 0.001$ ).

**CONCLUSION:** Survival in colorectal cancer patients has doubled over the past 20 years. This increase seems to be partly due to the generalization in the administration of chemotherapy and to the decrease of post surgical mortality.

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**Key words:** Colon cancer; Prognosis; Survival; Chemotherapy; Surgery

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## INTRODUCTION

Colorectal cancer (CRC) is the second most common form of cancer and the second leading cause of cancer death in both men and women in most developed countries including Spain. Mortality has increased by an annual average of 2.6% for men and 0.8% for women since 1975, without variations<sup>[1]</sup>. It is estimated that CRC caused 11 900 deaths in Spain in 2000, which represents 11% of the total deaths from cancer in men and 15% of those in women<sup>[1]</sup>.

The primary treatment for this condition is surgical resection. Despite resection of all macroscopic tumors, patients whose primary tumor has penetrated the serosa or that have regional lymph node metastases at the time of surgery have high recurrence rates. An effective adjuvant program to eradicate microscopic tumor foci is clearly needed for such high-risk patients<sup>[2]</sup>. A major advance in adjuvant treatment of CRC came with trials that explored the combination of 5-fluorouracil and levamisole or leucovorin (FL). Therapy with FL reduced the overall death rate by 33% relative to surgery alone in patients with stage III disease. During the last few years, sequential advances in chemotherapy after surgical resection (adjuvant chemotherapy) have had an irrefutable and substantial benefit<sup>[3]</sup>.

The aim of the present study was to evaluate CRC survival over the last 20 years.

## MATERIALS AND METHODS

### Study population

A total of 1990 patients diagnosed with CRC between 1980 and 1994 and 2001 were included in a prospective and consecutive manner. Patients were enrolled in two time periods: Group I included 1119 patients recruited between 1980 and 1994. Group II included 871 patients recruited during 2001. Patients were recruited as part of the EPICOLON project<sup>[4,5]</sup>. Age, sex, tumor features (location, TNM stage, differentiation) and type of chemotherapy administered, if any, was collected for all patients. Stage was defined according to the 4th Edition of the TNM classification<sup>[6]</sup>.

EPICOLON was a prospective, multicenter, nationwide study that was set up to record consecutive cases of CRC in 25 hospitals in Spain over one year. The initial aim of the study was to determine the incidence and characteristics of familial forms of CRC in Spain.

Patients went through periodical follow up in medical consultations or by telephone. Patients with familial adenomatous polyposis or inflammatory bowel disease

were excluded.

In order to evaluate survival trends, Group I was divided in three subgroups: patients diagnosed between 1980 and 1985 (343); patients diagnosed between 1986 and 1990 (392); and patients diagnosed between 1991 and 1994 (384).

Death during the first 30 d post surgery was considered postoperative mortality.

All patients provided written informed consent before enrollment in the study. The study was approved by the Institutional Review Board or Ethics Committee at each center and complied with the provisions of the Good Clinical Practice guidelines.

### Statistical analysis

Continuous variables are defined with (mean  $\pm$  SD). Discrete variables are defined by absolute and relative frequencies. The function of survival is calculated by the Kaplan Meier estimator. The accumulated probability of survival is presented at 3, 5 and 10 years of monitoring. The Log-Rank test is used to evaluate the differences in survival between the different categories of independent variables. Multivariable models are constructed using Cox regression analysis and the independent effect of each variable on mortality is estimated using Relative Risks (Hazard Ratio) and the respective confidence intervals of 95%. The SPSS v.15 program was used for analysis.

## RESULTS

### Tumor features, follow up and treatment

The average follow up time was 21 mo for Group I and 50 mo for Group II. Average follow up for deceased patients was 12 mo for Group I and 16 mo for Group II. For patients still alive, follow up was 67 mo for Group I and 60 mo for Group II.

The characteristics of the patients are described in Table 1. Both groups were similar regarding tumor stages at diagnose and tumor location. Significant differences were observed in sex and age: the average age of Group I was 66.7 years compared to 69 years in Group II; the percentage of men was 61% and 57%, respectively. Slight differences were also observed in tumor differentiation: Group I had 4% of poorly differentiated tumors and Group II 8%. The greatest differences were found in the proportion of patients that received chemotherapy: 17% in Group I and 47% in Group II.

### Mortality

The probability of survival at 3 and 5 years was approximately 20% higher in Group II than in Group I. Three year survival was 44% for Group I and 65% for Group II. Five year survival was 35% for Group I and 57% for Group II.

Overall median survival was significantly longer in Group II than in Group I (73 mo *vs* 25 mo,  $P < 0.001$ ).

Table 1 Characteristics of the patients *n* (%)

	Group I ( <i>n</i> = 1119)	Group II ( <i>n</i> = 871)	<i>P</i> value
Age mean (SD)	66.7 (12.4)	69.1 (11.5)	0.000
Sex			0.027
Males	638 (57)	531 (61)	
Females	481 (43)	340 (39)	
Tumor localization			
Distal to splenic flexure	831 (75)	644 (74)	
Proximal to splenic flexure	272 (25)	227 (26)	
TNM			0.11
I	190 (17)	122 (14)	
II	403 (36)	348 (40)	
III	302 (27)	244 (28)	
IV	224 (20)	157 (18)	
Tumor differentiation			0.000
Poor	45 (4)	70 (8)	
Well-moderate	1074 (96)	801 (92)	
Receiving chemotherapy	190 (17)	409 (46.9)	0.000

Table 2 Mortality variation in relation to 4 independent risk factors

	Hazard ratio (95% CI)
Stage	
II vs I	1.6 (1.2-2.1)
III vs I	3.5 (2.7-4.6)
IV vs I	13.7 (10.4-18.0)
Group	
I vs II	2.0 (1.7-2.4)
Chemotherapy	
No vs Yes	1.6 (1.3-1.9)
Grade	
I, II vs III	1.5 (1.1-1.9)

Three years after cancer diagnosis, 21% more patients were alive in Group II than in Group I.

Multivariate analysis showed 4 independent factors associated with a higher mortality risk (Table 2).

**Survival in Group I:** In Group I survival increased over the years in parallel to chemotherapy use (Figure 1). Ten percent of patients (35 patients) received chemotherapy between 1980 and 1985, 16% (63) in the period between 1986 and 1990 and 24% (92) in the period between 1991 and 1994. The median survival was 17 mo for the period between 1980 and 1985, 28 mo for the period between 1986 and 1990 and 34 mo for the period between 1991 and 1994 (Figure 1).

### Survival and stages

Survival was greater for group II than for group I for all tumor stages (Figure 2). Postoperative mortality was 8% for Group I and 2% for Group II (Table 3). However, when patients in Group I who had died within 30 d of emergency surgery were excluded the postoperative mortality rate was equal to that in Group II. Postoperative mortality in patients operated electively in Group I was 3% (5 patients) for stage I, 2% (10 patients) for stage II, 3% (10 patients) for stage III and 4% (10 patients) for stage IV.

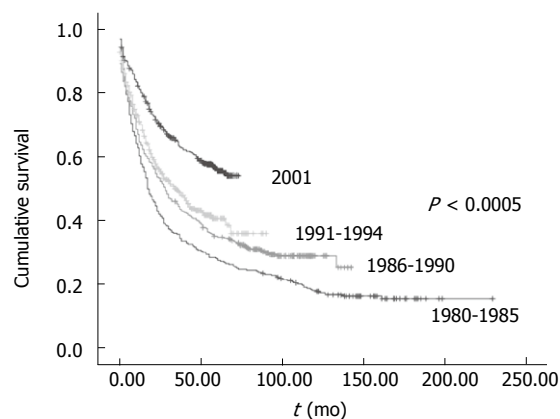


Figure 1 Kaplan-Meier analysis of overall survival in the different groups.

These data were similar to Group II. Twenty six percent more patients received chemotherapy in Group II than in Group I for stage III CRC. For the other tumor stages the percentage of patients that received chemotherapy was also greater in Group II, but to a smaller degree than stage III: 3% for stage I, 14% for stage II and 11% for stage IV.

## DISCUSSION

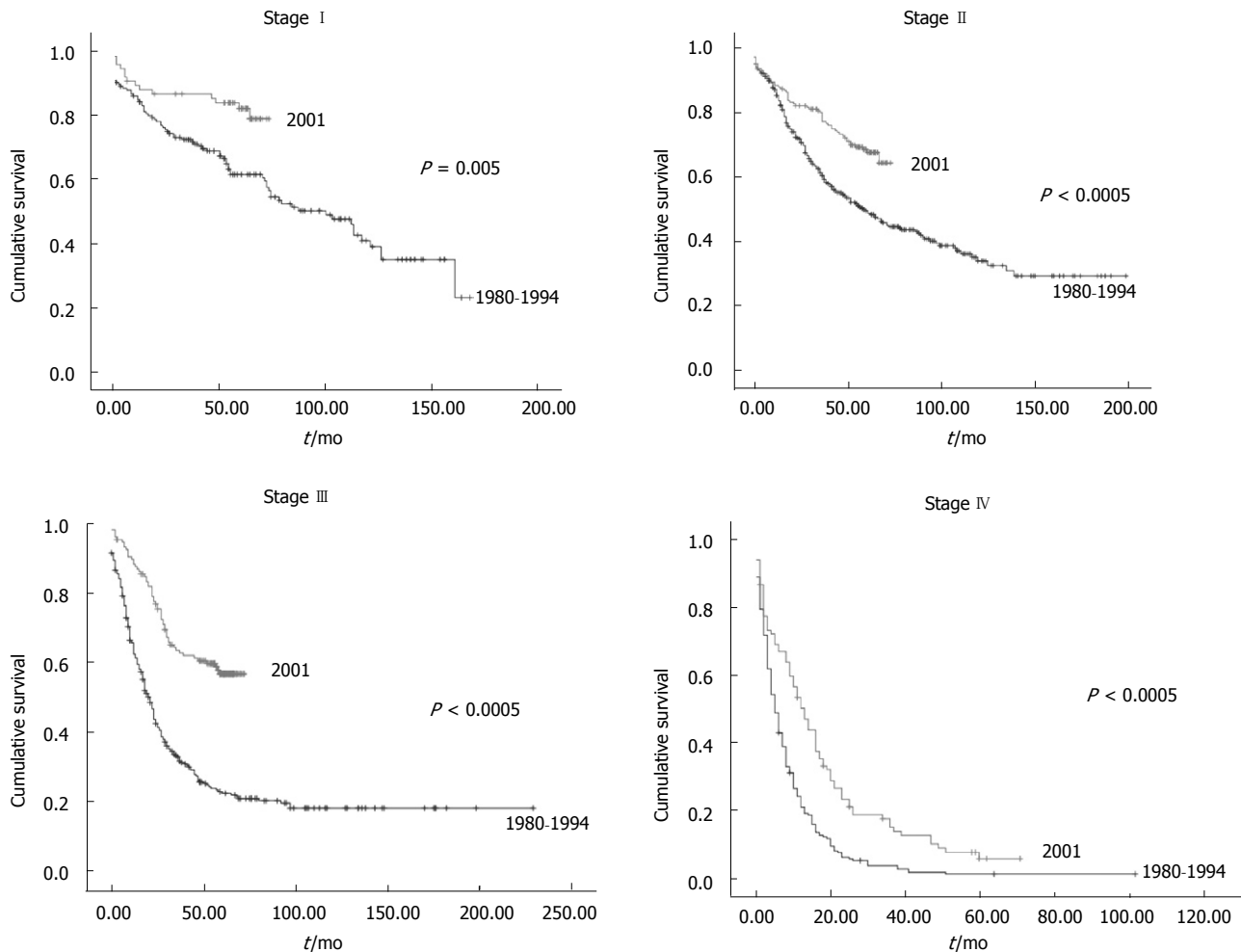
The last few years have seen great advances in treatment of CRC. The survival has increased substantially. In our study survival rate almost doubled over the last 20 years, from 35% to 57% at 5 years. Determining factors in this increased survival have been the generalization of chemotherapeutic agents and surgical advances, especially pertaining to rectal cancer.

The first chemotherapeutic agent used in the treatment of CRC was fluorouracil (FL) in 1958<sup>[7]</sup>. Since then and up to the last decade, FL has been the only drug used in CRC treatment. In 1988 a meta-analysis on the effect of FL showed a 10% reduction in the risk of death and an increase of 2.3% in survival after 5 years. In a subgroup of patients the risk of death was reduced by 17% and global survival after 5 years improved up to 34%<sup>[7]</sup>. Based on the results of this and other studies, the National Cancer Institute and the American Society of Clinical Oncology recommended FL-based post surgical treatment in their annual conference in 1997. In stage III disease, FL increases overall five year survival from about 51% to 64%<sup>[8]</sup>. The use of adjuvant FL in patients with stage II disease is controversial. FL in advanced CRC prolongs median survival by approximately 5 mo (6 mo without treatment to 11 mo with FL)<sup>[9]</sup>.

The US Food and Drug Administration (FDA) has approved a host of new chemotherapeutic agents for advanced colon cancer and for relapsing disease. Since the year 2000, irinotecan was approved as a first line treatment in metastatic CRC. In 2002, oxaliplatin was approved for use along with other drugs as adjuvant treatment of relapsing CRC. Sequential exposure to various combinations of FL, irinotecan and oxaliplatin extends median overall survival by approximately 20 mo<sup>[10]</sup>. The

**Table 3** Comparison of age, rectal localization, post-surgery mortality and administration of chemo and/or radiotherapy *n* (%)

	Stage I		Stage II		Stage III		Stage IV	
	Group I ( <i>n</i> = 190)	Group II ( <i>n</i> = 122)	Group I ( <i>n</i> = 403)	Group II ( <i>n</i> = 348)	Group I ( <i>n</i> = 302)	Group II ( <i>n</i> = 244)	Group I ( <i>n</i> = 224)	Group II ( <i>n</i> = 157)
Age	66.7	70	66.7	70	66.7	70	67.5	70
Rectal localization	93 (49)	65 (44)	121 (30)	115 (33)	103 (34)	81 (33)	67 (30)	49 (31)
Postoperative mortality	17 (9)	2 (2)	20 (5)	7 (2)	21 (7)	5 (2)	29 (13)	6 (4)
Chemotherapy and/or radiotherapy	10 (5)	10 (8)	48 (12)	90 (26)	91 (30)	137 (56)	54 (24)	55 (35)

**Figure 2** Kaplan-Meier analysis of overall survival and the stage of colorectal cancer according to the group of patients.

FDA approved capecitabine in 2005. Bevacizumab, a monoclonal humanized antibody united with the vascular endothelial growth factor (VEGF) was approved by the FDA for metastatic colon cancer in 2005. The availability of these new agents has resulted in a sustained increase in the use of chemotherapy for CRC patients from 17% in the period of 1980 to 1994 to 50% in 2001. This has been key to the increase of survival, fundamentally in stage III and slightly less in the other CRC stages.

The other important factor that has contributed to an increased survival has been surgical improvements, specifically in the rectal area. Surgery-related mortality has consistently decreased over the years. For example,

a Belgian study showed a decrease in surgical mortality from 20.1% between 1973 and 1979 to 7.8% between 1980 and 1986<sup>[11]</sup>. In our hospital, mortality decreased from 9.1% in 1981 to 8% in 1991<sup>[12]</sup>. In the last decade, the postoperative mortality for CRC varied between 1% and 9.9%<sup>[12-18]</sup>. Emergency surgery for CRC is associated with a high postoperative morbidity and mortality<sup>[14,18,19]</sup>. In rectal cancer, the number of abdomino-perineal resections has decreased from 26.3% in 1981 to 17.2% in 1991<sup>[12]</sup>. Between 1987 and 1992, the mortality in the Istituto di Patologia Speciale Chirurgica of the University of Bologna was 7.6% in anterior resections and 14.2% in abdomino-perineal resections<sup>[20]</sup>. Our study



also observed a significant decrease in the percentage of abdomino-perineal resections, from 30% in 1980 to 18% in 1994. The use of mechanical sutures in rectal cancer operations has allowed a higher rate of sphincter preservation after low anterior resection<sup>[21]</sup>. On the other hand, the number of anterior rectal resections has increased due to the use of mechanical sutures in the middle third of the rectum.

The availability since 1994 of self-expanding stents for obstructive colorectal cancer<sup>[22]</sup> has resulted in a dramatic decrease in the number of urgent palliative surgeries in patients with metastases and a subsequent decrease in the associated postoperative mortality in our study and others<sup>[23]</sup>.

Other factors that we have not analyzed and may also have played a significant role in the increased survival include the use of new antibiotics, improvements in the pre- and post-surgical care of patients, the improved application of radiotherapy and chemotherapy in rectal cancer, the surgical treatment of metastases and the improvement in bowel cleansing. Another limitation of our study is that the Group II cohort was not entirely similar to that of Group I, however, it was a cohort suitable for comparing with Group I (20 years earlier) and showing the differences in different variables such as survival, postoperative mortality or the application of chemotherapy-radiotherapy. In both groups of patients data were collected consecutively and protocols followed strictly.

In conclusion, we observed the survival has increased steadily over the years and is now almost double that 20 years ago. This increase has been in parallel with an increase in the administration of adjuvant chemotherapy and a decline in postoperative mortality.

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## COMMENTS

### Background

Colorectal cancer (CRC) is the second leading cause of cancer mortality. In the last two decades major advances have occurred in treatment. Adjuvant chemotherapy has extended its indications and incorporated new drugs. Surgery outcomes have also significantly improved, with less emergency operations, lower postoperative mortality and a decrease in the number of abdomino-perineal resections. There are few studies geared at analyzing these factors and their influence on CRC survival looking at a 20 year time span.

### Research frontiers

Numerous clinical trials have shown that chemotherapy and surgical advances have dramatically improved the prognosis in patients with CRC. Few studies outside the mentioned clinical trials have examined the impact of these advances on clinical outcome over the past two decades.

### Innovations and breakthroughs

This study has quantified the effect of new adjuvant chemotherapy and improved surgical techniques on the prognosis of patients with CRC.

### Applications

These data show that the increased use of chemotherapy for CRC and better surgical techniques are directly linked to improved survival.

### Peer review

This article compares survival differences between two cohorts of patients operated by colorectal cancer from 1980 to 1994 and 2001. It emphasizes the

importance of the highest percentage of patients receiving chemotherapy and better postsurgical mortality in the last group doubled over the last 20 years.

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## Surgical outcome after docetaxel-based neoadjuvant chemotherapy in locally-advanced gastric cancer

Roberto Biffi, Nicola Fazio, Fabrizio Luca, Antonio Chiappa, Bruno Andreoni, Maria Giulia Zampino, Arnaud Roth, Jan Christian Schuller, Giancarla Fiori, Franco Orsi, Guido Bonomo, Cristiano Crosta, Olivier Huber

Roberto Biffi, Fabrizio Luca, Division of Abdomino-pelvic Surgery, European Institute of Oncology, 20141 Milan, Italy  
 Nicola Fazio, Maria Giulia Zampino, Division of Medical Oncology, European Institute of Oncology, 20141 Milan, Italy  
 Antonio Chiappa, Bruno Andreoni, Division of General and Laparoscopic Surgery, European Institute of Oncology, 20141 Milan, Italy

Arnaud Roth, Department of Oncosurgery, Geneva University Hospitals, 1211 Geneva, Switzerland

Jan Christian Schuller, Statistics Unit, SAKK (Swiss Group for Clinical Cancer Research), 3000 Bern, Switzerland

Giancarla Fiori, Cristiano Crosta, Division of Endoscopy, European Institute of Oncology, 20141 Milan, Italy

Franco Orsi, Guido Bonomo, Interventional Radiology Unit, European Institute of Oncology, 20141 Milan, Italy

Olivier Huber, Department of Surgery, Geneva University Hospitals, 1211 Geneva, Switzerland

**Author contributions:** All authors provided substantial contributions to the conception and design of the study, and also to acquisition and interpretation of data, gave approval of the version to be published; Biffi R contributed to the study design and wrote the manuscript; Biffi R, Luca F, Chiappa A, Andreoni B and Huber O performed the surgical procedures and were involved in editing the manuscript; Fazio N, Roth A and Zampino MG provided medical oncology treatments and contributed to the study design; Fiori G and Crosta C provided endoscopic ultrasonography assessment and critical review of the obtained data; Orsi F and Bonomo G performed CT scans, revised the data and co-ordinated all imaging studies; Schuller JC contributed to the study design, performed statistical analysis and provided important intellectual content.

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Correspondence to: Roberto Biffi, MD, Director, Division of Abdomino-pelvic Surgery, European Institute of Oncology, Via Ripamonti 435, I-20141 Milan, Italy. roberto.biffi@ieo.it  
 Telephone: +39-2-57489609 Fax: +39-2-94379215

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mortality of a docetaxel-based chemotherapy regimen randomly administered before or after gastrectomy in patients suffering from locally-advanced resectable gastric cancer.

**METHODS:** Patients suffering from locally-advanced (T3-4 any N M0 or any T N1-3 M0) gastric carcinoma, staged with endoscopic ultrasound, bone scan, computed tomography, and laparoscopy, were assigned to receive four 21 d/cycles of TCF (docetaxel 75 mg/m<sup>2</sup> day 1, cisplatin 75 mg/m<sup>2</sup> day 1, and fluorouracil 300 mg/m<sup>2</sup> per day for days 1-14), either before (Arm A) or after (Arm B) gastrectomy. Operative morbidity, overall mortality, and severe adverse events were compared by intention-to-treat analysis.

**RESULTS:** From November 1999 to November 2005, 70 patients were treated. After preoperative TCF (Arm A), thirty-two (94%) resections were performed, 85% of which were R0. Pathological response was complete in 4 patients (11.7%), and partial in 18 (55%). No surgical mortality and 28.5% morbidity rate were observed, similar to those of immediate surgery arm ( $P = 0.86$ ). Serious chemotherapy adverse events tended to be more frequent in arm B (23% *vs* 11%,  $P = 0.07$ ), with a single death per arm.

**CONCLUSION:** Surgery following docetaxel-based chemotherapy was safe and with similar morbidity to immediate surgery in patients with locally-advanced resectable gastric carcinoma.

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**Key words:** Gastric cancer; Docetaxel; Neoadjuvant chemotherapy; Laparoscopy; Endoscopic ultrasonography; Morbidity

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### Abstract

**AIM:** To investigate feasibility, morbidity and surgical

of Gastroenterology, Saiseikai Niigata Second Hospital, Teraji 280-7, Niigata, Niigata 950-1104, Japan

Biffi R, Fazio N, Luca F, Chiappa A, Andreoni B, Zampino MG, Roth A, Schuller JC, Fiori G, Orsi F, Bonomo G, Crosta C, Huber O. Surgical outcome after docetaxel-based neoadjuvant chemotherapy in locally-advanced gastric cancer. *World J Gastroenterol* 2010; 16(7): 868-874 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i7/868.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i7.868>

## INTRODUCTION

In spite of a declining incidence in the Western world, gastric cancer is still a major malignant disease in many populations, and the second leading cause for cancer mortality worldwide<sup>[1]</sup>. While localized disease, limited to the submucosa, can be best treated surgically, with a long-term survival of 70%-95%, the prognosis of locally-advanced tumor is poorer, due to a high unresectability rate at presentation, and a much higher relapse rate after radical surgery<sup>[2]</sup>, thus demanding further studies regarding adjuvant and neoadjuvant treatment. Docetaxel (Taxotere®; Sanofi-Aventis, Paris, France) has been approved for treatment of metastatic gastric cancer, when combined with cisplatin and infused fluorouracil (TCF regimen), showing superiority in survival, time to progression, and response rate (RR) *vs* cisplatin/fluorouracil (CF) in a randomized phase III trial<sup>[3]</sup>. A better RR for docetaxel/cisplatin (TC) *vs* epirubicin/cisplatin/protracted venous infusion fluorouracil (ECF) has been documented in a randomized phase II trial<sup>[4]</sup>. These data suggested investigational use TCF in a preoperative neoadjuvant setting. This analysis aimed to test the hypothesis that preoperative chemotherapy with TCF does not influence negatively the results of subsequent surgery, when compared to immediate surgery. Primary endpoints of this study were operative morbidity and mortality rates; secondary endpoints were surgical and pathological assessments of downstaging and assessment by the surgeon as to whether the surgery was curative or not.

## MATERIALS AND METHODS

### Patient selection and treatment

Patients with histologically-proven locally-advanced resectable gastric carcinoma (T3-4 any N M0 or any T N1-3 M0 as defined in the 1997 TNM classification) were screened for eligibility. Other inclusion criteria were: World Health Organization (WHO) performance status  $\leq 2$ ; age 18-75 years; adequate blood counts (white blood cell count  $\geq 4000/\text{mm}^3$ , platelets  $\geq 100\,000/\text{mm}^3$ ); normal renal (calculated creatinine clearance  $\geq 60$  mL/min) and liver function. Patients suffering from Siewert type I cardia location adenocarcinoma (extended mostly into the lower esophagus) were excluded. All patients underwent chest X-ray, gastric endoscopic ultrasound (EUS), spiral thoraco-abdominal computerized tomography (CT) scan, bone scintigraphy and staging laparoscopy to define

nodal status and rule out distant deposits and/or peritoneal seeding.

The trial was approved in all centers by relevant ethics committees. All patients gave written informed consent for participation in the trial.

Patients were stratified by center, tumor size, tumor location (cardia adenocarcinoma Siewert II and III *vs* tumors of the rest of the stomach) and nodal status (N+ *vs* N-). Patients received four 21-d/cycles of TCF (docetaxel 75 mg/m<sup>2</sup>, 1-h IV infusion, day 1; cisplatin 75 mg/m<sup>2</sup> 4-h IV infusion, day 1; and 5-fluorouracil (5-FU) 300 mg/m<sup>2</sup> per day continuous IV infusion, days 1 to 14), either preoperatively (Arm A) or postoperatively (Arm B). Just before starting chemotherapy all patients underwent placement of a totally implantable central venous port. In Arm A, a re-evaluation was performed after 2 cycles. If local progression had occurred, then the patient immediately underwent surgery. Otherwise two more TCF cycles were administered and surgery was performed within 3-5 wk after day 1 of the last cycle. In Arm B, surgery was scheduled to take place within 1 wk after randomization. Postoperative TCF was to be initiated 3 to 6 wk after surgery.

### Perioperative complications

Data about postoperative course and complications were reported on hospital cards by surgical teams. In addition, an epidemiology nurse was in charge of regularly collecting microbiology data with respect to nosocomial infections (surgical site, pulmonary, urinary, and/or intravascular catheter infections). Data on hospital infections were regularly submitted to the infection central committees on a 3-mo basis.

As conclusive assessments of surgical procedures remain difficult, and there is a lack of consensus on how to define complications and to stratify them by severity, we decided to apply a single classification, proposed by Dindo *et al*<sup>[5]</sup>, which is based on the evaluation of a cohort of 6336 patients and the results of a survey. In this classification, the therapy used to correct a specific complication is the cornerstone in ranking a complication. For example, life-threatening complications requiring an intermediate or intensive care management (IC/ICU) have to be differentiated from complications treated on the ward; as such complications are associated with a high mortality, stress for the patients, and substantial resource consumption. Therefore, registered complications in both groups were analyzed accordingly, with the exception of those classified as grade I (deviation from the normal postoperative course without the need for pharmacological treatment or surgical, endoscopic or radiological interventions). This grade also included wound infections treated at bedside.

### Surgery

A careful intraoperative staging of disease was first performed, in order to rule out peritoneal seeding, ovarian involvement, "drop" metastasis in the pelvis, or periaortic gross adenopathy. Attention was directed to the liver, greater omentum and root of the mesentery below the transverse colon. The stomach was always inspected and gently



**Table 1** Extent of lymphadenectomy D1 vs D2 according to the JRS GC<sup>[5,6]</sup>

	D1	D2
Upper third	1-2-3-4	D1+5-6-7-8-9-10-11-110
Middle third	1-3-4-5-6	D1+2-7-8-9-10-11
Lower third	3-4-5-6	D1+1-7-8-9

JRSGC: Japanese Research Society for the Study of Gastric Cancer.

palpated to assess the location and the extent of the tumor and to exclude direct invasion of adjacent structures. Frozen sections of every suspect tissue were obtained (e.g. preaortic, infracolic nodes); intraoperative, histology-proven recognition of metastatic spread was considered an exclusion criterion. The extent of gastrectomy depended on the proximal distance of the tumor from the cardia; therefore, total gastrectomy was performed in all patients with cardia locations and in those having antral and body tumors in whom a 6-cm gross proximal margin could not be obtained. Subtotal distal gastrectomy was performed in the others (almost exclusively small-size body or antral locations). Proximal gastric resection was never carried out, as total gastrectomy with 2-3 cm extent to abdominal esophagus was chosen for all cardia locations.

Lymphadenectomy included excision of all N1 and most N2 stations (stations 7, 8, 9 and station 11), according to the classification of the Japanese Research Society for the Study of Gastric Cancer (JRSGC)<sup>[6,7]</sup> (Table 1). Hepatoduodenal ligament nodes (station 12) were also dissected, limiting the lymphadenectomy to the 12a station (left side of the hepatic artery), and leaving undissected the parts b and p of the station (right side of the ligament and just posteriorly to the portal vein, respectively). Lymph nodes of the surgical specimen were routinely dissected by experienced pathologists, using standard techniques; in some cases, depending on the pathologist's judgement, clearing fixatives were used prior to the dissection. Splenectomy was only carried out in cases of direct invasion of the spleen by the tumor, or gross appearance of metastatic nodes at station 10 (splenic hilum). Caudal pancreas was always preserved, according to the Maruyama's technique, even when splenectomy was performed, unless tumor direct involvement was clinically evident.

Surgeons were asked to document the extent of node dissection and to state whether the procedure was likely to be curative as follows: (1) Absolutely curative: absence of hepatic and/or peritoneal metastasis; serosa not involved; no infiltration within 10 mm of the proximal resection line; (2) Relatively curative: as above, but serosa involved, and/or cancer infiltrates within 10 mm of the proximal resection line, and/or nodal involvement (N stage) equals D number; and (3) Non-radical: resection line involvement; any residual disease after resection.

Reconstruction technique (Roux-en-Y, Braun or others) was entirely left to the discretion of the surgeon.

### Pathology

Pathological response to chemotherapy was centrally evaluated and classified as follows: (1) Complete

response (pCR): No residual tumor could be found after *in toto* examination of the potential tumor site. Acellular mucus or acellular necrosis in the gastric wall or in lymph nodes was not considered as residual tumor and therefore was not taken into consideration for staging (neither for T, nor for N); (2) Partial microscopic response: Microscopic residual tumor (persistence of microscopic islands of tumor cells); (3) Partial macroscopic response: Macroscopic residual tumor, but overt necrosis or calcification, or downstaging of the tumor; and (4) No response: Only minor necrosis and no downstaging of the tumor.

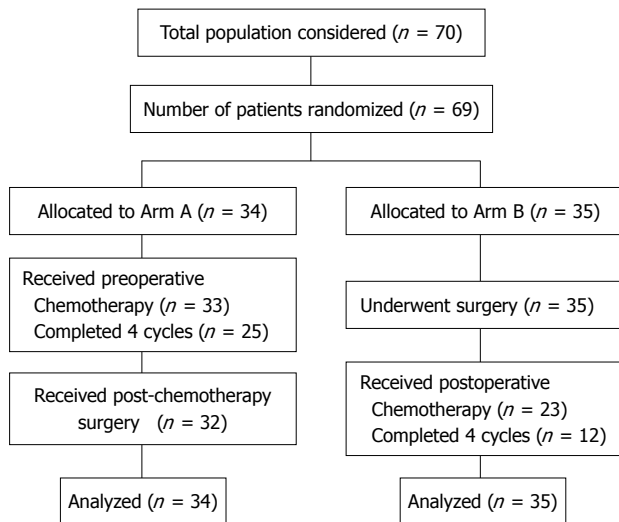
### Statistical analysis

Initially a target sample size at 240 patients was set, assuming a 3-year event-free survival rate of 20% in arm B and 35% in arm A (+ 15%). Trial was prematurely stopped at 70 randomized patients, due to insufficient accrual. Only two centers out of nine showed a good accrual rate, whereas most participating groups were not ready to be involved in such a multi-disciplinary approach. Moreover, some patients refused to participate to this kind of trial because they wanted to be operated on as soon as possible. For this reason, these study results are underpowered to detect any possibly significant differences in the experimental groups. Nevertheless, results were descriptively compared between the two arms on an intention-to-treat basis, using Mann-Whitney *U*-test and Kruskal-Wallis test to compare means and medians, respectively.  $\chi^2$  or Fisher's exact test were used to compare proportions. All tests were two-sided. A *P* value less than 0.05 was assumed significant. Intention-to-treat principle was adopted.

## RESULTS

This trial was activated in November 1999 and closed in November 2005 due to insufficient accrual. From December 1999 to August 2005 a total of 70 patients were enrolled from 9 Institutions in three countries. Eighty-five percent of included patients were from two Institutions; from Milan (IEO-European Institute of Oncology) and Geneva (University Hospitals of Geneva). One patient withdrew consent, did not receive any chemotherapy, and was excluded from the analysis. Of the remaining 69 patients, 34 were randomized to Arm A (TCF followed by surgery) and 35 to Arm B (surgery followed by TCF). One patient in Arm A did not receive any chemotherapy because he died before starting. This patient was included in the analysis, in agreement with the intention-to-treat principle. A trial profile, conforming to the Consolidated Standards of Reporting Trials (CONSORT) is shown in Figure 1. The two groups of patients were similar with respect to various characteristics (Table 2).

Table 3 shows details about surgical procedures performed and pathology reports. Thirty-two patients in Arm A (94%) underwent laparotomy: 29 (85%) had an R0 resection, and two a non-radical resection; one had no resection due to unsuspected peritoneal carcinomatosis. All 35 patients in Arm B underwent laparotomy; 32



**Figure 1** Trial profile conforming to the Consolidated Standards of Reporting Trials (CONSORT) guidelines.

**Table 2** Patient characteristics

	Arm A (n = 34)	Arm B (n = 35)
Age (yr): median (range)	57 (25-75)	59 (39-76)
Male (%)	68	71
PS 0/1/2 (%)	91/6/3	86/14/0
Tumor site (%)		
Cardia	21	20
Fundus/body	38	40
Antrum/pylorus	41	40
Stage (by EUS + CT scan)		
I B	2	1
II	14	13
III	18	21

EUS: Endoscopic ultrasound; CT: Computerized tomography.

(91%) had an R0 resection, two a non-radical resection and one no resection due to peritoneal carcinomatosis. In Arm A, pathological response was complete in 4 patients (11.7%), and partial (macro- or microscopic) in 18 (55%). The respective proportions of total *vs* subtotal gastrectomies, D  $\geq$  2 *vs* D1 lymph node dissections, median number of excised lymph nodes and of metastatic nodes were very similar in the two arms of the study, and all differences were not statistically significant.

Postoperative mortality and morbidity events are detailed in Table 4; they are stratified by severity, applying the classification proposed by Dindo *et al*<sup>[5]</sup>. In Arm A these included four septic intraabdominal complications (one anastomotic leak, two abdominal abscesses and one infected fluid peritoneal collection), one gastrojejunal anastomosis bleeding, one pneumonia requiring ICU admission, one pulmonary embolism, one urinary infection, and one fever of unknown origin. Morbidity events in Arm B included three septic intraabdominal complications (one anastomotic leak, one abdominal abscess, and one infected fluid peritoneal collection), and six extra abdominal infections (one infected mediastinal collection, three pneumonias requiring ICU admission,

**Table 3** Details of surgical procedures and pathology

	Arm A (n = 32)	Arm B (n = 35)
Complete resection R0 (%)	29 (85)	32 (91)
Non-radical resection (%)	2 (5.8)	2 (5.7)
No resection pM1 (peritoneum) (%)	1 (2.9)	1 (2.8)
pCR n (%)	4 (11.7)	NA
pPR n (%)	18 (55)	NA
Total gastrectomy	20	24
Subtotal gastrectomy	11	10
D-2 lymphadenectomy	29	31
D-3 lymphadenectomy	-	2
Excised lymph nodes median (range)	20 (9-39)	26 (13-76)
Metastatic lymph nodes median (range)	1 (0-23)	5 (0-50)

pCR: Pathological complete response; pPR: Pathological partial response; NA: Not available.

**Table 4** Postoperative morbidity and mortality, ranked according to Dindo *et al*<sup>[5]</sup>

Type of complication	Arm A (n = 32)	Arm B (n = 35)
Anastomotic leak	1-IVb	1-IVb
Abdominal abscess	2-IIIa	1-IIIa
Infected peritoneal collection	1-IIIa	1-IIIa
Anastomotic bleeding	1-IIIb	-
	(re-operation)	
Pneumonia requiring ICU	1-IVa	3-IVa-IVa, V (re-operation, death)
Pulmonary embolism	1-II	-
Urinary infection	1-II	-
Fever of unknown origin	1-II	-
Mediastinal infected collection + MOF	-	1-V (re-operation, death)
Central venous catheter-related blood stream infection	-	2-II
Total <sup>a</sup>	9 (28.5%), 1 re-operation	9 (25.7%), 2 re-operations, 1 death

<sup>a</sup>P = 0.86. ICU: Intensive care unit; MOF: Multiple organ failure.

and two central venous catheter-related blood stream infections). Three re-operations were performed, one in Arm A due to anastomotic hemorrhage, and two in Arm B, due to infected mediastinal collection and pleural empyema complicating severe pneumonia, respectively. Overall, 9 morbidity events occurred in each arm (28.5% in Arm A and 25.7% in Arm B). Two postoperative deaths occurred, in Arm B, as a consequence of multiple organ failure (MOF) complicating mediastinal infected fluid collection, in spite of re-operation. All these differences were not statistically significant ( $P = 0.86$ ).

A total of 189 TCF cycles were administered; 118 in Arm A and 71 in Arm B (Table 5). In Arm A, 25 patients (74%) received all 4 cycles, two patients 3 cycles, and six patients 2 cycles. In Arm B, only 12 patients received all 4 cycles (34%), five patients 3 cycles, two patients 2 cycles, four patients 1 cycle and 12 patients received no cycle. A 64-year-old female patient who had received one cycle of preoperative TCF died after severe worsening of performance status and dyspnoea. Excluding this case, serious adverse events (SAEs) oc-

Table 5 Treatment administration and SAEs

	Arm A ( <i>n</i> = 33)	Arm B ( <i>n</i> = 23)
Total number of cycles	118	71
Causes of treatment failure		
Progression of disease	1	0
G4 toxicity	2	6
Death	1	1
Patient refusal	1	1
Investigator's decision	2	3
Other	1	1
Total	8	12
Severe adverse events (% of cycles) <sup>a</sup>	13 (11)	16 (23)
No. of patients involved (% of pts. treated) <sup>b</sup>	10 (30)	14 (60)

<sup>a</sup>*P* = 0.07, <sup>b</sup>*P* = 0.15. SAE: Severe adverse event.

curred more frequently in Arm B. In Arm A, 13 SAEs in 10 patients were observed (13 SAEs out of 118 cycles = 11%), 7 of them infectious (3 febrile neutropenia). In Arm B, 16 SAEs occurred in 14 patients (16 SAEs out of 71 cycles = 23%). All these differences were not statistically significant (*P* = 0.07 and 0.15, respectively).

A 58-year-old male patient in Arm B died suddenly 38 d after gastrectomy from severe arrhythmia and pulmonary infection. Table 5 details reasons for cessation of therapy in the two arms of the study.

## DISCUSSION

Prognosis of locally-advanced gastric cancer is generally poor in Western surgical and population-based series, with 5-year overall survival rates of 25% or less<sup>[8]</sup>, in spite of complete excision of the gastric and nodal components of the disease<sup>[2]</sup>. This is the consequence of a high relapse rate after radical surgery, and has prompted many studies in the last decade, aimed at improving these results by means of adjuvant and neoadjuvant treatments<sup>[9-12]</sup>. Both these approaches remain controversial and are under current investigation. A large randomized trial [Medical Research Council Adjuvant Gastric Infusional Chemotherapy (MAGIC)]<sup>[13]</sup>, demonstrated a survival benefit with the use of perioperative chemotherapy (i.e. pre- and postoperatively delivered) as compared with surgery alone. Similarly, the FFGD 9703 trial<sup>[14]</sup> showed an improvement in both disease free survival (DFS) and overall survival (OS) with the use of perioperative chemotherapy (FP regimen: 5-FU continuous infusion + cisplatin) as compared to surgery. To date, no trial has so far investigated the effects of the same chemotherapy regimen given either pre- or postoperatively.

Although our study is underpowered to detect any possible significant difference in short-term postoperative outcome, it gives some preliminary answers to questions not yet available in the medical literature that could be interesting for future studies. The first relevant information provided by the present study is the safety of surgery following a preoperative docetaxel-based chemotherapy regimen. In fact, we did not register any mortality and we had a 28.5% morbidity rate, without any significant

difference between pre- and postoperative administration of chemotherapy. Although our patient population was slightly different from that of the MAGIC and FFGD trials, since Type I Siewert adenocarcinomas of the lower third of the esophagus were excluded, results of our study compare favorably with those of the MAGIC trial, where a 45% morbidity rate and 5% mortality rate were observed. In the FFGD trial, postoperative morbidity was 21% in the surgical arm, and 28% in the perioperative chemotherapy arm, whereas surgical mortality was 5% for both groups. A possible favorable factor was that 85% of patients in our series were operated on in two high-volume institutions where D2 gastrectomy is routinely carried out as standard treatment of gastric cancer. Our results support the conclusions that D2 gastrectomy can be considered a safe treatment of gastric cancer in Western patients, at least when performed in experienced centers<sup>[15]</sup>, and that gastric cancer resection should probably be added to the growing list of procedures which are safer when performed in high-volume institutions<sup>[16,17]</sup>. This could explain why reports from single large volume institutions continue to demonstrate low operative mortality after D2 radical gastrectomy, while randomized trials show no survival benefit and severely increased surgical mortality after this procedure<sup>[18,19]</sup>.

In addition, our data indicate that chemotherapy-related SAEs tended to be more frequent in Arm B (adjuvant) than in Arm A (neoadjuvant), suggesting that lower patient tolerance to treatment is a key factor in determining higher toxicity. This could be explained by an increased risk of gastrointestinal toxicity after surgery, when patients are already deeply affected in their eating capacity by the gastrectomy. For instance, it was shown that, in a population of 23 patients followed for dietary intake and nutritional status after total gastrectomy, no patient reached recommended dietary allowances by first monthly follow up<sup>[20]</sup>.

Our trial confirms the difficulties in administering intensive adjuvant chemotherapy in gastric cancer. In the MAGIC trial, 34% of patients who completed preoperative chemotherapy and surgery did not start postoperative chemotherapy, mostly owing to early progression, patient refusal and/or surgical complication. In the weekly-PELF trial<sup>[21]</sup>, only 14% of experimental arm patients completed the scheduled adjuvant treatment without time and/or dose modifications. Even without preoperative chemotherapy, 12 patients in this series did not receive any adjuvant chemotherapy. The most frequent reasons were patient refusal and medical decision. However, even when adjuvant chemotherapy was started, only 34% of the patients received all the four cycles. In the ACT-GC Group Trial (Adjuvant Chemotherapy Trial of TS-1 for Gastric Cancer), evaluating an oral fluoropyrimidine as adjuvant agent, enrollment was stopped after 1 year as a consequence of the higher rate of overall survival in the S-1 treated group than in controls who had only surgery<sup>[22]</sup>. Nevertheless, among the 517 patients who received S-1, 71 refused to continue treatment because of adverse events, and in 72 the



decision of the investigators was to terminate treatment because of adverse events or complications (143/571, 25.04%). The dose of S-1 was reduced in 219 of the 517 treated patients (42.4%).

Laparoscopy has been reported to improve clinical staging when compared to conventional methods, identifying unexpected peritoneal or liver metastases in up to 20% of operable patients<sup>[23,24]</sup>; consequently, a significant proportion of patients can avoid unnecessary laparotomy<sup>[25]</sup>. Although in our study staging laparoscopy has been confirmed as an effective tool to demonstrate peritoneal deposits even when missed by preoperative CT scan, minimal peritoneal deposits were found and biopsied at laparotomy in 3 patients previously judged peritoneal seeding-free at laparoscopy. These false-negative results of laparoscopy occurred within the omentum and/or the lesser sac, emphasizing the limits of staging laparoscopy to demonstrate a minimal peritoneal spread in these difficult locations. Similarly, in a recent report, the sensitivity for detecting peritoneal carcinomatosis was 85% for laparoscopy<sup>[26,27]</sup>. The possible role of the preoperative PET scan in reducing the rate of false negative results of staging laparoscopy is currently under investigation, with conflicting preliminary evidence<sup>[28,29]</sup>; it seems a priori highly improbable that such small-volume disease could be detected by this imaging technique.

Finally, our data confirm the huge difficulties in performing this kind of study, which requires a high level of cooperation between different disciplines. Principal investigators analyzed the reasons for the slow accrual of patients for their neoadjuvant study with FAMTX<sup>[30]</sup> for operable gastric cancer, and observed that around half of the participating centers were not ready for such a multi-disciplinary approach, not believing in the potential efficacy of the neoadjuvant treatment. Moreover, several patients refused to participate in this kind of trial because they wanted to be operated on as soon as possible.

In conclusion, our study does not provide information on efficacy of preoperatively-delivered TCF, due to early discontinuation for slow accrual. It is also underpowered to detect any possible significant differences in short-term postoperative outcome. Nevertheless, data regarding TCF efficacy and feasibility in the preoperative setting and TCF feasibility in the adjuvant setting could be interesting for future studies. In particular, neoadjuvant TCF achieved promising results with a 12% pCR rate. This evidence prompts further studies, since patients achieving a pCR tend to have a much better outcome, as underlined in a recent phase II trial of preoperative chemo-radiation therapy for resectable gastric cancer<sup>[31]</sup>. Surgery was safe after TCF preoperative chemotherapy, while toxicity (especially gastrointestinal) made adjuvant postoperative TCF more difficult to administer fully compared to the neoadjuvant setting. These data are consistent with the results of the recent FFCD trial<sup>[14]</sup>, where postoperative chemotherapy was completed in less than 50% of the patients. This should be carefully considered when an intensive adjuvant chemotherapy regimen is planned.

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## COMMENTS

### Background

In spite of a declining incidence in the Western world, gastric cancer is still a major malignant disease in many populations, and the second leading cause for cancer mortality worldwide. While localized disease, limited to the submucosa, can be best treated surgically, with a long-term survival rate of 70%-95%, the prognosis of locally-advanced tumor is poorer, due to a high unresectability rate at presentation, and a much higher relapse rate after radical surgery. Docetaxel (Taxotere®; Sanofi-Aventis, Paris, France) has been approved for treatment of metastatic gastric cancer, when combined with cisplatin and infused fluorouracil (TCF regimen), showing superiority in survival, time to progression, and response rate (RR) vs cisplatin/fluorouracil (CF) in a randomized phase III trial.

### Research frontiers

The above mentioned results obtained in metastatic disease suggested the investigational use of the TCF regimen in a preoperative neoadjuvant setting. The present trial aimed to test the hypothesis that preoperative chemotherapy with TCF does not influence negatively the results of subsequent surgery, when compared to immediate surgery.

### Innovations and breakthroughs

This trial proved that surgery is safe after TCF preoperative chemotherapy, while toxicity (especially gastrointestinal) makes adjuvant postoperative TCF more difficult to administer fully compared to the neoadjuvant setting. Moreover, neoadjuvant TCF achieved promising results with a 12% pCR (pathological complete response) rate.

### Applications

Obtained data regarding TCF efficacy and feasibility in the preoperative setting and TCF feasibility in the adjuvant setting could be interesting for future studies. In fact, patients achieving a pCR tend to have a much better oncology outcome. Finally, data here presented are consistent with the results of the recent FFCD trial, where postoperative chemotherapy was completed in less than 50% of the patients. This should be carefully considered when an intensive adjuvant chemotherapy regimen is planned.

### Peer review

This is an interesting report of the efficacy of neoadjuvant chemotherapy for advanced gastric cancer.

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## A novel cleansing score system for capsule endoscopy

Sung Chul Park, Bora Keum, Jong Jin Hyun, Yeon Seok Seo, Yong Sik Kim, Yoon Tae Jeon, Hoon Jai Chun, Soon Ho Um, Chang Duck Kim, Ho Sang Ryu

Sung Chul Park, Bora Keum, Jong Jin Hyun, Yeon Seok Seo, Yong Sik Kim, Yoon Tae Jeon, Hoon Jai Chun, Soon Ho Um, Chang Duck Kim, Ho Sang Ryu, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Korea University Anam Hospital, Institute of Digestive Disease and Nutrition, Korea University College of Medicine, Seoul 136-705, South Korea

Author contributions: Park SC and Keum B wrote the article; Hyun JJ, Seo YS, Kim YS and Jeon YT performed the research; Chun HJ, Um SH, Kim CD and Ryu HS analyzed the data.

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Correspondence to: Bora Keum, MD, PhD, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Korea University College of Medicine, 126-1 5-ga, Anam-dong, Seongbuk-gu, Seoul 136-705,

South Korea. [borakeum@korea.ac.kr](mailto:borakeum@korea.ac.kr)

Telephone: +82-2-9206555 Fax: +82-2-9531943

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### Abstract

**AIM:** To suggest a new cleansing score system for small bowel preparation and to evaluate its clinical efficacy.

**METHODS:** Twenty capsule endoscopy cases were reviewed and small bowel preparation was assessed with the new scoring system. For the assessment, two visual parameters were used: proportion of visualized mucosa and degree of obscuration. Representative frames from small bowel images were serially selected and scored at 5-min intervals. Intraclass correlation coefficient (ICC) was obtained to assess the reliability of the new scoring system. For efficacy evaluation and validation, scores of our new scoring system were compared with another previously reported cleansing grading system.

**RESULTS:** Concordance with the previous system, inter-observer agreement, and intra-patient agree-

ment were excellent with ICC values of 0.82, 0.80, and 0.76, respectively. The intra-observer agreements at four-week intervals were also excellent. The cut-off value of adequate image quality was found to be 2.25.

**CONCLUSION:** Our new scoring system is simple, efficient, and can be considered to be applicable in clinical practice and research.

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**Key words:** Capsule endoscopy; Cleansing score system

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### INTRODUCTION

Capsule endoscopy (CE) was introduced as a method for investigating the full length of the small intestine<sup>[1-3]</sup>. However, there are limitations such as impaired visualization by air bubbles, food residue, bile, and blood clots. Unfortunately, current CE does not have functions which allow suctioning of fluid or washing the small bowel mucosa during the examination. Therefore, since some of the lesions can be overlooked and missed, adequate bowel cleansing is mandatory for a successful CE<sup>[4]</sup>. There have been many studies on the necessity and methods of bowel preparation for CE<sup>[1,5-8]</sup>. Howev-

er, the benefits of bowel preparation prior to CE remain controversial and it is unclear which method is best.

One of the reasons for this controversy is that the current grading systems have not been standardized and therefore, a generally accepted grading system is not available. The presence of numerous grading systems has caused difficulties in comparing the results of studies on small bowel preparation prior to CE<sup>[4,9]</sup>. In addition, most of the previously reported grading systems are time-consuming, complicated, and difficult to apply in the clinical setting. The aim of this study is to suggest a new cleansing score system for small bowel preparation and to evaluate its clinical efficacy.

## MATERIALS AND METHODS

Twenty CE cases were reviewed according to the protocol by three examiners who had experience in interpreting more than 50 cases of CE. These examiners separately assessed small bowel cleanliness by using the grading system described below. The software program Rapid Reader 4 (Given Imaging Ltd, Yoqneam, Israel) was used to review and score the images.

### Scoring system

Two visual parameters were used in our scoring system. The first parameter was the proportion of visualized mucosa. This was scored using a 4-step scale ranging from 0 to 3: score 3, greater than 75%; score 2, 50% to 75%; score 1, 25% to 50%; score 0, less than 25% (Table 1, Figure 1). The second parameter was the degree of obscuration by bubbles, debris, and bile *etc.* This was also scored using a 4-step scale ranging from 0 to 3: score 3, less than 5%, no obscuration; score 2, mild (5% to 25%) obscuration; score 1, moderate (25% to 50%) obscuration; score 0, severe (greater than 50%) obscuration (Table 1, Figure 1). Images from the entire small bowel were serially selected at 5-min intervals (1 frame/5 min) by manual mode using the RAPID system. If the capsule got stuck or remained in the same place for more than 5 min, the frames were scored only once and not repeatedly.

Mean scores of each parameter were obtained by summing the scores of all selected images and dividing them by the number of examined frames. The representative values for each parameter were then calculated by the overall average of two mean scores.

### Efficacy evaluation

First, 20 CE cases were reviewed and frames were selected twice: once according to our scoring system and once according to one of the previously reported systems which evaluated CE images for 2 min in every 5-min period (e.g. 240 frames/5 min or 40% of the small bowel images)<sup>[6]</sup>. The images from selected frames were then graded using the aforementioned parameters. The concordance of scores between the two grading systems was analyzed.

Second, the reliability of our grading system was

Table 1 CE image scoring system

The proportion of visualized mucosa	
Score 3	≥ 75%
Score 2	50%-75%
Score 1	25%-50%
Score 0	< 25%
The degree of bubbles, debris and bile	
Score 3	< 5%, no obscuration
Score 2	5%-25%, mild obscuration
Score 1	25%-50%, moderate obscuration
Score 0	≥ 50%, severe obscuration

CE: Capsule endoscopy.

evaluated by assessing the inter-observer, intra-patient, and intra-observer agreement. For the assessment on inter-observer agreement, three examiners each scored the same selected frames separately at 5-min intervals which were then compared. For the evaluation of intra-patient agreement, each examiner reviewed the same case after choosing their own starting frame within the first 5 min of the capsule's entrance into the duodenum, from where the ensuing frames were picked up at 5-min intervals and scored accordingly. For the analysis on intra-observer agreement, the same frames from the same cases were scored once again after four weeks and scores were compared with the previous results.

Third, in order to determine the cut-off value for adequate cleansing, our scoring system was compared with another grading system, which examined all of the images obtained from the entire small bowel. This grading system defined small bowel mucosa as "clean" if less than 25% of the mucosal surface was covered by intestinal contents or food debris, and cleanliness was graded as "adequate" if the time the mucosa appeared clean was greater than 90% of the total examination time<sup>[5]</sup>. Overall adequacy was compared with that of our scoring system and the results were analyzed using the receiver operating characteristic (ROC) curve.

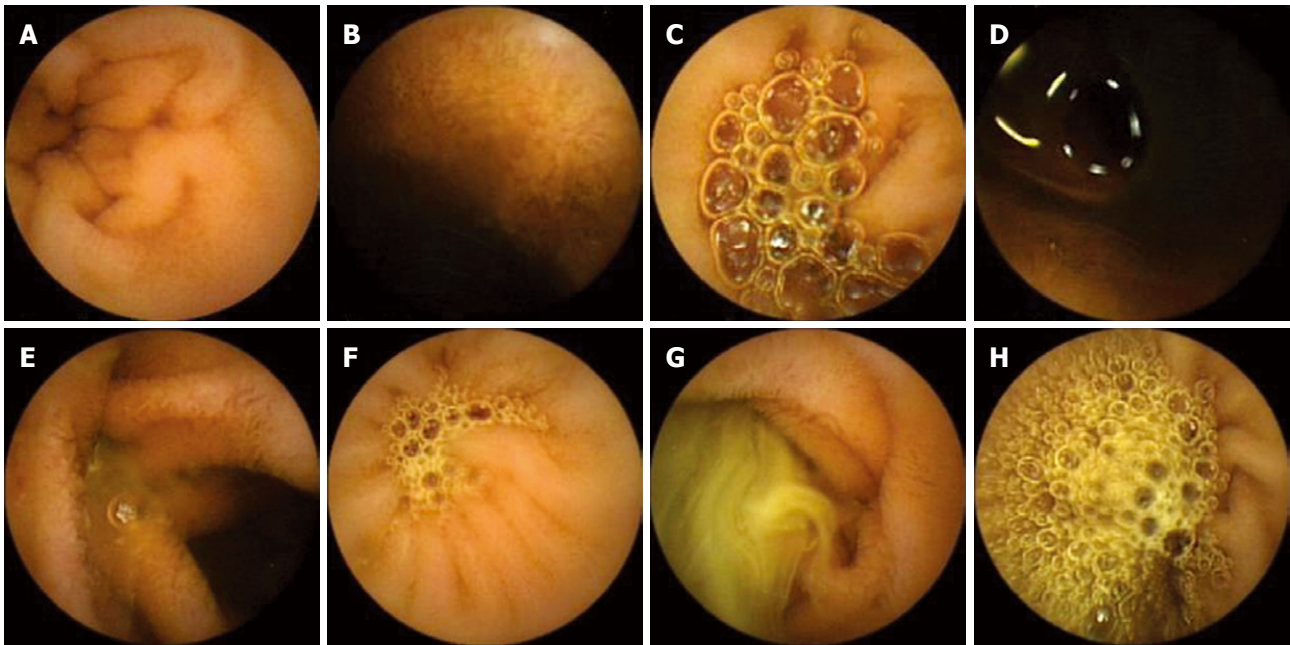
### Statistical analysis

Concordance was determined by using the intraclass correlation coefficient (ICC). An ICC value less than 0.40 was considered poor, between 0.40 and 0.75 was considered fair to good, and greater than 0.75 was considered excellent<sup>[6,10,11]</sup>. The cut-off value of the cleansing scores according to our scoring system, with optimal sensitivity and specificity, was determined using the ROC curve. Area under the curve (AUC) was used for assessing the overall accuracy of our scoring system which employed the ROC curve. All statistical analyses were performed with SPSS version 12.0 (SPSS Inc, Chicago, IL, USA).

## RESULTS

Twenty cases were selected from previously diagnosed patients. Mean age of the subjects was 48.5 (21-80) years and 80% (18/20) were male. Their indications for CE





**Figure 1** Images of scores according to the proportion of the visualized mucosa (A-D) and the degree of obscuration (E-H). A: Score 3; B: Score 2; C: Score 1; D: Score 0; E: Score 3; F: Score 2; G: Score 1; H: Score 0.

**Table 2** Agreement between our scoring system A and previous scoring system B

Parameter	System	Score (median and IQR)	Inter-system ICC (95% CI)
Visualized mucosa	A	2.44 (2.20-2.62)	0.72 (0.27, 0.89)
	B	2.24 (2.11-2.48)	
Degree of obscuration	A	2.13 (1.92-2.35)	0.86 (0.53, 0.95)
	B	1.98 (1.87-2.22)	
Overall average	A	2.30 (2.09-2.50)	0.82 (0.33, 0.94)
	B	2.12 (1.97-2.36)	

A: 1 frame per 5 min; B: 2 min per 5 min. IQR: Interquartile range; ICC: Intraclass correlation coefficient.

**Table 3** Inter-observer agreement of our scoring system among three examiners

Parameter	Observer	Score (median and IQR)	Inter-observer ICC (95% CI)
Visualized mucosa	A	2.61 (2.40-2.81)	0.88 (0.47, 0.99)
	B	2.62 (2.42-2.82)	
	C	2.44 (2.20-2.62)	
Degree of obscuration	A	2.34 (2.17-2.55)	0.71 (0.39, 0.87)
	B	2.28 (2.12-2.56)	
	C	2.13 (1.92-2.35)	
Overall average	A	2.47 (2.32-2.63)	0.80 (0.35, 0.93)
	B	2.47 (2.26-2.62)	
	C	2.30 (2.09-2.50)	

were gastrointestinal bleeding (12/20, 60%), iron deficiency anemia (4/20, 20%), abdominal pain (3/20, 15%), and diarrhea (1/20, 5%). Cases were prepared with 4 L of polyethylene glycol (PEG) four hours before examination without prokinetic agent or simethicone. The concordance between our cleansing score system and a previously reported system, which selected the frames and evaluated them for 2 min in every 5-min period, was excellent with an ICC value of 0.82 [95% confidence interval (CI), 0.33-0.94, Table 2).

As for the assessment on reliability, inter-observer and intra-patient agreement were excellent with ICC values of 0.80 (95% CI: 0.35-0.93, Table 3) and 0.76 (95% CI: 0.41-0.93, Table 4), respectively. The data regarding the assessment on intra-observer agreement was available from three examiners and the results were also excellent with ICC values of 0.80, 0.82, and 0.92, respectively (Figure 2).

To assess the overall adequacy of small bowel cleansing, the ROC curve was generated to determine the cut-

off value of image quality. The cut-off value, estimated by the ROC curve at an optimal level of sensitivity and specificity, was 2.25 with 85% sensitivity and 87% specificity. The AUC of the scoring system was 0.925 (95% CI: 0.859-0.990, Figure 3).

## DISCUSSION

CE has provided a new perspective for diagnosing, treating, and monitoring small bowel diseases, such as obscure GI bleeding, Crohn's disease, celiac sprue, polyposis syndromes, and small-bowel tumors.

However, CE has several limitations, one of which is image quality. Although 12 h-fasting or PEG ingestion is used for small bowel preparation in CE, air bubbles, intestinal secretions, bile or food residue occasionally cover the small bowel mucosa and obscure the view. The capsule endoscope is not equipped with functions to allow suctioning, inflating, and washing the lumen of the small intestine. Therefore, some parts of the lumen will not be



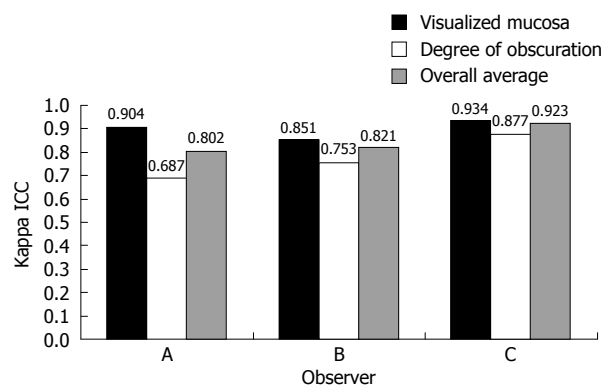


Figure 2 Intra-observer agreements between the scores at 4-wk intervals for two parameters and the overall average.

Table 4 Intra-patient agreement of our scoring system among different starting frames

Parameter	Frame	Score (median and IQR)	Intra-patient ICC (95% CI)
Visualized mucosa	A	2.33 (1.93-2.59)	0.95 (0.81, 0.98)
	B	2.39 (1.88-2.66)	
	C	2.31 (1.90-2.44)	
Degree of obscuration	A	2.13 (1.97-2.61)	0.72 (0.39, 0.87)
	B	2.01 (1.52-2.34)	
	C	2.01 (1.57-2.20)	
Overall average	A	2.28 (2.08-2.61)	0.76 (0.41, 0.93)
	B	2.45 (1.70-2.50)	
	C	2.16 (2.09-2.50)	

visualized and examiners are unable to observe the entire small bowel mucosa thoroughly. This means that small bowel preparation plays an important role because it can improve image quality by cleansing the small bowel. There have been many studies on the preparation for CE, however, there is no standardized procedure for small bowel preparation for CE. In addition, preparation type, doses, and time of administration differ among centers.

To assess the effect of bowel preparation on the cleansing of the small bowel mucosa objectively, scoring systems for grading small bowel cleanliness have been introduced<sup>[1,5,6,12-14]</sup>. Prior studies on preparation, formulated and used their own scoring systems to grade small bowel cleanliness, thus making it difficult to compare the effect of preparation among studies<sup>[4,9]</sup>. For example, the grading system by Viazis *et al*<sup>[5]</sup> simply graded the small bowel cleanliness as “adequate” or “inadequate” and the time, during which the small bowel mucosa appeared unclean, was recorded using a timer; the concordance among the investigators was excellent (92.5%). However, thousands of frames per case had to be reviewed with this system. Therefore, grading the cleanliness is time-consuming and laborious. In the studies by Niv *et al*<sup>[11,7]</sup> the capsule images were graded according to the proportion of the small bowel transit time during which the intraluminal fluid interfered with visualization and interpretation. Although concordance on the quality of bowel preparation was excellent (kappa statistic = 0.91,  $P < 0.001$ ), this grading sys-

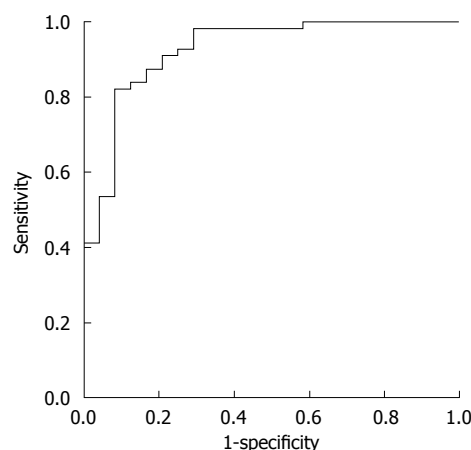


Figure 3 The area under the curve (AUC) was 0.925 (95% confidence interval, 0.859-0.990) in receiver operating characteristic (ROC) curve of image quality scores for grading small bowel cleansing.

tem, in which the entire small bowel could be examined, is also time-consuming.

The study by Dai *et al*<sup>[13]</sup> assessed the visibility of the small bowel as a percentage of the visualized intestinal wall during 10-min video segments at 1-h intervals. In the study by Shiotani *et al*<sup>[6]</sup> individual frames were examined for 2 min of every 5-min period and scored using four visual parameters: circumference, bubbles, debris, and lightning. In recent studies, five video segments (each 5 or 10 min long) were selected from all the videos<sup>[15-17]</sup>. However, no standard guidelines for grading small bowel cleanliness are currently available. Most of the reported grading systems are time-consuming, complicated, and difficult to apply clinically. In addition, the reliability and efficacy of these grading systems have rarely been evaluated. Therefore, we designed a novel, simple, and time-sparing grading system of small bowel preparation for CE. In our scoring system, one frame was selected every 5 min (1 frame/5 min) to reduce the duration of grading small bowel cleansing. As a result, the duration of grading was greatly shortened compared with prior systems, which evaluated the entire small bowel mucosa. Our scoring system was compared with a previous method, which analyzed the frames for 2 min of every 5-min period; this method is a grading system that has recently been reported, but is time-consuming in that a substantial number of frames have to be analyzed. The concordance between this grading system and our scoring system was excellent.

Validity and reliability are two minimum qualities required for a grading system. Without reliability, even a valid scale can differ among study groups<sup>[18]</sup>. In a recent study, the quantitative index (QI) was better than the qualitative evaluation (QE) for intra-observer and inter-observer reliability<sup>[19]</sup>. To make the scoring system more reliable, we selected two parameters: the proportion of visualized mucosa and the degree of obscuration by bubbles, debris, bile, *etc.* For each parameter, small bowel cleanliness can be graded objectively by scoring the im-

ages according to the percent of the area. As a result, although the agreement on the degree of obscuration tended to be lower than that of the visualized mucosa in some cases, inter-observer, intra-patient, and intra-observer agreements were excellent. If the examiners in our study had received a calibration exercise before grading cleanliness, the concordance may have been better.

To determine whether our grading system was reasonable for assessing the adequacy of small bowel preparation, we compared our system with the grading system by Viazis *et al.*<sup>[5]</sup>, and the AUC of the ROC curve was 0.925. This means that our grading system had good discriminative power. From the ROC curve, we found that the cut-off value of image quality score on adequate bowel cleansing was 2.25, which was considered to have optimal sensitivity and specificity. Therefore, this value may be used as a criterion for determining the overall adequacy of small bowel preparation for CE.

In conclusion, our novel scoring system for CE was simple, objective, and efficient. It showed excellent inter-observer, intra-patient, and intra-observer agreement. In addition, a cut-off value for the adequacy of small bowel preparation was also proposed. Therefore, our scoring system may be useful in clinical practice and in studies determining the optimal small bowel cleansing method.

## COMMENTS

### Background

For successful capsule endoscopy (CE), adequate bowel cleansing is mandatory. However, the benefits of bowel preparation prior to CE remain controversial and it is unclear which method is best. One of the reasons for this controversy is that the current grading systems have not been standardized and a generally accepted grading system is not available.

### Research frontiers

Most of the reported grading systems are time-consuming, complicated, and difficult to apply clinically. In addition, the reliability and efficacy of these grading systems have rarely been evaluated. Therefore, the authors designed a novel, simple, and time-sparing grading system of small bowel preparation for CE. For assessment, two visual parameters were used: the proportion of visualized mucosa and degree of obscuration. Representative frames from small bowel images were serially selected and scored at 5-min intervals. Intraclass correlation coefficient (ICC) was obtained to assess the reliability of the new scoring system. For efficacy evaluation and validation, scores of this new scoring system were compared with those of another previously reported cleansing grading system. Concordance with the previous system, inter-observer agreement, and intra-patient agreement were excellent with ICC values of 0.82, 0.80, and 0.76, respectively. The intra-observer agreements at four-week intervals were also excellent. The cut-off value for adequate image quality was shown to be 2.25.

### Innovations and breakthroughs

In this scoring system, one frame was selected every 5 min (1 frame/5 min) to reduce the duration of grading small bowel cleansing. As a result, the duration of grading was greatly shortened compared with prior systems, which evaluated the entire small bowel mucosa. From the receiver operating characteristic (ROC) curve, the found that the cut-off value of image quality score on adequate bowel cleansing was 2.25, which was considered to have optimal sensitivity and specificity. Therefore, this value may be used as a criterion to determine the overall adequacy of small bowel preparation for capsule endoscopy.

### Applications

The new scoring system is simple, efficient, and may be useful in clinical practice and in studies to determine the optimal small bowel cleansing method.

## Terminology

ICC: A descriptive statistic that can be used when quantitative measurements are made on units that are organized into groups. It describes how strongly units in the same group resemble each other. Its major application is in the assessment of consistency or reproducibility of quantitative measurements made by different observers measuring the same quantity.

## Peer review

In this manuscript, authors have reported a novel cleansing score system for capsule endoscopy. This new scoring system is simple, and efficient. Furthermore, it showed good inter-observer, intra-patient, and intra-observer agreement.

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## Etiological and clinicopathologic characteristics of intrahepatic cholangiocarcinoma in young patients

Hua-Bang Zhou, Hui Wang, Dong-Xun Zhou, Hao Wang, Qing Wang, Shan-Shan Zou, He-Ping Hu

Hua-Bang Zhou, Hui Wang, Dong-Xun Zhou, Hao Wang, Qing Wang, Shan-Shan Zou, He-Ping Hu, Department II of Comprehensive Treatment, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200438, China

Author contributions: Zhou HB and Hu HP designed the study; Wang H, Zhou DX, Wang H, Wang Q, Zou SS analyzed data; Zhou HB and Hu HP wrote the paper.

Correspondence to: Dr. He-Ping Hu, Professor, Department II of Comprehensive Treatment, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200438, China. [hp-hu@medmail.com.cn](mailto:hp-hu@medmail.com.cn)

Telephone: +86-21-81875141 Fax: +86-21-65566851

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### Abstract

**AIM:** To investigate the prevalence, risk factors, and clinicopathologic characteristics of intrahepatic cholangiocarcinoma (ICC) in young patients.

**METHODS:** A retrospective analysis was performed in ICC patients referred to the Eastern Hepatobiliary Surgery Hospital in Shanghai, China. Among 317 consecutively enrolled patients, 40 patients were aged  $\leq 40$  years (12.61%). We compared the risk factors and clinicopathologic characteristics of these patients (group I:  $n = 40$ ) with those aged  $> 40$  years (group II:  $n = 277$ ).

**RESULTS:** Group I had distinct features compared with group II, including a low frequency of hepatolithiasis ( $P = 0.000$ ); a high positive rate of serum hepatitis B surface antigen ( $P = 0.000$ ) and hepatitis B virus (HBV)-associated cirrhosis ( $P = 0.038$ ); a high frequency of  $\alpha$ -fetoprotein ( $> 400 \mu\text{g/L}$ ) ( $P = 0.011$ ); a low frequency of carbohydrate antigen 19-9 ( $> 37 \text{ U/mL}$ ) ( $P = 0.017$ ); and a high frequency of liver histological inflammation ( $P = 0.002$ ). Although there was no significant difference between the two groups in regards

to hepatic schistosomiasis, alcohol-associated cirrhosis and cirrhosis due to other causes ( $P > 0.05$ ), they only occurred in the elderly group.

**CONCLUSION:** The risk factors are significantly different between young and elderly ICC patients. HBV and HBV-associated cirrhosis are the most important risk factors for young ICC patients.

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**Key words:** Intrahepatic cholangiocarcinoma; Young patients; Clinicopathologic features; Hepatitis B virus; Risk factor

**Peer reviewer:** Yoshio Shirai, Associate Professor, Division of Digestive and General Surgery, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Niigata City 951-8510, Japan

Zhou HB, Wang H, Zhou DX, Wang H, Wang Q, Zou SS, Hu HP. Etiological and clinicopathologic characteristics of intrahepatic cholangiocarcinoma in young patients. *World J Gastroenterol* 2010; 16(7): 881-885 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i7/881.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i7.881>

### INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC) is a fatal cancer of the biliary epithelium, arising within the intrahepatic bile ducts. Globally, ICC is the most common primary hepatic malignancy after hepatocellular carcinoma (HCC). The incidence rates of ICC vary greatly among different areas of the world; this variation is related to the distribution of risk factors. Viral infection [hepatitis B virus (HBV) or hepatitis C virus (HCV)]<sup>[1]</sup>, primary sclerosing cholangitis (PSC)<sup>[2]</sup>, liver fluke infestation (particularly the endemic *Opisthorchis viverrini*)<sup>[3,4]</sup>, and hepatolithiasis are known as the risk factors for ICC<sup>[5,6]</sup>. ICC, similar to HCC, affects



predominantly those individuals aged > 40 years<sup>[7]</sup>; however, younger patients have recently been diagnosed with ICC. The risk factors and clinicopathologic features of young ICC patients have not yet been studied.

The aim of the present study was to investigate the prevalence, risk factors, and the clinicopathologic characteristics of ICC in young patients, and to compare these findings with the characteristics and risk factors associated with elderly patients with ICC.

## MATERIALS AND METHODS

We performed a retrospective analysis using medical records of the patients initially diagnosed with ICC in the Eastern Hepatobiliary Surgery Hospital of the Second Military Medical University, Shanghai, China from January 2003 to December 2006. The diagnosis of ICC was confirmed pathologically. We evaluated the age and sex distribution of the patients, and compared the risk factors and clinicopathologic characteristics of patients aged ≤ 40 years (group I) with those of patients aged > 40 years (group II).

To clarify a difference in the risk factors between the two groups, we analyzed HBV or HCV infection, liver cirrhosis, hepatolithiasis, and hepatic schistosomiasis (our previous study verified that these are the main risk factors for ICC patients from China). The presence of a seropositive HBsAg and/or anti-HCV (Abbott Laboratories, North Chicago, IL, USA) and HCV RNA (real time PCR; Abbott, IL, USA) was interpreted as an indication of chronic hepatitis infection. The diagnosis of ICC was confirmed by pathology. Liver function and serum tumor marker (carbohydrate antigen 19-9 and  $\alpha$ -fetoprotein) concentrations were evaluated in all the patients.

To determine the pathological characteristics, the diameter of the largest tumor was measured directly in the surgical specimens from patients who had undergone hepatic resection. The WHO tumor classification was used for pathological grading of the tumor (well, moderate or poor differentiation). When histological diversity was observed in a tumor, the higher grade, according to the classification system, was taken as the overall grade. The tumor mass was estimated by computed tomography (CT), and enlarged lymph nodes were defined as lymph nodes 0.1 cm in diameter at the portal, celiac, retrocrural or retroperitoneal lymph node stations. Ultrasound, CT scan, or surgery was used to diagnose portal vein thrombosis.

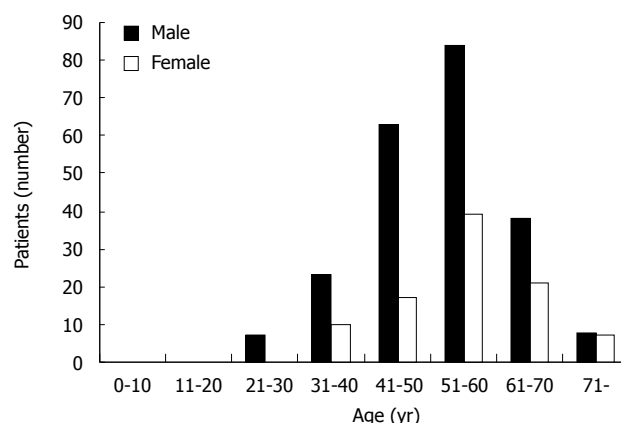
### Statistical analysis

Statistical analysis was performed using the  $\chi^2$  test to compare discrete variables, and analysis of variance (ANOVA) was made to compare continuous variables. SPSS for Windows (version 16.0) was used.  $P < 0.05$  was considered as significant difference.

## RESULTS

### Age and sex distribution

A total of 317 patients (223 men and 94 women, a male



**Figure 1** Age and sex distribution of intrahepatic cellular carcinoma (ICC) between 2003 and 2006 at the Eastern Hepatobiliary Surgery Hospital in Shanghai, China.

**Table 1** Risk factors for intrahepatic cholangiocarcinoma *n* (%)

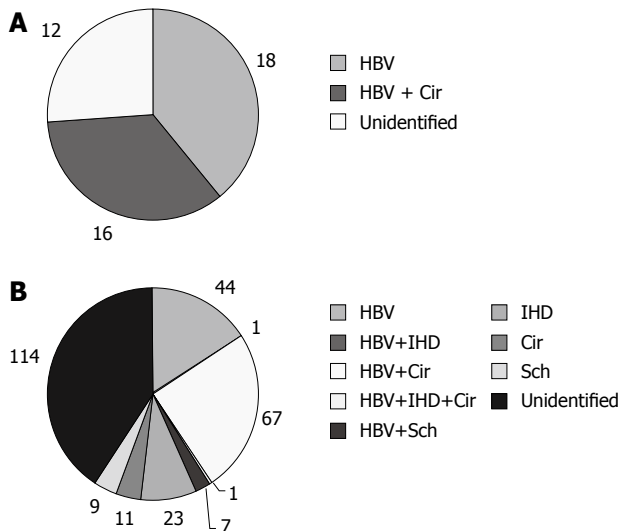
Risk factors	Group I ( <i>n</i> = 40)	Group II ( <i>n</i> = 277)	<i>P</i> value
HBV infection	34 (85.0)	120 (43.3)	0.000
HCV infection	0 (0.0)	1 (0.4)	0.703
Liver cirrhosis			0.038
Related to HBV	16 (40.0)	68 (24.5)	0.347
Related to alcohol	0 (0.0)	6 (2.2)	0.392
Other causes	0 (0.0)	5 (1.8)	
Total	16 (40.0)	79 (28.52)	
Hepatolithiasis	0 (0.0)	25 (9.0)	0.000
Liver schistosomiasis	0 (0.0)	16 (5.8)	0.119

HBV: Hepatitis B virus; HCV: Hepatitis C virus.

to female ratio, 2.37:1) initially diagnosed with ICC were enrolled in the present study. The mean age was  $53.05 \pm 10.53$  years (range 21-78). Most of ICC developed during the 4th-7th decades, with a peak at 54 years of age. Forty (12.61%) patients were aged ≤ 40 years, including 30 men and 10 women (Figure 1).

### Risk factor distribution

The risk factors for ICC are listed in Table 1. Of the 40 young ICC patients, 34 (85.0%) were seropositive for HBsAg, 16 (40.0%) had liver cirrhosis related to HBV, and none had intrahepatic-duct stones (IHD stones), liver schistosomiasis, or HCV (Figure 2A). In group II, 120 (43.3%) patients were seropositive for HBsAg, 1 (0.4%) was seropositive for anti-HCV and HCV RNA, 79 (28.5%) had liver cirrhosis, including 68 (24.5%) whose cirrhosis was related to HBV, 6 (2.2%) with liver cirrhosis related to alcohol, 5 (1.8%) with liver cirrhosis due to other causes (1 with liver cirrhosis related to HCV, 1 with nonalcoholic liver cirrhosis, and 3 with occult liver cirrhosis), 25 (9.0%) had IHD stones, and 16 (5.8%) had liver schistosomiasis (Figure 2B). It is worth mentioning that the HCV that is prevalent in Japan and some Western countries has been found to be the significant cause of ICC. In our series, only one case of ICC had HCV.



**Figure 2** Distribution of potential risk factors for ICC among young ICC patients (A) and elderly ICC patients (B). HBV = Seropositivity for HBsAg; Cir = Liver cirrhosis; IHD = Intrahepatic duct stone (Hepatolithiasis); Sch = Liver schistosomiasis.

### Clinical features

For further comparison between the two groups, the following clinical variables were investigated: gender, total bilirubin (TBIL) ( $> 20 \mu\text{mol/L}$  *vs*  $\leq 20 \mu\text{mol/L}$ ), albumin, alanine aminotransferase (ALT) ( $> 42 \text{ U/L}$  *vs*  $\leq 42 \text{ U/L}$ ), aspartate aminotransferase (AST) ( $> 37 \text{ U/L}$  *vs*  $\leq 37 \text{ U/L}$ ),  $\gamma$ -glutamyl-transferase ( $\gamma$ -GT) ( $> 64 \text{ U/L}$  *vs*  $\leq 64 \text{ U/L}$ ), alkaline phosphatase (ALP) ( $> 119 \text{ U/L}$  *vs*  $\leq 119 \text{ U/L}$ ),  $\alpha$ -fetoprotein (AFP) ( $> 400 \mu\text{g/L}$  *vs*  $\leq 400 \mu\text{g/L}$ ), and carbohydrate antigen 19-9 (CA19-9,  $> 37 \text{ ng/mL}$  *vs*  $\leq 37 \text{ ng/mL}$ ). AFP ( $> 400 \mu\text{g/L}$ ) and CA19-9 ( $> 37 \text{ U/mL}$ ) were significantly different between group I and group II by univariate analysis (Table 2).

### Pathological characteristics

Table 3 reveals the pathological characteristics in the two groups. There was significantly more histological inflammation (present *vs* no hepatitis) in the younger patient group ( $P = 0.002$ ). No difference was detected between the two groups with regard to the tumor number ( $< 2$  *vs*  $\geq 2$ ), location (right lobe, left lobe, or both lobes), tumor size (main tumor or the largest one), capsule (present *vs* absent), tumor differentiation (well, moderate or poor), portal vein invasion (invasion *vs* no invasion), microscopic satellite lesion (tiny nodule present around the main tumor *vs* no satellite), and immunohistochemical examination (cytokeratin 18 and cytokeratin 19).

## DISCUSSION

Although several risk factors have been associated with ICC, such as viral infection (HBV and/or HCV), liver cirrhosis, IHD stones, and liver parasite infestation, the distribution characteristics of these risk factors in young and elderly ICC patients and the mechanism responsible for ICC remain unknown. Here, we not only demonstrated different etiological characteristics

**Table 2** Comparison of clinical features of ICC between the two groups *n* (%)

	Group I ( <i>n</i> = 40)	Group II ( <i>n</i> = 277)	<i>P</i> value
Gender (M/F)	30/10	193/84	0.491
ALT ( $> 42 \text{ U/L}$ )	15 (60.00)	89 (32.13)	0.499
AST ( $> 37 \text{ U/L}$ )	12 (30.00)	103 (37.18)	0.377
TBIL ( $> 20 \mu\text{mol/L}$ )	7 (17.50)	62 (22.38)	0.484
Albumin (g/L)	$43.82 \pm 4.27$	$42.07 \pm 5.12$	0.236
GGT ( $> 64 \text{ U/L}$ )	19 (47.50)	176 (63.54)	0.051
ALP ( $> 119 \text{ U/L}$ )	15 (11.19)	134 (48.38)	0.198
AFP ( $> 400 \mu\text{g/L}$ )	7 (17.50)	17 (6.14)	0.011
CA19-9 ( $> 37 \text{ U/mL}$ )	14 (35.00)	153 (55.23)	0.017

M: Male; F: Female; TBIL: Total bilirubin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AFP:  $\alpha$ -fetoprotein; ALP: Alkaline phosphatase;  $\gamma$ -GT:  $\gamma$ -glutamyl-transferase; CA 19-9: Carbohydrate antigen 19-9. Group I: ICC patients aged  $\leq 40$  years; Group II: ICC patients aged  $> 40$  years.

**Table 3** Comparison of pathological features of ICC between the two groups *n* (%)

	Group I ( <i>n</i> = 40)	Group II ( <i>n</i> = 277)	<i>P</i> value
Tumor location			0.827
Right lobe	25 (6.25)	160 (57.76)	
Left lobe	14 (35.00)	111 (40.07)	
Both lobes	1 (2.50)	6 (15.00)	
Tumor size (cm)	$6.80 \pm 3.92$	$6.67 \pm 3.38$	0.892
Tumor number			0.474
1	36 (90.00)	258 (93.14)	
$\geq 2$	4 (10.00)	19 (6.86)	
Histological inflammation	15 (37.50)	47 (16.97)	0.002
Capsule	5 (12.50)	35 (12.64)	0.981
Tumor differentiation			0.506
Well	0 (0.00)	9 (3.25)	
Moderate	29 (72.50)	191 (68.95)	
Poor	11 (27.50)	71 (25.63)	
Microvascular invasion	10 (25.00)	41 (14.80)	0.101
Perineural infiltration	0 (0.00)	4 (1.44)	0.444
Portal vein invasion	3 (7.50)	30 (10.83)	0.519
Lymphatic metastasis	8 (20.00)	46 (16.61)	0.594
Microscopic satellite lesion	7 (17.50)	69 (24.91)	0.305
Immunohistochemical examinations			
Ck18 positive	38 (95.00)	269 (97.11)	0.475
Ck19 positive	40 (100.00)	267 (93.39)	0.222

Ck: Cytokeratin.

of ICC between young and elderly patients, but also the clinicopathologic features of young patients.

Our study focused on patients with ICC who were  $\leq 40$  years of age, and the “young patients” were defined as those  $\leq 40$  years of age at diagnosis. We chose the cut-off age of 40 years based on the recommended age for starting regular HCC screening for men in Asia<sup>[8]</sup> and according to Lam *et al*<sup>[9]</sup>. The age distribution of patients with ICC in China is described by a triangular-shaped curve (Figure 1). Only 12.61% of patients were  $\leq 40$  years of age.

HBV or HCV infection was strongly associated

with ICC risk. Korean investigators performed a case-control study comparing 41 cases of ICC with 406 controls without cancer and found that 13.8% of cases and 3.5% of controls were anti-HCV positive and 12.5% of cases and 2.3% of controls were HBsAg positive<sup>[10]</sup>. In a Japanese hospital-based study, investigators found that 36% of 50 patients with ICC but only 3% of 205 controls (surgical patients who did not have primary liver cancer) were HCV seropositive [OR (odds ratio) = 16.87; 95% CI (confidence interval): 5.69-50.00]<sup>[11]</sup>. Through a population based cohort study including 146 394 HCV-infected and 572 293 HCV-uninfected patients, El-Serag *et al.*<sup>[12]</sup> showed a strong association of ICC with HCV infection (hazard ratio = 2.55, 95% CI: 1.31-4.95), but the association was not observed in extrahepatic cholangiocarcinoma. Our previous study also found that the incidence of HBV infection in ICC patients was significantly higher than that in non-cancer individuals (48.6% *vs* 6.6%), indicating that chronic HBV infection was independently the most important risk factor for ICC in Chinese population (OR = 9.669, 95% CI: 6.329-14.770). Our findings were consistent with that reported by Zhou *et al.*<sup>[13]</sup> recently. In our study, 85.0% of young ICC patients had chronic HBV infection, which is significantly higher than that of elderly ICC patients (43.3%). It is interesting to note that HBV infection was also the only risk factor identified in young ICC patients. It is worth mentioning that the HCV that is prevalent in Japan and some Western countries has been proven to be the significant cause of ICC. In our series, only one case of ICC had HCV.

Cirrhosis, of any cause, has also been associated with intrahepatic cholangiocarcinoma<sup>[5]</sup>. A cohort study of over 11 000 patients with cirrhosis, followed up over 6 years, showed a 10-fold risk compared with the general population<sup>[14]</sup>. A prospective controlled study from Japan reported the risk of developing cholangiocarcinoma in patients with cirrhosis related to HCV as 3.5% at 10 years, 1000 times higher than in the general population<sup>[15]</sup>. The data obtained from our previous study also showed that cirrhosis, particularly HBV-associated cirrhosis, was an important risk factor for ICC in Chinese population. In the present study, the etiological distribution of cirrhosis was significantly different between young ICC patients and elderly ICC patients. HBV-associated cirrhosis had a higher incidence in young ICC patients than in the elderly group, while alcoholic cirrhosis and cirrhosis due to other causes only occurred in elderly ICC patients.

Hepatolithiasis is rare in Western countries, but relatively common in some parts of Asia, and is associated particularly with peripheral intrahepatic cholangiocarcinoma<sup>[4]</sup>. In Taiwan, up to 70% of patients with intrahepatic cholangiocarcinoma undergoing resection reportedly have intrahepatic biliary stones, and in Japan this figure is 6%-18%<sup>[16]</sup>. Biliary stones are thought to cause bile stasis, predisposing to recurrent bacterial infections and subsequent inflammation, a potential cofactor for cholangiocarcinogenesis. In our cohort,

hepatolithiasis only occurred in elderly ICC patients. The result indicates hepatolithiasis may be not a risk factor of ICC development for young patients.

A large body of experimental and epidemiological data suggests a pathogenic association between liver parasite infection, especially *Opisthorchis viverrini* (and less definitively *Clonorchis sinensis*) and intrahepatic cholangiocarcinoma<sup>[1]</sup>. Our previous study showed that hepatic schistosomiasis had a higher incidence in ICC patients than in no-cancer individuals. The data suggests that hepatic schistosomiasis was a risk factor for ICC development (OR = 11.06, 95% CI: 3.368-36.337). Although there was no significant difference between the young ICC patients and elderly ICC patients in regards to hepatic schistosomiasis ( $P > 0.05$ ), similar to alcohol-cirrhosis and cirrhosis due to other causes, hepatic schistosomiasis also only occurred in the elderly group.

$\alpha$ -fetoprotein (AFP), a 70-kDa glycoprotein, is normally produced during fetal development by the liver and yolk sac. The protein levels drop off rapidly after birth, and by the second year of life only trace amounts are detectable in the serum. AFP is increased in the majority of patients with HCC and is useful in the diagnosis and follow-up of cases. Studies suggest that, in patients with suspected HCC clinically, AFP levels  $> 400$  ng/mL should strongly confirm the presence of HCC *via* a tissue diagnosis<sup>[17,18]</sup>. Some cancers may originate from cancer stem cells, which may form *via* the carcinogenesis of normal stem cells<sup>[19-21]</sup>. It has been suggested that hepatocytes and cholangiocytes arise from the same pool of hepatic precursor cells, also called oval cells. Carcinogenesis of such hepatic precursor cells may cause ICC<sup>[22]</sup>. Hepatic progenitor cells were also shown to strongly express AFP mRNA and produce AFP during differentiation<sup>[23]</sup>. Compared with the elderly ICC patients, young ICC patients exhibited a higher incidence of AFP  $> 400$   $\mu$ g/L (17.50% *vs* 6.14%). Our data indicated that the neoplastic transformation of oval cells may be one of the mechanisms for ICC development and that the oval cell precursor retains its ability to produce AFP in the process of malignant transformation.

In a recent prospective study, serum CA19-9 was found to be useful in diagnosing cholangiocarcinoma, in deciding whether the tumor had been radically resected and in monitoring the effect of treatment. Serum CA19-9 concentrations were significantly elevated in patients with cholangiocarcinoma compared with patients with HCC, benign biliary disease or healthy individuals. After curative resection, serum CA19-9 decreased to a preoperative level<sup>[24]</sup>. In the present study, young ICC patients exhibited a lower serum CA19-9 level, similar to HCC.

In conclusion, the risk factors for ICC are different between young and elderly patients. HBV infection and HBV-associated cirrhosis may be the main risk factors for young ICC patients. Young ICC patients share etiological and many clinicopathologic similarities with HCC patients. These results indicated that ICC in young patients and HCC have a common process of

carcinogenesis (through a similar long-term inflammatory carcinogenic process) and that both may arise from hepatic progenitor cells.

## COMMENTS

### Background

Although several risk factors have been associated with the development of Intrahepatic cholangiocarcinoma (ICC), such as hepatitis B virus (HBV), hepatitis C virus (HCV) or cirrhosis, the risk factors and clinicopathologic features of young ICC patients have not been fully studied.

### Applications

ICC in young patients and hepatocellular carcinoma (HCC) shared a common process of carcinogenesis (through a similar long-term inflammatory carcinogenic process) and that both may arise from hepatic progenitor cells. However, this presumption awaits verification by more studies.

### Peer review

The authors clearly demonstrated that the risk factors for ICC differed significantly between young patients and elderly patients in a Chinese population. They also showed that HBV infection was closely associated with the development of ICC in young patients.

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## Does *Helicobacter pylori* infection play a role in iron deficiency anemia? A meta-analysis

Xin-Hua Qu, Xiao-Lu Huang, Ping Xiong, Cui-Ying Zhu, You-Liang Huang, Lun-Gen Lu, Xu Sun, Lan Rong, Liang Zhong, Da-Yu Sun, Hai Lin, Ming-Ci Cai, Zhi-Wei Chen, Bing Hu, Lian-Ming Wu, Yi-Bin Jiang, Wei-Li Yan

Xin-Hua Qu, Xiao-Lu Huang, Cui-Ying Zhu, Ming-Ci Cai, Zhi-Wei Chen, Bing Hu, Lian-Ming Wu, Wei-Li Yan, Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200127, China

Ping Xiong, Shanghai No. 9 People's Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200011, China  
You-Liang Huang, Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN 46202-5120, United States

Lun-Gen Lu, Shanghai Institute of Digestive Diseases, Shanghai 200001, China

Xu Sun, Lan Rong, Liang Zhong, Da-Yu Sun, Yi-Bin Jiang, Department of Gastroenterology, Huashan Hospital affiliated to Fudan University, Shanghai 200040, China

Hai Lin, Shanghai Health Development Research Center, Shanghai 200040, China

**Author contributions:** Qu XH, Huang XL, Xiong P and Zhu CY contributed equally to this work; Qu XH, Huang XL, Xiong P, Zhu CY, Jiang YB and Yan WL designed the research; Qu XH, Huang XL, Cai MC, Chen ZW, Hu B and Wu LM performed the research; Qu XH, Huang XL, Xiong P, Lu LG, Sun X; Rong L, Zhong L, Sun DY, Lin H collected data; Qu XH, Huang XL, Xiong P, Zhu CY and Lu LG analyzed the data; Qu XH, Huang XL, Xiong P, Huang YL, Lu LG and Yan WL wrote the paper.

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**Correspondence to:** Wei-Li Yan, Associate Professor, Renji Hospital, Shanghai Jiaotong University School of Medicine, 1630 Dongfang Road, Shanghai 200127, China. [w1\\_yan67@126.com](mailto:w1_yan67@126.com)  
Telephone: +86-21-26370067 Fax: +86-21-58394262

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association between *Helicobacter pylori* (*H. pylori*) and iron deficiency anemia (IDA).

**METHODS:** A defined search strategy was used to search Medline, Embase, the Cochrane Library, Clinical Trials, Cochrane Central Register of Controlled Trials, Premedline and Healthstar. Odds ratio (OR) was used to evaluate observational epidemiology studies, and weighted mean difference (WMD) was used to demonstrate the difference between control and intervention groups.

**RESULTS:** Fifteen observational studies and 5 RCTs were identified and used for calculation. The pooled OR for observational studies was 2.22 (95% CI: 1.52-3.24,  $P < 0.0001$ ). The WMD for hemoglobin (HB) was 4.06 g/L (95% CI: -2.57-10.69,  $P = 0.01$ ), and the WMD for serum ferritin (SF) was 9.47  $\mu$ g/L (95% CI: -0.50-19.43,  $P < 0.0001$ ). Results were heterogeneous for all comparisons.

**CONCLUSION:** This meta-analysis on observational studies suggests an association between *H. pylori* and IDA. In RCTs, eradication of *H. pylori* can improve HB and SF levels but not significantly.

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**Key words:** *Helicobacter pylori*; Iron-deficiency anemia; Meta-analysis; Hemoglobins; Odds ratio

**Peer reviewers:** Francesco Manguso, MD, PhD, UOC di Gastroenterologia, AORN A. Cardarelli, Via A. Cardarelli 9, Napoli, 80122, Italy; Morten Hylander Møller, Anaesthesiology and Intensive Care Medicine, Copenhagen University Hospital Herlev, Skolevej 14B, Holte, 2840, Denmark

### Abstract

**AIM:** To perform a meta-analysis of observational studies and randomized controlled trials (RCTs) on the

Qu XH, Huang XL, Xiong P, Zhu CY, Huang YL, Lu LG, Sun X, Rong L, Zhong L, Sun DY, Lin H, Cai MC, Chen ZW, Hu B, Wu LM, Jiang YB, Yan WL. Does *Helicobacter pylori* infection play a role in iron deficiency anemia? A meta-analysis. *World*

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## INTRODUCTION

Anemia, defined as a hemoglobin concentration below established cut-off levels, is a widespread public health problem with major consequences for human health as well as social and economic development<sup>[1]</sup>. The World Health Organization (WHO) estimates that about 2 billion people in the world are suffering from this disease, and that approximately 50% of all anemia cases are diagnosed as iron deficiency anemia (IDA)<sup>[2,3]</sup>. IDA affects the work capacity of patients and may contribute to mortality, thus limiting economic development. The overall death rate from IDA has been underestimated in most surveys from many developing and developed countries<sup>[4,5]</sup>. WHO suggested that researchers and clinical doctors should investigate the etiology of IDA and develop therapeutic strategies because timely treatment restores personal health and increases national productivity by 20%<sup>[6,7]</sup>.

It is known that a variety of causes such as inadequate iron intake, chronic blood loss, chronic disease, malabsorption, hemolysis, or a combination of these, can induce IDA<sup>[5,8-10]</sup>. Among possible causes, the involvement of *Helicobacter pylori* (*H. pylori*) infection remains controversial<sup>[11-13]</sup>. *H. pylori* is a highly prevalent microbial infection. Over 50% people in the world are infected by *H. pylori*. In Africa, Mexico, South America and Central America, *H. pylori* infection reaches 70%-90% of the population<sup>[14,15]</sup>. *H. pylori* has been considered as a major cause for the development of peptic ulcer disease, gastric malignancy and dyspeptic symptoms<sup>[16-19]</sup>. Recent studies have shown that *H. pylori* can also cause other extragastric diseases<sup>[20-22]</sup>. However, knowledge regarding any relation between *H. pylori* infection and IDA is limited. Moreover, studies regarding the role of *H. pylori* infection in IDA and the effectiveness of the eradication of *H. pylori* in the treatment of IDA are controversial.

This clinical research question is addressed by this meta-analysis. The aim of the study was to evaluate the association between *H. pylori* infection and IDA and examine the effect of *H. pylori* eradication on serum hemoglobin (HB) and serum ferritin (SF) levels. Observational epidemiological studies have demonstrated an association between *H. pylori* and IDA by comparing IDA risk between *H. pylori*-infected and non-infected participants. Randomized controlled trials (RCTs) have established a cause and effect relationship between *H. pylori* and IDA. In this meta-analysis, we hypothesized that there is a significant difference in IDA risk between *H. pylori*-infected and non-infected participants, and that *H. pylori* eradication therapy can significantly increase HB and SF concentration, thus alleviating IDA. We tested our

hypothesis by pooling the results of studies on *H. pylori* and IDA.

## MATERIALS AND METHODS

### Search strategy and identification of studies

We searched, without language restrictions, for all publications on *H. pylori* and IDA between January 1966, and June 2009. Searches were performed on Medline, Embase, Clinical Trials, Database of Abstracts of Reviews of Effects (DARE), Cochrane Central Register of Controlled Trials (CENTRAL), the Cochrane Database of Systematic Reviews, Premedline, Healthstar, by using the MeSH heading: “*Helicobacter pylori*”, “iron-deficiency anemia”, “anemia”, “iron” and “hemoglobin” and the non-MeSH terms “sideropenic refractory anemia” and “serum ferritin”. The reference lists of major textbooks, review articles, and of all the included articles identified by the search were then individually searched to find other potentially eligible studies. Information about unpublished and ongoing RCTs was sought from authors of the included RCTs, and experts in the field.

### Selection criteria and validity assessment

The present meta-analysis followed the Quality of Reports of Meta-Analyses of RCTs (QUOROM) guideline for RCTs and observational studies in epidemiology [Meta-analysis of Observational Studies in Epidemiology (MOOSE) and Methodological Index for Non-Randomized Studies (MINORS)] guideline in observational studies<sup>[23-25]</sup>. To avoid selection bias, selection criteria were established before searching. Two reviewers (Qu XH and Huang XL) identified articles eligible for further review by performing an initial screen of the abstracts or titles of the search results. The second screening was based on a full-text review according to the selection criteria. The observed agreement between reviewers for eligibility of articles was 96.3%, corresponding to modest agreement ( $\kappa = 0.40$ ). Discrepancies were resolved by discussion and consultation with other reviewers (Xiong P and Zhu CY).

**Observational epidemiology studies:** Observational epidemiology studies (cross-sectional, case-control, or cohort) investigating the prevalence of IDA in *H. pylori*-positive patients and negative controls were included in this meta-analysis. Duplicate publications and those studies in which patients had other underlying common IDA causes (e.g. aspirin/NSAID use, colonic carcinoma, gastric carcinoma, angiodysplasia) were excluded.

**Randomized controlled trials:** In order for an RCT to be included, its participants must have had both *H. pylori* infection and IDA/iron deficiency (ID). At least 2 authors independently assessed the methodological quality of included RCTs by Jadad scores<sup>[26]</sup>. In addition, for a study

to be eligible for inclusion, the use of therapy to eradicate *H. pylori* in intervention groups and administration of oral ferrous sulfate to both intervention and control groups were required. Discrepancies in data extraction were resolved by discussion among authors (Qu XH and Huang XL).

### Data abstraction

For observational epidemiology studies, we collected information on the year of publication, location of the study, age groups, number of cases and controls, country and region, number of IDA positive and negative patients, test method for *H. pylori*.

For RCTs, we collected information on references, year of publication, sample size, age group, treatment therapies, *H. pylori* testing methods and changes in mean  $\pm$  SD of HB and SF in both the intervention and control groups.

### Statistical analysis

For observational epidemiology studies, we recorded the prevalence of IDA in *H. pylori*-positive patients and controls for each study as an odds ratio (OR) and 95% CI and the weight of the studies. We used the heterogeneity  $\chi^2$  (Cochran Q) statistic to formally analyze heterogeneity across included studies. Meta-analysis was performed using Review Manager Version 5 (Cochrane Collaboration and Update Software) for observational studies<sup>[27]</sup>.

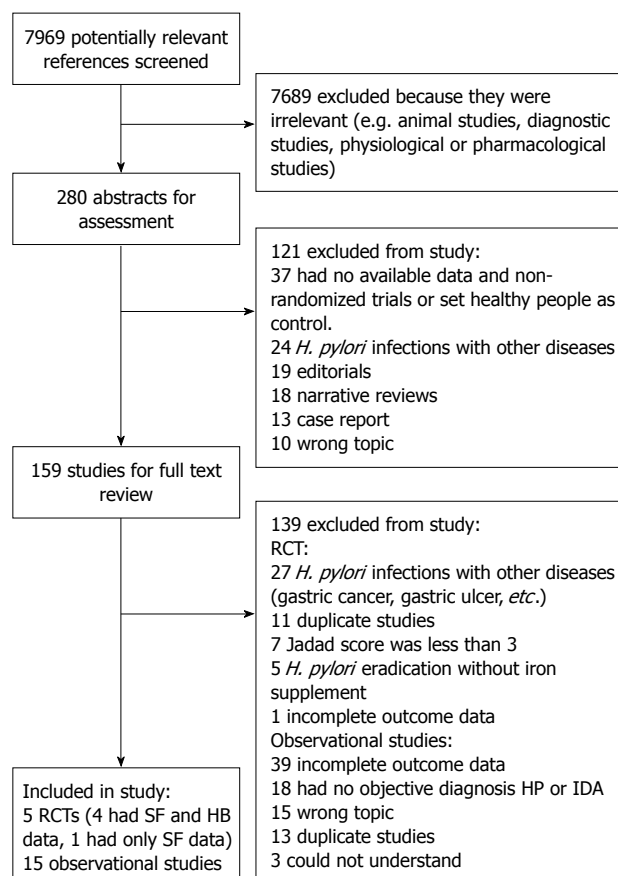
For RCTs, we collected changes in HB and SF concentration after *H. pylori* eradication and evaluated them by using weighted mean difference (WMD) with 95% CI. A  $\chi^2$  test was used to assess heterogeneity of the studies. If the studies were found to be heterogeneous, (i.e.  $\chi^2 > df$ ), we utilized the DerSimonian and Laird random-effects model<sup>[28]</sup> rather than a fixed effects model to reassess the pooled estimates. The source of heterogeneity was investigated as described below. Publication bias was performed by both Review Manager Version 5 and STATA version 10. We also performed the Duval and Tweedie nonparametric “trim and fill” procedure<sup>[29]</sup> to further assess the possible effect of publication bias in our meta-analysis.

### Subgroup analysis

Subgroup analysis was performed to assess the factors that might impact the pooled estimates and to investigate the source of heterogeneity. Sensitivity analysis was also conducted to test whether the analysis was robust by changing statistical methods, reanalyzing the data, and comparing the 2 results by the *t* test.

### Publication bias

Funnel plots and Begg’s test<sup>[30]</sup> are thought to detect the existence of publication bias of pooled ORs within observational studies. Small studies are scattered widely at the bottom of the graph, while the spread narrows for larger studies. When a funnel plot seemed to be



**Figure 1** Flow chart showing the trial flow for selection of RCTs and observational studies to be included. RCT: Randomized controlled trial; HP: *H. pylori*; IDA: Iron deficiency anemia; HB: Hemoglobin; SF: Serum ferritin.

asymmetrical, we used Duval and Tweedie’s nonparametric “trim and filled” method as a sensitivity analysis to reassess the pooled estimates<sup>[29]</sup>. This method considers the possibility of hypothetical “missing” studies that might exist and recalculates the results with the imputed missing studies.

## RESULTS

### Search results

The search strategy retrieved 7969 potentially relevant references. Of these, 7689 were not relevant, e.g. animal studies, physiological or pharmacological studies. The remaining 280 references were assessed by screening their abstracts, and we excluded any references that were editorials or narrative reviews. One hundred and fifty nine studies were subjected to a full text review and excluded according to the selection criteria as described earlier. Supplementary studies were identified that had been published only as abstracts from conference proceedings of scientific meetings. We then excluded all RCTs with a Jadad score under 3 to ensure the quality of eligible trials. Fifteen observational studies<sup>[31–45]</sup> and 5 RCTs<sup>[46–49]</sup> (4 of which<sup>[46–49]</sup> provided both HB and SF data, and one study<sup>[40]</sup> provided only SF data) meeting our criteria were included in our meta-analysis (Figure 1).

Table 1 Summary characteristics of studies and participants

Ref.	Participants	Age group (yr)	Male (%)	Prevalence of <i>H. pylori</i> infection (%)	<i>H. pylori</i> test methods
Observational studies					
Milman <i>et al</i> <sup>[31]</sup>	2264	30-60	1153 (51)	32.0	Sampling mode Hospital/health examination
Choe <i>et al</i> <sup>[32]</sup>	375	10-15	205 (55)	16.8	High school/questionnaires
Cuoco <i>et al</i> <sup>[33]</sup>	362	16-58	115 (32)	21.0	Hospital diagnosed patients
Choe <i>et al</i> <sup>[34]</sup>	660	15-17	376 (57)	29.5	High school/physical examination
Nahon <i>et al</i> <sup>[35]</sup>	210	57.4 (21.4) (SD)	80 (38)	NA	Hospital diagnosed patients
Choe <i>et al</i> <sup>[36]</sup>	937	10-18	475 (51)	20.8	-/physical examination
Choi <sup>[37]</sup>	674	9-12	344 (51)	13.6	Middle-class families/physical examination
Ciacchi <i>et al</i> <sup>[38]</sup>	55	> 17	22 (40)	60.0	Hospital diagnosed patients
Hershko <i>et al</i> <sup>[39]</sup>	210	16-77	NA	NA	Hospital diagnosed patients
Gessner <i>et al</i> <sup>[40]</sup>	690	7-11	NA	87.0	8 most populous villages/physical examination
Baggett <i>et al</i> <sup>[41]</sup>	668	7-11	354 (53)	86.5	10 predominantly villages/physical examination
Cardenas <i>et al</i> <sup>[42]</sup>	7462	≥ 3	NA	27.1	NHANES/questionnaire, laboratory, examination data
Süoglu <i>et al</i> <sup>[43]</sup>	70	4-16	NA	50.0	Hospital diagnosed patients
Haghi-Ashtiani <i>et al</i> <sup>[44]</sup>	209	2-14	111 (45)	47.8	Hospital diagnosed patients
Mulayim <i>et al</i> <sup>[45]</sup>	117	NA	0 (0)	61.5	Hospital diagnosed patients
Randomized controlled trials					
Choe <i>et al</i> <sup>[46]</sup>	13	10-17	NA	19.7	Treatment therapies B+A+M
Gessner <i>et al</i> <sup>[40]</sup>	201	7-11	NA	87.0	L+A+C
Chen <i>et al</i> <sup>[47]</sup>	86	18-76	50 (58)	NA	B+A+M
Vijayan <i>et al</i> <sup>[48]</sup>	22	> 13	NA	NA	L+T+C
Sarker <i>et al</i> <sup>[49]</sup>	99	2-5	46 (46)	NA	O+A+C

UBT: Urea breath test; IgG: Immunoglobulin G; NHANES: National Health and Nutrition Examination Survey; B: Bismuth; A: Amoxicillin; M: Metronidazole; L: Lansoprazole; C: Clarithromycin; T: Tinidazole; O: Omeprazole.

### Study characteristics and quality

In total, 15 183 patients from 20 studies (15 observational and 5 RCTs) were included in the meta-analysis, and the characteristics of the sample are summarized in Table 1.

Of the observational studies, 4 studies with participants over 18 years old<sup>[31,35,38,39]</sup> and one study with patients aged 16-58 years old<sup>[33]</sup> were classified as adult groups. Three studies with patients aged 10-18 years old were classified as adolescent groups<sup>[32,34,36]</sup>. Two studies with patients younger than 11 years old were classified as child groups<sup>[41,49]</sup>. For the presence of *H. pylori*, 6 studies utilized serum immunoglobulin G (IgG)<sup>[31,32,34,36,37,42]</sup>, 5 utilized histological examination<sup>[33,35,38,43,44]</sup> and 3 used the urea breath test (UBT)<sup>[40,41,45]</sup>. UBT and serum IgG were utilized by Hershko *et al*<sup>[39]</sup> in their studies. Of the RCTs, 2 studies with participants aged 2-11 years old were classified as child groups<sup>[40,49]</sup> and 3 were classified as adolescent and adult groups<sup>[46-48]</sup>. All the RCTs used eradication triple therapy for *H. pylori* as intervention.

Study methodological quality is shown in Table 2 (observational studies) and Table 3 (RCTs). In observational studies, the MINORS quality score ranged from 6 to 14 points. Only 8 (53%) of the articles included employment outcomes as part of the main study aim. Five studies satisfied the criteria for inclusion. Most data were collected according to a protocol established before

the beginning of the study, and most studies have no exclusion or details about the reasons for exclusion. In RCTs, qualities of all the studies were evaluated by Jadad score. All of the studies included had a score greater than 3. Only one study fulfilled all of the evaluated quality criteria. All studies were randomized, and for 4 of them, the generation of allocation sequence was judged adequate. Only 2 studies were designed as double-blind, but placebo was offered in only one study. Three of the 5 RCTs had no exclusions, and one of the 5 RCTs gave details on the reasons for exclusion.

### Summary estimates

**Risk of IDA for *H. pylori*-positive versus *H. pylori*-negative patients:** We tested the heterogeneity of the 15 observational studies that provided information about prevalence OR, and the heterogeneity  $\chi^2$  statistic was 51.29 ( $P < 0.00001$ ). Therefore, the pooled estimates were evaluated under a random effects model instead of a fixed effects model. The pooled OR was 2.22 and the 95% CI was 1.52-3.24 ( $P < 0.0001$ ) suggesting that IDA is associated with *H. pylori* (Figure 2).

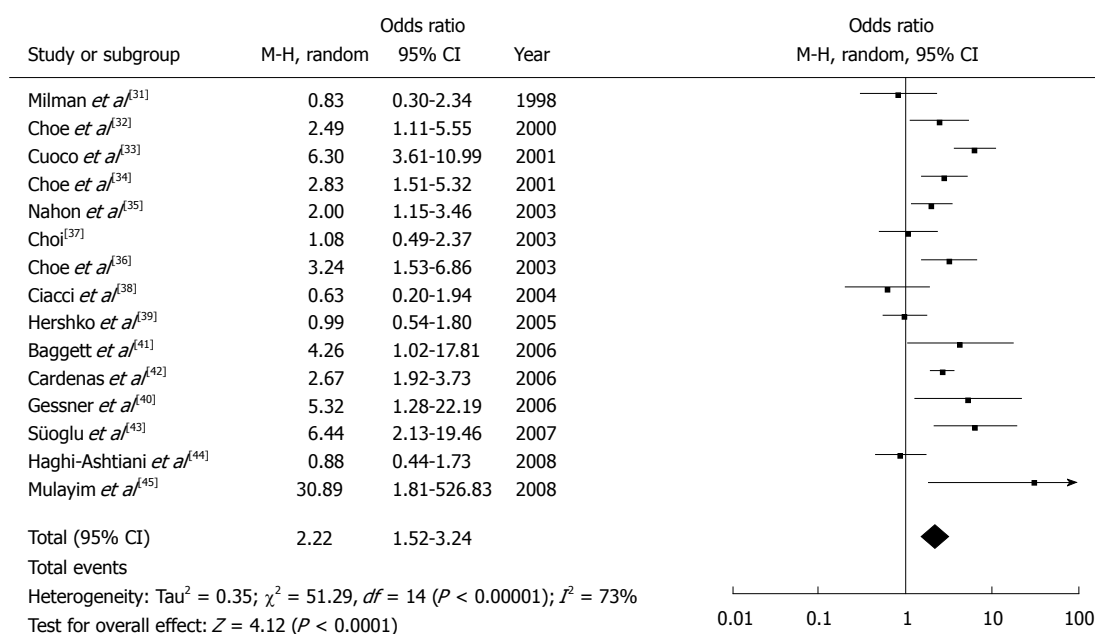
***H. pylori* eradication effect in IDA patients:** Four RCTs<sup>[46,48-49]</sup> reported blood parameter (HB levels and SF concentrations) differences and one RCT<sup>[40]</sup> reported



Table 2 Methodological quality assessment based on MINORS<sup>1</sup>

Source	Aim <sup>2</sup>	Rate <sup>3</sup>	Data <sup>4</sup>	Measure <sup>5</sup>	Bias <sup>6</sup>	Time <sup>7</sup>	Loss <sup>8</sup>	Size <sup>9</sup>	Total <sup>10</sup>
Milman <i>et al.</i> <sup>[31]</sup>	2	2	2	2	2	1	1	0	12
Choe <i>et al.</i> <sup>[32]</sup>	2	1	1	1	2	1	0	0	8
Cuoco <i>et al.</i> <sup>[33]</sup>	1	1	1	2	2	0	0	1	8
Choe <i>et al.</i> <sup>[34]</sup>	2	1	1	2	2	1	0	0	9
Nahon <i>et al.</i> <sup>[35]</sup>	1	2	2	1	2	1	1	0	10
Choe <i>et al.</i> <sup>[36]</sup>	2	2	1	1	0	0	0	0	6
Choi <sup>[37]</sup>	2	1	1	2	2	2	1	0	11
Ciacci <i>et al.</i> <sup>[38]</sup>	1	1	2	1	2	1	0	0	8
Hershko <i>et al.</i> <sup>[39]</sup>	1	1	1	1	2	1	1	0	8
Gessner <i>et al.</i> <sup>[40]</sup>	2	2	2	1	2	2	2	1	14
Baggett <i>et al.</i> <sup>[41]</sup>	2	1	2	1	2	1	1	0	10
Cardenas <i>et al.</i> <sup>[42]</sup>	2	2	2	1	2	1	1	1	12
Süoglu <i>et al.</i> <sup>[43]</sup>	1	1	1	1	2	1	0	0	7

<sup>1</sup> Assessed with the adapted Methodological Index for Non-Randomized Studies (MINORS)<sup>[25]</sup>; <sup>2</sup> Clearly stated aim (0,1,2 points); <sup>3</sup> Inclusion of consecutive patients and response rate (0,1,2); <sup>4</sup> Prospective collection of data (0,1,2); <sup>5</sup> Inclusion of employment measure (0,1,2); <sup>6</sup> Unbiased assessment of study end points (0 or 2); <sup>7</sup> Follow-up time appropriate (0,1,2); <sup>8</sup> Loss to follow-up (0,1,2); <sup>9</sup> Prospective calculation of the study size (0 or 1); <sup>10</sup> Total: minimum equals 0; maximum equals 15 points.



**Figure 2 Forest plot of the observational studies.** M-H, Random: Mantel-Haenszel heterogeneity random effects model. Horizontal lines = 95% CI. The rectangles represent the point estimates of the study and the size of the rectangle represents the weight given to each study in the meta-analysis. The diamond represents the summary estimate; the size of the diamond represents the CIs of the summary estimate.

Table 3 Quality evaluation of the included studies

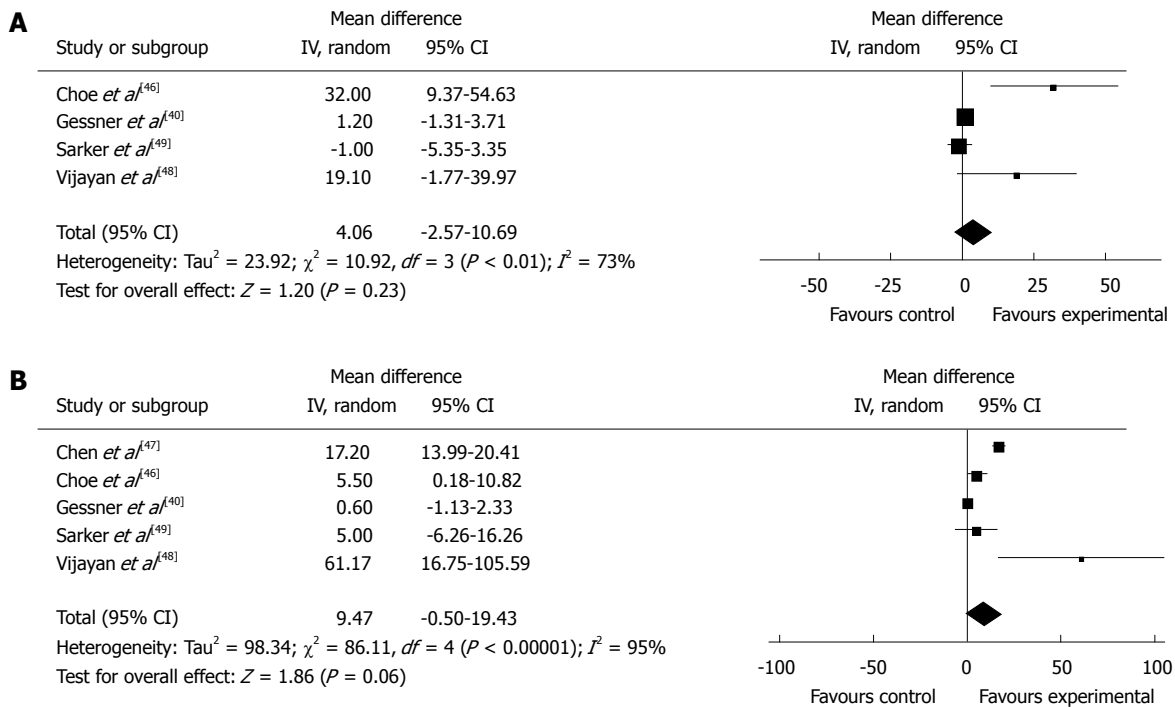
RCT	Randomization	Blindness	Withdraw and dropout	Total
Choe <i>et al.</i> <sup>[46]</sup>	2	2	0	4
Gessner <i>et al.</i> <sup>[40]</sup>	2	1	1	4
Chen <i>et al.</i> <sup>[47]</sup>	1	1	1	3
Vijayan <i>et al.</i> <sup>[48]</sup>	2	2	1	5
Sarker <i>et al.</i> <sup>[49]</sup>	2	0	1	3

SF concentration differences between the intervention and control groups after *H. pylori* eradication therapy. We pooled summary estimates to demonstrate the treatment

effect and underlying connection between *H. pylori* and IDA. The results showed that *H. pylori* eradication therapy can improve IDA. Four RCTs compared the increase in HB levels and 5 in SF concentrations achieved with *H. pylori* eradication (plus iron) treatment and with iron administration alone in patients with IDA, and found a greater effect in the eradication group (WMD of HB: 4.06 g/L, 95% CI: -2.57-10.69,  $P = 0.01$ ; WMD of SF: 9.47  $\mu\text{g/L}$ ; 95% CI: -0.50-19.43,  $P < 0.0001$ , Figure 3).

### Subgroup analysis

Subgroup analysis was performed to investigate the source of heterogeneity and detect the influential factors that could impact the summary estimates. The methodological



**Figure 3** The treatment effect and underlying connection between *H. pylori* and IDA. A: Weighted mean difference (WMD) forest plots of HB (g/L) involved in the meta-analysis; B: Forest plots of studies estimate changes in SF ( $\mu\text{g/L}$ ) level. IV, Random: Inverse variance heterogeneity random effects model. Horizontal lines = 95% CI. The size of the data marker corresponds to the weight of that study. The diamond represents the summary estimate. The result favors experimental groups.

and biological heterogeneity of the studies made it possible to explore the summary estimates in many different subgroups.

In observational studies, differences in the sensitivity of the *H. pylori* test methods could partly result in different pooled ORs. The 15 observational studies utilized 3 methods, enzyme-linked immunosorbent assay (ELISA) serum IgG, histological biopsy and UBT, to test for the presence of *H. pylori*. The pooled OR of ELISA serum IgG was lowest (OR = 2.16, 95% CI: 1.49-3.14,  $P = 0.10$ ) and the pooled OR of UBT was highest (OR = 5.88, 95% CI: 2.27-15.23,  $P = 0.47$ ). The pooled ORs were consistent with the sensitivity of these methods.

Subgroup analysis for different age groups revealed a significant difference between children, adolescents and adults in the association between *H. pylori* and IDA. The pooled OR of children younger than 11 years was 4.76 (95% CI: 1.73-13.08,  $P = 0.83$ ). Adolescents yielded a pooled OR of 2.85 (95% CI: 1.68-4.31,  $P = 0.89$ ), while adults had a pooled OR of 1.55 (95% CI: 0.67-3.62,  $P < 0.0001$ ).

In RCTs, factors included in the subgroup analysis were age and therapy of each study. The summary estimate from children was 0.65 g/L (95% CI: -1.52-2.82,  $P = 0.39$ ) for HB changes, significantly different from the pooled estimate of 25.03 g/L (95% CI: 9.69-40.37,  $P = 0.41$ ) from adolescent and adult groups, indicating that adult IDA patients react more strongly to *H. pylori* eradication therapy. The WMD for SF changes was 0.70  $\mu\text{g/L}$  (95% CI: -1.01-2.41,  $P = 0.45$ ) in children while the WMD was 14.79  $\mu\text{g/L}$  (95% CI: 2.53-27.05,  $P =$

0.0001) in adolescent and adult patients.

To examine the method of therapy, we separated the studies into a bismuth triple therapy group and a proton pump inhibitor (PPI) triple therapy group. Bismuth triple therapy showed an obvious advantage (WMD of SF = 11.55  $\mu\text{g/L}$ , 95% CI: 0.09- 23.01,  $P = 0.0002$ ) over PPI triple therapy (WMD of SF = 7.15  $\mu\text{g/L}$ , 95% CI: -6.45-20.75,  $P = 0.002$ ), particularly when used together with oral ferrous sulfate for *H. pylori* patients with IDA.

We analyzed the association between *H. pylori* and IDA in developed areas and less developed areas, large sample subgroups and small sample subgroups, and did not find any significant difference between those parameters. Table 4 shows the summary estimates of subgroups of both RCTs and observational studies.

### Sensitivity analysis

We chose to change the weights of every observational study involved so as to detect the stability of this meta-analysis. We then reanalyzed the data using different statistical methods. The pooled OR using a fixed effects model was 2.34 (95% CI: 1.97-2.78), which is not a significant change from the original random effects model ( $P = 0.74$ ).

### Publication bias

Visual inspection of the Begg's funnel plot revealed asymmetry ( $P < 0.01$ ). This raises the possibility of publication bias, so we undertook a sensitivity analysis using the trim and fill method. This method conservatively imputes hypothetical negative unpublished studies

Table 4 Summary of subgroup analyses of both observational studies and experimental studies

	Subjects ( <i>n</i> )		95% CI	Heterogeneity ( $\chi^2$ )	<i>P</i> <sup>1</sup>
Observational studies					
Odds ratio (OR)					
<i>H. pylori</i> test methods					
ELISA serum IgG	12372	2.16	1.49-3.14	9.27	0.10
Histological biopsy	906	2.17	0.90-5.26	28.85	< 0.0001
UBT	1475	5.88	2.27-15.23	1.53	0.47
Age					
Child	1358	4.76	1.73-13.08	0.05	0.83
Adolescent	1972	2.85	1.68-4.31	0.22	0.89
Adult	3101	1.55	0.67-3.62	28.41	< 0.0001
Randomized controlled trials <sup>2</sup>					
Weighted mean difference (WMD)					
Hemoglobin (g/L)					
Age					
Child	300	0.65	-1.52-2.82	0.74	0.39
Adolescent and adult	35	25.03	9.69-40.37	0.67	0.41
Therapy					
PPI	322	0.95	-2.98-4.87	3.71	0.16
Bismuth	13	32.00	9.37-54.63	-	-
<i>H. pylori</i> test methods					
Histological biopsy	223	7.03	-9.41-23.47	2.79	0.10
UBT	35	25.03	9.69-40.37	0.67	0.41
Serum ferritin ( $\mu$ g/L)					
Age					
Child	300	0.70	-1.01-2.41	0.57	0.45
Adolescent and adult	121	14.79	2.53-27.05	17.91	0.0001
Therapy					
PPI	322	7.15	-6.45-20.75	7.68	0.002
Bismuth	99	11.55	0.09-23.01	13.61	0.0002

<sup>1</sup>*P*-value tested for heterogeneity of subgroups; <sup>2</sup>Data were derived from hemoglobin changes and serum ferritin changes.

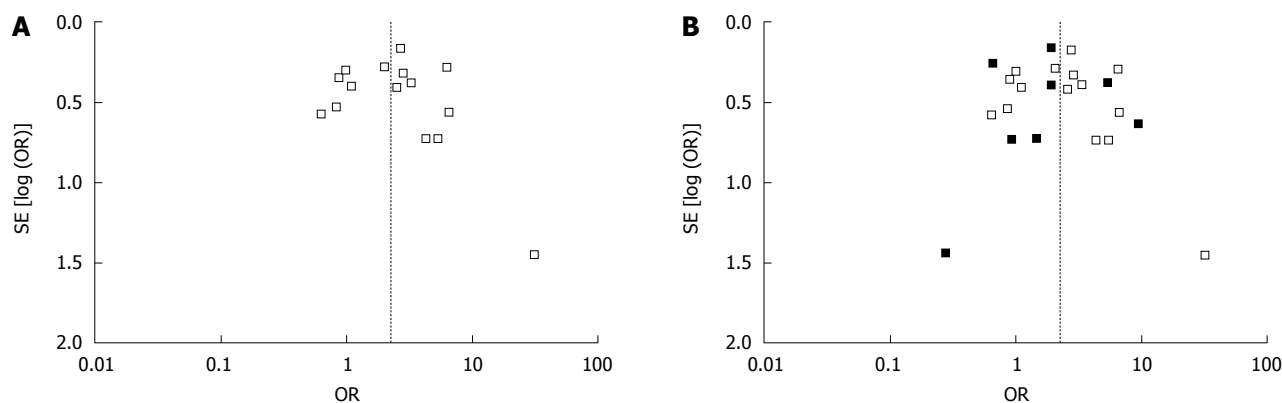


Figure 4 Funnel plots without (A) and with (B) trim and fill. The pseudo 95% CI is computed as part of the analysis that produces the funnel plot, and corresponds to the expected 95% CI for a given standard error (SE). OR: Odds ratio.

to mirror the positive studies that cause funnel plot asymmetry. The adjusted summary OR is based on the eventually filled funnel plot (4.32, 95% CI: 3.00-5.66,  $P < 0.001$ ), which continued to show a statistically significant association between *H. pylori* and IDA (Figure 4).

## DISCUSSION

Our results from a meta-analysis of 15 observational epidemiological studies revealed a correlation between *H. pylori* and IDA (OR, 2.22; 95% CI: 1.52-3.24,  $P < 0.0001$ ), although some studies reported only a slight association. In addition, In RCTs, eradication of *H. pylori*

can improve HB and SF levels but not significantly (WMD of HB: 4.06 g/L, 95% CI: -2.57-10.69,  $P = 0.01$ ; WMD of SF: 9.47  $\mu$ g/L, 95% CI: -0.50-19.43,  $P < 0.0001$ ).

Dufour *et al*<sup>[50]</sup> first reported that *H. pylori* eradication had a positive effect on sideropenic refractory anemia, indicating a possible underlying association between *H. pylori* and IDA. A large population-based study from the USA reported that *H. pylori* infection was an independent risk factor for IDA in 7462 children, adolescents, and adults<sup>[42]</sup>. This research reported that *H. pylori* infection was associated with an increased risk of IDA (OR, 2.6; 95% CI: 1.5-4.6). Compared to former studies, our meta-analysis was a detailed and comprehensive investigation

procedure. It included RCTs of highly detailed power and large-scale observational epidemiology studies.

It is reported that *H. pylori* infection is observed in over 50% people in the world with peaks of 70%-90% for some countries. Moreover, IDA affects 2 billion people in the world. When 2 diseases have such a high prevalence in the population they may appear to be associated with each other. Recent studies regarding the role of *H. pylori* infection in IDA are controversial. However, whether eradication of *H. pylori* prevents IDA has been widely debated. This meta-analysis was performed to clarify this issue: whether iron deficiency could specifically be related to *H. pylori* infection. The observational studies in our meta-analysis prove the association between *H. pylori* and IDA. In fact, in the RCTs of our meta-analysis, after iron replacement, HB and SF were not different between the groups with or without *H. pylori* eradication. However, these data should be interpreted with caution because of the marked heterogeneity among studies. We carefully performed subgroup analysis, and found age and therapies had an impact on the increase in the levels of HB and SF. These results should be investigated further in the future. Larger scale RCTs should be recommended to test the results of our meta-analysis.

As with all meta-analyses, the results we obtained could be impacted by 3 factors: heterogeneity within the studies involved, bias (including selection bias<sup>[51,52]</sup> and detection bias<sup>[53,54]</sup>), and publication bias<sup>[55-57]</sup>. The generation of heterogeneity could occur by virtue of the methodological and biological heterogeneity of the studies analyzed, such as differences in diagnostic methods, the population under study, the sample size, and language of publication. Each of the subgroups described above contributed partly to the heterogeneity of the observational studies.

We assessed the included studies with caution. For observational studies, the MINORS quality score ranged from 6 to 14 points. In RCTs, quality of all the studies were evaluated by the Jadad score. All of the studies included had a score greater than 3. Our test for heterogeneity was significant, and hence we utilized a random effects model that accounted for inter-study variation. Compared with the fixed effects model, the random effects model evenly distributes weight among studies, minimizing the impact of heterogeneity<sup>[58]</sup>.

We used age as one subset determinant for the pooled estimates. A significant association between *H. pylori* and IDA was found in children younger than 11 years. The common causes of IDA, such as colonic carcinoma, gastrectomy or menstruation, were usually absent in children. Thus, *H. pylori* infection can be treated as the only indicator for refractory IDA in children, and *H. pylori* infection should be considered first in children with IDA<sup>[59-61]</sup>. The same scenario occurred in adolescents, as adolescents are particularly susceptible to ID. Because of the requirement for a large amount of iron to sustain their growth, dietary deficiency, and menstrual blood loss, girls should be more strongly affected by *H. pylori*

infection<sup>[10,34,62]</sup>. In adults, no such strong association was found. The possible explanation for this phenomenon is that *H. pylori* plays a smaller part in the etiology of IDA in adults<sup>[1,5,63]</sup>. In RCT subgroup analysis, eradication therapy for *H. pylori* did not demonstrate the same curative effect on IDA in children as in adults. This may arise from the special characteristics of children (quickly growing blood volume and large requirement)<sup>[59-61]</sup>.

Different diagnostic methods for *H. pylori* contribute to the variation of the pooled estimates because of their different sensitivities. The pooled OR value increased with the sensitivity of the diagnostic method. Therefore, UBT, which has the highest sensitivity of the 3 tests used<sup>[64-66]</sup>, obtained the highest pooled OR, while ELISA serum IgG tests yielded the lowest pooled OR. Recent guidelines have indicated that UBT is regarded as a gold standard diagnostic method and the most reliable nonendoscopic test for the existence of *H. pylori*<sup>[67]</sup>.

The way in which RCTs chose to eradicate *H. pylori* can make a difference in pooled analyses. Bismuth-based triple therapy indicated a much better response to iron intake than PPI-based triple therapy. The work done by McColl and Hutchinson may explain this phenomenon<sup>[68,69]</sup>. It was reported that PPI therapy lowers the concentration of vitamin C in gastric juice and reduces the bioavailability of ingested vitamin C thus resulting in low absorption of nonheme iron. It may also retard the clinical response to iron supplementation. Vitamin C, as an essential factor in alimentary iron absorption, not only converts ferric iron to the ferrous form, which maintains solubility at the alkaline pH of the duodenum, but also chelates with ferric chloride which is also stable at a pH > 3. PPI can also reduce the absorption of vitamin B<sub>12</sub>, a significant factor in iron absorption, probably by inhibiting intragastric proteolysis.

Publication bias was tested by Begg's test and illustrated by funnel plots. The results indicated the existence of publication bias. The funnel plot showed that there were some missing small sample studies. Therefore, meta-analysis would underestimate the association between IDA and *H. pylori*. The "trim and fill" method helped to resolve this problem by imputing the hypothetical studies symmetrically and reassessing the pooled estimates as a sensitivity analysis. The filled funnel plot showed a strong association between IDA and *H. pylori*. Possible sources of asymmetry in funnel plots were explored: variations in sample size, etc, could contribute to publication bias.

Sensitivity analysis was performed in several ways to test concordance of the results by changing the statistical methods used. The sensitivity analyses that were performed did not materially change the results, increasing the confidence that can be placed in these results when applying the conclusion in practice.

Our study has limitations. We have excluded trials that studied the relationship between *H. pylori* and iron deficiency. However, these studies have been reviewed elsewhere<sup>[70]</sup>. Furthermore, in the experimental studies,



eradication treatment without ferrous sulfate but with placebo groups were excluded because the number of studies was not adequate. This kind of design can illustrate the role that *H. pylori* plays in IDA in a better way, and we expect further investigations will take that design into consideration. Lastly, results were markedly heterogeneous for all comparisons. These data should be interpreted with caution because of the marked heterogeneity among studies.

In conclusion, our meta-analysis of 15 observational studies demonstrated an association between *H. pylori* and IDA. In addition, the meta-analysis of RCTs showed that eradication of *H. pylori* can improve HB and SF levels, though not significantly. From the analysis, we also concluded that IDA could not specifically be related to *H. pylori* infection. We do not recommend a strategy of population-based screening and treatment for *H. pylori* infection to prevent IDA. This concept should be discussed in the future. UBT is the most reliable nonendoscopic test for the existence of. Bismuth-based triple therapy has a better response to increase HB and SF levels than PPI-based triple therapy. There are no significant differences between less developed areas and developed areas in the association between *H. pylori* infection and IDA.

## COMMENTS

### Background

Both *Helicobacter pylori* (*H. pylori*) and iron deficiency anemia (IDA) have high prevalence worldwide. The relationship between these 2 remains controversial. Recent guidelines for *H. pylori* and IDA both focus on the role *H. pylori* plays in the process of IDA.

### Research frontiers

The interaction between *H. pylori* and IDA is a current 'hot topic'. *H. pylori* infection impairs iron absorption causing a considerable decrease in the concentration of gastric juice ascorbic acid (vitamin C) that is the best promoter of nonheme iron absorption. It thus causes the decline of hemoglobin in red blood cells and directly leads to anemia. The gastric colonization by *H. pylori* increases lactoferrin uptake from neutrophils and increases iron demand.

### Innovations and breakthroughs

To the best of the authors' knowledge, this is the first published meta-analysis assessing the association between *H. pylori* infection and IDA (evaluating children, adolescents and adults) and assessing the effect of *H. pylori* eradication on hemoglobin (HB) and serum ferritin (SF) levels.

### Applications

The research showed an association between *H. pylori* and IDA. Eradication of *H. pylori* can improve HB and SF levels but not significantly. The authors do not recommend a strategy of population-based screening and treatment for *H. pylori* infection to prevent IDA.

### Peer review

This is a good and interesting meta-analysis.

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## Betaine inhibits Toll-like receptor 4 expression in rats with ethanol-induced liver injury

Qing-Zhao Shi, Lu-Wen Wang, Wei Zhang, Zuo-Jiong Gong

Qing-Zhao Shi, Lu-Wen Wang, Wei Zhang, Zuo-Jiong Gong, Department of Infectious Diseases, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China

**Author contributions:** Gong ZJ designed the study, supervised and collected and analyzed the data; Shi QZ and Wang LW performed the majority of experiments and wrote the manuscript; Zhang W participated in the design and coordination of the work involved; all authors have approved the final draft submitted.

**Correspondence to:** Dr. Zuo-Jiong Gong, Department of Infectious Diseases, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China. [zjgong@163.com](mailto:zjgong@163.com)

Telephone: +86-27-88041911 Fax: +86-27-88042922

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### Abstract

**AIM:** To test whether ethanol feeding could induce Toll-like receptor 4 (TLR4) responses, assess the hepatoprotective effect of betaine and its inhibitive effect on TLR4 in animal models of alcoholic liver injury.

**METHODS:** Forty-eight female Sprague-Dawley rats were randomly divided into four groups as control, model, low and high dose betaine groups. Except control group, all rats were fed with high fat-containing diet plus ethanol and fish oil gavages for 8 wk. Betaine was administered intragastrically after exposure of ethanol for 4 wk. The changes of liver histology were examined. The expression of TLR4 mRNA and protein was detected by RT-PCR and Western blotting, respectively. The serum aminotransferase activity [alanine transaminase (ALT), aspartate aminotransferase (AST)], serum endotoxin, and liver inflammatory factors [tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interferon- $\gamma$  (IFN- $\gamma$ ), interleukin-18 (IL-18)] were also assayed.

**RESULTS:** Compared with control group, rats of model group developed marked liver injury, accompanied by an increase of ALT ( $159.41 \pm 7.74$  U/L vs  $59.47 \pm$

$2.34$  U/L,  $P < 0.0001$ ), AST ( $248.25 \pm 1.40$  U/L vs  $116.89 \pm 3.48$  U/L,  $P < 0.0001$ ), endotoxin ( $135.37 \pm 30.17$  ng/L vs  $44.15 \pm 7.54$  ng/L,  $P < 0.0001$ ), TNF- $\alpha$  ( $20.81 \pm 8.58$  pg/mL vs  $9.34 \pm 2.57$  pg/mL,  $P = 0.0003$ ), IFN- $\gamma$  ( $30.18 \pm 7.60$  pg/mL vs  $16.86 \pm 9.49$  pg/mL,  $P = 0.0039$ ) and IL-18 ( $40.99 \pm 8.25$  pg/mL vs  $19.73 \pm 9.31$  pg/mL,  $P = 0.0001$ ). At the same time, the expression of TLR4 mRNA and protein was markedly induced in the liver after chronic ethanol consumption ( $1.45 \pm 0.07$  vs  $0.44 \pm 0.04$ ,  $P < 0.0001$ ;  $1.83 \pm 0.13$  vs  $0.56 \pm 0.08$ ,  $P < 0.0001$ ). Compared with model group, betaine feeding resulted in significant decreases of ALT ( $64.93 \pm 6.06$  U/L vs  $159.41 \pm 7.74$  U/L,  $P < 0.0001$ ), AST ( $188.73 \pm 1.11$  U/L vs  $248.25 \pm 1.40$  U/L,  $P < 0.0001$ ), endotoxin ( $61.80 \pm 12.56$  ng/L vs  $135.37 \pm 30.17$  ng/L,  $P < 0.0001$ ), TNF- $\alpha$  ( $9.79 \pm 1.32$  pg/mL vs  $20.81 \pm 8.58$  pg/mL,  $P = 0.0003$ ), IFN- $\gamma$  ( $18.02 \pm 5.96$  pg/mL vs  $30.18 \pm 7.60$  pg/mL,  $P = 0.0008$ ) and IL-18 ( $18.23 \pm 7.01$  pg/mL vs  $40.99 \pm 8.25$  pg/mL,  $P < 0.0001$ ). Betaine also improved liver steatosis. The expression levels of TLR4 mRNA or protein in liver tissues were significantly lowered ( $0.62 \pm 0.04$  vs  $1.45 \pm 0.07$ ,  $P < 0.0001$ ; and  $0.65 \pm 0.06$  vs  $1.83 \pm 0.13$ ,  $P < 0.0001$ ). There was a statistical difference of TLR4 mRNA and protein expression between high- and low-dose betaine groups ( $0.62 \pm 0.04$  vs  $0.73 \pm 0.05$ ,  $P < 0.0001$ , and  $0.65 \pm 0.06$  vs  $0.81 \pm 0.09$ ,  $P < 0.0001$ ).

**CONCLUSION:** Betaine can prevent the alcohol-induced liver injury effectively and improve the liver function. The expression of TLR4 increases significantly in ethanol-fed rats and betaine administration can inhibit TLR4 expression.

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**Key words:** Betaine; Toll-like receptor 4; Alcoholic liver injury; Expression

**Peer reviewers:** Dr. BS Anand, Professor, Digestive Diseases Section (111D), VA Medical Center, 2002 Holcombe Blvd., Houston, TX 77030, United States; Dr. Radha Krishna Yellapu,



MD, DM, Department of Hepatology, Mount Sinai, 121 E 97 Street, NY 10029, United States

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## INTRODUCTION

Previous studies have shown that the chronic ingestion of ethanol can induce functional and structural changes in liver. Bacterial lipopolysaccharide (LPS; endotoxin), an abundant and essential component of the outer membrane of gram negative bacteria, causes liver injury in many experimental models<sup>[1,2]</sup>. Chronic alcohol administration increases gut-derived endotoxin in the portal circulation, thereby activating Kupffer cells to produce several proinflammatory cytokines such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin (IL)-1<sup>[3,4]</sup>.

Toll-like receptor 4 (TLR4), a transmembrane protein with a cytoplasmic domain that bears homology to the IL-1 receptor, is expressed in monocytes and macrophages, including Kupffer cells<sup>[5]</sup>. Recently, TLR4 has been shown to mediate LPS-induced signal transduction in peripheral blood monocytes<sup>[6]</sup>. Furthermore, it has been shown that Kupffer cell activation by LPS is dependent on the presence of a functional TLR4<sup>[7]</sup>. It has been confirmed that TLR4 is involved in the mechanism of early alcohol-induced liver injury<sup>[8-11]</sup>. Ethanol administration can lead to the synthesis of TLR4 protein and its gene expression in Kupffer cells, indicating that TLR4 may play a major role in the development of alcohol-induced liver injury.

Betaine, also known as trimethyl glycine, is the only methyl donor, which can replace folate or S-adenosylmethionine in the human body<sup>[12,13]</sup>. Betaine is quaternary ammonium salt soluble alkaloids, which participates in the methionine recycling and phosphatidylcholine synthesis<sup>[14,15]</sup>. Many studies including our previous study have indicated that betaine can prevent the alcohol-induced liver injury effectively and improve the liver function<sup>[16,17]</sup>. The hepatoprotective mechanism of betaine is related to the inhibition of inflammatory factor, the decrease of lipid peroxidation, the promotion of endoplasmic reticulum stress and the prevention of apoptosis<sup>[18-21]</sup>.

In the present study, we employed the intragastric ethanol-fed rat model, which reproduces the pathological features of early alcohol-induced liver injury, to observe the changes of TLR4 expression and study the effect of betaine in alcohol-induced liver injury animal models.

## MATERIALS AND METHODS

### Chemicals and reagents

Betaine hydrochlorides (99% of purity) were kindly presented by Juhua Group Co. (Zhejiang, China). Ferrous

sulfate was obtained from Shanghai reagent chemicals Co. Ltd (Shanghai, China). Fish oil and 560 mL/L of alcohol were purchased from supermarkets. Limulus amoebocyte lysate assay kit for serum endotoxin assay was purchased from BioWhittaker Inc. (USA). Enzyme-linked immunosorbent (ELISA) kits for rat TNF- $\alpha$ , IFN- $\gamma$ , and IL-18 detection were purchased from Shanghai Senxiong Biotech industry Co. Ltd. (Shanghai, China). TRIzol reagent was purchased from Invitrogen Co. (USA). DL1000 DNA ladder marker was purchased from TaKaRa Biotech Co. Ltd. (Japan). M-MLV reverse transcriptase, deoxyribonucleotide (dNTP, 10 mmol/L), oligo (dT)<sub>15</sub> primer, Taq DNA polymerase, RNasin were purchased from Promega Biotech Co. Ltd. (USA). Polymerase chain reaction (PCR) primers for TLR4 and GAPDH were synthesized by Sai-Bai-Sheng Biocompany (Shanghai, China).

### Animal models

Forty-eight female specific pathogen free (SPF) Sprague-Dawley rats, weighing  $150 \pm 10$  g, were purchased from the Experimental Animal Center of Wuhan University. After acclimation for 1 wk, animals were randomly divided into four groups as control, model, low dose and high dose betaine groups. Each group contains 12 rats. Except rats of control group fed with ordinary diet and administrated intragastrically with physiological saline, the rats of the other three groups were fed with fat-rich diet containing common animal feeds, lard and whole milk powder (80:10:10), and were administrated intragastrically with ethanol and 0.5 mL fish oil. The initial dose of ethanol was 6 g/kg per day (solutions maximally containing 560 mL/L alcohol). Within the first week, the dose was increased progressively to a maintenance dose of 8 g/kg per day that was continued for 8 more weeks. After exposure of ethanol for 4 wk, the rats of low dose and high dose groups were administrated intragastrically with betaine 200 and 400 mg/kg per day, respectively. Animals were weighted three times per week. At the end of the experiment, animals were anaesthetized with urethane (20%, 1.0 g/kg) and sacrificed by bleeding from femoral arteries. Blood samples were collected. Immediately after exsanguination, the livers were harvested. Small portions of the livers were kept frozen at  $-70^{\circ}\text{C}$  for reverse transcriptase-polymerase chain reaction (RT-PCR), whereas other portions were separated and immersed in 10% buffered formalin solution for histological examination. All animals were given humane care in compliance with the institutional guidelines.

### Liver function assay

Blood samples were allowed to clot, and the sera were isolated by centrifugation at  $1000 \times g$  for 10 min and kept at  $-20^{\circ}\text{C}$  before determination. Serum alanine transaminase (ALT), aspartate transaminase (AST) and albumin (ALB) were determined by routine laboratory methods using a Hitachi Automatic Analyzer (Hitachi, Inc. Japan).

### Determination of serum endotoxin, TNF- $\alpha$ , IFN- $\gamma$ and IL-18

Serum levels of endotoxin, TNF- $\alpha$ , IFN- $\gamma$  and IL-18 were measured using commercial kits according to the manufacturer's protocol.

### Detection of liver TLR4 mRNA in liver tissues

Total RNA was extracted from approximately 100 mg frozen liver tissue using TRIzol reagent according to the manufacturer's protocol. The concentration of total RNA was assayed by ultraviolet spectrophotometric measurements at wavelength of 260 nm, and its purity was estimated by the ratio of  $A_{260}/A_{280}$ . The total RNA was reversely transcribed into single-stranded complementary DNA (cDNA) using the following methods: 2  $\mu$ g RNA, 0.5  $\mu$ g oligo(dT)<sub>15</sub> primer and DEPC (diethylpyrocarbonate)-treated water were added to reach a total volume of 15  $\mu$ L mixture at 70°C for 5 min, then rapidly chilled on ice. Finally, 5  $\mu$ L 5  $\times$  reaction buffer, 1.25  $\mu$ L dNTP (10 mmol/L, each), 25 units of RNasin, 200 units of M-MLV reverse transcriptase and DEPC-treated water were added to reach a total volume of 25  $\mu$ L mixture and incubated at 42°C for 60 min, then terminated by placing it on ice after deactivation at 85°C for 5 min. The cDNA was amplified by PCR. The amplification primers for rat TLR 4 were 5'-ACTCGAGCCAGAATGAGGACT-3' and 5'-ACTGCCATGTCTGAGCAATCT-3', for rat GAPDH were 5'-TCCCTCAAGATTGTCAGCAA-3' and 5'-AGATCCACAACGGATACATT-3'. The 50  $\mu$ L PCR reaction mix contained 10 mmol/L dNTP, 2.5 mmol/L MgCl<sub>2</sub>, 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 25 pmol/L of sense and antisense primers, and 2U of Taq DNA polymerase. Amplification was performed with 35 cycles with initial incubation at 94°C for 3 min and final extension at 72°C for 7 min, each cycle consisted of denaturation for 45 s at 94°C, annealing for 45 s at 55°C, and extension for 1 min at 72°C. The PCR products were 237 bp and 309 bp for TLR 4 and GAPDH, respectively. In all experiments, possible contamination with genomic DNA was excluded by PCR amplification in the absence of reverse transcriptase. The PCR products were electrophoresed on 2% agarose gel. Semiquantitative evaluation was performed using the Gel Doc 2000 System (BioRad Laboratories GmbH, München, Germany). GAPDH was used as a positive internal control and was positive for each specimen. Its expression was used as a correction factor for TLR 4 mRNA, thus the results were calculated as the ratio of the intensity of bands of TLR 4 cDNA per GAPDH cDNA on the gel.

### Western blotting assay of TLR4 protein in liver tissues

Liver tissue samples of 100 mg were crushed in a liquid nitrogen-cooled grinding bowl and then were lysed in cold RIPA buffer (25 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) (Pierce Biotechnology, Inc., USA), supplemented with Halt™ Protease Inhibitor Cocktail (Pierce Biotechnology, Inc., USA). Whole cell lysates were ob-

tained by subsequent centrifugation at  $15000 \times g$  for 10 min at 4°C. Protein concentrations were determined using Bradford Protein Assay Kit with bovine serum albumin (BSA) as standard (SinoBio Biotech Co., Ltd. Shanghai, China). Fifty  $\mu$ g of protein extracts were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a Protran® nitrocellulose membrane (Schleicher & Schuell BioScience GmbH, Whatman Group, Germany). The membrane was incubated with the rabbit anti-TLR4 polyclonal antibody (BioChain, USA) at 4°C overnight after being blocked with a 10% BSA solution. The membrane was washed with TBST buffer (20 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 0.1% Tween-20) and incubated with a secondary goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) for 2 h at room temperature, and finally detected by chemiluminescence using Enhanced NuGlo™ Chemiluminescent Substrate Kit (Alpha Diagnostic Intl. Inc., USA) followed by autoradiographic and densitometric analysis.  $\beta$ -actin was used as an internal control.

### Histological examinations of liver

The liver specimens were fixed in 10% formaldehyde for 12-24 h, embedded in paraffin, sliced into sections of 5  $\mu$ m thickness and stained with hematoxylin-eosin (HE). Histological assessment was performed by three pathologists independently. The severity of steatosis was scored as 0 (no hepatocytes), 1 (less than 25% of hepatocytes), 2 (26%-50%), 3 (51%-75%), and 4 (greater than 75% of hepatocytes). The severity of the inflammation was scored as 0 (none), 1 (minimal), 2 (mild), 3 (moderate) and 4 (severe) based on the degree of portal and lobular inflammation and the evidence of piecemeal and spotty necrosis. The degree of necrotic hepatocytes was also scored as 0 to 4 (none, minimal, mild, moderate, and severe, respectively) based on the evidence of piecemeal and spotty necrosis.

### Statistical analysis

All data were presented as mean  $\pm$  SE. Differences among groups were assessed using unpaired Student's *t* test and one-way ANOVA. *P* value less than 0.05 was considered to be statistically significant. Calculations were performed with the SPSS11.0 statistical software package.

## RESULTS

### General conditions of rats

During the experiment, 4 rats in the model group died because fluids were poured mistakenly into trachea when they were administrated intragastrically. The other 44 rats survived.

### Changes of weight and liver index of rats

The changes of the rat weight in models were significantly

**Table 1** Changes of weight and liver index in rats

Groups	<i>n</i>	Weight change(g)	Liver index (Liver wet weight/rats weight × 100)
Control	12	27.27 ± 1.55 <sup>b</sup>	3.65 ± 0.22 <sup>b</sup>
Model	8	18.44 ± 1.16	5.49 ± 0.34
Low dose betaine	12	19.38 ± 1.95	3.83 ± 0.14 <sup>b</sup>
High dose betaine	12	19.34 ± 1.38	3.75 ± 0.68 <sup>b</sup>

<sup>b</sup>*P* < 0.01 compared with model group.**Table 2** Changes of liver function in rats

Groups	<i>n</i>	ALT (U/L)	AST (U/L)	ALB (g/L)	ALB/GLB
Control	12	59.47 ± 2.34 <sup>b</sup>	116.89 ± 3.48 <sup>b</sup>	38.1 ± 0.16	1.40 ± 0.34
Model	8	159.41 ± 7.74	248.25 ± 1.40	36.1 ± 1.22	1.55 ± 0.06
Low dose betaine	12	62.82 ± 7.78 <sup>b</sup>	189.25 ± 5.9 <sup>b</sup>	37.8 ± 2.36	1.50 ± 0.03
High dose betaine	12	64.93 ± 6.06 <sup>b</sup>	188.73 ± 1.11 <sup>b</sup>	36.4 ± 3.17	1.46 ± 0.12

<sup>b</sup>*P* < 0.01 compared with model group. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALB: Albumin; GLB: Globulin.**Table 3** Changes of rat serum endotoxin, TNF- $\alpha$ , IFN- $\gamma$  and IL-18

Groups	<i>n</i>	Endotoxin (ng/L)	TNF- $\alpha$ (pg/mL)	INF- $\gamma$ (pg/mL)	IL-18 (pg/mL)
Control	12	44.15 ± 7.54 <sup>b</sup>	9.34 ± 2.57 <sup>b</sup>	16.86 ± 9.49 <sup>b</sup>	19.73 ± 9.31 <sup>b</sup>
Model	8	135.37 ± 30.17	20.81 ± 8.58	30.18 ± 7.60	40.99 ± 8.25
Low dose betaine	12	87.36 ± 15.93 <sup>b</sup>	12.61 ± 1.70 <sup>b</sup>	22.63 ± 4.90 <sup>b</sup>	26.51 ± 5.59 <sup>b</sup>
High dose betaine	12	61.80 ± 12.56 <sup>b,d</sup>	9.79 ± 1.32 <sup>b,d</sup>	18.02 ± 5.96 <sup>b,d</sup>	18.23 ± 7.01 <sup>b,d</sup>

<sup>b</sup>*P* < 0.01 compared with model group; <sup>d</sup>*P* < 0.01 compared with low dose betaine group. TNF- $\alpha$ : tumor necrosis factor- $\alpha$ ; IL-10: Interleukin-10; IL-18: Interleukin-18; IFN- $\gamma$ : Interferon- $\gamma$ .

lower than that in controls (*P* < 0.01). Compared with the model group, there were no significant differences in betaine intervention groups (*P* > 0.05). Liver index was significantly higher in models than in controls (*P* < 0.01). Compared with the model group, liver index decreased significantly in the betaine intervention groups (*P* < 0.01), and there was no statistical difference between high dose betaine group and low dose betaine group (*P* > 0.05) (Table 1).

### Changes of liver function

The changes of the ALT and AST in models were significantly higher than in controls (*P* < 0.01). Compared with the model group, the ALT and AST levels were significantly lowered in betaine intervention groups, indicating that the betaine can greatly improve the alcohol-induced liver injury, and there was no statistical difference between high dose betaine group and low dose betaine group (*P* > 0.05). There were no significant differences in ALB and A/G between model group and betaine intervention groups (*P* > 0.05) (Table 2).

### Changes of serum endotoxin, TNF- $\alpha$ , IFN- $\gamma$ and IL-18

The levels of serum endotoxin, TNF- $\alpha$ , IFN- $\gamma$  and IL-18 were significantly higher in model group than in

control group (*P* < 0.01). Compared with model group, serum endotoxin, TNF- $\alpha$ , IFN- $\gamma$  and IL-18 significantly decreased in betaine intervention groups (*P* < 0.01). There was a statistical difference between high dose betaine group and low dose betaine group (*P* < 0.01) (Table 3).

### Expressions of TLR4 mRNA and protein in liver tissue of rats

The software Quantiscan was used to analyze the absorbance of the products of TLR4 mRNA or protein quantitatively. The expression of TLR4 mRNA and protein was both significantly higher in model group than in normal (*P* < 0.01). Compared with the model group, the expression of TLR4 mRNA and protein was significantly reduced in betaine intervention groups (*P* < 0.01). There was a statistical difference of TLR4 expression between high dose betaine group and low dose betaine group (*P* < 0.01) (Table 4 and Figure 1).

### Histopathological changes of liver

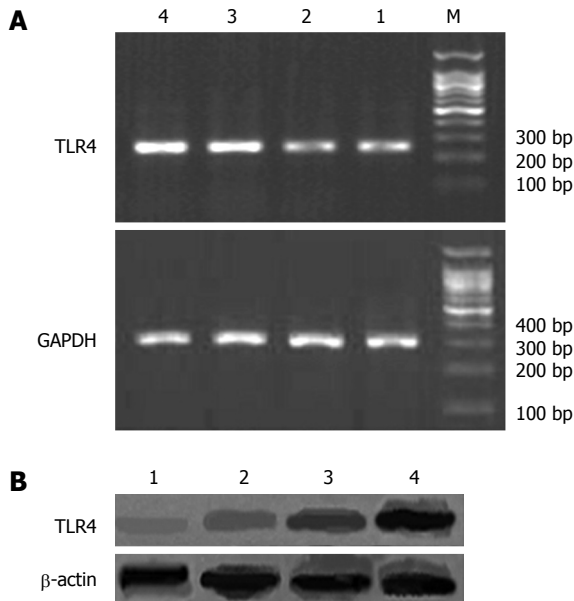
The liver structure in control group was normal, and no obvious inflammation and hepatic steatosis were observed (Figure 2A). In model group, the structure of hepatic cord was deranged, and various degrees of diffuse hepatic



**Table 4** Changes of TLR4 expression in rat liver tissues

Groups	<i>n</i>	TLR4 mRNA	TLR4 protein
Control	12	0.44 ± 0.04 <sup>b</sup>	0.56 ± 0.08 <sup>b</sup>
Model	8	1.45 ± 0.07	1.83 ± 0.13
Low dose betaine	12	0.73 ± 0.05 <sup>b</sup>	0.81 ± 0.09 <sup>b</sup>
High dose betaine	12	0.62 ± 0.04 <sup>b,d</sup>	0.65 ± 0.06 <sup>b,d</sup>

<sup>b</sup>*P* < 0.01 compared with model group; <sup>d</sup>*P* < 0.01 compared with low dose betaine group. TLR 4: Toll-like receptor 4.

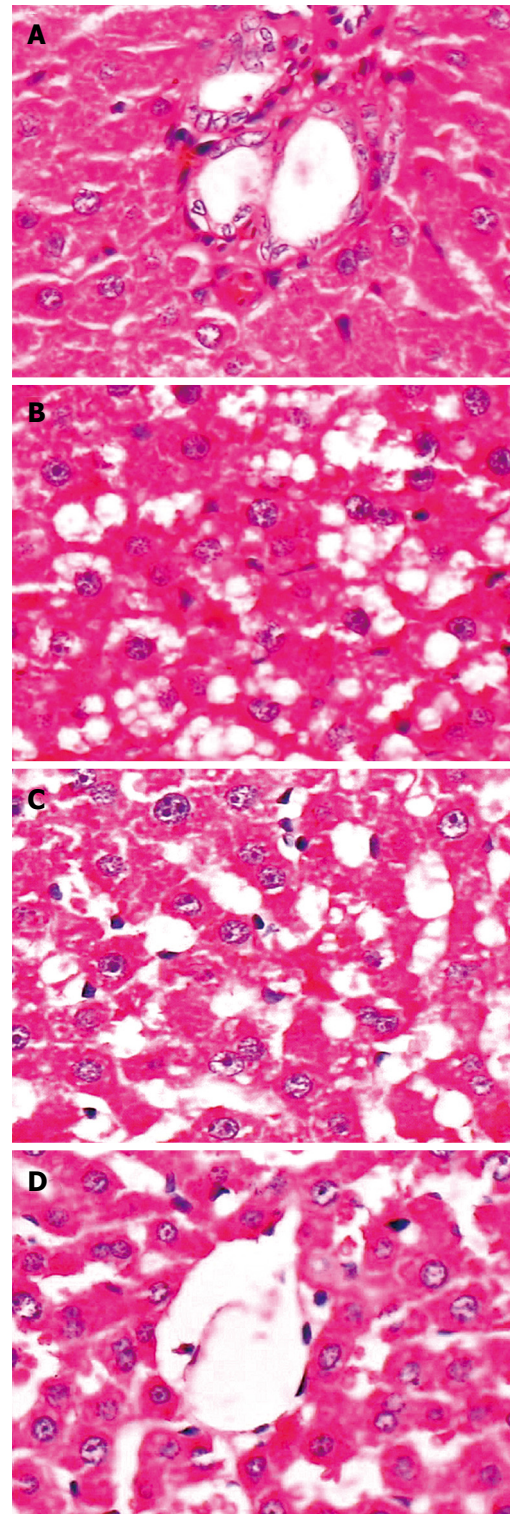


**Figure 1** Expression of TLR4 in rat liver tissues. A: RT-PCR assay of TLR4 mRNA; B: Western blotting assay of TLR4 protein. 1: Control; 2: High dose betaine group; 3: Low dose betaine group; 4: Model; M: DNA marker.

steatosis and intralobular inflammation could be found obviously (Figure 2B). Compared with model group, the degree of hepatic steatosis and inflammation was greatly reduced in betaine intervention groups. The improvement of liver histopathology in the high dose betaine group was most significant (Figure 2C and D).

## DISCUSSION

In this study, by establishing the intragastric fat-rich and ethanol diet mouse model, we found that the rats in model group had lower weight and higher liver index, obvious liver injury and hepatic steatosis, higher serum endotoxin, TNF- $\alpha$ , IFN- $\gamma$  and IL-18 levels compared with the rats in control group. Endotoxemia and oxidative stress are two key factors for the progression of alcoholic liver diseases<sup>[22]</sup>. There are solid data supporting the hypothesis that endotoxin is indeed involved in alcoholic liver injury. First, it has been shown that excessive alcohol intake increases gut permeability of normally nonabsorbed substances<sup>[23,24]</sup>. Second, intestinal gram-negative bacteria, as well as blood endotoxin levels, are increased both in alcoholic patients and in the Tsukamoto-French enteral



**Figure 2** Histopathological changes of rat liver after betaine intervention. A: In control group, liver structure was normal, without obvious inflammation and hepatic steatosis; B: In model group, the structure of hepatic cord was deranged, with various degrees of diffuse hepatic steatosis and intralobular inflammation; C: In low dose betaine group, the degree of hepatic steatosis and inflammation was greatly reduced compared with model group; D: High dose betaine group, showing more significant improvement of hepatic steatosis and inflammation than the low dose betaine group. Original magnification  $\times 400$ .

alcohol feeding model<sup>[25,26]</sup>. Third, intestinal sterilization with antibiotics and displacement of gram-negative



bacteria with lactobacillus treatment prevents alcohol-induced liver injury<sup>[27,28]</sup>. Alcohol can increase the levels of circulating endotoxin in the portal blood. Once bound to LPS-binding protein (LBP), this complex is formed with the endotoxin receptor and CD14, activates Kupffer cells *via* TLR4<sup>[29]</sup>. Kupffer cell activation leads to the up-regulation of key cytokines, including TNF- $\alpha$ . Besides direct toxic effects on hepatocytes, TNF- $\alpha$  can indirectly damage the liver by increasing expression of intercellular adhesion molecule-1 (ICAM-1) on endothelial cells, as well as increasing the production of chemoattractant molecules from inflammatory cells<sup>[30]</sup>.

In this study, we also found that the rats in model group fed with ethanol had a significantly higher expression of TLR4 mRNA and protein than normal rats. Many studies have confirmed that TLR4 is critical for early alcoholic liver injury<sup>[8-11]</sup>. It was shown that a functional mutation in TLR4 prevents early alcohol-induced liver injury in mice. Specifically, no differences in alcohol levels or plasma endotoxin were observed between the groups fed with ethanol. Moreover, a functional mutation in the TLR4 receptor prevents all downstream events, including increased TNF- $\alpha$  expression, inflammation, and liver injury<sup>[11]</sup>. These results support the hypothesis that endotoxin and TLR4 play a major role in the development of early alcohol-induced liver injury. CD14, a glycosylphosphatidylinositol-anchored receptor for LPS, is important in mediating the effects of LPS/LPB complexes on peripheral blood monocytes<sup>[31]</sup>, and it is known that ethanol increases expression of CD14 on Kupffer cells<sup>[32]</sup>. However, CD14 lacks the ability to transduce LPS-induced cytoplasmic signals across a cell membrane, because it is not a transmembrane protein<sup>[33]</sup>. It had been suggested that LPS-induced inflammatory cell activation *via* CD14 also requires TLR4, which associates with CD14 on the cell surface, mediating LPS-induced signal transduction<sup>[34]</sup>. The finding that alcoholic liver injury is blocked in both CD14 and TLR4-deficient mice suggests that both of these receptors are necessary to initiate liver damage caused by alcohol<sup>[35]</sup>. Therefore, pharmacologic manipulation and targeting of the endotoxin-CD14/TLR4 signaling pathways could prove to be useful in alcoholic liver disease.

Previous studies have shown that betaine can prevent the alcohol-induced liver injury effectively and improve the liver function, which is related to the inhibition of inflammatory factor, the decrease of lipid peroxidation, the rivalry of endoplasmic reticulum stress and the prevention of apoptosis<sup>[16-21]</sup>. Our study indicates that in rats with alcohol-induced liver injury, betaine feeding can decrease the levels of serum ALT, AST, endotoxin, TNF- $\alpha$ , IFN- $\gamma$  and IL-18, and reduced the expressions of TLR4, and improved the degree of hepatic steatosis and inflammation in liver tissues. It is suggested that betaine can prevent the alcohol-induced liver injury effectively and improve the liver function. The hepatoprotective mechanism of betaine is probably related to the inhibition of endotoxin/TLR4 signaling pathways.

In summary, the results of this study show that the

expression of TLR4 increased significantly in ethanol-fed rats. Betaine administration can inhibit TLR4 expression, which may be one of mechanisms of alcoholic liver injury protected by betaine.

## COMMENTS

### Background

Chronic ethanol ingestion increases gut-derived endotoxin (LPS) in the portal circulation, thereby activating Kupffer cells to produce proinflammatory cytokines and induce liver injury. Toll-like receptor 4 (TLR4), *via* mediating LPS-induced signal transduction, plays a major role in the development of alcohol-induced liver injury. Blocking TLR4 signaling pathways is a therapeutic target of alcoholic liver disease. Many studies reported that betaine can prevent the alcohol-induced liver injury effectively and improve the liver function, but there are few reports about the effects of betaine on TLR4 and endotoxin in alcohol-induced liver injury.

### Research frontiers

Betaine is the only methyl donor, which can replace folate or S-adenosylmethionine to participate in methionine recycling and phosphatidylcholine synthesis in the human body. The hepatoprotective effect and mechanism of betaine is a research hotspot in the area of prevention and cure of alcoholic liver disease. Current studies show that the hepatoprotective mechanism of betaine is related to the inhibition of inflammatory factor, the decrease of lipid peroxidation, the promotion of endoplasmic reticulum stress and the prevention of apoptosis. However, whether the inhibition of TLR4 expression and reduction of endotoxin are involved in hepatoprotective effect of betaine in the alcoholic liver injury remains unclear.

### Innovations and breakthroughs

In the present study, the authors employed the intragastric ethanol-fed rat model, which reproduces the pathological features of early alcohol-induced liver injury, to observe the changes of TLR4 and endotoxin, and to study the protective effect of betaine on alcohol-induced liver injury. The authors found that the ethanol-fed rats had obvious liver injury and hepatic steatosis, higher serum endotoxin and inflammatory factor (TNF- $\alpha$ , IFN- $\gamma$  and IL-18) levels, and significantly higher TLR4 expression, whereas betaine feeding can improve the liver function, reduce the expressions of TLR4 and endotoxin levels, and improve the degree of hepatic steatosis and inflammation in liver tissues.

### Applications

The study results suggest that betaine can prevent the alcohol-induced liver injury effectively, and one of the hepatoprotective mechanisms of betaine is probably related to the inhibition of endotoxin/TLR4 signaling pathways.

### Terminology

Betaine, also known as trimethylglycine, is a chemical compound similar to folic acid and S-adenosylmethionine. These compounds function as "methyl donors" that carry methyl molecules throughout the body, thus helping in the completion of several vital chemical processes. Toll-like receptor 4 (TLR4): TLR4 is a member of the Toll-like receptors (TLRs) family which can recognize pathogen-associated molecular patterns (PAMPs) that are expressed on infectious agents, and mediate the production of cytokines necessary for the development of effective immunity, and thereby plays a fundamental role in pathogen recognition and activation of innate immunity.

### Peer review

This is a well conducted and well written study. The experiments are described in detail, the results are shown nicely and the figures are impressive. This study for the first time shows that Betaine reduces the expression of TLR4 in rats with ethanol-induced liver injury, and proposes that the hepatoprotective mechanism of betaine is secondary to inhibition of endotoxin/TLR4 signaling pathways.

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## Role of *RECK* methylation in gastric cancer and its clinical significance

Yun-Yi Du, Dong-Qiu Dai, Zhi Yang

Yun-Yi Du, Dong-Qiu Dai, Zhi Yang, Department of Surgical Oncology, the First Affiliated Hospital China Medical University, Shenyang 110001, Liaoning Province, China  
 Author contributions: Du YY performed the whole experiment, wrote the manuscript; Dai DQ designed the experiment, provided financial support, conducted the whole study; Yang Z participated in the study.

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Correspondence to: Dong-Qiu Dai, Professor, Department of Surgical Oncology, the First Affiliated Hospital China Medical University, Shenyang 110001, Liaoning Province, China. daidq63@163.com

Telephone: +86-24-83283556 Fax: +86-24-83283556

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### Abstract

**AIM:** To investigate the relation between *RECK* methylation and clinicopathological characteristics of gastric cancer patients and evaluate the role of *RECK* methylation in peritoneal metastasis of gastric cancer.

**METHODS:** Methylation of *RECK* gene in 40 paired samples of gastric cancer and its corresponding adjacent normal mucosa, lymph nodes and peritoneal irrigation fluid was detected by methylation-specific polymerase chain reaction.

**RESULTS:** Aberrant methylation of *RECK* gene was detected in 27.5% (11/40) of the adjacent normal mucosa samples, in 47.5% (19/40) of gastric cancer samples, in 57.1% (12/21) of the lymph node samples, and in 35% (14/40) of peritoneal irrigation fluid samples, respectively, with a significant difference between the adjacent normal mucosa and lymph node samples ( $P = 0.023$ ). Presence of *RECK* methylation in the primary tumor samples was significantly correlated with tumor invasion ( $P = 0.023$ ). The accuracy of *RECK*

methylation in peritoneal lavage fluid samples for the diagnosis of peritoneal metastasis of gastric cancer was 72.5% (26/40), with a sensitivity of 66.7% (6/9) and a specificity of 74.2% (23/31).

**CONCLUSION:** Aberrant methylation of *RECK* gene may provide useful information for the early diagnosis and treatment of peritoneal metastasis of gastric cancer.

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**Key words:** *RECK* gene; Hypermethylation; Gastric cancer; Metastasis; Peritoneal lavage fluid

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### INTRODUCTION

Gastric cancer seriously threatens the human health worldwide. There is increasing evidence that almost all gastric cancers have epigenetic abnormalities that drive cancer development and progression in collaboration with genetic changes. Aberrant methylation in the promoter CpG island of tumor suppressor genes (*TSG*) where DNA is transcribed into RNA causes its silence. Transcription of DNA is the first major step in decoding DNA into a protein. DNA methylation can inactivate tumor suppressor genes<sup>[1]</sup>. It has been shown that aberrant methylation and diminished expression of DNA in the promoter CpG island occur in a number of tumor-related genes in gastric cancer<sup>[2]</sup>. For example, *RASSF1A*, a candidate tumor suppressor gene, is

hypermethylated in gastric cancer<sup>[3,4]</sup>, *TIMP-3*, a silenced tumor suppressor gene, encodes a protease inhibitor that may inhibit tissue invasion<sup>[5]</sup>, and *RECK*, a newly discovered metastasis suppressor gene, is silenced with aberrant CpG island hypermethylation in some common tumors<sup>[6-8]</sup>. However, the relation between methylation of *RECK* gene and gastric cancer has not been fully studied.

In this study, *RECK* gene methylation was detected in samples of primary tumor tissue and its adjacent normal mucosa, metastatic lymph nodes and peritoneal irrigation fluid by methylation-specific PCR in order to find the relation between *RECK* methylation and clinicopathological characteristics of gastric cancer and the role of *RECK* methylation in diagnosis of peritoneal metastasis of gastric cancer.

## MATERIALS AND METHODS

### Patients

Forty patients including 28 males and 12 females at the age of 34-78 years underwent resection of their gastric cancer at the First Affiliated Hospital of China Medical University from July 2008 to January 2009. All patients did not receive chemotherapy or radiotherapy before operation.

### Samples

Physiological saline (50 mL) was injected into the Douglas cavity at the beginning of operation and aspirated after gentle stirring, and then peritoneal lavage fluid was collected from the cavity before operation. Half of the peritoneal lavage fluid was examined using conventional cytological methods with Papanicolaou's staining and intact cells were harvested from the other half centrifuged at 2000 r/min for 20 min as previously described<sup>[9,10]</sup> and stored in liquid nitrogen. Samples of primary tumor tissue and its paired adjacent normal mucosa and metastatic lymph nodes were taken immediately after resection of gastric cancer and stored in liquid nitrogen until use. The diagnosis of gastric cancer was made with hematoxylin and eosin (HE) staining. Paired adjacent normal mucosa samples were obtained at least 3 cm from the distal negative surgical margin to confirm the absence of malignancy. Lymph node samples were also stained with HE to confirm the occurrence of metastasis. Differentiation of tumor cells was detected and the tumor was staged following the guidelines of International Union against Cancer (UICC).

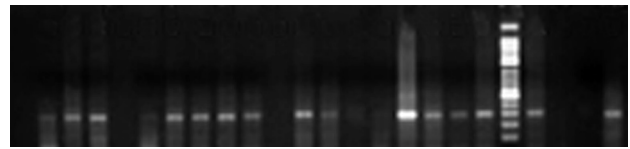
### DNA extraction and bisulfite treatment

DNA was extracted from the genome with the hydroxy-benzene-chloroform extraction method, stored at -70°C, and treated with bisulfite to convert the unmethylated cytosine to uracil.

### Methylation-specific PCR

DNA was purified using a Wizard DNA clean-up system

9 8 7 6 5 4 3 2 1 Ma MP UP  
M U M U M U M U M U M U M U M U M U



**Figure 1** PCR showing methylation of *RECK* in primary tumor and its paired adjacent normal mucosa and metastatic lymphnode samples. M: Methylation; U: Unmethylation; Ma: 50 bp DNA ladder marker; MP: Methylation positive control; UP: Unmethylation positive control; 1-9: Sample number.

(Promega) according to its manufacturer's instructions. A 20  $\mu$ L reaction volume was consisted of 3  $\mu$ L DNA, 2  $\mu$ L 10  $\times$  PCR buffer, 0.8  $\mu$ L dNTP, 0.4  $\mu$ L primers, 0.15  $\mu$ L Tap enzyme, and 13.25  $\mu$ L double-distilled water. PCR conditions were as follows: pre-denaturation at 94°C for 10 min, followed by 40 cycles at 94°C for 30 s, at 54°C for 20 s, at 72°C for 30 s, and a final extension at 72°C for 5 min. Methyltransferase Sss I -treated DNA in peripheral blood cells from healthy people was used as a methylation positive control, untreated DNA served as an unmethylation positive control, and double-distilled water served as a negative control<sup>[4]</sup>. The sequences of primers are as follows: unmethylation primer: UF\_*RECK* (5'-GGTTAGTTTTTTTATTT-TAGTGGTTTGA-3') and UR\_*RECK* (5'-ATTTC-CAAAACCTCCCAAAAACA-3'), methylation primer: MF\_*RECK* (5'-GTTAGTTTTTTTATTT-TATTTTAGTGGTTTCA-3') and MR\_*RECK* (5'-TC-CAAAACCTCCCGAAAACGAAAACG-3')<sup>[8]</sup>. The PCR products (205 bp and 201 bp) were subjected to 2.5% agarose gel electrophoresis at 120 V for 40 min and quantified with the Fluor Chen 2.0 system.

### Statistical analysis

Statistical analysis was performed using the SPSS13.0 software package.  $\chi^2$  test and Fisher's exact test were adopted to verify the difference.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Clinicopathological characteristics of gastric cancer patients and *RECK* methylation

The methylation of *RECK* in samples of primary tumor tissue and its paired adjacent normal mucosa and metastatic lymphnode was detected by methylation special-PCR (Figure 1).

Methylation of *RECK* was found in 47.5% (19/40) of primary tumor tissue samples, in 27.5% (11/40) of paired adjacent normal mucosa samples, in 57.1% (12/21) of metastatic lymphnode samples, respectively. A significant relation was found between adjacent normal mucosa and metastatic lymphnode samples. *RECK* methylation was related with tumor invasion ( $P = 0.023$ ) but not with other clinicopathological characteristics of gastric cancer patients such as age,



Table 1 Relation between clinicopathological characteristics and *RECK* methylation in gastric cancer patients

Variable	Patients (n)	<i>RECK</i> methylation	P value
Age (yr)			0.689
≤ 65	32	16	
> 65	8	3	
Tumor size (cm)			0.121
≤ 5	24	9	
> 5	16	10	
Borrmann classification			0.199
1+2	19	7	
3+4	21	12	
Tumor differentiation			0.935
Well	25	12	
Moderate/poor	15	7	
Tumor invasion			0.023
T1+T2	28	10	
T3+T4	12	9	
Nodal status			0.199
N-	19	7	
N+	21	12	

TNM was staged according to the guideline of International Union against Cancer (UICC). N-: Without nodal metastasis; N+: With nodal metastasis.

tumor size, tumor differentiation, and Borrmann classification (Table 1).

**Relation between peritoneal metastasis of gastric cancer and *RECK* methylation in peritoneal lavage fluid**

In this study, the promoter of *RECK* gene was hypermethylated in 35% (14/40) of the samples. Among the 14 samples, peritoneal metastasis of gastric cancer was observed in 9. The diagnostic accuracy of *RECK* methylation in peritoneal lavage fluid for peritoneal metastasis of gastric cancer was 72.5%, with a sensitivity of 66.7%, a specificity of 74.2%, a PPV of 47.1%, and a NPV of 95.7% (Table 2).

*RECK* methylation in peritoneal lavage fluid was found in tumors with lymph node metastasis (42.6%) and without lymph node metastasis (26.3%), although the difference between them was not statistically significant (Table 3).

**DISCUSSION**

*RECK* gene was discovered on chromosome region 9p13-p12 by Takahashi *et al*<sup>[11]</sup> in 1998. It encodes a membrane-anchored glucose protein with a relative molecular mass of 110000. *RECK* protein is an important mediator of tissue remodeling to inhibit *MMP-2*, *MMP-9* and *MT1-MMP* after transcription<sup>[12,13]</sup>. *RECK* protein limits tumor invasion and metastasis and angiogenesis through negatively regulated *MMPs*. It has been shown that several common tumors, such as colorectal, breast, and lung carcinomas, are linked to down-regulation of *RECK*<sup>[14-16]</sup>. In these tumors, *RECK* is down-regulated most likely as a result of inhibition at the *Sp1* promoter site<sup>[17]</sup>. It was reported that down-regulation of the *RECK* gene is mediated by promoter methylation which causes

Table 2 Relation between *RECK* methylation in peritoneal lavage and peritoneal metastasis

PLM	Peritoneal metastasis	
	+	-
+	6	8
-	3	23

PLM: Methylation in peritoneal lavage.

Table 3 Relation between *RECK* methylation in peritoneal lavage and clinicopathological factors

Variable	Patients (n)	<i>RECK</i> methylation in peritoneal lavage	P value
Tumor invasion			0.193
T1+T2	28	8	
T3+T4	12	6	
Nodal status			0.273
N-	19	5	
N+	21	9	

its silence, just as other tumor suppressor genes<sup>[7,8,18]</sup>. Epigenetic alteration induced by DNA methyltransferases (DNMT) catalyzing methylation at 5 positions of cytosine ring using S-adenosylmethionine as the donor molecule for the methyl group plays an important role in tumorigenesis and progression<sup>[1]</sup>. The mechanism underlying *RECK* down-regulation appears to be multifactorial, and more studies are required to define its reasons.

In this study, *RECK* methylation was observed in samples of primary tumor tissue and its paired adjacent normal mucosa and metastatic lymph nodes from gastric cancer patients, indicating that *RECK* methylation in primary tumor tissue samples (47.5%) and in metastatic lymph node samples (57.1%) is much higher than that in paired adjacent normal mucosa samples (27.5%) ( $P = 0.023$ ) and that *RECK* methylation is correlated with tumor invasion ( $P = 0.023$ ). No significant difference was found in other factors, including age, tumor size, tumor differentiation, nodal status, Borrmann classification. However, Song *et al*<sup>[19]</sup> found that *RECK* expression is negatively related with lymph node metastasis and tumor stage in gastric cancer patients, which may be due to the small sample size, contamination of normal tissues, technical limitations<sup>[7]</sup>, and down-regulation of *RECK*. Cho *et al*<sup>[7]</sup> showed that *RECK* promoter is methylated in 44% of tumor tissue samples and down-regulation of *RECK* is significantly correlated with promoter methylation ( $P < 0.05$ ), suggesting that *RECK* methylation plays a significant role in inhibiting tumorigenesis and metastasis.

Methylation alteration occurs not only in solid cancer tissues but also in various remote samples from cancer patients. It has been recently reported that DNA methylation can act as a promising biomarker in early diagnosis and prognosis of gastric cancer<sup>[20]</sup>. In our

study, *RECK* methylation in peritoneal lavage fluid was related with peritoneal metastasis of gastric cancer. Peritoneal metastasis of gastric cancer with cytologically positive peritoneal lavage was found in 9 of 14 patients with promoter hypermethylation. *RECK* promoter hypermethylation in peritoneal lavage showed a higher sensitivity (66.7%) for the diagnosis of peritoneal dissemination of gastric cancer than cytology. The reasons why methylation alteration acts as a biomarker are as follows. First, the methylation signal can act as a marker at a low concentration. Second, the methylation pattern and underlying DNA are more stable than RNA level and molecules<sup>[10]</sup>. However, methylation alteration in peritoneal lavage has a lower specificity for the diagnosis of peritoneal dissemination of gastric cancer, which can be explained as follows. First, most cells in peritoneal lavage are mesothelial cells leading to false positive *RECK* methylation. Second, the discrepancy of methylation profile exists sometimes in peritoneal lavage and cancer tissue. In order to solve these problems, serial test, *RECK* methylation and other examinations, such as carcino-embryonic antigen in peritoneal lavage, can be used in the diagnosis of peritoneal dissemination of gastric cancer. In our study, *RECK* methylation in peritoneal lavage fluid was more frequently found in tumors with lymph node metastasis than in tumors without lymph node metastasis, suggesting that *RECK* methylation in peritoneal lavage can be considered a biomarker for predicting peritoneal metastasis of gastric cancer.

In summary, hypermethylation of *RECK* promoter is a common event in gastric cancer patients. *RECK* methylation in peritoneal lavage fluid acts as a biomarker of peritoneal metastasis of gastric cancer. Promoter hypermethylation of *RECK* gene provides a new tool for the prevention and treatment of gastric cancer. Further study is needed on the mechanism underlying *RECK* hypermethylation in gastric cancer patients.

## COMMENTS

### Background

Gastric cancer is a common tumor which seriously threatens the human health worldwide. DNA methylation in the promoter CpG island of tumor suppressor genes is one of the reasons for tumorigenesis and progression. It has been shown that DNA methylation, especially in body fluid, can act as a biomarker for predicting tumor metastasis.

### Research frontiers

*RECK* hypermethylation plays an important role in the epigenetic regulation of gene transcription. There is evidence that DNA promoter hypermethylation can cause transcription repression, contributing to tumorigenesis and progression. It has been recently shown that DNA methylation, especially in body fluid, can act as a biomarker for predicting tumorigenesis and prognosis. However, further study is needed on the mechanism underlying *RECK* hypermethylation.

### Innovations and breakthroughs

*RECK* methylation in gastric cancer and peritoneal lavage fluid was detected, showing that *RECK* methylation plays an important role in diagnosing peritoneal metastasis.

### Applications

Promoter hypermethylation of *RECK* gene provides a new tool for the prevention and treatment of gastric cancer. In addition, *RECK* methylation,

especially in peritoneal lavage fluid, can act as a biomarker for diagnosing peritoneal metastasis.

### Peer review

It is a very interested topic for the readers of *WJG*. The results of this study show that promoter hypermethylation of *RECK* gene provides a new tool for the prevention and treatment of gastric cancer and *RECK* methylation, especially in peritoneal lavage fluid, can act as a biomarker for diagnosing peritoneal metastasis, which are of great value for the diagnosis of gastric cancer.

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## Successful endoscopic procedures for intraductal papillary neoplasm of the bile duct: A case report

Kohei Tsuchida, Michiko Yamagata, Yasuyuki Saifuku, Dan Ichikawa, Kazunari Kanke, Toshimitsu Murohisa, Masaya Tamano, Makoto Iijima, Yukiko Nemoto, Wataru Shimoda, Toshiaki Komori, Hirokazu Fukui, Kazuhito Ichikawa, Hitoshi Sugaya, Kazuhito Miyachi, Takahiro Fujimori, Hideyuki Hiraishi

Kohei Tsuchida, Michiko Yamagata, Yasuyuki Saifuku, Dan Ichikawa, Kazunari Kanke, Toshimitsu Murohisa, Masaya Tamano, Makoto Iijima, Hitoshi Sugaya, Hideyuki Hiraishi, Department of Gastroenterology, Dokkyo Medical University, Tochigi 321-0293, Japan

Yukiko Nemoto, Wataru Shimoda, Toshiaki Komori, Kazuhito Miyachi, First Department of Surgery, Dokkyo Medical University, Tochigi 321-0293, Japan

Hirokazu Fukui, Kazuhito Ichikawa, Takahiro Fujimori, Department of Surgical and Molecular Pathology, Dokkyo Medical University, Tochigi 321-0293, Japan

**Author contributions:** Tsuchida K and Yamagata M designed the research and wrote the paper; Tsuchida K, Saifuku Y, Ichikawa D, Kanke K, Murohisa T, Tamano M, Iijima M, Nemoto Y, Shimoda W, Komori T and Miyachi K did the clinical work; Fukui H, Ichikawa K, Fujimori T analyzed the pathological data; Sugaya H and Hiraishi H reviewed the paper.

**Correspondence to:** Michiko Yamagata, MD, Department of Gastroenterology, Dokkyo Medical University, 880 Kitakobayashi, Mibu, Shimotsugun, Tochigi 321-0293, Japan. [myamagat@dokkyomed.ac.jp](mailto:myamagat@dokkyomed.ac.jp)

Telephone: +81-282-861111 Fax: +81-282-867761

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noted, clinical profiles and alternative therapies other than resection may be recommended. We report the case of a patient who experienced repetitive cholangitis for 10 years and was finally diagnosed with IPNB. Radical resection could not be recommended because of the age of the patient, therefore, endoscopic sphincterotomy was performed. Although an endoscopic retrograde biliary drainage catheter was placed several times for repetitive cholangitis, the patient has done well during follow-up. Our case may offer insights into the natural course and management decisions for the novel disease entity of IPNB.

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**Key words:** Biliary tract neoplasms; Papilloma; Endoscopic sphincterotomy; Endoscopic retrograde biliary drainage

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Tsuchida K, Yamagata M, Saifuku Y, Ichikawa D, Kanke K, Murohisa T, Tamano M, Iijima M, Nemoto Y, Shimoda W, Komori T, Fukui H, Ichikawa K, Sugaya H, Miyachi K, Fujimori T, Hiraishi H. Successful endoscopic procedures for intraductal papillary neoplasm of the bile duct: A case report. *World J Gastroenterol* 2010; 16(7): 909-913 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i7/909.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i7.909>

### Abstract

Attention has recently been focused on biliary papillary tumors as the novel disease entity intraductal papillary neoplasm of the bile duct (IPNB), which consists of papillary proliferation of dysplastic biliary epithelium. As even benign papillary tumors are considered as premalignant, some investigators recommend aggressive surgical therapy for IPNB, although no guidelines are available to manage this disease. Few reports have described long-term follow-up of patients with benign IPNB without radical resection. If patients with IPNB who are treated only with endoscopic procedures are

### INTRODUCTION

Recently, the novel disease entity of intraductal papillary neoplasm of the bile duct (IPNB) has been proposed to include biliary papillomatosis, which comprises multiple



biliary papillomas composed of papillary proliferation of the dysplastic biliary epithelium, and papillary cholangiocarcinoma<sup>[1]</sup>. IPNB is thought to represent the biliary counterpart of pancreatic intraductal papillary mucinous neoplasm (IPMN-P) and is thus considered premalignant<sup>[1,2]</sup>. The prognosis of patients with IPNB is good when curative surgery is performed, therefore, aggressive resection is recommended as the first choice<sup>[3-5]</sup>. However, little information is available regarding the prognosis of IPNB without curative surgery, as few patients with IPNB who have been followed for several years without surgery have been reported. Here, we present a patient with IPNB who was followed for 10 years without surgical resection, and has done well during the follow-up period. This case indicates a natural course of the novel entity IPNB and alternative therapies for this disease.

## CASE REPORT

A previously healthy 76-year-old woman developed acute cholecystitis caused by gallbladder stones, and cholecystectomy was performed in 1999. Cholangioscopic examination during surgery revealed a bile duct tumor with papillary proliferation protruding into the common bile duct (Figure 1A). The tumor was followed without resection, and a biopsy specimen showed tubular adenoma packed with small glandular or papillary components.

When the patient was admitted for acute cholangitis in 2004, the papillary tumor in the bile duct appeared unchanged. The patient recovered well until she became symptomatic again in September 2007, for which an endoscopic retrograde biliary drainage (ERBD) catheter (FLEXIMA™ Biliary Stent System, Boston Scientific Co., Natick, MA, USA) was placed. In December 2007, the patient developed recurrent cholangitis because of slippage of the ERBD catheter, which was re-inserted into the bile duct. In early January 2008, the ERBD catheter slipped, and the patient was seen for abdominal pain and fever. Laboratory test results included: alkaline phosphatase, 498 U/L (normal: 104-338 U/L);  $\gamma$ -glutamyltranspeptidase, 122 U/L (normal: 18-66 U/L); aspartate aminotransferase, 58 U/L (normal: 10-37 U/L); alanine aminotransferase, 71 U/L (normal: 3-34 U/L); carbohydrate antigen 19-9, 414 U/mL (normal: 0-37 U/mL); white blood cell count, 11 400/mm<sup>3</sup> (normal: 4000-9000/mm<sup>3</sup>); and C-reactive protein, 17.3 mg/dL (normal: 0-0.3 mg/dL).

Endoscopic retrograde cholangiography (ERC) revealed extrahepatic bile duct dilatation, which was more marked than that seen in 1999, and the presence of intraductal polypoid and amorphous filling defects (Figure 1B and C). Magnetic resonance cholangiopancreatography (MRCP) showed cystic lesions connected to the intrahepatic bile duct in the left liver. Cystic lesions were enlarged compared with those in 1999 (Figure 1D and E). Abdominal computed tomography (CT) showed both intrahepatic and extrahepatic bile ducts to be dilated and the presence of a 20-mm mass in the distal common bile duct. In addition, the cystic lesion in the left liver was enlarged compared to that in 1999 (Figure 1F-I). Con-

trast-enhanced harmonic endoscopic ultrasound (CEH-EUS) with Sonazoid® (Daiichi-Sankyo, Tokyo, Japan) showed flow signals inside the whole mass, which revealed the papillary structure (Figure 2A and B). CEH-EUS for biliary diseases was approved by the ethical committee of Dokkyo Medical University, and written informed consent was obtained from the patient before the examination. Intraductal ultrasonography (IDUS) showed a 10-mm papillary mass in the middle bile duct (Figure 2C), and multilobular lesions in the distal bile duct (Figure 2D). Duodenoscopy demonstrated a 10-mm discolored mass on the ampulla of Vater. A biopsy specimen from the discolored mass showed adenoma.

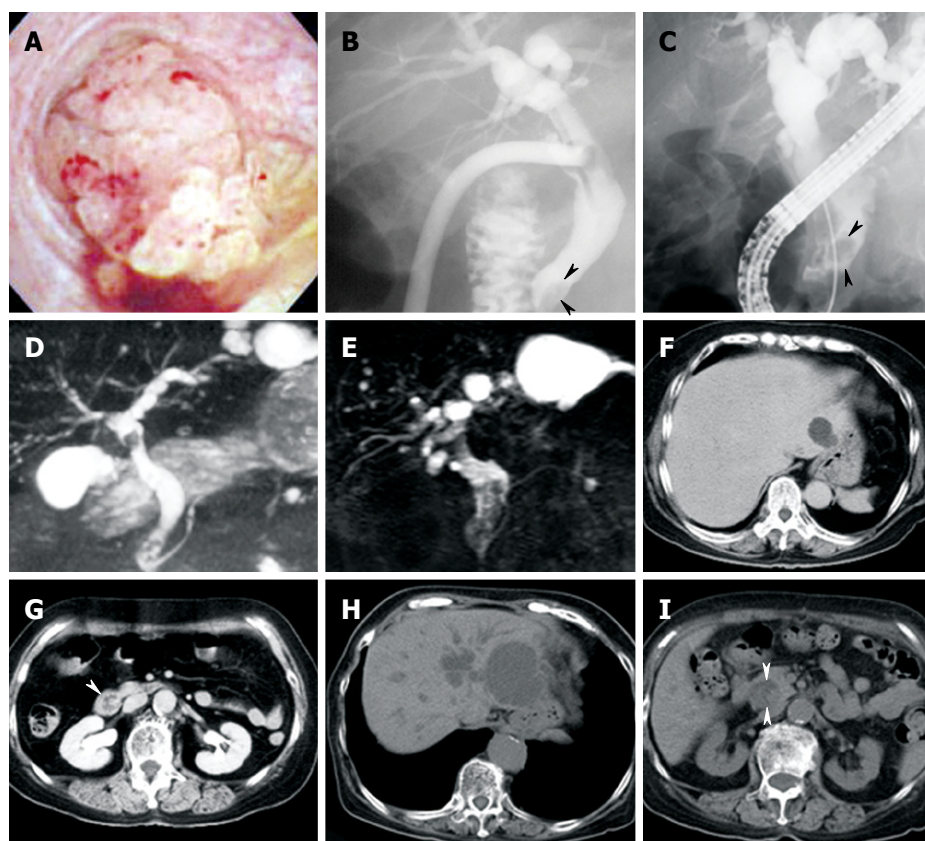
As the patient was diagnosed with cholangitis caused by obstruction of the bile duct by intraductal papillary tumor, endoscopic biliary sphincterotomy (EST) was performed (Figure 3). Radical resection was not recommended because of the age of the patient. Through the orifice of the ampulla of Vater, a soft, bead-like mass was extracted by balloon sweep and a net-type catheter.

Pathological diagnosis of the extracted mass was papillary neoplasia without invasive carcinoma (Figure 4A). Immunohistochemical analysis was performed for mucin core proteins and cytokeratins (CKs). Less than 5% of adenoma cells showed positivity for MUC1 in the apical membrane (Figure 4B). Negative results were obtained for MUC2. About 40%-50% of adenoma cells showed positivity for MUC5AC in the cytoplasm (Figure 4C). Both CK7 and CK20 were negative.

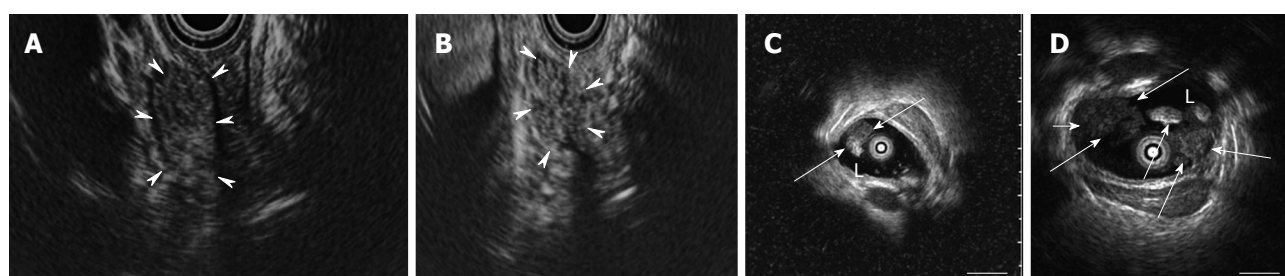
Although the patient was readmitted in May 2008 and in June 2009 with signs of cholangitis, for which no special procedures and placement of ERBD catheter were respectively performed, the patient has done well since EST.

## DISCUSSION

This case is compatible with the novel disease entity of IPNB<sup>[1]</sup>, and biliary papillomatosis can be diagnosed as discussed below. Biliary papilloma is a rare benign neoplasm that consists of papillary proliferation of atypical biliary epithelium along with delicate fibrovascular stalks<sup>[6]</sup>. Biliary papillomatosis is defined as the presence of more than three papillomas at different sites of the biliary tree. Some cholangiocarcinomas that show mainly papillary proliferation in the bile duct are designated as papillary cholangiocarcinoma. Zen *et al*<sup>[1]</sup> have proposed biliary papilloma(tosis) and papillary cholangiocarcinoma with or without mucus hypersecretion as belonging to the novel tumor entity of IPNB. In this case, cholangioscopy and tissue specimens demonstrated that the tumor originated from the biliary epithelium, and various imaging modalities revealed tumors with papillary formation. Two papillary tumors in the extrahepatic bile duct detected by IDUS, and an expanding cystic lesion in the liver contiguous with the intrahepatic bile duct suggested that at least three lesions were present at different sites of the biliary tree. To summarize, this case could be diagnosed as biliary papillomatosis, although whether malignant transformation was present is unknown.



**Figure 1** Images of cholangioscopic examination, ERC, MRCP and abdominal CT. A: Cholangioscopic examination performed in 1999. A multilobulated papillary tumor was seen protruding into the common bile duct; B: ERC performed in 1999; C: ERC performed in 2008. The extrahepatic bile duct became more dilated in 2008 than in 1999. A polypoid filling defect (arrow heads) could be detected in the distal bile duct; D: MRCP in 1999; E: MRCP in 2004. Cystic lesions connected to the intrahepatic bile ducts of the left liver became enlarged in 2004; F and G: Abdominal CT in 1999; H and I: Abdominal CT in 2004. Intra- and extrahepatic bile ducts became dilated in 2004. A mass in the distal bile duct (arrow heads) and a cystic lesion in the left liver were enlarged in 2004, compared with those in 1999.



**Figure 2** Images of ultrasonographic studies. A: EUS showed a mixed echoic mass in the distal bile duct (arrow heads); B: Papillary structure of the mass could be apparently observed on CEH-EUS with Sonazoid®; C: IDUS showed a 10-mm papillary mass (arrows) in the middle bile duct; D: In the distal bile duct, multilobar and papillary lesions (arrows) were observed. L: Lumen of the bile duct.

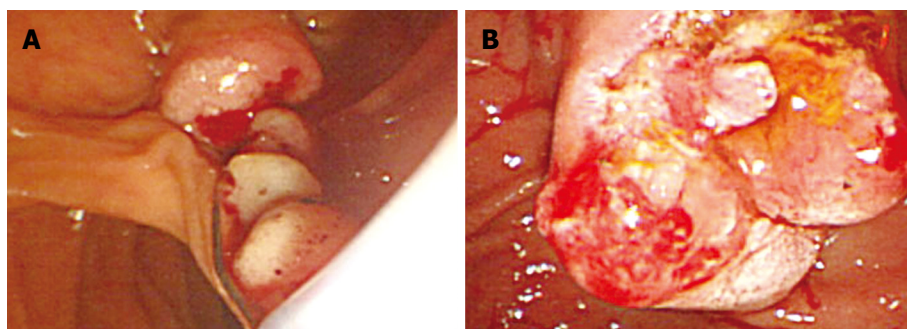
Occasional association with mucin hypersecretion is one of the pathological similarities between IPNB and IPMN-P<sup>[1]</sup>. Zen *et al*<sup>[1]</sup> have performed immunohistochemical analysis for mucin core proteins in patients with IPNB and compared the results with patients with IPMN-P. They have proposed the typical mucin and cytokeratin expression profile of IPNB as MUC1-negative, MUC2-positive, MUC5AC-positive, CDX2-positive, CK7-positive, CK20-positive. According to Shibahara *et al*<sup>[7]</sup>, patients with MUC1-positive expression show poorer survival than those with MUC1-negative expression in papillary cholangiocarcinoma. In addition, the same group has reported that IPMN-P tends to show MUC1-negative and MUC2-positive expression, in contrast to invasive carcinoma, which shows MUC1-positive and MUC2-negative expression<sup>[8]</sup>. The mucin and cytokeratin expression profile in the present case was MUC1-positive, MUC2-negative, MUC5AC-positive, CK7-negative, CK20-negative, which

differs from the typical profile reported by Zen *et al*<sup>[1]</sup>. Reasons for this difference might have included: (1) our specimens were taken by biopsy and immunohistochemical analysis was not performed for the entire lesion; (2) even in the original paper by Zen *et al*<sup>[1]</sup>, not all cases showed the typical profile; and (3) MUC1-positivity implies that our case could have malignant potential and needs cautious observation in the future.

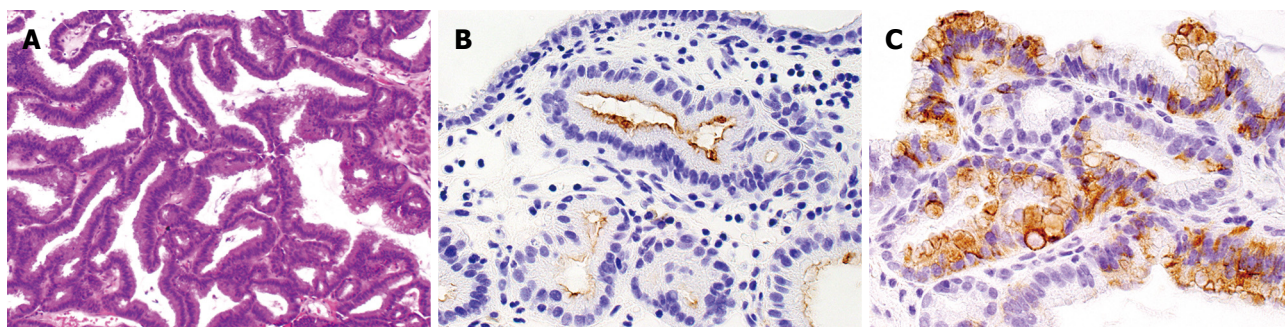
Surgical resection is often recommended because of the high malignancy rate, diffuse pattern of disease, and better survival after curative surgery<sup>[3-5]</sup>. Liver transplantation has been suggested as an alternative<sup>[9]</sup>, while many patients with biliary papillomatosis, which is a disease of the elderly (mean age at time of diagnosis, 63 years)<sup>[10]</sup>, would not be eligible for transplantation.

We consider that curative resection is not necessarily reasonable for every patient with IPNB for the following reasons. First, compared with the prognosis of usual





**Figure 3** EST was performed. A: A soft, bead-like mass was extracted through the orifice of the ampulla of Vater after EST; B: Image of the ampulla of Vater after extraction of the mass by balloon sweep and a net-type catheter.



**Figure 4** Histopathological findings. A: Hematoxylin-eosin staining; B: Immunohistochemical analysis for MUC1; C: Immunohistochemical analysis for MUC5AC.

**Table 1** Patients with IPNB treated by endoscopic procedures reported between 2005 and 2009

Author	Gender	Age (yr)	Follow-up after diagnosis of IPNB	Treatment	Reason surgery was not performed	Outcome
Bechmann <i>et al</i> <sup>[10]</sup>	Male	65	10 years	Whipple, right hepatectomy, PDT	Patient's age	Death without cholestasis
Park <i>et al</i> <sup>[11]</sup>	Female	78	3 wk	EST, EPBD	Unknown	Well for a short time
Brauer <i>et al</i> <sup>[12]</sup>	Male	86	1 mo	APC	Diffuse involvement of biliary system, patient's age and comorbidities	Death due to hepatic encephalopathy
Jazrawi <i>et al</i> <sup>[13]</sup>	Male	37	6 mo	Extrahepatic bile duct resection, APC	Patient's refusal	Being evaluated for liver transplantation due to disease progression
Current case	Female	86	11 years	Cholecystectomy, EST, ERBD	Patient's age	Being well irrespective of slow disease progression

EST: Endoscopic sphincterotomy; EPBD: Endoscopic papillary balloon dilatation; Whipple: Pancreaticoduodenectomy; PDT: Photodynamic therapy; APC: Argon plasma coagulation; ERBD: Endoscopic retrograde biliary drainage.

cholangiocellular carcinoma, that of biliary papillomatosis including benign and malignant cases is much better<sup>[2]</sup>. This implies that the prognosis of benign biliary papillomatosis, in particular, may be good enough to be observed without radical resection, although precise information on the clinical course of patients with biliary papillomatosis is currently unclear. Second, the Whipple procedure and hemihepatectomy are the therapies of choice depending on the location and extension of the disease. In addition, malignant change is observed in 40%-50% of cases<sup>[11]</sup>, which means that half of the cases remain benign. If radical resection were recommended for all patients, the therapy might be too invasive for the potentially large population of patients with benign disease.

A search of the English-language literature published in the past 5 years was performed using the MEDLINE

database with keywords of 'biliary papillomatosis' and 'intraductal papillary neoplasm of the bile duct'. Five patients with pathologically benign IPNB<sup>[10-13]</sup>, including the present case, have been followed up using endoscopic procedures (Table 1). Two patients had been followed for > 10 years. Among these five patients, surgery was not considered a viable option because of the age of the patient in three cases, small range of the disease in one case, and for unknown reasons in the other.

The therapy offered to these patients was EST plus additional endoscopic papillary balloon dilation in two cases, argon plasma coagulation in two cases, and photodynamic therapy (PDT) in one case. Prognosis of these patients was unchanged with occasional cholangitis in two cases, exacerbation in one, death from another disease in one, and unknown in one. Based upon the summaries of

these patients, cases in which malignant transformation cannot be ascertained pathologically could be cautiously followed using endoscopic procedures, and using radical resection as the gold standard is unnecessary. In addition, another patient similar to our own was followed up for 10 years, and treated using endoscopic procedures for recurrent biliary papillomatosis, because there was no surgical option left. As IPNB is considered as the biliary counterpart of IPNM-P<sup>[1,7]</sup>, a follow-up period of over a decade for some patients with IPNB, as in our case, is not necessarily inappropriate.

To conclude, we encountered a patient with IPNB who was treated only with endoscopic procedures for 10 years, which suggests that some patients with benign IPNB could be followed conservatively without radical resection. Moreover, the present case might partly demonstrate the natural course of patients with the novel disease entity of IPNB.

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## Monocyte chemotactic protein-1 gene polymorphism and spontaneous bacterial peritonitis

Levent Filik

Levent Filik, Gastroenterology Division, Ankara Research Hospital, Ankara, TR06600, Turkey

Author contributions: Filik L wrote the paper.

Correspondence to: Dr. Levent Filik, Associate Professor, Gastroenterology Division, Ankara Research Hospital, Ankara, TR06600, Turkey. [leventfilik@yahoo.co.uk](mailto:leventfilik@yahoo.co.uk)

Telephone: +90-505-2653075 Fax: +90-312-5954272

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### Abstract

I read with great interest the article by Gäbele *et al* published in issue 44 of *World J Gastroenterol* 2009. The results of their study indicate that -2518 *Monocyte chemotactic protein-1* (MCP-1) genotype AA is a risk factor for spontaneous bacterial peritonitis in patients with alcoholic cirrhosis. However, there are some items that need to be discussed.

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**Key words:** Spontaneous bacterial peritonitis; *Monocyte chemotactic protein-1*; Polymorphism

**Peer reviewers:** Dr. Sang Geon Kim, PhD, MS, BS, Professor, Chairman, College of Pharmacy, Seoul National University, Sillim-dong, Kwanak-gu, Seoul 151-742, South Korea; Robert Flisiak, PhD, Department of Infectious Diseases, Medical University of Bialystok, 15-540 Bialystok, Zurawia Str., 14, Poland

Filik L. *Monocyte chemotactic protein-1* gene polymorphism and spontaneous bacterial peritonitis. *World J Gastroenterol* 2010; 16(7): 914 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i7/914.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i7.914>

### TO THE EDITOR

I read with great interest the article by Gäbele *et al*<sup>[1]</sup>

published in issue 44 of *World J Gastroenterol* 2009. The article provides important data. The results of their study indicate that the -2518 *Monocyte chemotactic protein-1* (MCP-1) genotype AA is a risk factor for spontaneous bacterial peritonitis (SBP) in patients with alcoholic cirrhosis. The authors suggested that the reduced MCP-1 ascites level may a cause for patients with SBP compared to those with G allele. However, there are some items that need to be discussed. It is debatable to get this conclusion unless ascites MCP-1 levels are measured before and after the treatment of SBP. It has been reported that the MCP-1 level in both sera and ascites is higher in SBP than in non-SBP patients, and decreases after treatment<sup>[2]</sup>. Infection other than SBP data is also missed in that article. For example, urinary tract infection and even asymptomatic bacteriuria may precede SBP. It is not easy to decide if MCP-1 polymorphism causes urinary tract infection and subsequently SBP, because MCP-1 plays a role even in asymptomatic bacteriuria<sup>[3]</sup>. Another issue of my concern is the number of SBP episodes. No data in relation with repeated SBP were provided in the article. Did the authors observe repeated SBP episodes in the patients with genotype AA over a 6-year period between 2001-2007? Did the patients respond to the antibiotic therapy well in a similar time interval?

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**Tamara M Alempijevic, MD, PhD, Assistant Professor**, Clinic for Gastroenterology and Hepatology, Clinical Centre of Serbia, 2 Dr Koste Todorovica St., 11000 Belgrade, Serbia

**Fernando Alvarez, Professor**, Service de gastroentérologie, hépatologie et nutrition, Hôpital Sainte-Justine, 3175 Côte Ste-Catherine, Montréal, Québec, Canada H3T 1C5, Canada

**Giedrius Barauskas, Professor**, Department of Surgery, Kaunas University of Medicine, Eiveniu str. 2, Kaunas, LT-50009, Lithuania

**Albert J Bredenoord, Dr., MD**, Department of Gastroenterology, St Antonius Hospital, PO Box 2500, 3430 EM, Nieuwegein, The Netherlands

**Yeun-Jun Chung, MD, PhD, Professor, Director**, Department of Microbiology, Integrated Research Center for Genome Polymorphism, The Catholic University Medical College, 505 Banpo-dong, Socho-gu, Seoul 137-701, South Korea

**Da-Jun Deng, Professor**, Department of Cancer Etiology, Peking University School of Oncology, 1 Da-Hong-Luo-Chang Street, Western District, Beijing 100034, China

**Francesco Feo, Professor**, Department of Biomedical Sciences, Section of Experimental Pathology and Oncology, University of Sassari, Via P. Manzella 4, 07100 Sassari, Italy

**Valeria Ghisetti, Dr.**, Laboratory of Microbiology, Molinette Hospital, Corso Bramante 88 / 90, 10126 Torino, Italy

**Peter Raymond Gibson, Professor**, Department of Medicine, Box Hill Hospital, Box Hill, Victoria 3128, Australia

**Chakshu Gupta, MD, FCAP**, Pathology and Laboratory Medicine,

Heartland Regional Medical Center, 5325 Faraon Street, St. Joseph, 64506 Missouri, United States

**Eva Herrmann, Professor**, Department of Internal Medicine, Biomedicine Saarland University, Faculty of Medicine, Kirrberger Str., 66421 Homburg/Saar, Germany

**Yi-Min Mao, Professor**, Department of Gastroenterology, Shanghai Research Institute of Gastroenterology, Renji Hospital, Shanghai Jiao-Tong University School of Medicine, No.145, Shandong Zhong Road, Shanghai 200001, China

**Kazuichi Okazaki, Professor**, Third Department of Internal Medicine, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi City, Osaka, 570-8506, Japan

**Fabio Pace, Professor**, Division of Gastroenterology, "L. Sacco" University Hospital, University of Milan, Via G. B. Grassi, 74, Milano 20157, Italy

**George Papatheodoridis, MD, Assistant Professor** in Medicine & Gastroenterology, 2nd Department of Internal Medicine, Athens University Medical School, Hippokraton General Hospital of Athens, 114 Vas. Sophias Ave., 115 27 Athens, Greece

**Raymund R Razonable, MD**, Division of Infectious Diseases, Mayo Clinic, 200 First Street SW, Rochester, Minnesota 55905, United States

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**Xiang-Dong Wang, MD, PhD, Professor**, Zhongshan Hospital, Fudan University, Shanghai 230003, China

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## Meetings

### Events Calendar 2010

January 25-26  
Tamilnadu, India  
International Conference on Medical  
Negligence and Litigation in Medical  
Practice

January 25-29  
Waikoloa, HI, United States  
Selected Topics in Internal Medicine

January 26-27  
Dubai, United Arab Emirates  
2nd Middle East Gastroenterology  
Conference

January 28-30  
Hong Kong, China  
The 1st International Congress on  
Abdominal Obesity

February 11-13  
Fort Lauderdale, FL, United States  
21th Annual International Colorectal  
Disease Symposium

February 26-28  
Carolina, United States  
First Symposium of GI Oncology at  
The Caribbean

March 04-06  
Bethesda, MD, United States  
8th International Symposium on  
Targeted Anticancer Therapies

March 05-07  
Peshawar, Pakistan  
26th Pakistan Society of  
Gastroenterology & Endoscopy  
Meeting

March 09-12  
Brussels, Belgium  
30th International Symposium on  
Intensive Care and Emergency  
Medicine

March 12-14  
Bhubaneswar, India  
18th Annual Meeting of Indian  
National Association for Study of  
the Liver

March 23-26  
Cairo, Egypt  
14th Pan Arab Conference on  
Diabetes PACD14

March 25-28  
Beijing, China  
The 20th Conference of the Asian

Pacific Association for the Study of  
the Liver

March 27-28  
San Diego, California, United States  
25th Annual New Treatments in  
Chronic Liver Disease

April 07-09  
Dubai, United Arab Emirates  
The 6th Emirates Gastroenterology  
and Hepatology Conference, EGHG  
2010

April 14-17  
Landover, Maryland, United States  
12th World Congress of Endoscopic  
Surgery

April 14-18  
Vienna, Austria  
The International Liver Congress™  
2010

April 28-May 01  
Dubrovnik, Croatia  
3rd Central European Congress  
of surgery and the 5th Croatian  
Congress of Surgery

May 01-05  
New Orleans, LA, United States  
Digestive Disease Week Annual  
Meeting

May 06-08  
Munich, Germany  
The Power of Programming:  
International Conference on  
Developmental Origins of Health  
and Disease

May 15-19  
Minneapolis, MN, United States  
American Society of Colon and  
Rectal Surgeons Annual Meeting

June 04-06  
Chicago, IL, United States  
American Society of Clinical  
Oncologists Annual Meeting

June 09-12  
Singapore, Singapore  
13th International Conference on  
Emergency Medicine

June 14  
Kosice, Slovakia  
Gastro-intestinal Models in  
the Research of Probiotics and  
Prebiotics-Scientific Symposium

June 16-19  
Hong Kong, China  
ILTS: International Liver  
Transplantation Society ILTS Annual  
International Congress

June 20-23  
Mannheim, Germany  
16th World Congress for  
Bronchoesophagology-WCBE

June 25-29  
Orlando, FL, United States  
70th ADA Diabetes Scientific  
Sessions

August 28-31  
Boston, Massachusetts, United States  
10th OESO World Congress on  
Diseases of the Oesophagus 2010

September 10-12  
Montreal, Canada  
International Liver Association's  
Fourth Annual Conference

September 11-12  
La Jolla, CA, United States  
New Advances in Inflammatory  
Bowel Disease

September 12-15  
Boston, MA, United States  
ICAAC: Interscience Conference  
on Antimicrobial Agents and  
Chemotherapy Annual Meeting

September 16-18  
Prague, Czech Republic  
Prague Hepatology Meeting 2010

September 23-26  
Prague, Czech Republic  
The 1st World Congress on  
Controversies in Gastroenterology &  
Liver Diseases

October 07-09  
Belgrade, Serbia  
The 7th Biannual International  
Symposium of Society of  
Coloproctology

October 15-20  
San Antonio, TX, United States  
ACG 2010: American College of  
Gastroenterology Annual Scientific  
Meeting

October 23-27  
Barcelona, Spain  
18th United European  
Gastroenterology Week

October 29-November 02  
Boston, Massachusetts, United States  
The Liver Meeting® 2010--AASLD's  
61st Annual Meeting

November 13-14  
San Francisco, CA, United States  
Case-Based Approach to the  
Management of Inflammatory Bowel  
Disease

December 02-04  
San Francisco, CA, United States  
The Medical Management of HIV/  
AIDS



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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 Xiaohua 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

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- 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 1, 274 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]

## Instructions to authors

### No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA 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

#### Author(s) and editor(s)

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek 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

#### 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

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### EDITING

Editorial Board of *World Journal of Gastroenterology*, Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China  
Telephone: +86-10-5908-0039  
Fax: +86-10-8538-1893  
E-mail: [wjg@wjgnet.com](mailto:wjg@wjgnet.com)  
<http://www.wjgnet.com>

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Telephone: +86-10-8538-1892  
Fax: +86-10-8538-1893  
E-mail: [baishideng@wjgnet.com](mailto:baishideng@wjgnet.com)  
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### SUBSCRIPTION

Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China  
Telephone: +86-10-8538-1892  
Fax: +86-10-8538-1893  
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Telephone: +86-10-5908-0039  
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## Computed tomographic colonography: Hope or hype?

Otto Schiueh-Tzang Lin

Otto Schiueh-Tzang Lin, Digestive Diseases Institute, Virginia Mason Medical Center, Seattle, WA 98101, United States; Department of Medicine, University of Washington School of Medicine, Seattle, WA 98101, United States

Author contributions: Lin OST wrote this editorial.

Correspondence to: Otto Schiueh-Tzang Lin, MD, MSc, Digestive Diseases Institute, Virginia Mason Medical Center, 1100 Ninth Avenue, Seattle, WA 98101,

United States. [otto.lin@vmmc.org](mailto:otto.lin@vmmc.org)

Telephone: +1-206-6257373-67694 Fax: +1-206-2236379

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### Abstract

Computed tomographic colonography (CTC) is a promising emerging technology for imaging of the colon. This concise review discusses the currently available data on CTC technique, test characteristics, acceptance, safety, cost-effectiveness, follow-up strategy, and extracolonic findings. In summary, CTC technique is still evolving, and further research is needed to clarify the role of automated colonic insufflation, smooth-muscle relaxants, intravenous and oral contrast, software rendering, and patient positioning. Currently, full bowel preparation is still required to achieve optimal results. The sensitivity for detecting large polyps (> 1 cm) can be as high as 85%, with specificity of up to 97%. These test characteristics are almost comparable to those of conventional colonoscopy. Patient acceptance of CTC is generally higher than that for colonoscopy, especially in patients who have never undergone either procedure. CTC is generally safe, although uncommon instances of colonic perforation have been documented. In terms of cost-effectiveness, most decision analyses have concluded that CTC would only be cost-effective if it were considerably cheaper than conventional colonoscopy. The proper follow-up strategy for small polyps or incidental extracolonic findings discovered during CTC is still under debate. At present, the exact clinical role of virtual colonoscopy still awaits determination. Even though widespread CTC screening is not available today,

in the future there may eventually be a role for this technology. Technological advances in this area will undoubtedly continue, with multi-detector row CT scanners allowing thinner collimation and higher resolution images. Stool-tagging techniques are likely to evolve and may eventually allow for low-preparation CTC. Perceptual and fatigue-related reading errors can potentially be minimized with the help of computer-aided detection software. Further research will define the exact role of this promising technology in our diagnostic armamentarium.

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**Key words:** Computed tomographic colonography; Colonoscopy; Colonic neoplasms; Cancer screening; Colonic polyps

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### INTRODUCTION

First described in 1994<sup>[1]</sup>, virtual colonoscopy, more properly termed computed tomographic colonography (CTC), uses dedicated processing software to generate 2- and 3-D reconstructions of the colon and rectum, based on data obtained by high-definition CT of the abdomen and pelvis. In recent years, there have been rapid advances in this technology, heightening its potential as a less invasive means of visualizing the colon. However, there remain numerous pitfalls to its widespread use. This article gives a succinct review of CTC technique, test characteristics, acceptance, safety and cost-effectiveness, to give an informed understanding of the potential of this promising new imaging modality.

## TECHNIQUE

CTC technique is constantly evolving, and there is still considerable variation depending on institutions. Currently, in most facilities, a full bowel preparation is required for CTC because retained stool cannot be reliably differentiated from polyps. The colon is distended with gas during the scan, as visualization is compromised in underinflated segments. The degree of insufflation may be controlled by the technician, the patient or an automated insufflation device. In most centers, room air is used for colonic distension, but carbon dioxide may be better tolerated because it diffuses through the colonic wall more quickly<sup>[2]</sup>. Smooth-muscle relaxants can theoretically reduce artifacts from colonic motility<sup>[3]</sup>, while the use of intravenous contrast may result in better differentiation of polyps from colonic fluid<sup>[4]</sup>. In addition, oral iodinated contrast can be ingested to change the attenuation of residual colonic fluid; however, studies have not demonstrated any significant improvement in accuracy<sup>[5]</sup>.

During colonography, the abdomen is scanned during one or two breath-holds that last < 2 min. Scans are performed in the craniocaudal direction, with the patient in the prone and supine positions. The incorporation of the prone position has been shown to improve distension of colonic segments and allow for displacement of fluid and stools<sup>[5]</sup>. Studies have suggested that scanning in the supine and left lateral decubitus positions improves visualization even further<sup>[6]</sup>. The best results for CTC require the use of multidetector (4-8 channels) scanners with 1.25-2.5 mm collimation, and reconstruction intervals of 1 mm. Standard helical images of the colon are processed by imaging software using one of three rendering techniques: surface rendering, volume rendering, or perspective rendering. In addition to 2-D axial, coronal and sagittal images, 3-D rendered views of the colon that simulate endoluminal views during colonoscopy can be reproduced. These allow both antegrade and retrograde “fly throughs” of the colon, with the ability to examine the proximal aspect of the haustral folds, a potential blind spot for conventional colonoscopy.

## TEST CHARACTERISTICS

Ever since publication of the initial study on the sensitivity and specificity of CTC compared to conventional colonoscopy<sup>[7]</sup>, numerous studies have reported widely disparate estimates of CTC test performance, probably due to differences in examination techniques, patient populations, reference standards, and examiner experience or skill. Many of the earlier studies used single-row scanners and showed mediocre or poor CTC sensitivity, especially for small polyps. More recent studies have used multi-detector scanners and have adopted more rigorous study designs, and some have reported favorable test performance characteristics. The results of the six largest studies to date in western populations are as follows.

In 2003, Pickhardt *et al*<sup>[8]</sup> presented a landmark study that showed that, under optimal conditions, CTC had comparable sensitivity and specificity to conventional colonoscopy. For detecting large polyps  $\geq 10$  mm in size, the sensitivity of CTC was 92%; for smaller polyps ( $\geq 6$  mm), the sensitivity was 86%. In this study, the investigators achieved excellent results by performing solid-stool tagging with oral barium and opacification of colonic fluid with iodinated contrast, post-procedure “electronic cleansing” with software that digitally removed opacified colonic fluid, and primary reading of 3-D images, with 2-D images used for problem solving. Segmental unblinding was adopted to provide an enhanced gold standard, and indeed CTC detected several lesions that were missed by conventional colonoscopy, as described by the authors in a follow-up study<sup>[9]</sup>. More recently, the multicenter ACRIN study, which featured the largest sample size to date (2531 subjects), reported a sensitivity of 90% and specificity of 86% for large polyps<sup>[10]</sup>, while another study on 1103 Italian patients achieved a sensitivity of 85% and specificity of 87% for advanced neoplasia<sup>[11]</sup>. However, three other large studies have reported less impressive results, with sensitivities of 63%, 55% and 59% for the detection of large polyps ( $\geq 10$  mm)<sup>[12-14]</sup>. Large-scale CTC screening has also been used in Asian populations, with variable degrees of success<sup>[15,16]</sup>.

Four meta-analyses have been published to summarize the available data. An earlier meta-analysis that involved 14 studies reported a pooled sensitivity of 81% for large polyps ( $\geq 10$  mm) and 43% for small polyps ( $\leq 5$  mm)<sup>[17]</sup>, while another meta-analysis of 24 studies reported a sensitivity of 93% for large polyps<sup>[18]</sup>. These two reviews did not include many of the more recent studies. A subsequent, more comprehensive meta-analysis included 33 studies (comprising a total of 6393 patients), and calculated pooled sensitivities ranging from 48% for small polyps ( $\leq 5$  mm) to 85% for large polyps ( $\geq 10$  mm). Specificity was more consistent, between 92% and 97%<sup>[19]</sup>. The most recent meta-analysis included 30 studies and used a summary receiver operating characteristic method for combining data, and reached similar conclusions<sup>[20]</sup>.

Technical factors that can limit the accuracy of CTC include poor bowel preparation, inadequate colonic distension, breath-hold artifacts and suboptimal image resolution. The sigmoid colon is often a problematic area, although diverticulosis does not appear to adversely affect the accuracy of CTC<sup>[21]</sup>. The rectum is another site with high miss rates for polyps because it is difficult to achieve adequate air insufflation there<sup>[2,22]</sup>. Studies have now confirmed that flat adenomatous lesions are common in western patients, and many of these may feature advanced neoplastic histology<sup>[23]</sup>. Such flat lesions may be difficult to recognize on CTC. Perceptual failure, when the polyp is evident on the scan but is not recognized as such by the reader, is thought to be correlated with inadequate training, limited experience and reader fatigue<sup>[24]</sup>.



## PATIENT ACCEPTANCE

At present, it is unclear if patients find CTC preferable to colonoscopy. In general, colonoscopy is perceived as being more invasive. However, colonoscopy offers the advantage of a “one-stop” diagnostic and therapeutic procedure, and its discomfort is mitigated by the use of conscious sedation in most developed countries. We recently presented data on a systematic review and meta-analysis on patient acceptance of CTC compared with conventional colonoscopy<sup>[25]</sup>; we reviewed 19 studies and found that, in general, patients preferred CTC over colonoscopy, although there was significant heterogeneity between studies (risk difference of 24%,  $P < 0.001$ ). Bowel preparation is universally perceived as the worst part of both procedures. There have been efforts to improve the accuracy of low-dose bowel preparation CTC<sup>[26-31]</sup>. If this becomes a commonly available procedure, patients will likely find CTC much more acceptable.

## SAFETY

CTC may not be as free from procedural complications as previously assumed<sup>[32]</sup>. Several cases of CTC-induced perforation have now been reported. These cases mostly have been associated with ulcerative colitis<sup>[33]</sup>, Crohn's disease<sup>[34,35]</sup> or rectosigmoid obstruction<sup>[36]</sup>, but occurrences in patients with normal colons have occurred as well<sup>[37,38]</sup>. Reviews in the United Kingdom and Israel have suggested that the rate of serious complications may be as high as 0.06%-0.08%<sup>[39,40]</sup>, which approaches the complication rates reported for conventional colonoscopy. There also have been concerns about radiation exposure. The surface radiation dose received during CTC is approximately 0.44 rem, which is roughly equivalent to that of two abdominal radiographs<sup>[41]</sup>. Although this is a relatively small dose, multiple repeated scans at regular intervals for surveillance purposes can still lead to cumulative radiation doses that may be of concern<sup>[42]</sup>. Low radiation dose protocols have been investigated<sup>[43]</sup>, but these do not appear to reduce overall radiation exposure in practice<sup>[44]</sup>.

## COST-EFFECTIVENESS

The cost-effectiveness of screening with CTC is uncertain. Several decision analysis computer simulation studies have tried to assess this question<sup>[45-48]</sup>. Sonnenberg has compared the cost-effectiveness of screening CTC and colonoscopy, and has found that to achieve cost-effectiveness similar to colonoscopy, CTC needs to have a compliance rate that is 15%-20% better than colonoscopy, or cost 54% less. Ladabaum's analysis has found that, if the sensitivities of the two tests are equal, conventional colonoscopy is more cost-effective than CTC unless CTC costs are 40% lower than those of colonoscopy<sup>[45]</sup>. The greater the prevalence of polyps in the screened population, the greater the cost advantage of conventional colonoscopy. A Canadian analysis has

concluded that CTC marginally increases mortality, with projected deaths due to missed adenomas exceeding deaths prevented by avoiding perforation<sup>[46]</sup>. The most recent study has found that CTC would only be cost-effective if its cost is  $< 43\%$  of the cost of colonoscopy<sup>[48]</sup>. However, the cost-effectiveness of CTC may be better if the analysis takes into consideration clinically useful extracolonic findings<sup>[49]</sup>. The use of computer-assisted detection may also improve cost-effectiveness<sup>[50]</sup>. Even though studies at expert centers have reported that only 7.9% of patients screened with CTC needed to be referred for colonoscopy<sup>[51]</sup>, in routine clinical practice, the referral rate may be considerably higher (perhaps as high as 15%-20%). Therefore, further studies are needed to investigate this issue, preferably using real cost data in a cohort of prospectively followed patients.

## FOLLOW-UP STRATEGIES

The proper approach to diminutive polyps ( $\leq 5$  mm in size) seen on CTC, in which the risk of cancer is extremely low<sup>[52]</sup>, is also unclear at this time. Referral of all patients with diminutive polyps for follow-up colonoscopy would dramatically increase the cost of CTC screening. The alternative approach, that is, following the polyp using repeat CTC at shorter intervals, would also be expensive and increase radiation exposure. Several decision analyses have simulated CTC screening with non-reporting of diminutive polyps and have reached conflicting conclusions regarding cost-effectiveness, outcomes and safety<sup>[53-56]</sup>. It has been estimated that up to 33% of screening patients with high-risk neoplastic lesions would be interpreted as normal if American College of Radiology guidelines on CTC reporting were followed (these guidelines recommend that polyps  $< 6$  mm be ignored)<sup>[57,58]</sup>. This is because a significant fraction (almost 7%) of relatively small polyps may harbor advanced neoplastic tissue<sup>[59]</sup>. Furthermore, some surveys have indicated that most patients and physicians favor reporting of diminutive adenomas found during CTC<sup>[60]</sup>. It is also of concern that polyp size or location reported at CTC may not be accurate, when compared with pathological assessment or colonoscopic evaluation<sup>[61,62]</sup>.

## EXTRACOLONIC FINDINGS

Extracolonic abnormalities have been found during CTC in up to 50% of patients<sup>[63-65]</sup>. Although incidental detection of previously unsuspected pathology may benefit some patients, others will be subjected to needless anxiety and testing for what will ultimately turn out to be clinically insignificant lesions or false-positive results. Some studies have suggested that this may have significant cost implications<sup>[66,67]</sup>, while others have not found this to be a problem<sup>[68,69]</sup>.

## CONCLUSION

At present, the exact clinical role of virtual colonoscopy

still awaits determination. Two types of patients for whom CTC is clearly useful are those with incomplete colonoscopy due to colonic tortuosity, and those with obstructive cancer that precludes passage of a colonoscope<sup>[70,71]</sup>, although there is some concern that patients with incomplete colonoscopies might sometimes harbor occult perforation<sup>[37]</sup>. Currently, screening CTC is not covered by Medicare or any other public or private health insurance plan. The only exception is a limited program in Wisconsin; review of data from this program has shown similar detection rates of advanced neoplasia for colonoscopic screening *vs* CTC screening<sup>[51]</sup>. Even though widespread CTC screening is not available today, in the future there may eventually be a role for this technology. As a result of its general acceptance by patients, CTC offers the possibility of increasing the overall prevalence of colon cancer screening. One approach would be to offer CTC as the primary screening modality for all patients, followed by same-day colonoscopy if lesions are found<sup>[72]</sup>; alternatively, a risk-stratified strategy using colonoscopy for high-risk patients and CTC for low-risk patients might be more resource-efficient<sup>[73]</sup>. Currently, there appears to be enough multi-detector CT scanning capability in the United States to handle large-scale screening requirements, if needed<sup>[74,75]</sup>. Of course, a prerequisite for CTC screening programs is adequate training of all radiologists; gastroenterologists can also potentially be trained to read CTC results<sup>[76]</sup>. Although decision analyses have suggested that screening CTC can result in a decrease in colonoscopy volume<sup>[77]</sup>, in practice, this has not been observed because the decrease in the number of primary screening colonoscopies is compensated for by an increase in colonoscopies for positive CTCs<sup>[78]</sup>.

Technological advances in this area will undoubtedly continue, with multi-detector row CT scanners allowing thinner collimation and higher resolution images. Stool-tagging techniques are likely to evolve and may eventually allow for low-preparation or preparationless CTC. Perceptual and fatigue-related reading errors potentially can be minimized with the help of computer-aided detection software<sup>[79,80]</sup>. Further research will define the exact role of this promising technology in our diagnostic armamentarium.

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## Folate and fiber in the prevention of colorectal cancer: Between shadows and the light

Wan Du, Wen-Ying Li, Rong Lu, Jing-Yuan Fang

Wan Du, Wen-Ying Li, Rong Lu, Jing-Yuan Fang, Shanghai Institute of Digestive Disease, Shanghai Jiaotong University School of Medicine Renji Hospital, Shanghai 200001, China  
Author contributions: Du W, Li WY, Lu R and Fang JY compiled the data; Du W and Fang JY designed the editorial; Du W and Fang JY wrote the editorial.

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Correspondence to: Jing-Yuan Fang, Professor, Shanghai Institute of Digestive Disease, Shanghai Jiaotong University School of Medicine Renji Hospital, 145 Shandong Middle Road, Shanghai 200001, China. [jingyuanfang@yahoo.com](mailto:jingyuanfang@yahoo.com)

Telephone: +86-21-63200874 Fax: +86-21-63266027

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**Peer reviewers:** Peter L Lakatos, MD, PhD, Assistant Professor, 1st Department of Medicine, Semmelweis University, Koranyi S 2A, Budapest H1083, Hungary; Won Ho Kim, MD, Professor, Department of Internal Medicine, Yonsei University College of Medicine, 134 Shinchon-dong Seodaemun-ku, Seoul 120-752, South Korea; Dr. Abdul-Wahed Meshikhes, MD, FRCS, Chairman & Consultant Surgeon, Department of Surgery, King Fahad Specialist Hospital, Amir Bin Thabit St, Dammam, 31444, Eastern Province, Saudi Arabia

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### Abstract

Colorectal cancer (CRC) is one of the most common malignancies and causes of cancer deaths throughout the world. Endoscopy has its functional and financial limitations; therefore, chemoprevention might be crucial in reducing the incidence of CRC. Although a number of studies have demonstrated the potential chemopreventive effects of folate (or folic acid), many challenges still remain. The relationship between folate intake and CRC risk is a complex association that might depend on many factors including gender, age, alcohol consumption, and smoking, all of which interfere with folate metabolism. The supplementary dose of fiber, the length of time required to observe the effects, and confounding factors exposed during the trial might also influence these findings. Therefore, more evidence from clinical studies is needed regarding the mechanisms that underlie the actions of bioactive food components in minimizing the risk of CRC.

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**Key words:** Folate; Fiber; Butyrate; Prevention; Colorectal cancer

### INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies and causes of cancer deaths in Europe<sup>[1]</sup> and the United States<sup>[2]</sup>. However, there has been a notable rise in the incidence of CRC in Asia over the last few decades<sup>[3]</sup>. Almost all CRCs develop from normal colonic epithelial cells with an adenomatous polyp as an intermediate step. Current efforts are directed towards detection and removal of adenomatous polyps. Therefore, endoscopy has become a part of routine clinical practice. Unfortunately, the polyp often remains indolent for years and is generally discovered only during routine screening. Even after removal, polyps can still lead to recurrence. Endoscopy has its financial limitations; therefore, chemoprevention might be crucial in reducing the incidence of CRC. In this context, chemoprevention refers to the use of chemical compounds to prevent, inhibit, or reverse carcinogenesis. The focus of chemoprevention in CRC is bioactive food components.

Bioactive food components are constituents in commonly consumed foods or dietary supplements, which result in the promotion of better health. It is clear that

bioactive food components can play an important role and influence cancer outcomes<sup>[4]</sup>, as previously demonstrated by cytochrome p450 inhibitors, inducers of cell cycle arrest and apoptosis, and inhibitors of angiogenesis<sup>[5]</sup>. Bioactive food components involved in CRC chemoprevention include folate, fiber, and short-chain fatty acids (SCFA, e.g. butyrate, which is decomposed product of fiber in large intestine), methionine, bilineurine, vitamin D, and calcium. Epigenetic changes play a very important role in the evolution from normal intestinal epithelium to colon cancer<sup>[6]</sup>. The main chemoprevention drugs for epigenetic changes are folate and butyrate<sup>[6]</sup>. This review discusses the effect of folate and fiber on CRC chemoprevention.

## FOLATE

Folate is a water-soluble B vitamin found abundantly in fresh fruits and leafy green vegetables, and provides one-carbon groups in methylation of DNA<sup>[7]</sup>, and contributes to DNA synthesis and replication as well as epigenetic regulation of gene expression<sup>[8]</sup>. Therefore, folate deficiency might impair these processes and cause chromosomal breaks, as well as deleterious alterations in gene expression. For example, folate depletion has been shown to induce the upregulation of p16<sup>INK4A</sup>, p21<sup>WAF1</sup> and p53 tumor suppressor genes, which are involved in DNA damage signaling, inhibition of cell-cycle progression through checkpoints, and apoptosis<sup>[9]</sup>. Folate deficiency also induces genomic instability and aberrant DNA methylation, which might contribute to colorectal carcinogenesis<sup>[10]</sup>. However, whether folate can prevent the occurrence of CRC should take the following into consideration:

Firstly, several epidemiologic and clinical studies have found a relationship between folate deficiency and CRC risk<sup>[7,8,11-13]</sup>. Low levels of folate in the diet or blood have been shown to be associated with a higher risk of CRC<sup>[14,15]</sup>. In contrast, high intake of dietary folate has been inversely associated with the risk of CRC<sup>[8,14]</sup>. Multiple case-control and observational cohort studies suggest a reduction of 30%-40% in CRC risk for participants with high levels of folate intake compared to those with low levels<sup>[11-13]</sup>. According to some data<sup>[16]</sup>, the risk of CRC decreases 11% for every 400 µg of total folate ingested. It has also been shown to elicit various responses that might be beneficial in reducing the risk of CRC, where these effects might be mediated through increased concentrations of colonic mucosal folate<sup>[17]</sup>.

Secondly, although a protective role against CRC has been suggested for high dietary folate intake, epidemiological evidence has not consistently shown a protective effect of high folate intake against CRC<sup>[8,18,19]</sup>. As such, high folate intake might enhance colorectal tumor recurrence and progression<sup>[20-22]</sup>. Animal studies have suggested that high-dose folic acid might promote colorectal tumorigenesis<sup>[23]</sup>, as there is a very close relationship between increases in the incidence of CRC and the remarkable increase in dietary folate intake and blood

folate levels<sup>[22]</sup>. A large, placebo-controlled multicenter trial has shown that high-dose folate might potentially increase the risk of neoplastic transformation<sup>[24]</sup> and one study has reported a significantly increased risk of CRC in subjects with high plasma folate concentrations<sup>[6]</sup>.

Thirdly, in two large prospective cohorts<sup>[25]</sup>, the Nurses' Health Study and Health Professionals Follow-Up Study, higher prediagnostic levels of plasma folate were not associated with an increased risk of CRC-specific or overall mortality. Moreover, a low folate diet induced genomic uracil misincorporation and hypomethylation in Big Blue Mice and uracil DNA glycosylase deficiency (Ung<sup>-/-</sup>) mice, which is insufficient to promote tumor development<sup>[26]</sup>. A multicenter, randomized, double-blind trial has shown that folate supplementation was found to have no effect on adenoma recurrence [relative risk (RR) = 1.07, 95% CI: 0.85-1.34]<sup>[27]</sup>.

Why did above-mentioned differences emerge? Some scholars have suggested that dietary folate supplementation protection against colonic carcinogenesis might depend on the stage of colorectal carcinogenesis, and would protect against carcinogenesis in normal colorectal tissue, but that folate might enhance pre-existing lesions<sup>[23,24,28]</sup>. Findings from several studies indicate that any relationship between folate intake and CRC risk is a complex association that might depend on many factors, including gender, age, alcohol consumption, and smoking, all of which interfere with folate metabolism (Figure 1).

## Gender

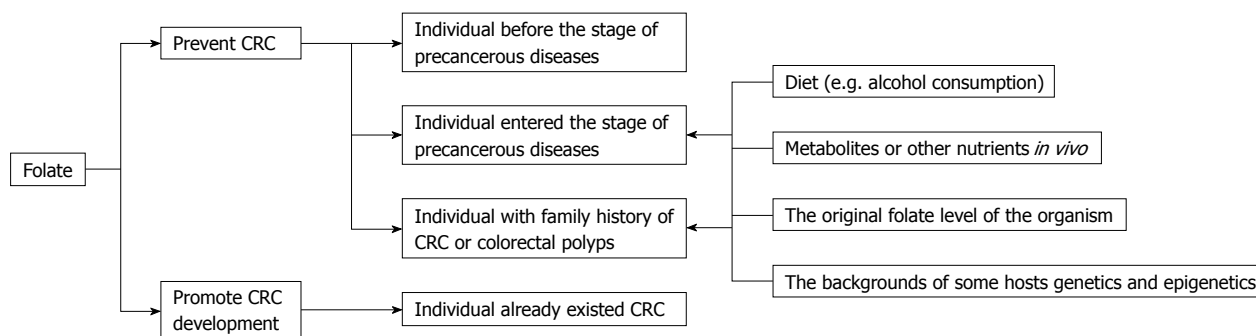
One of the earliest epidemiological papers put forth a hypothesis that men might benefit from folate more than women in colorectal neoplasia. There was a significantly reduced risk for adenomatous polyps in men, but no such association was observed for women<sup>[29]</sup>. Another study, based on the data from the National Health and Nutrition Examination Survey I (NHANES I), revealed that dietary intake of greater than 249 µg/d of folate in men was inversely associated with colon cancer, but not in women<sup>[30]</sup>. However, most investigations with regard to the relationship between colorectal neoplasia and gender revealed that men and women have the same susceptibility<sup>[31,32]</sup>.

## Age

Lower folate intake might have a more pronounced effect on aging colonic mucosa; a folate-depleted diet reduced colonic folate concentrations in older, but not younger rats<sup>[33]</sup>. Age related changes in the metabolism of folate might explain the link between age and CRC risk.

## Alcohol

Alcohol consumption was found to be a risk factor for developing CRC. There might also be an interaction between low folate levels and high alcohol consumption and CRC<sup>[34]</sup>. Folate absorption in the intestine and its availability in the body can be modified by alcohol consumption<sup>[35]</sup>.



**Figure 1** Folate supplementation protection against colorectal cancer (CRC) might depend on the stage of colorectal carcinogenesis and might be influenced by many factors.

### Folate stores

Folate stores might influence tumor responsiveness to chemotherapy. Even under conditions of high systemic folate status, transient localized folate depletion might occur<sup>[36]</sup>, especially in colonic epithelium that demonstrates rapid rates of proliferation<sup>[37]</sup>. Localized inflammation is also known to deplete folate<sup>[38]</sup>. These factors could act together to substantially reduce localized intracellular folate bioavailability.

### Metabolites

Scientists have shown that folate deficiency is significantly more frequently associated with oncogenesis when combined with hyperhomocysteinemia. Patients with folate deficiency associated with hyperhomocysteinemia had 17 times as many carcinogenic lesions as patients with normal homocysteinemia, regardless of the folate status of the disease<sup>[39]</sup>. Inflammatory bowel disease patients with folate deficiency and hyperhomocysteinemia might be associated with increased colorectal carcinogenesis<sup>[39]</sup>. A study in the general population has shown that low folate status with hyperhomocysteinemia increased the risk of recurrence of adenoma<sup>[40]</sup>.

Apart from the above-mentioned factors, distinctions between dietary folate levels and chemical drug effects, a patient's folate levels before treatment, the occurrence of folate supplementation in patients and some genetic and epigenetic discrepancies of organisms might also be causes of variances in these findings.

In summary, we think that folate deficiency makes the body inclined to generate DNA repair defects and heterotypic cells due to abnormal methylation, which develops in the early stage of abnormal cells. Folate supplementation might have preventive effects on the individual before the stage of precancerous diseases, but for those who have entered this stage (such as adenomatous polyposis), the effect is still difficult to determine. Therefore, folate should be included in the discussion of those factors that might promote CRC tumor development. On the other hand, even in high-risk groups with a family history of CRC or colorectal polyps (for example, adenomatous polyposis and Peutz-Jaegher hamartoma syndrome), folate is supplied to prevent CRC. Precancerous diseases are still influenced by a number of factors,

such as diet, metabolites or other nutrients *in vivo* (such as choline and vitamin B12), folate levels of the organism, and the predisposed backgrounds of some host's genetics and epigenetics.

### FIBER

The protective effects of bioactive food components against the development of CRC are due to not only their folate content, but also their fiber content. Fiber has been investigated as a CRC chemopreventive agent in many clinical trials. As such, it is speculated that increased dietary fiber intake can reduce the risk of CRC through a variety of mechanisms<sup>[6,41-43]</sup>. These include dilution or adsorption of fecal potential carcinogens, which potentially inhibit chemically-induced carcinogenesis; decreasing the exposure period of colonic epithelial cells to carcinogens, co-carcinogens, or promoters through reduction of contact time between intraluminal contents and the colonic mucosa; alterations in bile acid metabolism, by binding potential carcinogens like secondary bile acids; increasing the production of SCFA; and promoting a favorable colonic microflora by modifying its metabolic activities and composition.

Studies on the protective role of dietary fiber and its main end product of intestinal microbial fermentation, butyrate, against CRC remain controversial. Butyrate, an SCFA, is an important energy source for intestinal epithelial cells and plays a role in the maintenance of colonic homeostasis<sup>[44]</sup>. It induces potent effects, such as inhibition of inflammation and carcinogenesis of colonic mucosa, reinforcing various components of the colonic defense barrier, decreasing oxidative stress, as well as promotion of cancer cell growth arrest, differentiation, and apoptosis<sup>[45]</sup>.

Several studies have linked higher fiber intake to a reduced risk for CRC<sup>[46,47]</sup>. A multiethnic cohort study<sup>[48]</sup> showed that dietary fiber was inversely associated with a CRC risk after adjustment for age and ethnicity in men (RR = 0.49, 95% CI: 0.41-0.60, highest vs lowest quintile) and women (RR = 0.75, 95% CI: 0.61-0.92). After further adjustment for lifestyle and dietary factors, the inverse association remained significant in men (RR = 0.62, 95% CI: 0.48-0.79), but not in women (RR =

0.88, 95% CI: 0.67-1.14). These authors presumed that this phenomenon is related to women's hormone use. The data from the Japan Collaborative Cohort Study supported the potential protective effects of dietary fiber against CRC, mainly against colon cancer<sup>[49]</sup>. Other studies showed that high fiber and calcium intakes were more markedly associated with a lower risk of CRC in patients carrying the D1822V APC allele [odds ratio (OR): 0.50, 95% CI: 0.27-0.94 for fiber; OR: 0.51, 95% CI: 0.28-0.93 for calcium] than in those without this allele<sup>[50]</sup>.

The role of fiber in the prevention of colorectal diseases remains controversial. There is some evidence that fiber is not effective for primary prevention of CRC<sup>[51,52]</sup>. In a large prospective cohort study, total dietary fiber intake was not associated with CRC risk either<sup>[53]</sup>. Another continued follow-up study failed to show any effect of a low-fat (20% of total energy), high-fiber (18 g/1000 kcal), high-fruit and -vegetable (3.5 servings/1000 kcal) eating pattern on adenoma recurrence even after 8 years of follow-up<sup>[54]</sup>.

When analyzing the differences in the above-mentioned studies, the following aspects should be addressed: (1) Patients with different types of CRCs might be associated with the histone acetylation state of the promoters of tumor-related genes (especially apoptosis-related genes); (2) The findings are different between *in vitro* and *in vivo* experiments, possibly due to the direct administration of butyrate to *in vitro* cells compared to preventing CRC or adenoma with a high-fiber diet *in vivo*. If we use sodium butyrate interference directly in patients, we might discover different effects; (3) The timing of the different interventions will produce different results: in the early stage of DNA damage, butyrate supplementation can induce chromosomes to remain in the open stage, *via* inhibition of histone deacetyltransferase, where it is easy to repair DNA damage. Therefore, the best opportunity for butyrate prevention of CRC might be in the early stages of polyp formation, rather than during the transformation stage of adenomatoid polypus to adenocarcinoma; (4) The status of DNA mismatch repair might affect the results of butyrate prevention of CRC<sup>[55]</sup>; (5) The dose of sodium butyrate suitable for research *in vitro* and *in vivo* might be different<sup>[56]</sup>; (6) The concentration of SCFA formed in the intestinal tract is variable; (7) The complexity of dietary interactions might have an effect; and (8) The duration of the study might also influence the protective role of fiber against CRC<sup>[44]</sup>. Although the effects of increased dietary fiber intake on reducing the risk of CRC has not been unified in studies conducted to date, more studies are needed to increase knowledge in this area.

## CONCLUSION

Although treatment continues to improve, CRC remains a major cause of death throughout the world. A number of studies have demonstrated the potential chemopreventive effect of bioactive food components, but many challenges

still remain. For example, there is very little evidence from clinical studies, such that most of the available information is based on epidemiological and experimental data. The value of folate and fiber has not yet been confirmed in epidemiological and clinical studies; therefore, it cannot be accepted as standard medical practice and cannot replace endoscopic screening. Therefore, we should lucubrate the timing of medication, drug dose, interaction mechanisms, and molecular regulatory network of folate and fiber to further advance the field of CRC chemoprevention research. We should then be able to identify compounds and/or molecules using high-throughput techniques, and validate them using the large number of clinical cases resources. Thus we will obtain new individual clinical application programs of CRC chemoprevention to combat this major health problem and minimize the risk of CRC. Future studies and clinical trials must be conducted in a variety of study settings and among different population groups, which will help to elucidate the role of bioactive food components in CRC.

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Shahid A Khan, BSc (Hons), MBBS, MRCP, PhD, Series Editor

## Surgical treatment for liver cancer

Nicole C Tsim, Adam E Frampton, Nagy A Habib, Long R Jiao

Nicole C Tsim, Adam E Frampton, Nagy A Habib, Long R Jiao, Department of Hepatopancreatobiliary Surgery, Division of Surgery, Hammersmith Hospital campus, Imperial College London, Du Cane Road, London W12 0HS, United Kingdom  
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Correspondence to: Long R Jiao, MD, FRCS, Reader and Consultant HPB Surgeon, Department of Hepatopancreatobiliary Surgery, Division of Surgery, Hammersmith Hospital campus, Imperial College London, Du Cane Road, London W12 0HS, United Kingdom. [l.jiao@imperial.ac.uk](mailto:l.jiao@imperial.ac.uk)

Telephone: +44-20-83833937 Fax: +44-20-83833212

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### INTRODUCTION

Over the past 25 years, hepatic resection has evolved from a high risk, resource intensive procedure with limited application, to a safe and commonly performed operation with broad indications. We have seen a dramatic improvement in perioperative outcome, including reductions in mortality, blood loss, transfusion rates, and hospital stay. These improved perioperative results are largely responsible for the emergence of hepatic resection as a viable and effective treatment option for selected patients with liver tumors. Continued advances in imaging technology, along with a heightened awareness of the clinical and tumor-related variables that dictate outcome, have allowed better preoperative assessment of disease extent and improved patient selection. Advances in other areas, such as minimally invasive and ablative techniques, have increased the treatment options and have had some impact on the approach to patients with liver tumors; however resection remains the most effective therapy. The current status of partial hepatectomy is not the result of any randomised trial demonstrating greater efficacy over another therapy and recurrence rates remain high. Further improvements in survival will require more effective systemic agents and as better adjuvant and neo-adjuvant therapies emerge, the results of resection are likely to improve. The indications for its application will then perhaps extend to patients currently considered to have unresectable disease.

### PRIMARY HEPATOCELLULAR CARCINOMA

Worldwide, primary hepatocellular carcinoma (HCC)

### Abstract

Primary liver cancer is amongst the commonest tumors worldwide, particularly in parts of the developing world, and is increasing in incidence. Over the past three decades, surgical hepatic resection has evolved from a high risk, resource intensive procedure with limited application, to a safe and commonly performed operation with a range of indications. This article reviews the approach to surgical resection for malignancies such as hepatocellular cancer, metastatic liver deposits and neuroendocrine tumors. Survival data after resection is also reviewed, as well as indications for curative resection.

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**Key words:** Liver cancer; Surgical resection; Indications; Hepatocellular carcinoma; Colorectal liver metastases; Neuroendocrine tumors; Non-colorectal non-neuroendocrine

**Peer reviewer:** Dr. Fabio Marra, Dipartimento di Medicina Interna, University of Florence, Viale Morgagni, 85, I-50134 Florence, Italy

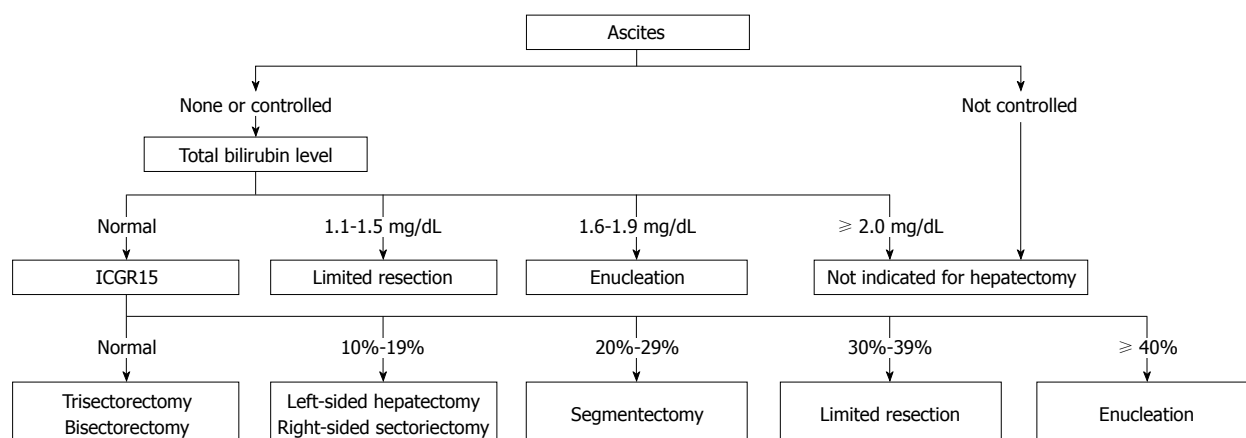


Figure 1 A decision algorithm for the selection of hepatic resection procedure<sup>[8]</sup>.

is among the most frequently encountered solid organ tumors, responsible for approximately 250 000 new cases annually. Previously considered uncommon in western countries, the incidence and mortality related to HCC is increasing, due to the increasing incidence of hepatitis C virus infection. The treatment of HCC, unlike other hepatic malignancies, is often complicated by the coexistence of chronic liver disease and cirrhosis, the presence of which frequently limit treatment options. The only curative treatments are liver resection (LR) or liver transplantation (LT). Improvements in operative technique and postoperative care now mean that a 10% mortality rate for the resection of cirrhotic livers, with up to 30% to 50% 5 years survival rates are to be expected<sup>[1]</sup>.

### Indications and decision making for surgical resection

When HCC arises in non-cirrhotic liver, it is often diagnosed when the tumor becomes large and symptomatic. In the absence of diffuse bilobar disease, or extra-hepatic metastases, aggressive surgical management is indicated. In this situation, removal of the tumor can be considered as these patients are usually in good general condition and surgical resection tends to involve only tumor mass rather than functional hepatic parenchyma. Although their survival rates are lower than patients with small uninodular tumors, resection is safe and they can expect a 5-year survival rate of up to 39%<sup>[2,3]</sup>.

Most HCCs however occur in patients with chronic liver disease or cirrhosis. This often results in important changes in portal haemodynamics and a reduction in the functional liver parenchyma. The Child-Pugh<sup>[4]</sup> classification is the most useful tool in evaluating cirrhotic patients with impaired liver function. Other sophisticated techniques for determining hepatic reserve, for example the plasma retention rate of indocyanine green at 15 min (ICG15)<sup>[5]</sup>, preoperative portal pressure assessment<sup>[6]</sup>, and 3-dimensional-CT reconstruction of the liver, can be used in deciding whether to proceed with surgery<sup>[7]</sup>.

Resection of HCC is only considered in Child-Pugh A patients. However, Child-Pugh is only used in cirrhosis and liver damage at resection can vary widely from periportal

fibrosis to extensive fibrosis/cirrhosis. Therefore the operating surgeon must modify their technique using as many preoperative investigation results as possible before proceeding. A decision algorithm combining the presence or absence of ascites, total serum bilirubin levels and ICG retention at 15 min has been proposed by Makuuchi *et al*<sup>[8]</sup> (Figure 1). Patients with ICG15s of 20%-30% and more than 30% can only be subjected to one segmentectomy or limited resection, respectively<sup>[9]</sup>.

Other modalities like hepatic venous pressure gradient have also been proposed. Patients with a gradient below 10 mmHg are eligible for resection and those above 10 mmHg are referred for non-operative management. Factors such as tumor size, the depth and distance of the tumor from the major vessels or the presence of intra-hepatic metastases should also be taken into consideration. Patients with large tumors require careful selection. In clinical practice all patients benefit most from multidisciplinary discussion.

LR for HCC in a cirrhotic liver is contraindicated in the presence of severe liver functional impairment such as ascites, jaundice, Child-Pugh B and C, and liver atrophy. In these cases, there is an increased risk of liver decompensation or failure in the postoperative period. Other factors that preclude resection are the presence of portal vein thrombosis (reflecting extensive disease)<sup>[10]</sup>, lymph node metastases, extra-hepatic localizations and intra-hepatic diffuse disease. All these situations render any treatment palliative. The use of laparoscopic ultrasound as a preoperative assessment tool has further reduced LR rates by as much as 63%<sup>[11]</sup>. Table 1 summarizes the current indications for curative resection of HCC.

### Results after surgical resection

The choice of treatment modality [e.g. hepatic resection, LT, or radiofrequency ablation (RFA)] can influence patient survival and this in turn is governed by the size and distribution of lesions. Improved patient selection leads to optimal survival rates for each subgroup of patients with HCC. The available literature is broadly divided into small ( $\leq 5$  cm) or 3 or less tumors ( $\leq 3$  cm)



**Table 1** Indication for curative resection of HCC

Early stage HCC
Satisfactory liver function tests
Single nodule $\leq 5$ cm; 3 nodules $\leq 3$ cm
Okuda <sup>[59]</sup> stage 1 or 2
Child-Pugh <sup>[4]</sup> A or B
WHO Performance Score 0
No portal hypertension
No portal thrombosis
Normal bilirubin

HCC: Hepatocellular carcinoma.

and large ( $\geq 5$  cm) tumors, all with differing survival rates. Large multicentre series involving Asia, Europe, USA and France<sup>[3,12,13]</sup>, have demonstrated 3-, 5- and 10-year hepatic resection survival rates of 38%-64%, 36%-41%, and 14%, respectively. Individual centres have published series of 5-year survival rates of 40%-50%<sup>[1,14-19]</sup>, and 10-year survival rates of 8%-17%<sup>[16,20,21]</sup>. Table 2 shows the survival data for patients who underwent hepatic resection for HCC from large published series. When resecting early HCC, Poon *et al.*<sup>[22]</sup> achieved a 70% 5-year survival rate and a 60% 5-year recurrence rate. Furthermore, when this cohort of patients experienced recurrence, 79% of them were eligible for salvage transplantation. It is important to note the perioperative mortality rates in hepatectomy patients range between 0.9%-6.4% and recurrence rates between 30%-55% (intra-hepatic recurrence being the most likely site)<sup>[3,13-16,20,23]</sup>.

### Disease recurrence

Microscopic vascular invasion, fibrosis and underlying cirrhosis have been consistently shown to be independent risk factors for decreased overall long-term survival in multivariate analysis<sup>[3,13-16,21,24,25]</sup>. Multiplicity and size of tumors ( $> 5$  cm) are also negative predictors of prognosis. Margin positivity is associated with local recurrence<sup>[1,26]</sup>, however the absolute width of the margin is less important<sup>[27,28]</sup>. Poon *et al.*<sup>[22]</sup> also demonstrated in their paper that recurrence is most likely disseminated by both the portal venous system for intra-hepatic metastases and multicentric carcinogenesis for multi-segmental metastases, while proportionally less recurrence occurs locally. Therefore the authors propose that the presence of venous invasion and satellite nodules, factors that are incorporated in the pathological tumor-node-metastasis staging system, are more important predictors of recurrence irrespective of the margin status<sup>[29]</sup>. A surgeon's technique and the extent of tissue dissection has been shown to indirectly influence survival, as blood transfusion perioperatively predicts poor prognosis in multivariate analysis<sup>[18,24,29]</sup>. With regards to percutaneous RFA, the preliminary results from a recent nationwide study involving 7185 patients from multiple centres in Japan showed that surgical resection was a significant negative factor for recurrence as compared with RFA, however there was no difference in the overall survival rates<sup>[30]</sup>.

### LT

LT can have the benefit of being curative and treating any underlying cirrhosis, however there are many contraindications to transplantation, as well as a worldwide shortage of suitable allografts. Comparing orthotopic LT to LR in cirrhotic patients with small ( $\leq 3$  cm) uninodular or binodular metastasis, the 3-year disease-free survival rate without recurrence is significantly better in the transplant group (83% *vs* 18%)<sup>[31]</sup>. Mazzaferro *et al.*<sup>[32]</sup> first published their results in 1996 where the 4-year survival rates post-transplant in selected patients with isolated small tumors ( $\leq 5$  cm) or two to three nodules  $\leq 3$  cm were up to 75%. Since their landmark publication, LT is universally accepted as the first line treatment for patients who fulfil the Milan criteria.

The use of LR in the presence of cirrhosis is limited because of significant morbidity, which could be related to the laparotomy itself<sup>[33]</sup>, and high recurrence rates. However, LT is also limited due to the shortage of organ donors<sup>[34]</sup> and the resultant risk of tumor progression with increased waiting times<sup>[33]</sup>. Controversy still remains over the treatment of patients with preserved liver function who could tolerate LT or resection. Therefore the use of primary resection followed by salvage transplantation (if required) for intrahepatic HCC recurrence has been proposed as an acceptable approach in such patients<sup>[34,35]</sup>. Some authors recommend the use of other neoadjuvant antitumoral procedures during the waiting time to LT, such as transarterial chemoembolization, percutaneous RFA or percutaneous ethanol injection. Whilst effective, they have the disadvantage of not being able to perform a full pathological examination of a surgical specimen, which allows assessment of prognostic factors of HCC (i.e. microvascular invasion, satellite nodules, tumor differentiation and molecular markers of recurrence), thereby further helping the transplant decision-making process<sup>[33]</sup>.

## SECONDARY TUMORS

### Colorectal liver metastases (CLM)

Stage IV colorectal carcinoma is by far the most common indication for hepatic resection in Western countries. However no single consensus exists on the indications for surgical resection. Surgery is the treatment of choice in patients with colorectal cancer, but over half of all patients develop liver metastases. It has been postulated that because haematogenous spread usually occurs in a stepwise fashion, initially to the liver, with subsequent intra-hepatic spread *via* the portal vein and further spread to the systemic circulation, surgical resection of isolated hepatic metastases from colorectal cancer may be curative. The natural history of colorectal cancer is variable, with a median survival without treatment of only 8 mo. Patients with isolated metastases have a better prognosis than those with more extensive metastatic disease<sup>[36]</sup>. However few patients with liver only metastases survive for 5 years. Around 20%-30% of these are potentially

Table 2 Survival data for patients who underwent hepatic resection for HCC and colorectal liver metastases

	Number of patients	Morbidity (%)	Mortality (%)	Recurrence (%)	DFS 5 yr (%)	Survival (%)			
						1 yr	3 yr	5 yr	10 yr
Hepatic resection for HCC									
Ng <i>et al</i> <sup>[3]</sup>	784	23	2	34	40	88	76	58	NA
Esnaola <i>et al</i> <sup>[12]</sup>	586	NA	4	NA	NA	NA	NA	36	14
Wayne <i>et al</i> <sup>[13]</sup>	249	NA	6	NA	NA	NA	NA	41	NA
Shimozawa <i>et al</i> <sup>[16]</sup>	135	25	2	82	30	NA	73	55	18
Ercolani <i>et al</i> <sup>[17]</sup>	224	36	NA	42	27	83	63	43	NA
Borie <i>et al</i> <sup>[25]</sup>	107	NA	6	NA	NA	NA	NA	33	NA
Zhang <i>et al</i> <sup>[24]</sup>	412	NA	3	71	26	80	53	30	NA
Hepatic resection for colorectal liver metastases									
Gomez <i>et al</i> <sup>[50]</sup>	501	NA	4	87	13.4	NA	NA	62	NA
Arru <i>et al</i> <sup>[45]</sup>	297	17	1	NA	NA	91	51	28	17
Aoki <i>et al</i> <sup>[44]</sup>	187	NA	NA	74	19	NA	49	30	22
Fong <i>et al</i> <sup>[26]</sup>	1001	NA	3	NA	NA	89	57	37	22
Kato <i>et al</i> <sup>[60]</sup>	585	NA	NA	NA	NA	NA	NA	33	NA
Mala <i>et al</i> <sup>[61]</sup>	137	NA	3	NA	NA	NA	NA	29	NA
Rees <i>et al</i> <sup>[42]</sup>	107	3	1	NA	NA	94	56	37	NA

DFS: Disease free survival; NA: Not available.

resectable and the selection criteria for surgery are constantly changing<sup>[37,38]</sup>. Chemotherapy is palliative when used alone, but can prolong survival in inoperable disease. Used in combination with surgery it may prolong time to recurrence after resection or downsize to resectability in patients previously judged inoperable<sup>[39]</sup>. With regards to percutaneous RFA, a recent systematic review by Stang *et al*<sup>[40]</sup> found that RFA of unresectable CLM is a useful adjunct to surgery and/or chemotherapy and can prolong time without toxicity and survival.

Surgical resection of hepatic metastases can be undertaken safely in the majority of patients and the median postoperative 30 d mortality is 2.8%<sup>[26]</sup>. The most common reported causes of postoperative death include hepatic failure, postoperative haemorrhage and sepsis.

The outcome of resection of colorectal liver metastasis is encouraging, with postoperative mortality in large series ranging from 0.2%-3.5%<sup>[26,41-45]</sup>. The 1-year overall survival rate is 89%-97%<sup>[26,42,46]</sup>, while the 5-year survival rate ranges between 15%-50%<sup>[26,41,45,47-49]</sup>. Five years disease free survival rates are on average 19% in radically resected patients. Some studies with longer follow-up periods report a 10-year overall survival rate of 17%-33%<sup>[14,44,45]</sup>. The rate of complication in individual studies is more variable, ranging from 3%-17%<sup>[42,45]</sup>. The recurrence rate with 5-year period of follow-up is consistent around 62%-74%<sup>[44,50]</sup>.

The factors which are predictive of poor prognosis include poor tumor differentiation<sup>[44,45,50]</sup>, increasing size and number of metastases<sup>[26,42,44,50]</sup>, Dukes' staging, presence of extra-hepatic metastasis (other than resectable pulmonary metastasis), elevated carcinoembryonic antigen (CEA) levels and positive nodal status. Fong *et al*<sup>[26]</sup> summarised the above with the Clinical Risk Score which includes 5 risk factors (size of metastasis > 5 cm, extra-hepatic metastasis, disease-free interval between primary tumor to diagnosis of metastasis, multiple metastasis

and CEA level greater than 200 ng/mL). A score of 5 is predictive of poor prognosis, whilst patients with 0-2 are more likely to have favourable outcomes. Gomez *et al*<sup>[50]</sup> also recently demonstrated in their paper that increased inflammatory markers and number of blood transfusions perioperatively are related to the early development of recurrence. Table 2 shows the survival data for patients who underwent hepatic resection for CLM from published large series.

### Neuroendocrine tumors (NET)

Cytoreductive therapy is effective in the management of metastatic NET to the liver, independent of their functioning status<sup>[51]</sup>. In functioning tumors, clinical endocrinopathies are relieved in most patients and this response usually lasts for several months. Major morbidity and mortality are not greater than the average complication rate for resection for non-neuroendocrine metastatic tumors at major centres; therefore surgical outcomes appear to justify operative intervention<sup>[51]</sup>. Patients whose primary tumor can be controlled, whose metastases outside the liver are limited, and who have a good performance status are candidates for resection. Directed anatomic and non-anatomic hepatic resections and RFA therapy can effectively reduce the amount of active disease, thereby improving hormone control and patient survival, with very low morbidity and mortality in comparison to other tumor types<sup>[51]</sup>. A full symptomatic response in up to 90% patients with a median overall survival of 48 mo adds many months of symptom-free survival to the lives of most patients. In many patients undergoing a major hepatic resection, concurrent resection of the primary tumor is also performed<sup>[51]</sup>. Resection in selected patients is not more complicated or risky than resection for other metastatic tumors. In addition, endocrinopathies do not increase anaesthetic or operative risk in selected populations. The best post-operative

results are the product of managing these patients over time, becoming familiar with their clinical syndromes and ensuring the early detection of both local recurrence and the development of resistant disease.

After complete removal of the primary tumor, LT seems to be very attractive as a means of eradicating metastatic NET. Unfortunately, there is very sparse evidence for any benefit of LT over LR. Current indications for LT include those with unresectable disease, no extrahepatic or resectable extrahepatic metastatic spread, progressive hepatic metastases, refractory symptoms to medical therapy or interventional procedures, and deposits exhibiting Ki-67 levels  $< 10\%$ <sup>[52,53]</sup>. It has also been suggested that LT should be reserved for patients with non-carcinoid (i.e. non-serotonin secreting) tumors, as the overall survival may be better than LR alone. In a recent article by Frilling *et al.*<sup>[53]</sup>, the 5-year tumor free survival was 48.3% amongst the 16 Type III (disseminated metastatic spread) patients that they transplanted. They concluded that LT for metastatic NET achieves excellent long-term palliation for highly selected patients<sup>[54]</sup>. Current methods to detect the spread of NET disease that were not readily available in the past, such as magnetic resonance imaging (MRI) and indium-111 pentetreotide (Octreoscan), may decrease the application of LT and allow for a better selection of candidates. The option of LT is still open for improvement and is dependent on organ availability and better staging of the disease.

Metastases from NET are hypervascular thus favouring the application of MRI as the single imaging method. MRI not only evaluates the location and characteristics of the lesions, but also determines their relationship with major vessels and bile ducts. Spiral CT scan has also been used extensively in the past with acceptable results. Indium-111 pentetreotide functions on the basis of somatostatin receptors present in these tumors, but its use has not been established definitely in the work-up of these patients. The best use of indium-111 pentetreotide is in the evaluation of disease beyond the primary and liver locations, for example to exclude bone metastases. Its use therefore will likely affect the preoperative work-up of candidates for operative management.

In general, surgery is appropriate for patients with metastatic NET for the following two reasons: (1) many of them still have the primary tumor in place and resection should be undertaken to avoid acute complications and (2) the addition of adjunctive ablative therapies to surgical resection accomplishes the control of  $\geq 90\%$  of the bulk of the tumor. It is important to note that even when complete resections are performed the recurrence rates for metastatic NET are extremely high (76% *vs* 91% for incomplete resection at 5 years)<sup>[55]</sup>; overall 5-year recurrence rates are up to 88%<sup>[51]</sup>. In practical terms, patients with metastatic NET are seldom cured. The best hope physicians can offer these patients is an extended survival period with minimal endocrine symptoms and decreased requirements of somatostatin analogues<sup>[51]</sup>.

### Non-neuroendocrine, non-colorectal liver metastases (NCNN)

The role of metastasectomy for colorectal and neuroendocrine liver secondaries is well established. Significant palliation and survival have been reported after aggressive surgical resection. The indication for the surgical resection of liver secondaries from NCNN tumors is less well defined. In the past, patients with metastatic liver disease were not considered curable and their life expectancy was limited. However, progress in chemotherapy has spurred the development of surgical strategies to cope with patients presenting with liver secondaries from other primary tumors. Diversity of tumor types and a wide variation in available adjuvant treatment schedules makes it difficult to draw conclusions from the published data, but LRs have been performed for metastatic spread from gastrointestinal stromal tumor, renal, lung, thyroid, parotid haemangiopericytoma, ovarian, cervical, Ampulla of Vater, pancreatic and melanoma primaries to name a few. Survival is directly related to the nature of the primary tumor. Reports to date suggest no survival advantage in resection of liver metastases from oesophageal, stomach, small intestine or pancreas. Indeed, 3 and 5 years survival rates for resected metastatic breast tumors are 53.9% and 24.6%, genitourinary tumors 50.4% and 37.8%, and leiomyosarcoma 63% and 36%, respectively<sup>[56-58]</sup>.

### CONCLUSION

Surgical resection is the mainstay of treatment for primary and secondary liver tumors. With the recent increased use of newer surgical techniques, for example laparoscopic LR using RFA for the transection of the liver parenchyma, morbidity and mortality rates from this surgery have improved. Patients with HCC meeting the local criteria for transplantation should be considered for this. Alternatively, if LT is not an easily accessible option or the patient is ineligible for transplantation, then hepatic resection and/or other antitumor treatments should be considered. The use of cytoreductive surgery in metastatic NET may successfully control symptoms and prolong survival. For patients with CLM, hepatic resection remains the best treatment option. The results for the resection of NCNN metastases are not as encouraging and are dependent on the tumor of origin.

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## Difficult treatment decisions in autoimmune hepatitis

Albert J Czaja

Albert J Czaja, Division of Gastroenterology and Hepatology, Mayo Clinic College of Medicine, 200 First Street S.W., Rochester, MN 55905, United States

Author contributions: Czaja AJ researched the topic, wrote the entire article, provided the references and designed the tables.

Correspondence to: Albert J Czaja, MD, Division of Gastroenterology and Hepatology, Mayo Clinic College of Medicine, 200 First Street S.W., Rochester, MN 55905, United States. [czaja.albert@mayo.edu](mailto:czaja.albert@mayo.edu)

Telephone: +1-507-2848118 Fax: +1-507-2840538

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### Abstract

Treatment decisions in autoimmune hepatitis are complicated by the diversity of its clinical presentations, uncertainties about its natural history, evolving opinions regarding treatment end points, varied nature of refractory disease, and plethora of alternative immunosuppressive agents. The goals of this article are to review the difficult treatment decisions and to provide the bases for making sound therapeutic judgments. The English literature on the treatment problems in autoimmune hepatitis were identified by Medline search up to October 2009 and 32 years of personal experience. Autoimmune hepatitis may have an acute severe presentation, mild inflammatory activity, lack autoantibodies, exhibit atypical histological changes (centrilobular zone 3 necrosis or bile duct injury), or have variant features reminiscent of another disease (overlap syndrome). Corticosteroid therapy must be instituted early, applied despite the absence of symptoms, or modified in an individualized fashion. Pursuit of normal liver tests and tissue is the ideal treatment end point, but this objective must be tempered against the risk of side effects. Relapse after treatment withdrawal requires long-term maintenance therapy, preferably with azathioprine. Treatment failure or an incomplete response warrants salvage therapy that can include conventional medications in modified dose or empirical

therapies with calcineurin inhibitors or mycophenolate mofetil. Liver transplantation supersedes empirical drug therapy in decompensated patients. Elderly and pregnant patients warrant treatment modifications. Difficult treatment decisions in autoimmune hepatitis can be simplified by recognizing its diverse manifestations and individualizing treatment, pursuing realistic goals, applying appropriate salvage regimens, and identifying problematic patients early.

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**Key words:** Autoimmune hepatitis; Fulminant hepatitis; Salvage therapy; Treatment end points

**Peer reviewer:** Tom H Karlsen, MD, Institute of Immunology, Rikshospitalet University Hospital, N-0027 Oslo, Norway

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### INTRODUCTION

Corticosteroid therapy is established as an effective treatment for autoimmune hepatitis<sup>[1-3]</sup>. It induces clinical, laboratory and histological remission in 80% of patients within 3 years<sup>[2,4]</sup>; the 10- and 20-year life expectancies of treated patients exceed 80%<sup>[5-7]</sup>; hepatic fibrosis is reduced or prevented in 79%<sup>[8,9]</sup>; and variceal hemorrhage, death from hepatic failure, and deteriorations warranting liver transplantation occur in less than 5%<sup>[10,11]</sup>. These successes are tempered by the development of severe treatment-related side effects in 13%<sup>[12,13]</sup>, treatment failure in 9%<sup>[14]</sup>, incomplete response in 13%<sup>[15]</sup>, and relapse after drug withdrawal in 50%-86%<sup>[2,4,16-18]</sup>. Efforts are ongoing to improve results by refining current treatment strategies<sup>[19]</sup> and by introducing different pharmacological agents, such as cyclosporine<sup>[20]</sup>, tacrolimus<sup>[21,22]</sup>, mycophenolate mofetil<sup>[23,24]</sup> and budesonide<sup>[25,26]</sup>. The benefits

from these efforts have not been fully realized, and the management algorithm is still in flux.

Treatment decisions in autoimmune hepatitis are complicated by the diversity of clinical presentations associated with the disease, uncertainties about the natural history of asymptomatic mild disease, evolving recommendations regarding treatment end points, varied nature of individuals refractory to or intolerant of the conventional therapy, and plethora of alternative immunosuppressive agents<sup>[27-30]</sup>. Diagnostic and therapeutic guidelines have been promulgated to codify the recognition and treatment of autoimmune hepatitis, but clinical judgment remains the essence of successful therapy<sup>[31,32]</sup>. Decisions to start or withdraw medication, manage a sluggish or absent response, and institute unfamiliar empirical therapy in problematic patients are difficult because they are highly individualized and not amenable to rigorous study.

In this review, the difficult treatment decisions in autoimmune hepatitis are described and the bases for making a sound judgment are provided. Treatment decisions can be guided but not codified, and every management strategy must be directed by the status of the individual patient.

## DECISION TO TREAT ACUTE SEVERE (FULMINANT) HEPATITIS WITH CORTICOSTEROIDS

Autoimmune hepatitis can have an acute severe (fulminant) presentation<sup>[33-36]</sup>, or a previously indolent chronic disease can exacerbate spontaneously and appear acute<sup>[37]</sup>. The diagnosis can be unsuspected if this propensity is not realized. Furthermore, the presence of centrilobular zone 3 necrosis on histological examination can suggest an acute viral or toxic injury<sup>[38-42]</sup>. The centrilobular zone 3 pattern can transform to the classical pattern of interface hepatitis as the disease evolves<sup>[39]</sup>, and its presence early in the disease should not delay the diagnosis or therapy.

The key to recognizing acute severe autoimmune hepatitis is to remember it in the differential diagnosis and to make the designation after viral, drug-induced, toxic and metabolic disorders have been systematically excluded<sup>[31,43]</sup>. The diagnosis may include atypical histological findings (centrilobular zone 3 necrosis) or absent classical features (autoantibodies or hypergammaglobulinemia), but it is justified by the completeness of the exclusion effort<sup>[44,45]</sup>.

Autoantibodies and hypergammaglobulinemia, especially increased serum IgG level, support the diagnosis of autoimmune hepatitis, but they are neither specific nor required for the diagnosis<sup>[31,45,46]</sup>. Seronegative patients can respond well to corticosteroid treatment, and those with severe presentations should not be denied this potential benefit because of their non-classical phenotype<sup>[47-50]</sup>. Confidence in the diagnosis can be enhanced

**Table 1** Conventional corticosteroid treatment regimens for autoimmune hepatitis<sup>[19,54,55]</sup>

Schedule	Monotherapy	Combination therapy	
	Prednisone only <sup>1</sup> (mg/d)	Prednisone <sup>1</sup> (mg/d)	Azathioprine (mg/d)
Induction period			
Week 1	60	30	50
Week 2	40	20	50
Week 3	30	15	50
Week 4	30	15	50
Maintenance period			
Fixed doses until end point	20	10	50
Conditions that favor each regimen	Cytopenia (severe) Absent thiopurine methyltransferase activity Pregnancy Malignancy (active) Short trial ( $\leq 6$ mo) Acute severe onset	Elderly/postmenopausal state Osteoporosis Brittle diabetes Obesity Acne Emotional instability/psychosis Hypertension Prolonged therapy ( $\geq 6$ mo)	

<sup>1</sup>Prednisolone can be used in place of prednisone in equivalent doses.

by applying the comprehensive scoring system developed by the International Autoimmune Hepatitis Group (IAIHG)<sup>[31]</sup>. Atypical or absent classical features can be assessed in the context of other findings that may have sufficient strength to carry the diagnosis<sup>[46]</sup>.

Corticosteroid therapy is effective in 36%-100% of patients with acute severe (fulminant) presentations<sup>[35,51,52]</sup>, and this range of response may reflect in part the timeliness of treatment<sup>[53]</sup> (Table 1). The response to corticosteroid therapy should be evident quickly<sup>[56,57]</sup>, and the failure of any laboratory test of liver inflammation to improve within 2 wk in a patient with acute severe disease is a justification for considering liver transplantation<sup>[53,56]</sup> (Table 2).

There are no clinical or laboratory features prior to therapy that reliably predict a treatment non-response<sup>[56]</sup>, but the model of end stage liver disease (MELD) can be useful in assessing risk and quantifying improvement or deterioration. MELD scores  $\geq 12$  points at presentation have a sensitivity of 97% and specificity of 68% for treatment failure in autoimmune hepatitis, and patients with such scores warrant close scrutiny<sup>[14]</sup>. Infection has been associated with protracted corticosteroid therapy in patients with acute severe (fulminant) presentations<sup>[52]</sup>, and treatment should be discontinued promptly whenever there is evidence that the disease is worsening or if there has been no improvement after 2 wk<sup>[53,56]</sup>.

## DECISION TO TREAT ASYMPTOMATIC MILD AUTOIMMUNE HEPATITIS

Autoimmune hepatitis may be asymptomatic in 25%-34% of patients<sup>[66,67]</sup>, and 25%-85% of individuals can be clas-

**Table 2** Difficult treatment decisions before starting conventional corticosteroid therapy

Problem	Response
Acute severe (fulminant) presentation	Prompt institution of conventional corticosteroid therapy with prednisone monotherapy <sup>[44,51-53]</sup> Azathioprine, 50 mg/d, can be added later if treatment is to be continued for $\geq 3$ mo <sup>[55]</sup> Liver transplantation evaluation if laboratory indices worsen at any time during treatment, especially progressive hyperbilirubinemia, or no improvement after 2 wk <sup>[56]</sup>
Asymptomatic mild or mild disease	Institute conventional corticosteroid therapy with prednisone in combination with azathioprine <sup>[58,55]</sup> Consider empirical treatment with budesonide, 3 mg <i>tid</i> , in conjunction with azathioprine, 50 mg/d, if preexistent osteopenia, diabetes, hypertension, obesity, or emotional instability <sup>[25,26]</sup>
Autoantibody-negativity	Exclude viral, drug, toxic, metabolic causes and celiac disease <sup>[31,43]</sup> Apply codified scoring criteria of IAIHG for probable or definite diagnosis <sup>[31,46]</sup> Institute conventional corticosteroid therapy with prednisone in combination with azathioprine or a higher dose of prednisone alone <sup>[19,47-50]</sup>
Overlap syndromes	Conventional corticosteroid therapy alone or in combination with azathioprine if serum alkaline phosphatase level $< 2$ times ULN <sup>[59,62]</sup> Add ursodeoxycholic acid, 13-15 mg/kg per day, to corticosteroid regimen if serum alkaline phosphatase level $\geq 2$ times ULN <sup>[60,63]</sup> Consider ursodeoxycholic acid alone, 13-15 mg/kg per day, if predominant features of PBC with minimal features of autoimmune hepatitis <sup>[64,65]</sup>

IAIHG: International Autoimmune Hepatitis Group; ULN: Upper limit of the normal.

sified as having mild disease by clinical, laboratory and histological findings<sup>[58,68,69]</sup>. Asymptomatic patients are typically men, and they have lower serum aspartate aminotransferase (AST) levels at presentation than symptomatic patients<sup>[66]</sup>. Histological features are similar between symptomatic and asymptomatic patients, including the occurrence of cirrhosis, and 26%-70% of asymptomatic patients become symptomatic during follow-up<sup>[66,67]</sup>. The asymptomatic state is meta-stable, and its presence does not exclude the existence of severe liver inflammation at presentation, especially in children, or its development later<sup>[58]</sup>.

The natural history of mild autoimmune hepatitis is unknown, and patients with mild laboratory and histological disease can have 10- and 15-year survivals that exceeds 80% without treatment<sup>[67,70]</sup>. These results are better than those in untreated patients with severe disease, in whom the early mortality is 40%-50%<sup>[1-3]</sup>, and they suggest that some patients with mild autoimmune hepatitis can do well without treatment. The difficulty is in identifying this safe population of patients. The lack of codified treatment guidelines and concerns about treatment-related side effects have resulted in highly individualized and inconsistent management strategies for these patients<sup>[30,58]</sup>.

Untreated mild autoimmune hepatitis does not have a uniformly benign prognosis. Cirrhosis develops in 49% of untreated patients within 15 years<sup>[70]</sup>; liver failure and hepatocellular carcinoma are possible<sup>[58]</sup>; asymptomatic patients frequently become symptomatic<sup>[66,67]</sup>; and 10-year mortality exceeds 10%<sup>[67]</sup>. Spontaneous resolution is possible, but untreated patients with mild autoimmune hepatitis improve less commonly (12% *vs* 63%,  $P = 0.006$ ) and more slowly than treated patients, and they have a lower 10-year survival (67% *vs* 98%,  $P = 0.01$ )<sup>[58]</sup>. The rapidity of improvement rather than the severity of inflammation may be important in preventing disease progression in mild disease, and protection can be most reliably obtained by instituting treatment<sup>[11]</sup>.

Autoimmune hepatitis is by nature a labile and aggressive disease, and phases of mild disease activity can be interspersed with phases of severe activity that can be aggressive<sup>[71,72]</sup>. In this context, the true existence of mild autoimmune hepatitis can be questioned, and treatment criteria based on perceptions of disease severity at any single time point fail to recognize this fluctuating nature. The uncertainty that mild disease remains mild indefinitely favors therapy for all such patients. The urgency rather than the need for treatment may be all that is decreased in these individuals (Table 2).

Until randomized clinical trials are performed that compare treatment against no treatment, the management strategy in patients with mild autoimmune hepatitis should lean toward conventional therapy<sup>[58]</sup> (Table 1). This option eliminates concern regarding unsuspected disease progression, and the treatment response is likely to be rapid and well-tolerated.

## DECISION TO TREAT AUTOANTIBODY-NEGATIVE AUTOIMMUNE HEPATITIS

Autoantibodies in autoimmune hepatitis are signatures of the disease, but they are not pathogenic or requisites for its occurrence<sup>[73]</sup>. They can appear and disappear during the illness<sup>[74]</sup>; they do not correlate closely with laboratory or histological indices of liver inflammation<sup>[74,75]</sup>; and they cannot be used to reliably monitor disease behavior<sup>[74,75]</sup>. Patients may have all the features of autoimmune hepatitis except the autoantibodies, and they can respond as well to corticosteroid therapy as patients with classical autoantibody-positive disease<sup>[47,50]</sup>.

Seronegative individuals may have escaped detection by testing for the conventional autoantibodies, or their serological signature may be undiscovered. These patients may express conventional autoantibodies later in the course of their disease<sup>[74]</sup>, or their diagnosis can be supported by testing for the non-classical autoantibodies, including antibodies to soluble liver antigen (anti-SLA)<sup>[76]</sup> and atypical anti-neutrophil cytoplasmic antibodies<sup>[77]</sup>. Celiac disease must also be excluded since celiac liver disease can have acute, acute severe (fulminant), and chronic presentations that may respond to gluten restriction<sup>[78-81]</sup>. IgA antibodies to tissue transglutaminase or



endomysium should be sought in all seronegative patients with active liver disease of undetermined cause<sup>[82-84]</sup> (Table 2).

Confidence in the diagnosis of autoantibody-negative autoimmune hepatitis can be strengthened by applying the comprehensive scoring system of the IAIHG<sup>[31]</sup>. Seronegative patients can frequently be categorized as having autoimmune hepatitis by this method<sup>[46]</sup>. Once the diagnosis has been made by the exclusion of other conditions that it might resemble, corticosteroid treatment should be started with regimens identical to those used in classical autoimmune hepatitis<sup>[19]</sup> (Table 1). Treatment should not be extended beyond 3 mo if there has been no improvement, and the accuracy of the original diagnosis and the legitimacy of the treatment regimen should be reassessed if the disease worsens despite compliance with the medication schedule.

## DECISION TO TREAT OVERLAP SYNDROMES

Patients with autoimmune hepatitis may have findings that suggest concurrent primary sclerosing cholangitis (PSC)<sup>[85-87]</sup>, primary biliary cirrhosis (PBC)<sup>[59,63,88,89]</sup>, or a cholestatic syndrome in the absence of PSC and PBC<sup>[90,91]</sup>. Overlap syndromes lack codified clinical or pathological definitions, and they do not have a particular etiological agent or distinctive pathogenic mechanism<sup>[92,93]</sup>. The designations are arbitrary and imprecise, and the clinical phenotypes of patients with the same overlap designation are commonly different<sup>[60,92-96]</sup>.

Twenty percent of patients with autoimmune hepatitis have antimitochondrial antibodies (AMAs)<sup>[61,97-100]</sup>; 19% have a disproportionate elevation of the serum alkaline phosphatase level<sup>[61]</sup>; 15% have increased serum levels of IgM<sup>[61]</sup>; 9% have histological features of bile duct injury<sup>[61,91,101,102]</sup>; and 8% have antibodies to the E2 subunit of the pyruvate dehydrogenase complex<sup>[103]</sup>. Any or all of these features suggest an overlap syndrome with PBC.

Similarly, 16% of patients with autoimmune hepatitis have concurrent inflammatory bowel disease<sup>[104,105]</sup>; 10% (adults) to 50% (children) have biliary changes reminiscent of PSC by magnetic resonance imaging or retrograde endoscopic cholangiography<sup>[106,107]</sup>; and 13% fail to respond to corticosteroids<sup>[14]</sup>. Any or all of these features suggest an overlap syndrome with PSC.

The overlap syndrome with PSC may be associated with intrahepatic bile duct changes (small duct PSC)<sup>[108,109]</sup> or extrahepatic bile duct changes with or without intrahepatic findings (classical PSC). Small duct PSC is probably an early stage of classical PSC as protracted follow-up demonstrates late involvement of the extrahepatic bile ducts in many patients<sup>[108,109]</sup>. The occurrence of intrahepatic biliary changes in patients with predominant features of autoimmune hepatitis could represent coincidental bile duct injury associated with the exuberant inflammatory process<sup>[90,91,102]</sup> or an overlap syndrome with small duct PSC or AMA-negative PBC<sup>[110]</sup>.

The overlap syndromes are important because they are common, occur in 18% of adults with autoimmune liver disease, and they can respond poorly to corticosteroid therapy<sup>[62]</sup>. Adults with autoimmune hepatitis, ulcerative colitis and PSC by cholangiography enter remission less frequently during corticosteroid therapy than patients with normal cholangiograms (59% *vs* 94%,  $P < 0.05$ ), and they fail treatment more commonly (41% *vs* 6%,  $P < 0.05$ )<sup>[104]</sup>. The inflammatory bowel disease is not a determinant of response since patients with ulcerative colitis and normal cholangiograms respond as well to corticosteroid therapy as counterparts without inflammatory bowel disease<sup>[104]</sup>. Cholangiography is important to make these distinctions, and it should be performed in all patients with autoimmune hepatitis and inflammatory bowel disease. Forty-two percent of these individuals will have biliary changes of PSC<sup>[104]</sup>.

The variant syndromes should be suspected when patients with autoimmune hepatitis manifest clinical, laboratory or histological features of cholestasis or respond poorly to conventional corticosteroid therapy<sup>[62]</sup>. The serum alkaline phosphatase level is useful in distinguishing classical autoimmune hepatitis from its overlap syndromes with PBC and PSC<sup>[62]</sup>. Serum alkaline phosphatase levels more than fourfold higher than the upper limit of the normal (ULN) do not occur in classical autoimmune hepatitis, and the presence of an abnormality of this degree in a patient with other features of autoimmune hepatitis compels a search for underlying PBC or PSC<sup>[61]</sup>. In children, the serum  $\gamma$  glutamyl transferase (GGT) level is a more reliable indicator of cholestasis than the serum alkaline phosphatase level. Bile duct changes are common in advanced fibrotic liver disease regardless of type, and these biliary distortions detected by magnetic resonance imaging must be distinguished from PSC<sup>[111]</sup>.

Management of the overlap syndromes is empirical and based on the predominant manifestations of the disease (Table 2). Adults with autoimmune hepatitis and features of PBC who have serum alkaline phosphatase levels less than twofold higher than ULN can be treated with corticosteroids<sup>[59,61,62]</sup>. Adults with higher serum alkaline phosphatase levels and those with florid duct lesions on histological examination are candidates for treatment with corticosteroids and ursodeoxycholic acid<sup>[63,112,113]</sup>. Ursodeoxycholic acid alone may be effective in some patients who have predominant features of PBC<sup>[64,65]</sup>.

Adults with autoimmune hepatitis and PSC are commonly given a trial of prednisone and ursodeoxycholic acid<sup>[87]</sup>, but in adults with mainly hepatitis features, corticosteroid therapy alone may be beneficial<sup>[86]</sup>. These patients typically respond less well to treatment than those with mixed features of autoimmune hepatitis and PBC<sup>[62,96]</sup>. Patients with the cholestatic syndrome in the absence of PBC and PSC can be treated with prednisone, ursodeoxycholic acid, or both depending on the serum alkaline phosphatase level<sup>[102]</sup>. Multicentered collaborative

investigations are needed to codify diagnostic criteria and establish confident treatment algorithms for these non-classical syndromes (Table 2).

The diagnosis of an overlap syndrome implies that its clinical phenotype is outside the boundaries of classical disease, but the point at which this occurs is unknown<sup>[94,113]</sup>. The features of the classical autoimmune liver diseases are not disease-specific, and they are commonly shared<sup>[62,88,114,115]</sup>. This commonality of manifestations can cluster in different densities in individual patients and suggest another disease. The overlap syndromes are probably atypical manifestations of a classical disease rather concurrent diseases or a distinctive pathological entity<sup>[92,94,113]</sup>. The diagnostic scoring systems of the IAIHG are not discriminative diagnostic indices, and they cannot be used to declare an overlap syndrome, especially because the definition of such an entity has not been codified<sup>[88,116,117]</sup>.

## DECISION TO STOP TREATMENT

Twenty-one percent of patients with autoimmune hepatitis achieve a sustained long-term remission after initial corticosteroid treatment, and 28% who relapse after drug withdrawal achieve this same result after retreatment<sup>[18]</sup>. Autoimmune hepatitis can enter a sustained inactive state after treatment<sup>[15,18,67,118]</sup>, and this possibility has justified efforts to terminate therapy in all patients despite their well-recognized high frequency of relapse<sup>[17,72]</sup>. Patients who sustain their remission after drug withdrawal have fewer laboratory abnormalities at the time of drug withdrawal than patients who relapse, and the ideal treatment end point is when normal liver tests and tissue have been achieved<sup>[15,119-123]</sup> (Table 3).

The key laboratory indices to monitor are the serum AST, alanine aminotransferase, bilirubin and  $\gamma$ -globulin levels<sup>[121]</sup>, and these tests should be normal prior to drug withdrawal. The ideal histological end points are normal liver architecture or inactive cirrhosis<sup>[15,123]</sup>. Relapse has been associated with residual plasma cell infiltration in the liver tissue despite the absence of other disruptive changes, and the plasma cells may indicate persistence of the immune response<sup>[119,123]</sup>. Plasma cell infiltration in the native liver has also been associated with recurrent autoimmune hepatitis after liver transplantation, and it may signal an active pathogenic process<sup>[134]</sup>.

Liver tissue examination immediately prior to drug withdrawal is the only confident method of confirming an ideal treatment end point, but it should not be performed for at least 3 mo after normalization of the laboratory indices. Histological improvement lags behind clinical and laboratory resolution by 3-8 mo<sup>[2]</sup>, and liver tissue examination before this interval will disclose histological features of interface hepatitis in 55% of instances<sup>[133]</sup>.

The presence of interface hepatitis on the follow-up tissue examination justifies the continuation of therapy for an additional 6 mo before reconsidering drug withdrawal. Another liver tissue examination is not neces-

**Table 3** Difficult treatment decisions during conventional corticosteroid therapy

Problem	Response
Determining treatment end point	Continue conventional therapy until normal serum AST, ALT, bilirubin and $\gamma$ -globulin levels and normal liver tissue or inactive cirrhosis (ideal end point) <sup>[119-121]</sup> Continue conventional therapy until serum AST $\leq$ 2 times ULN, bilirubin and $\gamma$ -globulin levels normal, and portal hepatitis or minimally active cirrhosis (satisfactory end point) <sup>[11,54,55]</sup> Decrease dose of culprit drug or discontinue its use if side effects emerge (drug toxicity end point) <sup>[13,55]</sup> Limit conventional corticosteroid treatment of patients aged $\geq$ 60 yr if an ideal or satisfactory end point has not been achieved $\leq$ 24 mo (incomplete response end point) <sup>[11,19,124,125]</sup>
Relapse after drug withdrawal	Institute original therapy until clinical and laboratory resolution, then increase azathioprine dose to 2 mg/kg per day as dose of prednisone is withdrawn <sup>[126,127]</sup> Continue daily azathioprine in fixed dose indefinitely <sup>[126,127]</sup> Use low dose prednisone ( $\leq$ 10 mg/d) if severe cytopenia (leukocyte counts $< 2.5 \times 10^9$ /L or platelet counts $< 50 \times 10^9$ /L) or other azathioprine intolerances <sup>[13,55]</sup> Use low dose prednisone (2.5-5 mg/d) to supplement azathioprine maintenance if abnormal serum AST level <sup>[55,128]</sup>
Treatment failure	Prednisone, 60 mg/d, or prednisone, 30 mg/d, in combination with azathioprine, 150 mg/d, for at least 1 mo, then dose reductions by 10 mg for prednisone and 50 mg for azathioprine each month of laboratory improvement until conventional doses reached <sup>[54,55,129]</sup> Evaluate for liver transplantation if minimal criteria for listing (MELD $\geq$ 15 points) are met <sup>[130-132]</sup>
Incomplete response	Azathioprine (2 mg/kg per day) indefinitely after corticosteroid withdrawal <sup>[54,55,127]</sup> Low-dose prednisone ( $\leq$ 10 mg/d) if azathioprine intolerance <sup>[54,55,128]</sup> Adjustments to maintain serum AST level $\leq$ 3 times ULN <sup>[55,133]</sup>

MELD: Model of end stage liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

sary if the histological findings 6 mo earlier have shown improvement during treatment and the inflammatory changes have been mild. The frequency of relapse after full resolution of the laboratory and histological features can be reduced from 86%<sup>[17]</sup> to 60%<sup>[121]</sup>, and in some instances, as low as 20%<sup>[15]</sup>.

Full resolution of liver tests and tissue is an ideal treatment end point, but it may be achievable in only 40% of patients<sup>[121]</sup>. Relentless pursuit of an ideal end point may be hazardous because the likelihood of a full response must be balanced against the risk of treatment related side effects<sup>[12,13]</sup>. Seventy-seven percent of patients who respond will do so within 24 mo, and patients aged  $\geq$  60 years respond more quickly than adults aged  $\leq$  40 years<sup>[11]</sup>. The rapidity of response may reflect age-related differences in the vigor of the immune response (immune senescence)<sup>[135-137]</sup> or human leukocyte antigen (HLA) status<sup>[138]</sup>. Elderly patients more commonly have

**Table 4** Difficult treatment decisions after conventional corticosteroid therapy

Problem	Response
Empirical salvage drugs	Consider cyclosporine (5–6 mg/kg per day) <sup>[144–150]</sup> or tacrolimus (4 mg <i>bid</i> ) <sup>[21,22,151,152]</sup> if progressive disease on conventional treatment Consider mycophenolate mofetil (1 g <i>bid</i> ) if corticosteroid or azathioprine intolerance <sup>[23,24,153–159]</sup> Consider budesonide (3 mg <i>tid</i> ) as frontline therapy if mild disease or if azathioprine maintenance insufficient after relapse or incomplete response <sup>[25,26]</sup> Complete benefit-risk and cost analyses before use <sup>[160,161]</sup> Empirical trial must not supersede liver transplantation <sup>[55,130,131]</sup>
Liver transplantation	Consider if acute severe (fulminant) presentation unresponsive or worse within 2 wk of conventional treatment <sup>[52,53,56,57]</sup> Consider if treatment dependent $\geq 3$ yr and features of decompensation develop (ascites, encephalopathy or variceal bleeding) <sup>[130]</sup> Consider if failure to conventional therapy and MELD score $\geq 15$ points <sup>[52,131,132]</sup>
Elderly patients (aged $\geq 60$ yr)	Restrict conventional therapy to combination regimen <sup>[124]</sup> Limit initial treatment to $\leq 24$ mo <sup>[125]</sup> Institute azathioprine maintenance therapy (2 mg/kg per day) if initial response is incomplete at 24 mo <sup>[124]</sup> Consider liver transplantation if features of decompensation emerge <sup>[132]</sup>
Pregnant patients	Counsel regarding risks of prematurity and infant mortality <sup>[162–167]</sup> Institute high-risk obstetrical care <sup>[30,162]</sup> Avoid azathioprine if possible <sup>[165,168]</sup> Reduce doses of prednisone to lowest levels to stabilize if not resolve laboratory indices <sup>[169]</sup> Reestablish conventional prednisone doses prior to delivery <sup>[169]</sup> Be alert to post-partum flares <sup>[163,164,169]</sup>

HLA DRB1\*04 than young adults, and this phenotype has been associated with a quicker and better treatment response than other HLA phenotypes<sup>[138–142]</sup>. The inability to induce resolution within 24 mo of therapy portends the development of treatment-related side effects<sup>[13,143]</sup>, and it justifies a change in the end point strategy.

Improvements during the initial 24 mo of therapy may still be sufficient to consider drug withdrawal despite the absence of an ideal response. The disappearance of symptoms, improvement of the serum AST levels to less than twofold greater than ULN, normalization of the serum bilirubin and  $\gamma$ -globulin levels, and histological improvement to portal hepatitis or minimally active cirrhosis have been associated with a sustainable remission for at least 6 mo in 50% of cases, and these improvements during therapy constitute a satisfactory but not ideal end point<sup>[16,72]</sup>. A protracted interval of quiescent disease that requires no therapy is a desirable achievement, and it may be long-term despite the absence of an ideal response. Discontinuation of therapy after achieving satisfactory milestones should be consid-

ered at the 24-mo interval or at any earlier point in the course of treatment if signs of drug intolerance have emerged<sup>[54,143]</sup> (Table 3).

Failure to achieve an ideal or satisfactory response by 24 mo requires reassessment of the individual clinical situation. Ninety-four percent of patients aged  $\geq 60$  years who achieve an ideal or satisfactory end point do so within 24 mo<sup>[11]</sup>, and those elderly patients who have not done so are best treated with a long-term maintenance strategy designed to reduce or eliminate the corticosteroid dose and replace it with azathioprine<sup>[126–128]</sup> (Table 4). Similarly, patients who have shown little improvement during this interval or who are manifesting corticosteroid-related side effects should be treated with long-term azathioprine maintenance<sup>[126,127]</sup> (Table 3).

In contrast, 36% of adults aged  $\leq 40$  years achieve an ideal or satisfactory end point beyond 24 mo of therapy<sup>[11]</sup>, and their original treatment regimen can be maintained for an additional 12 mo if they are drug tolerant. Eighty-one percent of the adults aged  $\leq 40$  years who respond do so within 36 mo<sup>[11]</sup>, and those who do not are candidates for maintenance therapy with azathioprine. Most patients with autoimmune hepatitis do relapse and require long-term maintenance treatment<sup>[4,16,18]</sup>, but the patients who are able to achieve a sustained long-term remission should not be penalized by blanket assignment to continuous initial therapy<sup>[54]</sup>.

## DECISION TO TREAT AFTER RELAPSE

Relapse after drug withdrawal constitutes a recrudescence of inflammatory activity that is typified by the reappearance of interface hepatitis in the liver biopsy specimen<sup>[2]</sup>. Laboratory correlations with histological findings after drug withdrawal have indicated that an increase in the serum AST level to at least threefold higher than ULN is invariably associated with interface hepatitis, and liver tissue examination is not required to diagnose this occurrence<sup>[133]</sup>.

Reinstitution of the original treatment schedule rapidly suppresses the exacerbation, and another clinical, laboratory and histological remission can be achieved<sup>[16]</sup>. Subsequent treatment withdrawal is typically followed by another relapse, and the sequence of retreatment, drug withdrawal, and relapse can be repeated indefinitely<sup>[16]</sup>. With each exacerbation and retreatment, the frequency of achieving a sustained remission decreases (14% after three retreatments)<sup>[16]</sup>; the occurrence of drug-related side effects escalates (70% after two retreatments)<sup>[12]</sup>; and the cumulative frequencies of progression to cirrhosis (38%) and liver failure increase (20%)<sup>[170]</sup>. The optimal time to prevent these outcomes is after the first relapse, and repeated administrations of the original treatment regimen are not advised.

The preferred management of relapse is to institute long-term treatment with azathioprine after the first exacerbation<sup>[126,127]</sup> (Table 3). Clinical and laboratory resolution is achieved with conventional corticosteroid

treatment, and then the dose of prednisone is gradually withdrawn as the dose of azathioprine is increased to 2 mg/kg daily. Azathioprine is then continued indefinitely as a maintenance therapy. Eighty-seven percent of patients are able to sustain clinical and laboratory remission in this fashion over 10 years<sup>[126,127]</sup>. The most common side effect is arthralgia associated with corticosteroid withdrawal (63%). Myelosuppression and lymphopenia occur in 7% and 57% of patients, respectively, and malignancies of diverse cell types and uncertain association with therapy have developed in 8%<sup>[127]</sup>.

Prednisone in low dose can be used instead of azathioprine for long-term maintenance if there is preexistent or evolving cytopenia<sup>[128]</sup> (Table 3). The goal is to maintain the serum AST level below threefold greater than ULN on the least amount of medication. Eighty-seven percent of patients can be managed long-term on  $\leq 10$  mg/d prednisone (median dose, 7.5 mg/d)<sup>[128]</sup>. Observation intervals for up to 149 mo have indicated satisfactory outcomes that have justified continued application of the strategy. Side effects associated with the earlier conventional corticosteroid treatments improve or disappear in 85% of patients; new side effects do not develop; and survival is unaffected when compared with patients who receive standard dose corticosteroid therapy after relapse<sup>[128]</sup>. Recent studies in patients followed for as long as 43 years (median, 13.5 years) have confirmed that the low-dose prednisone strategy can be used effectively and safely in the long term<sup>[171]</sup>.

## DECISION TO TREAT THE ADVERSE RESPONSE

The unsatisfactory responses to initial corticosteroid therapy are treatment failure, incomplete response, and drug toxicity. Each adverse outcome justifies a treatment modification.

### Treatment failure

Treatment failure connotes clinical, laboratory, and histological worsening despite compliance with the original treatment schedule<sup>[129]</sup>. Nine percent of patients fail treatment<sup>[14,15,129]</sup>, and high-dose therapy with prednisone (30 mg/d) in conjunction with azathioprine (150 mg/d) or prednisone alone (60 mg/d) is the preferred initial approach to this problem<sup>[19,23,54,55]</sup> (Table 3). Doses of medication are maintained at this level for 1 mo before improvements in the laboratory tests justify an attempt at dose reduction. The dose of prednisone is reduced by 10 mg and the dose of azathioprine is reduced by 50 mg each month that the serum AST level improves, until the original conventional doses are reached<sup>[19,54,55]</sup>. Seventy percent of patients improve their clinical and laboratory findings within 2 years, but histological resolution is achieved in only 20%<sup>[129]</sup>. Most patients remain on therapy indefinitely. Manifestations of liver decompensation during high-dose therapy (encephalopathy,

ascites, or variceal hemorrhage) are indications for liver transplantation<sup>[130]</sup>.

Thirteen percent of patients have an incomplete response to conventional treatment<sup>[15,19,54,55]</sup>. The clinical, laboratory, and histological findings improve, but the improvements are insufficient to constitute an ideal or satisfactory end point. These patients are unlikely to enter remission if therapy is continued beyond 36 mo ( $< 3\%$  occurrence)<sup>[11,130]</sup>, and they are candidates for indefinite maintenance therapy with azathioprine alone<sup>[55,126,127]</sup> or low-dose prednisone<sup>[128,171]</sup> at that time (Table 3). Treatments should be adjusted to maintain the serum AST level below threefold greater than ULN if possible to reduce the likelihood of an aggressive histological lesion<sup>[133]</sup>.

### Drug toxicity

Drug toxicity compels dose reduction or premature discontinuation of the offending drug in 13% of patients<sup>[13]</sup>. Cytopenia, nausea, emotional lability, hypertension, cosmetic changes, and diabetes are typically dose-related, and these consequences can improve with dose reduction<sup>[53]</sup>. Severe reactions, including psychosis, extreme cytopenia (leukocyte counts  $< 2.5 \times 10^9/L$  or platelet counts  $< 50 \times 10^9/L$ ), and symptomatic osteopenia with or without vertebral compression, justify immediate discontinuation of the offending agent<sup>[55]</sup>. In these patients, the single tolerated drug (prednisone or azathioprine) is continued in adjusted dose to suppress inflammatory activity. Routine phenotyping or genotyping for thiopurine methyltransferase deficiency has not been predictive of azathioprine-induced toxicity at the low doses of azathioprine (50-150 mg/d) used to treat autoimmune hepatitis<sup>[172-174]</sup>. Accordingly, routine screening for this enzyme activity has not been established<sup>[13]</sup>.

## DECISION TO INSTITUTE EMPIRICAL SALVAGE THERAPY

Multiple immunosuppressive agents have emerged mainly from the transplantation arena, and they have site-specific actions of theoretical advantage in the treatment of autoimmune hepatitis<sup>[175-177]</sup>. Many such agents have been used empirically in small, single-institution, treatment trials with some success, and they have been proposed as salvage therapies<sup>[19]</sup>. None has been studied rigorously in controlled or comparative treatment trials; all must be used off-label in autoimmune hepatitis; and none has been incorporated into standard management algorithms. Target populations, dosing schedules, safety profiles and cost analyses are lacking, and the nature of the clinical situation that requires rescue is also unclear<sup>[153,160]</sup>.

The major clinical problems that warrant rescue are worsening of the liver disease despite compliance with the standard corticosteroid regimen (treatment failure) and corticosteroid or azathioprine intolerance (drug



toxicity)<sup>[153]</sup>. In the former instance, the patient must be rescued from the liver disease, and in the latter instance, the patient must be rescued from the treatment. There are conventional corticosteroid- and azathioprine-based strategies for each of these contingencies, but new pharmacological agents have a theoretical basis and burgeoning experience that support their use<sup>[19,55]</sup>.

The calcineurin inhibitor, cyclosporine, and the purine antagonist, mycophenolate mofetil, have generated the most interest (Table 4). Numerous studies have described successful salvage of patients with corticosteroid intolerance or treatment failure by administering cyclosporine<sup>[144-150]</sup>, and similar results in fewer studies have been described with tacrolimus<sup>[21,22,151,152]</sup>. In a representative study, cyclosporine improved the laboratory tests of liver inflammation, reduced the histological activity index, and was well tolerated when administered for 26 wk<sup>[20]</sup>.

Mycophenolate mofetil has induced clinical and laboratory improvements in 39%-84% of patients, and it has allowed discontinuation of corticosteroid treatment in most patients<sup>[23,24,151,154-159]</sup> (Table 4). Non-response or drug intolerance (nausea, vomiting, pancreatitis, rash, alopecia, deep venous thrombosis, and diarrhea) has been described in 34%-78% of patients treated with mycophenolate mofetil, and the potential benefits of this drug must be balanced against these deficiencies. Salvage therapy regardless of the drug is inconsistently effective, potentially toxic, interminable, and expensive<sup>[160]</sup>. Liver transplantation may offer the most reliable form of rescue, and it must be considered carefully as an alternative to empirical new drug therapy in every salvage situation<sup>[130]</sup> (Table 4).

The results of salvage therapy with cyclosporine, tacrolimus or mycophenolate mofetil can be improved by selecting the patients who are most likely to respond. The major reason for treatment failure with these agents is uncertainty about the correct target population and the proper timing, dosing and duration of treatment. Patients may advance quickly beyond drug rescue, and many patients may need a new liver rather than a new drug<sup>[53]</sup>. The ideal candidates for cyclosporine therapy are patients who have failed corticosteroid treatment or been intolerant of the conventional medications and who are still below minimal listing criteria for liver transplantation (MELD scores < 15 points)<sup>[131]</sup>. Transplantation should be considered at the first sign of liver decompensation (usually the development of ascites) during the new drug regimen<sup>[130]</sup> (Table 4).

Children with autoimmune hepatitis and cholangiographic features of sclerosing cholangitis (overlap syndrome) respond poorly to mycophenolate mofetil<sup>[24,178]</sup>, as do adult patients who are failing conventional treatment<sup>[158]</sup>. Therapy with mycophenolate mofetil should be considered mainly in adults with azathioprine intolerance<sup>[158]</sup> and children with non-response to conventional corticosteroid regimens<sup>[24]</sup>. The metabolism of mycophenolate mofetil is independent of the thiopurine methyl-

transferase pathway, and it can be considered in patients with known thiopurine methyltransferase deficiency.

Budesonide has promise as an alternative frontline therapy in treatment-naïve patients with autoimmune hepatitis<sup>[25,179,180]</sup>, but it has been variably successful as a salvage therapy in corticosteroid-treated patients with treatment failure or corticosteroid dependence<sup>[26,181]</sup>. Furthermore, it can be associated with glucocorticoid side effects, particularly in patients with cirrhosis and portosystemic shunting<sup>[161,181]</sup>. Similarly, treatment with ursodeoxycholic acid has not allowed consistent withdrawal from corticosteroid therapy or rescue from treatment failure<sup>[182]</sup>.

## DECISION TO TREAT THE ELDERLY

Twenty percent of adults with autoimmune hepatitis develop the disease after the age of 60 years<sup>[138,183,184]</sup>, and these patients have a greater degree of hepatic fibrosis at presentation than young adults aged < 40 years<sup>[185]</sup> and higher frequencies of ascites<sup>[184]</sup> and cirrhosis<sup>[138]</sup>. These findings suggest that the elderly have aggressive liver disease that is commonly indolent and unsuspected. Symptoms of fatigue and myalgia may be attributed to the aging process; concurrent immune diseases, such as rheumatoid arthritis, may mask the underlying liver disease; and liver test abnormalities may be ascribed to the medications used for other ailments. The proper diagnosis may also trigger concern about side effects associated with corticosteroid therapy and result in reluctance to treat the condition in a standard fashion<sup>[186]</sup>. These concerns are justified, but they do not mitigate the need for treatment or portend a dismal outcome.

The indications for treatment and the initial treatment regimens are the same for the elderly as for young adults<sup>[124]</sup>. The preferred schedule is prednisone in combination with azathioprine (Table 1). Elderly patients enter remission as commonly as young adults (61% *vs* 59%), and they fail treatment less often (5% *vs* 24%, *P* = 0.03)<sup>[138]</sup>. Relapse, sustained remission, death from liver failure or need for liver transplantation occur as commonly in the elderly as in young adults<sup>[138]</sup>, and the elderly respond more quickly to medication<sup>[11]</sup>. Patients aged ≥ 60 years enter remission within 6 mo more frequently than adults aged < 40 years (18% *vs* 2%, *P* = 0.02), and most have achieved an ideal or satisfactory end point of therapy within 24 mo (94% *vs* 64%, *P* = 0.003)<sup>[11]</sup>.

The development of side effects associated with medication relates mainly to the duration of initial therapy and the cumulative durations of subsequent corticosteroid treatment<sup>[125]</sup>. Protracted corticosteroid therapy for > 24 mo and retreatment with corticosteroids after multiple relapses must be avoided to reduce the occurrence of vertebral compression and progressive osteopenia<sup>[13]</sup>. The risk of treatment-related complications in the elderly underscores the importance of limiting corticosteroid therapy to < 24 mo. Azathioprine maintenance therapy (2 mg/kg per day) should be instituted if

treatment is to be extended beyond 24 mo or be required after the first relapse<sup>[30,124]</sup> (Table 4).

A bone maintenance regimen should also be prescribed for all elderly patients undergoing initial corticosteroid treatment<sup>[13,30,124]</sup>. Regular weight-bearing exercise should be emphasized, and calcium (1-1.5 g/d), vitamin D3 (400-800 U/d), and alendronate (70 mg/wk) should be considered as adjuvant therapies. An annual bone density assessment can guide the vigor of the bone maintenance schedule. Budesonide (3 mg *tid*) can be considered as an empirical supplement to long-term azathioprine maintenance if liver inflammation is controlled inadequately<sup>[19,124]</sup>. Liver transplantation is effective in rescuing elderly patients with liver failure who have been screened for other comorbidity. The 5-year survival after liver transplantation in carefully screened elderly patients is comparable to that of young adults (80% in patients aged 60-65 years and 73% in patients aged > 65 years *vs* 78% in patients aged 18-59 years). Elderly patients also have fewer episodes of acute cellular rejection<sup>[132]</sup>.

## DECISION TO TREAT PREGNANT WOMEN

Pregnancy complicates the management of autoimmune hepatitis because of the risks that the liver disease and its treatment pose for the mother and the fetus (Table 4). Perinatal mortality is 4%<sup>[162]</sup>; serious complications develop in 9%<sup>[163]</sup>; caesarian section is required in 17%; stillbirths occur in 5%; and fetal loss is 21%<sup>[164]</sup>. These outcomes are better than those in mothers with diabetes, but they do indicate the need for high-risk obstetrical care<sup>[164]</sup>. The presence of maternal antibodies to SLA and extractable nuclear antigens (Ro/SSA) is associated with a complicated course<sup>[163]</sup>.

Azathioprine is associated with congenital malformations in pregnant mice, and it is a category D drug for pregnancy<sup>[165]</sup>. The odds ratio for having a child with congenital malformations while taking azathioprine for inflammatory bowel disease is 3.4, whereas it is negligible in similarly treated pregnant women with systemic lupus erythematosus<sup>[166]</sup>. There have been no reports of congenital malformations in the children of mothers treated with azathioprine for autoimmune hepatitis<sup>[166]</sup>, and there have been no serious consequences associated with breast feeding of these infants<sup>[167]</sup>. Nevertheless, the placenta is only a partial barrier to the metabolites of azathioprine<sup>[168]</sup>; there have been no rigorously designed studies that confirm the safety of azathioprine in pregnant women with autoimmune hepatitis<sup>[166]</sup>; and azathioprine is not an essential medication in the management of the disease<sup>[19,30]</sup>. The preferred treatment during pregnancy is with prednisone alone.

Autoimmune hepatitis can improve during pregnancy possibly because the high blood levels of estrogen promote a cytokine shift from a type 1 cytotoxic profile to an anti-inflammatory type 2 profile<sup>[187,169]</sup>. The reduced inflammatory activity may allow a reduction in the dose of prednisone or its elimination<sup>[169]</sup>. Exacerbations of

disease activity are common after delivery (12%-86%), presumably because the falling blood concentrations of estrogen facilitate a cytokine shift back to the cytotoxic type 1 profile<sup>[163,164,169]</sup>. These flares must be anticipated, and conventional dosing with prednisone should be resumed during the third trimester (Table 4).

Women with autoimmune hepatitis should not be discouraged from pregnancy, but they must be counseled about the increased frequency of prematurity and fetal loss, the normal low occurrence of congenital defects, the theoretical hazards of azathioprine during pregnancy, the possibility of an exacerbation of the liver disease after delivery, the need for high-risk obstetrical care, and the reasons for regular medical assessment during and after the pregnancy<sup>[30]</sup>.

## CONCLUSION

Current corticosteroid regimens (Table 1) are effective in the management of most patients with autoimmune hepatitis, and new pharmacological agents with powerful site-specific actions promise to strengthen the therapeutic repertoire. These treatments must be adapted and integrated to satisfy individual clinical situations. Established therapies can be improved by defining end points that permit optimal opportunity for resolution without extending beyond achievable goals and introducing undue risk of drug toxicity. The ideal treatment end point is normalization of liver tests and liver tissue, and the expected duration of initial therapy to achieve this end point is  $\leq 24$  mo (Table 2).

Autoimmune hepatitis is by nature an aggressive liver disease with fluctuating activity. Mild asymptomatic disease may be a temporary condition, and corticosteroid therapy should be considered for all patients regardless of disease activity at presentation. Other variations in the clinical phenotype, including acute severe (fulminant) presentations, absence of autoantibodies, and cholestatic features (overlap syndromes), warrant management appropriate for the predominant manifestations of the disease (Table 2).

Relapse after drug withdrawal justifies a long-term maintenance regimen with azathioprine, and azathioprine can also be used as a single-drug therapy for patients with an incomplete response to conventional schedules. Treatment adjustments are warranted in elderly patients who respond slowly and in pregnant patients in whom azathioprine avoidance is prudent and postpartum exacerbations are possible (Tables 3 and 4).

Empiric salvage therapy includes the calcineurin inhibitors (cyclosporine and tacrolimus) and mycophenolate mofetil, and they can be introduced judiciously for otherwise refractory inflammation (cyclosporine or tacrolimus) or drug intolerance (mycophenolate mofetil) (Table 4). Salvage therapy is expensive, unproven, associated with its own toxicity, inconsistently effective, and poorly guided. It should never supersede indications for liver transplantation.

Treatment decisions in autoimmune hepatitis will not be difficult if they are guided by an awareness of the phenotypic diversity of the disease, realistic therapeutic expectations, willingness to make individualized adjustments according to the clinical need, and familiarity with the alternative empirical therapies.

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## Gastroenterology training in private hospitals: India vs South Africa

Chris Jacob Johan Mulder, Amarender Singh Puri, Duvvur Nageshwar Reddy

Chris Jacob Johan Mulder, Department of Gastroenterology, VU University Medical Center, 1007 MB Amsterdam, The Netherlands

Amarender Singh Puri, Department of Gastroenterology, GB Pant Hospital, New Delhi 110002, India

Duvvur Nageshwar Reddy, Asian Institute of Gastroenterology, Hyderabad 500082, India

Author contributions: Mulder CJJ wrote the paper; Puri AS and Reddy DN contributed data and helped to design the paper.

Correspondence to: Chris Jacob Johan Mulder, MD, PhD, Professor, Department of Gastroenterology, VU University Medical Center, PO Box 7057, 1007 MB Amsterdam, The Netherlands. [cjmulder@vumc.nl](mailto:cjmulder@vumc.nl)

Telephone: +31-20-4440613 Fax: +31-20-4440554

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future structure and logistics of training needs. This is required in all subspecialties including gastroenterology, as has been done in India. It is hoped that as a consequence well-trained doctors, similar to those in India, might move to provincial hospitals in rural areas, upgrading the medical services and keeping medical power in South Africa. South Africa should become a model for Sub-Saharan Africa, as India already is for South-East Asia.

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**Key words:** Brain drain; Gastroenterology; India; Manpower; Private hospitals; Specialist training; Subspecialties; Teaching hospitals

**Peer reviewer:** Yasuji Arase, MD, Department of Gastroenterology, Toranomon Hospital, 2-2-2 Toranomonminato-ku, Tokyo 105-8470, Japan

### Abstract

In South Africa, nurses and doctors are emigrating in significant numbers. Job satisfaction, safety and ensuring career progression are important in retaining doctors to make a career in Republic of South Africa (RSA). Due to budgetary constraints many hospitals have not been upgraded. Coming home after overseas training seems difficult. In RSA it takes a minimum of 13 years for a young specialist to become registered and 15 years for subspecialists. Career progression, creating more specialist trainees in public and private hospitals and shortening the period of professional training are potential solutions to the problem. India, which has a population of more than 1 billion people, is struggling with similar problems. For the past 10-15 years, private hospitals have assisted in manpower development for medical specialist and subspecialist careers. Currently their private sector trains 60% of their recognised (sub)specialties fellows. A national task force for specialist training in RSA should be instituted. It should discuss, based on the current status and projected specialist and subspecialist personnel requirements, the

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### INTRODUCTION

In South Africa, the health care system faces a variety of problems. There is an overall shortage and maldistribution of medical doctors and specialists, with remote areas the least well populated. In South Africa nurses and medical doctors are the professional categories that emigrate in the most significant numbers. The impact is felt beyond the emigrating numbers of the highly developed skill base the country loses when they leave. Globally, dissatisfaction with income in developing countries is one of the major causes of doctors leaving public service, going overseas and/or joining private hospitals.



It was reported that increases in salaries of doctors in the public sector in South Africa might lead to a number of private general practitioners returning to the public service<sup>[1]</sup>. However, many doctors state that other factors such as job satisfaction, safety, working conditions, and improved career opportunities in medical specialist training are more important to stop the exodus of doctors from South Africa to wealthier and safer countries. Ensuring career progression is another factor suggested to be important in retaining academic specialists and fellows to make a career in South Africa. It is therefore imperative to understand and study which specific factors are responsible for doctors leaving the country<sup>[2-4]</sup>.

New academic hospitals such as Inkosi Albert Luthuli Central Hospital in Durban (2002), Steve Biko Academic Hospital in Pretoria (2006) and Chris Hani Baragwanath Hospital (2010) in Johannesburg have been built or are in the process of being refurbished in an attempt to equalise the provision of tertiary health care. Due to limited national and provincial budget allocations, many urban regional hospitals have not been upgraded. They, like many of the rural hospitals built in the early and mid 1900s, are in a poor state of repair. The quality of these hospitals' patient and staff accommodation is often inadequate. A so-called "capital works" programme has been implemented by the post-apartheid government to build new clinics and hospitals to improve the physical infrastructure of existing health care facilities, but it is currently short of its targets for the workforce to man these institutions once they are built.

However, public hospitals are not the only health facilities with vacancies for nursing staff and medical doctors, especially medical specialists. Private hospitals in competition with the public hospitals also face the same problems although on a smaller scale.

Medical graduates looking for specialist training in South Africa recognise a lack of opportunities and are often forced to look and sample abroad for skills development. This whets their appetite to deliver high quality specialist care. Yet on return, the lack of facilities even at the top institutes leads to frustration in not being able to utilise their newly acquired specialist skills to the benefit of their own community. Completion of training overseas also makes it difficult for doctors who have become accustomed to life in their new environment to return home once their training has been completed. This "Brain-Drain" problem has been witnessed not only in South Africa but in Sub-Saharan Africa in general<sup>[5]</sup>.

Subspecialties have been considered a luxury in the past by the Government in South Africa. Due to this point of view there is a lack of structure and funding for training in subspecialties of Internal Medicine. These bars to pursuing a subspecialist career in Republic of South Africa result in a shortage of fellows in Internal Medicine finishing their training<sup>[6,7]</sup>, and competition for posts from within a common pool allocated to all the medical subspecialties.

One solution to this problem would be to change the training for subspecialties such as cardiology, rheu-

matology, pulmonology and gastroenterology to an Internal Medicine track of 2 years instead of the full (minimum 4 years) training in Internal Medicine currently required. This would negate the need for two sets of professional exams and shorten the training period. A common trunk training system of 2-3 years is the standard in Europe and the USA<sup>[8]</sup>. In India such a common trunk is still 3 years.

## GASTROENTEROLOGY

The number of gastroenterologists in Europe varies between 1:5000 and 1:58000 of the population<sup>[9]</sup>. In 2007 the number of gastroenterologists per 100000 of the population was 3.9 in the USA, 3.48 in France, 2.1 in Australia, 1.41 in the UK and 1.25 in The Netherlands<sup>[9,10]</sup>. In South Africa this figure was around 0.12. Comparisons between poor and rich countries are notoriously difficult. However, South Africa is both a third and first world country and has a lot of similarities in this respect with India. The Indian model will be discussed in this article.

Unfortunately, no model has been developed and published in South Africa to calculate the required numbers of medical specialists. The Cuban South African agreement, which was signed over a decade ago, notes that South African policymakers were attracted to the Cuban emphasis on prevention and primary care<sup>[2]</sup>. This agreement neglected specialist care for the underprivileged. It seems as if the focus of the government was on training medical doctors for the most basic health care level, instead of understanding which and how many medical specialists and subspecialists are needed in post-apartheid South Africa. There is some evidence that the current basic strategy is failing as the infant mortality rate, a parameter often used to measure quality of health care, has increased since 1994. This is in contrast to Europe, but also to countries like India where great emphasis is placed on specialty and subspecialty training. For instance, additional national training numbers have been awarded to trusts in the UK to help meet the requirements of the European Working Time Directive (EWTD). In addition, expansion in the UK is planned with the recommendation that there should be 2.5 gastroenterologists per 100000 of the population<sup>[11]</sup>. In comparison, in South Africa there are less than 0.22 gastroenterologists per 100000 inhabitants. (personal communication, Karin Fenton, SAGES, Cape Town).

## GUT FEELING

Most internists learn during the current tubal training programmes in South Africa a "gut-feeling" within 3-4 mo, especially in "tubal gut" gastroenterology. Only sixty-two consultant gastroenterologists are available in public and private hospitals around the country, and of these only 17 consultants in gastroenterology work in Academic Hospitals. Only 6 out of the 8 academic complexes teach internists the subspecialty of gastroenterology. There is a lack of internists in general,

of internists for vacancies in training posts, and a lack of dedicated funding for these trainings posts. There is also a lack of gastroenterologists who focus on hepatology and pancreatobiliary disease. Endoscopic ultrasound, therapeutic endoscopy, transplant immunology and nutritional support have developed considerably over the past decades abroad, but not in academic South Africa. In a rather strange contrast, some private institutions in South Africa have gained considerable expertise in some of these fields with the availability of highly specialised equipment and skilled manpower. The University of the Witwatersrand Donald Gordon Medical Center has started a University driven “private” superspecialist programme involving hepatology and liver transplantation. Specialist trainees in Internal Medicine and Anaesthesiology also rotate through the ICU at this private hospital.

The smallness of the gastroenterology community in South Africa, the total sum of equipment and expertise still available in academic institutions and private practise allows a unique opportunity to join forces for developing more training positions for the projected gastroenterology needs of the country in the decades to follow.

## NEW SUBSPECIALTY STRUCTURE PROPOSAL

We would like to propose a new training programme with a common trunk of Internal Medicine for 3 years for (“sub”) specialists in cardiology, pulmonology, rheumatology and gastroenterology. Recruitment to gastroenterology will probably entail 3 years of basic medical training in Internal Medicine. A “Brain-Drain” of young doctors willing to specialise abroad should be prevented by creating training posts in private hospitals. The private hospitals should be incorporated in these programmes, like the private hospitals in countries such as India (DN Reddy: personal communication).

A new “core” curriculum should include basic upper GI endoscopy and colonoscopy and a general GI training over 3 years. Additional clinical and endoscopy training should be organised in the final year, further specialisation in GI-oncology, interventional endoscopy or hepatology should be organised if necessary<sup>[8,10]</sup>.

## PRIVATE HOSPITALS AT “CROSSROADS”

With a government that is moving towards a National Health Insurance (NHI) system in South Africa, private hospitals need to work towards the goals of strengthening the existing health services in the country. The new African National Congress leadership seems more willing to listen to new ideas aimed at improving access and affordability and might move away from the current distrust and negativism that have soured the relationship between the government and private sector. Due to the financial situation in public hospitals, medical education and training are drifting to the private hospitals. Major

strides have been made by private hospital groups in addressing the medical needs of South Africa. The private hospital groups are now collectively training more nurses than government institutions. However, medical specialist training in private has not been officially defined nor is there any government policy to regulate its introduction. South Africa is living in 2 worlds when it comes to health services: those who have and those who have not (personal communication: Netcare CEO, Richard Friedland). It would be interesting to look to countries with a similar background between those who have and have not, like India.

## INDIA

India with a population of more than 1 billion people is struggling with similar problems. The Indian government has problems in managing and investing in multi-specialty hospitals. However, banks are encouraged to finance innovations in the private sector. Medical education state secretaries realise that joint ventures will enrich India’s health system, and subsequently poor people who can not get basic services will be helped. India is the most preferred treatment destination in the region due to major factors favouring its health care system. India’s private tertiary care medical institutions, with international accreditations, are among the best in the world. They are extremely well equipped with the latest technology and have excellent training standards.

For the last 10-15 years private hospitals in India assist in training and manpower development for medical specialist, nursing and administrative staff. Private hospitals train around 60% of (sub)specialties in India. To encourage the number of junior doctors aiming for subspecialisation, the National Board of Examinations was constituted. This Board is functioning like a National University “sans Frontiers”, where students are enrolled. These students are trained in smaller well-equipped non-academic hospitals. The fact that liver transplantation is being carried out only in the best private sector hospitals and not even in one academic hospital bears ample testimony to the level of competence at these hospitals<sup>[12]</sup>. In the early 1990s, the National Board of Examinations added the subspecialties to its list. Consequently a number of junior doctors moved to smaller cities, thereby upgrading the medical services in many provincial hospitals in rural areas. Up to 5-10 years ago the common trend was that Indian doctors training abroad were reluctant to return to India. This trend is also reversing with the set up of corporate hospitals all over India, which offer state-of-the-art equipment and good salaries for medical doctors.

This initiative was followed in the whole South-East region: since October 2007 the Main Board listed Pacific Healthcare Holdings Ltd., a leading Singapore provider of integrated multidisciplinary specialist healthcare services, organised trainee programmes in South-East Asia, in close cooperation with private hospitals in India.

## COMPARISON INDIA-SOUTH AFRICA

South Africa has many similarities with India - a large multicultural, multilingual population and coexisting Public (state-run) and Private Medical Care. The public health care system faces resource constraints just as in India. As such, comparisons between medical specialist training in South Africa and India may lead to ideas for improvement, which may be easily adaptable to local conditions.

### Medical education in South Africa

There are eight Medical Schools in South Africa, each attached to one of the provincial universities. Students apply to the school of their choice, and may be called in for an interview depending upon their matriculation results and racial background. There is a restructuring of admission policies to redress racial inequities and train more black doctors.

The young student enters medical school and typically undergoes a 6-year undergraduate training programme. Some years ago the Department of Health in collaboration with Health Professions Council extended internship to 2 years. The argument was that newly graduated doctors lacked skills and extra supervised exposure was needed. A compulsory community service was introduced. Specialist training can be completed in 4 years. Subspecialties require a further 2 years. Cardiology and Gastroenterology introduced abroad require 3-4 years instead of 2 years training<sup>[8]</sup>. A shorter common trunk system seems the way forward if the total training period is not to be lengthened.

## PROPOSAL FOR SUBSPECIALTY TRAINING PROGRAMME IN PRIVATE HOSPITALS

Differences between training in gastroenterology in USA/Europe *vs* India and South Africa are magnified by the obvious resource gap between Third and First world countries. Private hospitals should aim to identify shortages in specialist training positions and provide flexible funding options, thereby providing incremental growth in medical specialist training capacity in the public health system. This is already common practice in Europe.

The public and private sector have to co-operate in preparing for the increased number of medical trainees seeking training positions in developing countries.

A Task Force for specialist and subspecialist training in South Africa should be instituted to meet the current and future specialist training demands, as has been done in India and Eastern-Europe<sup>[13-15]</sup>. Redefining training programmes to train specialists more efficiently would be another goal based on a common trunk system for subspecialties of Surgery and Internal Medicine.

### Priority 2010-2015

This "priority specialty group for South Africa" should be

identified by Task Forces for Specialist training in close co-operation with the Colleges of Medicine of South Africa. A working group for this Task Force should be instituted with the following tasks: (1) Analysing the South African medical workforce flow data, identifying the numbers entering and leaving workforces in the public and private spheres; (2) Comparing workforce numbers in South Africa with India/Middle East *vs* Europe/USA; (3) Analysing the presence of specialities in different geographical locations; (4) Defining the minimal required service access in rural areas for the basic medical specialties such as Internal Medicine, Surgery, Neurology, Gynaecology and Paediatrics; (5) Discussing the current system of 13-15 years of training for a young specialist to become registered and look for a shorter medical training period to prevent the Brain Drain; (6) Defining the minimal numbers of trainees for subspecialty training in private hospitals and providing and defining funds for training in those private hospital settings in close cooperation with Academic Hospitals; (7) Acknowledging that the medical specialist trainee workforce is complementary to public hospital service. Therefore expansion of training opportunities outside traditional academic settings in private hospitals should be defined and organized. The Medical Board of the HPCSA should expand its role to control and regulate the quality of training, not only in the Public Hospitals but in the private hospitals and prioritizing disciplines facing immediate workforce shortage or maldistribution around the country especially in remote rural areas; and (8) Development of tertiary referral centres would prevent loss of foreign exchange by keeping private patients in the country instead of leaving and spending their money abroad.

## CONCLUSION

Medical specialist training is acknowledged as an essential component of health care delivery systems. South Africa, both a developing and developed country similar to India, has a shortage of medical specialists and subspecialists. It is therefore imperative that a national solution should be developed to address the challenges of maintaining an adequate specialist workforce<sup>[6,16,17]</sup>.

We acknowledge that the academic medical complexes have been and will continue to be the cornerstone of medical training around the world. Over recent years, it has become evident that clinical training will expand from the traditional settings of public hospitals into private hospitals. India showed us the way in how to develop this, keeping junior doctors in the country and giving them access to proper specialist training. This dual pathway for obtaining (sub)speciality training in India over the last 20 years has resulted in almost double the number of specialist doctors, who are now also working inside the smaller rural hospitals in India. Additionally, the massive increase in medical student numbers underscores the need to increase the capacity to train these students in specializations to prevent further "Brain-Drain".

The complexity of medical specialist training in South Africa requires the cooperation of many stakeholders such as, government agencies, the ministries of health and education, assurance companies, academic hospital complexes, the medical board of the health professions council, CMSA, public and private hospitals, fellows registrars and junior medical doctors. A National Task Force should clarify the necessary resource investments and define accountabilities. A Task Force should co-ordinate and govern, while maintaining appropriate flexibility<sup>[18]</sup>. We hope that the Medical Societies and Associations in South Africa will consider this expansion of specialist training into settings beyond the traditional teaching hospital model.

Can future specialist training in South Africa involve both, and possibly partner, the private sector and the public teaching system? South Africa could then become a model for Sub-Saharan Africa, as India already is for the poorer Asian countries.

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## Radical vs conservative surgery for hydatid liver cysts: Experience from single center

Sami Akbulut, Ayhan Senol, Arsenal Sezgin, Bahri Cakabay, Mehmet Dursun, Omer Satıcı

Sami Akbulut, Bahri Cakabay, Department of Surgery, Diyarbakir Education and Research Hospital, 21400 Diyarbakir, Turkey  
 Ayhan Senol, Department of Radiology, Diyarbakir Education and Research Hospital, 21400 Diyarbakir, Turkey  
 Arsenal Sezgin, Department of Pathology, Diyarbakir Education and Research Hospital, 21400 Diyarbakir, Turkey  
 Mehmet Dursun, Department of Gastroenterology, Veni Vidi Hospital, 21100 Diyarbakir, Turkey

Omer Satıcı, Department of Biostatistics, Dicle University, Faculty of Medicine, 21380 Diyarbakir, Turkey

**Author contributions:** Akbulut S and Cakabay B performed the surgical procedure; Akbulut S contributed writing the article and review of the literature, as well as undertaking a comprehensive literature search; Akbulut S and Sezgin A contributed design and manuscript preparation; Sezgin A provided the pathological information; Senol A provided the radiological information; Dursun M performed ERCP and contributed to design and manuscript preparation; Satıcı O provided the statistical information.

**Correspondence to:** Sami Akbulut, MD, Department of Surgery, Diyarbakir Education and Research Hospital, 21400 Diyarbakir, Turkey. [akbulutsami@gmail.com](mailto:akbulutsami@gmail.com)

Telephone: +90-412-2285434 Fax: +90-412-2295912

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### Abstract

**AIM:** To compare the efficacy and safety of radical and conservative surgical interventions for liver hydatid disease.

**METHODS:** The study comprised 59 patients in two groups who had undergone radical and conservative surgical procedures for liver hydatid disease in our department between 2004 and 2009. Preoperative diagnostic tools, medical treatments, demographic and clinical characteristics, postoperative follow-up, and recurrence were compared in both groups.

**RESULTS:** This non-randomized retrospective study

included 59 patients who had undergone liver hydatid disease surgery. The radical technique was used in 18 patients (mean age:  $42.1 \pm 13.5$  years, seven male, 11 female), and the conservative technique was used in 41 patients (mean age:  $43.5 \pm 13.9$  years, 17 male, 24 female). The follow-up period ranged from 3 to 58 mo. Although operative time was significantly shorter in the conservative group ( $P < 0.001$ ), recurrence was significantly reduced in the radical group ( $P = 0.045$ ). No statistically significant differences were found in terms of hospitalization duration, cyst count and size, location, postoperative complications, scolical solution usage, or follow-up duration between the two groups.

**CONCLUSION:** The more effective method for preventing postoperative recurrence is radical surgery. Endoscopic retrograde cholangiopancreatography for bile leakage in the early postoperative period may decrease the requirement for repeat surgery.

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**Key words:** Echinococcosis; Endoscopic retrograde cholangiopancreatography; Digestive system surgery

**Peer reviewers:** Hussein M Atta, MD, PhD, Department of Surgery, Faculty of Medicine, Minia University, Mir-Aswan Road, El-Minia 61519, Egypt; Giedrius Barauskas, Professor, Department of Surgery, Kaunas University of Medicine, Eiveniu str. 2, Kaunas, LT-50009, Lithuania; Dr. Cuneyt Kayaalp, MD, Professor, Department of General Surgery, Staff Surgeon of Gastrointestinal Surgery, Turgut Ozal Medical Center, Inonu University, Malatya 44315, Turkey

Akbulut S, Senol A, Sezgin A, Cakabay B, Dursun M, Satıcı O. Radical vs conservative surgery for hydatid liver cysts: Experience from single center. *World J Gastroenterol* 2010; 16(8): 953-959 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i8/953.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i8.953>

## INTRODUCTION

Hydatid cysts are common in societies where agriculture and raising animals are common, and hydatid disease continues to be a serious public health problem in many countries, including Turkey<sup>[1-3]</sup>. Hydatid cysts can develop in any organ of the body, but are most frequently seen in the liver (60%-70%) and lungs (20%-30%)<sup>[4-6]</sup>. Infection rates are lowest in urban environments; in endemic rural areas, prevalence rates of 2%-6% or higher have been recorded<sup>[7]</sup>. In Turkey, hydatid disease is more common in Eastern and Middle Anatolia and in the Marmara and Trakya regions<sup>[3]</sup>. The first step in the prevention of hydatid disease is basic hygiene and the second step involves the approach to treatment. No consensus exists regarding the optimal treatment, although medical treatment is effective against larval *Echinococcus granulosus* (*E. granulosus*). Surgical treatment varies from complete resection to minimally invasive procedures (e.g. percutaneous aspiration)<sup>[8-12]</sup>.

In this study, we present the surgical approach to hydatid disease patients at our clinic in Diyarbakir, eastern Eastern Anatolia, and a comparison of the efficacy and safety of radical surgery and conservative surgery for liver hydatid disease.

## MATERIALS AND METHODS

A total of 59 patients who had undergone surgery for liver hydatid cysts between January 2004 and July 2009 were investigated retrospectively. All the patients in the study were operated on by two surgeons. Patients were divided into two groups according to the type of surgery. The radical surgery group ( $n = 18$ ) consisted of patients whose treatment involved pericystectomy or left lobe segmentectomy. The conservative surgery group ( $n = 41$ ) included patients who underwent conservative surgery, including partial cystectomy plus external drainage plus omentopexy, or cystectomy plus external drainage. The data analyzed included patient age, sex, occupation, residence, symptoms, diagnostic tools used, type of cyst, type of surgical procedure, and maximum diameter of the cyst, postoperative complications, preoperative and postoperative anti-helminthic treatment, recurrence, and length of follow-up.

### Clinical presentation

For 43 (72.8%) patients, abdominal pain was the initial symptom of hydatid disease. Seven of 43 patients experienced recurrence of the disease between 5 and 22 years after one or more operations elsewhere. In 14 patients, the diagnosis was made incidentally during a medical checkup. Two patients were diagnosed with a hydatid cyst preoperatively during diagnostic laparoscopy for gynecological disease.

### Preoperative diagnosis

The preoperative evaluation of the patients included liver function tests, a complete blood count, indirect

**Table 1** Comparison of both groups according to Gharbi classification

Type of cyst	Gharbi classification	Radical surgery	Conservative surgery	Total
Type I	Pure fluid collection		Excluded	0
Type II	Fluid collection with a split wall	2	7	9
Type III	Fluid collection with septal	5	14	19
Type IV	Heterogenous acho pattern	4	8	12
Type V	Reflecting thick walls		Excluded	0
Type III-IV		4	7	11
Type III-IV-V		3	5	8

hemagglutination antibody (IHA), abdominal ultrasonography (USG), and abdominal computed tomography (CT). The cysts were classified according to the five categories described by Gharbi *et al*<sup>[13]</sup>. The distribution of the patients according the Gharbi classification is shown in Table 1. CT was performed in 19 patients who had more than one cyst, to obtain detailed information on the location of the cysts (Figure 1, Figure 2A and B). Magnetic resonance cholangiopancreatography was used to confirm the diagnosis in two patients who had suspicious dilatation of the common bile duct (CBD) and three patients with elevated liver function tests. Dilatation of the CBD was observed in only one patient on magnetic resonance imaging. This patient underwent a sphincterotomy *via* endoscopic retrograde cholangiopancreatography (ERCP) preoperatively.

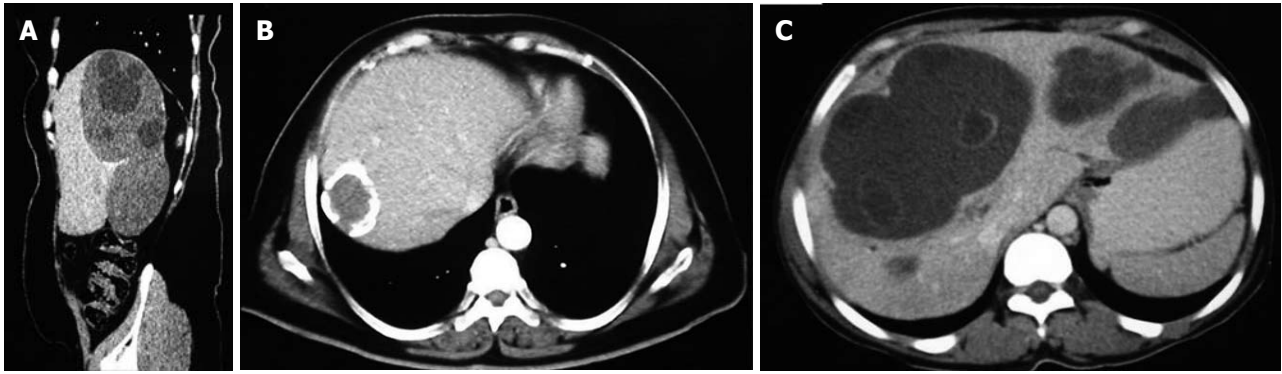
### Cyst characteristics

The size of the cysts was measured by USG and CT; the number of the cysts was counted radiologically and confirmation was made by sight during surgery.

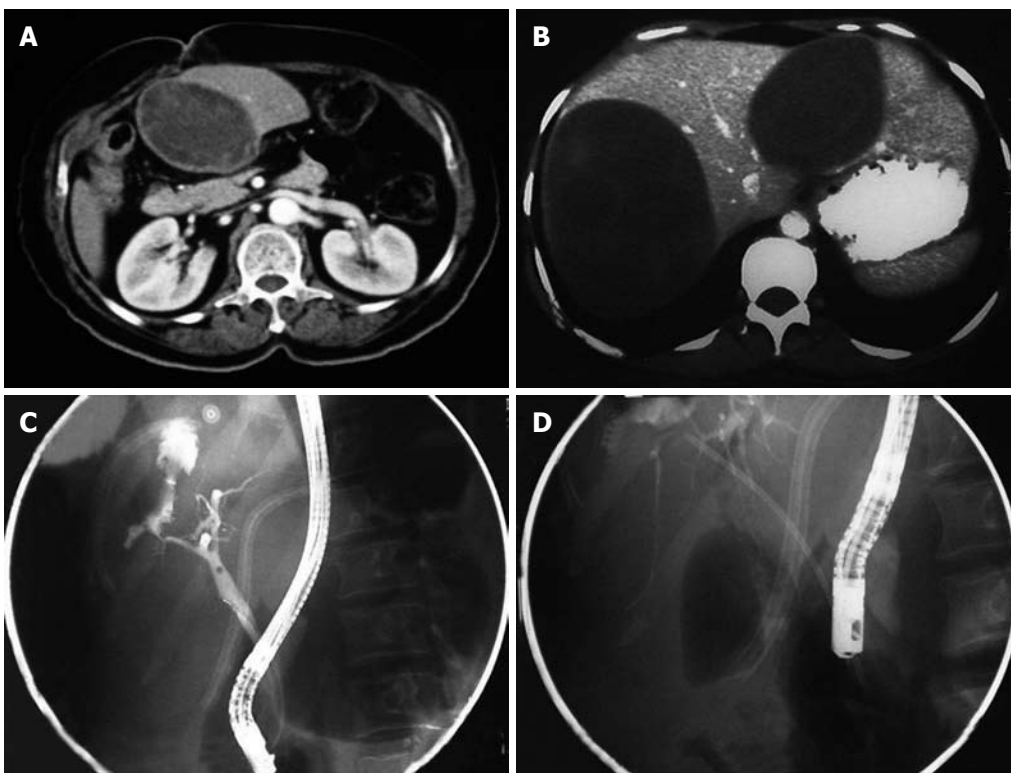
The mean  $\pm$  SD size of the cysts was  $16.28 \pm 6.97$  cm (range: 8-32 cm) in the RS group *vs*  $12.98 \pm 4.90$  cm (range: 6-26 cm) in the CS group. There was no significant difference in cyst count ( $P > 0.694$ ), cyst size ( $P > 0.088$ ) and in location ( $P > 0.650$ ) between the two groups. The other cyst characteristics are summarized in Table 2.

### Our approaches to liver hydatid cysts

As liver hydatid disease is endemic in the area where we work, a general consensus has been established for patients to be operated upon. Accordingly, the patients presenting at our clinic were categorized according to the following criteria Gharbi I : all the patients in this group were referred to other hospitals as there was the possibility of percutaneous drainage. We have recently started laparoscopy drainage techniques. This group of patients was not included in this study; (2) Gharbi II, III and IV group cases with cysts sized  $> 10$  cm were recommended for surgery; (3) Type II-IV cystic lesions in which there was compression underwent surgery regardless of size (e.g. in our cases where location was in the left lobe lateral segment and posterior to the gallbladder); (4) In



**Figure 1** Multi-detector computed tomography (CT) images in different planes following contrast injection. A: Sagittal plane multi-detector CT images showed cystic lesions in segments 6 and 7 of the right liver lobe; B: A cystic lesion with a calcified wall located in the subcapsular region in the right lobe posterior segment was seen in contrast-enhanced axial plane CT sections; C: Contrast-enhanced axial plane CT images showed widespread cysts with separate membranes in an enlarged liver.



**Figure 2** Contrast-enhanced axial CT and endoscopic retrograde cholangiopancreatography (ERCP) images of three different patients. A: A cystic lesion located in segment 3 of the left lobe was seen in contrast-enhanced axial plane CT images; B: A cystic lesion in the right lobe posterior superior and left lobe medial segment was seen in oral and intravenous contrast-enhanced axial plane CT images; C: Leakage of contrast material into the cyst cavity was observed after placing a cannula and injecting radiopaque material into the biliary tract; D: The same patient after performing a sphincterotomy and inserting a 7 Fr biliary stent.

the Gharbi V group, if the patient was symptomatic or if there was compression of the stomach, gallbladder or intestines, surgery was recommended (there were no such patients in this study). Otherwise type V cysts were kept under observation; and (5) Asymptomatic cysts < 5 cm were followed-up. If the size was seen to have increased, firstly albendazole treatment was administered. If the size continued to increase despite this, then surgery was planned.

#### **Albendazole treatment**

All patients with hydatid disease at our clinic were ad-

ministered 10 mg/kg albendazole for 14-21 d preoperatively. During this period, liver function tests were closely observed. For all patients undergoing surgery, the same treatment protocol was recommenced on postoperative day 1 and continued for 14-21 d. If patients experienced recurrence during follow-up, again 14-21 d treatment was administered preoperatively, and the postoperative treatment period was 2 mo.

#### **Surgical procedure**

In all patients, the pericystic area and operating field were covered with sponges soaked with 20% hypertonic saline

**Table 2** Comparison of surgical characteristics, follow-up time and postoperative complications among the both groups *n* (%)

Surgical features	Radical surgery	Conservative surgery	Total	<i>P</i>
Number of patients	18 (30.5)	41 (69.5)	59 (100)	
Operation time (min)	83.25 ± 12.09	51.97 ± 15.07	60.91 ± 19.92	< 0.001
Hospitalization time (d)	7.62 ± 1.49 (5-10)	7.24 ± 1.86 (4-21)	7.42 ± 1.69	NS
Cyst count	1.72 ± 1.0 (1-4)	1.99 ± 1.33 (1-6)	1.86 ± 1.24	NS
Cyst size (cm)	16.27 ± 7.1 (8-32)	12.98 ± 4.9 (6-26)	13.98 ± 5.83	NS
Location				NS
Right	9 (50.0)	23 (56.1)	32 (54.2)	
Left	5 (27.8)	7 (17.1)	12 (20.4)	
Bilateral	4 (22.2)	11 (26.8)	15 (25.4)	
Postoperative complication				NS
Biliary leakage	2 (11.1)	3 (7.3)	5 (8.5)	
Atelectasis	1 (5.5)	1 (2.4)	2 (3.3)	
Bilioma	0	1 (2.4)	1 (1.6)	
Pneumonia	0	2 (4.8)	2 (3.3)	
Wound infection	0	3 (7.3)	3 (5.0)	
Surgical procedures				NS
Pericystectomy	14 (77.7)	0	14 (23.7)	
Segmentectomy	4 (22.3)	0	4 (6.8)	
PC + D + Om	0	36 (87.8)	36 (61)	
PC + D	0	5 (12.2)	5 (8.5)	
Scolocidal solution				NS
Saline	12 (66.7)	29 (70.7)	41 (69.5)	
Betadine	6 (33.3)	12 (29.3)	18 (30.5)	
Follow-up time (mo)	30.72 ± 17.92	30.29 ± 15.08	30.42 ± 15.71	NS
Recurrence disease	0	7 (17)	7 (11.8)	0.045

NS: Not significant.

or 10% povidone-iodine (Betadine®) solution. Before injecting this solution into the cyst cavity, the cyst was punctured and as much fluid as possible was aspirated to prevent dilution of the agent. Then, 10% Betadine® or 20% hypertonic serum was instilled into the cyst cavity in a volume equal to the amount aspirated. After 15 min, the cyst fluid was aspirated again.

The surgical method was decided by collating the factors of patient age, general condition, systemic disease, American Society of Anesthesiologists (ASA) values, viral hepatitis markers, radiological images, liver function tests, blood group and the surgeon's experience.

**Radical surgery:** In the pericystectomy technique, the cyst was totally removed together with 1 cm of the liver parenchyma, without opening the cavity. In a left lobe lateral segmentectomy, to secure the vasculature of the left lobe lateral segment, the segmentectomy was performed after taking the mesentery. In both situations, one or two drains were placed in the operated area.

**Conservative surgery:** The anterior wall of the cystic lesion was removed as widely as possible. All the components of the cyst were removed from the interior. After

**Table 3** Comparison of both groups in terms of clinical and demographic characteristics *n* (%)

Demographic features	Radical surgery	Conservative surgery	Total	<i>P</i>
Number of patient	18 (30.5)	41 (69.5)	59 (100)	NS
Male	7 (38.9)	17 (41.5)	24 (40.6)	
Female	11 (61.1)	24 (58.5)	35 (59.4)	
Age (yr)	42.1 ± 13.87	43.5 ± 13.9	43.0 ± 13.77	NS
Distribution according to age				NS
< 40	6 (33.3)	17 (41.46)	23 (39.0)	
40-60	9 (50.0)	19 (46.34)	28 (47.5)	
> 60	3 (16.7)	5 (12.20)	8 (13.5)	
Residence				NS
Village	9 (50.0)	26 (63.4)	35 (59.3)	
Suburban	7 (38.9)	9 (22.0)	16 (27.1)	
Urban	2 (11.1)	6 (14.6)	8 (13.6)	
Occupation				NS
Housewife	7 (38.9)	21 (51.2)	28 (47.5)	
Shepherd	4 (22.2)	7 (17.1)	11 (18.6)	
Farmer	4 (22.2)	8 (19.5)	12 (20.3)	
Student	1 (5.6)	3 (7.3)	4 (6.8)	
Officer	2 (11.1)	2 (4.9)	4 (6.8)	

washing the operated area with saline or Betadine solution, one or two drains were placed. Omentopexy was not performed when cysts were located proximally but was performed when cysts were located inferior to the liver.

### Postoperative follow-up

Postoperative follow-up was carried out by physical examination, IHA, USG and CT. In order for there not to be any confusion as to whether there had been any recurrence of the disease in the future, all patients underwent IHA and abdominal CT examination at the end of the first postoperative month. Thus, we obtained baseline results for future comparison. The follow-up period was completed with IHA + USG at 6 mo, IHA + CT at 1 year, IHA + USG at 18 mo, IHA + CT at 2 years, IHA + CT at 3 years, and only USG at 4 years. During this period, any cases suspected of recurrence were examined more frequently.

### Statistical analysis

All data were collected and analyzed using SPSS version 11.5 (SPSS, Chicago, IL, USA). Comparison between two groups was done by independent sample Student's *t* test for continuous variables and  $\chi^2$  test for categorical variables. *P* < 0.05 was considered statistically significant.

## RESULTS

All demographic and clinical characteristics, operative factors, postoperative outcomes, and surgical complications of the patients in the two groups are presented in Tables 2 and 3. Group-CS comprised 41 patients, 17 male and 24 female, with a mean age of 43.5 ± 13.9 years (range: 17-67 years). Group-RS comprised 18 patients, seven male and 11 female, with a mean age of 42.1 ±



**Table 4** Postoperative complications and management

Complications	Radical surgery	Conservative surgery	Management
Biloma	0	1	Percutaneous drainage (other center)
Wound infection	0	3	Secondary healing therapy
Atelectasis	1	1	PEEP
Pneumonia	0	2	PEEP + antibiotherapy
Biliary leakage	2	3	3 cases: ERCP + sphincterotomy 1 case: ERCP + sphincterotomy + internal biliary stent 1 case: choledoc exploration + T-tube drainage

PEEP: Positive end-expiratory pressure.

13.5 years (range: 19-62 years). There was no significant difference in age ( $P > 0.703$ ), sex ( $P > 0.831$ ), residence ( $P > 0.416$ ) and occupation ( $P > 0.853$ ) between the two groups.

### Surgical characteristics

In the radical surgery group, 1-4 cysts occurred in 10, five, one and two patients, respectively. The cysts were located in the right lobe in nine patients, left lobe in five, and in both lobes in four. Fourteen patients with an exophytic cyst underwent complete pericystectomy, whereas four patients with two cysts in the left lateral segment underwent left lobe lateral segmentectomy.

In the conservative surgery group, 1-6 cysts occurred in 23, eight, four, three, two and one patients, respectively. The cysts were located in the right lobe in 23 patients, left lobe in seven, and in both lobes in 11. Partial cystectomy plus external drainage plus omentopexy was performed in 36 patients, whereas five patients had partial cystectomy plus external drainage.

In four patients, intraoperative observation revealed that the cyst cavity was connected to the biliary system. In one of these four patients, the diameter of the CBD was enlarged and exploration of the CBD and T-tube drainage were performed. In three of these four patients, the open biliary duct in the cyst was closed primarily. None of these four patients had postoperative bile leakage. Mean operation time of the radical and conservative surgery group, was  $83.25 \pm 12.0$  min and  $51.97 \pm 15.0$  min, respectively. The mean operation time was significantly longer in the radical than conservative surgery group ( $P < 0.001$ ). There was no significant difference in duration of hospitalization ( $P > 0.361$ ), and in scolicalid use ( $P > 0.756$ ) between the two groups.

### Early postoperative complications

The postoperative complications and the approach to these complications are summarized in Table 4. There was no significant difference in postoperative complications ( $P > 0.338$ ) between the two groups. On postoper-

ative days 2-4, five patients had drainage of 250-400 mL of bile. Since the drainage did not decrease after being monitored for 3 d, ERCP was performed and four patients underwent sphincterotomy. In the case shown in Figure 2C and D, because the open biliary duct in the cystic cavity was dilated, a sphincterotomy was performed in this patient and an internal biliary stent that reached the leaking biliary duct was placed. Seven days after drainage stopped, which was 3 mo after placement, the stent was removed. One patient had 300-350 mL of bile leakage and elevated liver function tests. In addition, the CBD was dilated on CT. This patient had a previous gastrojejunostomy, therefore, ERCP was unsuccessful and repeat surgery was performed. Exploration of the CBD and T-tube drainage were performed. The drainage stopped on postoperative day 18 and the tube was removed.

One patient developed a postoperative biloma that was confirmed by CT. This patient underwent percutaneous drainage elsewhere. Local infection developed in three patients, who were treated appropriately. Four elderly patients developed atelectasis and pneumonia on the fourth postoperative day. These patients were treated with respiratory physiotherapy and antibiotic therapy.

### Follow-up and recurrence

The follow-up duration ranged from 3 to 58 mo. Seven patients developed recurrences at 8-17 mo (mean: 13.1 mo) postoperatively. Although the recurrence rate was reduced significantly in the radical surgery group ( $P < 0.045$ ), no statistically significant difference was found in terms of follow-up duration between the two groups ( $P > 0.429$ ).

The preoperative IHA values were similar in all patients who had recurrences. Cystic lesions in all seven patients were confirmed using CT. Saline solution was used as the scolicalid agent in all patients who had recurrence. No recurrence was observed in patients who underwent radical surgery. Two of the seven patients refused repeat surgery, since they were asymptomatic. In the remaining five patients, the recurrent cystic lesions were treated with a partial cystectomy and drainage.

## DISCUSSION

Hydatid liver disease is still endemic in certain regions of the world. The incidence of hydatid disease in Turkey ranges from 2/1 000 000 to 1/2000 in different studies<sup>[14-17]</sup>. The symptoms of hydatid liver disease generally differ according to the location, size, and grade of the cyst<sup>[18,19]</sup>. Overall, 75% of the patients are identified incidentally and are asymptomatic<sup>[4,19]</sup>. In our study, 23.7% of the patients were identified incidentally at routine examinations and 72.8% of the patients were identified after complaining of abdominal pain. All symptomatic and asymptomatic patients with cysts  $> 5$  cm in diameter should be considered as candidates for surgery<sup>[19]</sup>. In our study, 72.8% of the patients were symptomatic, while

27.2% were asymptomatic. In all of the patients, the cysts found were  $\geq 6$  cm.

Which treatment modality should be used for liver hydatid cysts is still a subject of controversy. Agents which are effective on *E. granulosus* larvae, such as albendazole and mebendazole are used as medical therapy. It has been reported that this treatment is administered 7-21 d preoperatively and continues for 2-3 mo postoperatively<sup>[6,18,20,21]</sup>.

As this treatment is given both pre- and postoperatively, the risk of postoperative secondary disease developing has been seen to be low. When we look at choice of surgical treatment used, conservative surgery is the most common, then radical surgery, and more recently, laparoscopy. Conservative surgery is recommended for the elderly, those with high ASA levels, deeply located cysts  $\geq 10$  cm, more than one cyst at the same time, cysts located in both lobes and in the liver posterior segment. Radical surgery can be carried out by experienced hepatobiliary surgeons on younger patients, where the cysts are located in the anterior superior liver and in the left lobe lateral segment, those with exophytic location, alveolar hydatid cyst and cysts  $\geq 5$  cm in diameter.

Although the rate of recurrence is lower with radical surgery, application is limited as the associated morbidity and mortality rates are high<sup>[6,18,22]</sup>. In the radical surgery cases in our study, four were in the left lobe lateral segment with straightforward localization and the other 14 were exophytic locations, therefore, there was no mortality or morbidity related to surgery. The laparoscopic approach is a treatment method developed in recent years using an umbrella trocar to perform partial or total cystectomy<sup>[23]</sup>.

Topcu *et al*<sup>[2]</sup> have reported cysts with biliary system involvement that were  $> 10$  cm in diameter in 22.2% of patients. In our study, the biliary system was connected to the cyst cavity in nine patients (15.2%); this was discovered intraoperatively in four patients and postoperatively in five. Six of these cysts were in the right lobe and  $> 15$  cm in size.

Most studies assert that rupture of the cyst into the biliary system is the most important complication<sup>[2]</sup>. In our study, while exploring the CBD in a patient with a dilated CBD, a daughter vesicle was found and T-tube drainage was performed. The cysts in our study were larger than those reported in the literature, although no rupture into the biliary system was observed with these large cysts.

Ezer *et al*<sup>[18]</sup> found biliary leakage in 18.3% of their patients and five of the leaks closed spontaneously within 7 d. Kayaalp *et al*<sup>[24]</sup> found biliary leakage in 26% of their patients and seven of the leaks closed within 1 wk, while biliary fistulas occurred in five patients. In our study, biliary leakage occurred in only 8.5% of the patients and a biloma developed in one (1.6%) patient.

Recurrence is one of the major problems in the management of hepatic liver disease. Recurrent disease is defined as the appearance of new active cysts after

therapy for intra- or extrahepatic disease. The failure to achieve permanent control of the primary treated cyst is considered to be the cause of local recurrence. Local recurrence occurs after surgical or radiological intervention and manifests as the reappearance of live cysts at the site of a previously treated cyst, or the appearance of new extrahepatic disease resulting from procedure-related spillage. The incidence of recurrence after liver cyst hydatid surgery is reported to range from 0% to 25% in various studies<sup>[4,18,20,25-28]</sup>. Although this rate ranges from 0% to 4.65% in patients who have had radical surgery<sup>[4,26,28]</sup>, it ranges from 4.65% to 25% in those who have had conservative surgery<sup>[4,18,20,21,27,28]</sup>. In our study, recurrence occurred in none of the patients treated with radical surgery and 11.8% of those treated with conservative surgery. Recurrence may occur many years later, however, and longer follow-up is recommended when possible.

Postoperative recurrence has been reported to have developed at different periods from 4 mo to 35 years<sup>[4,18,20,26-28]</sup>. In our study, this period was found to be a mean 13.1 mo. Our experience indicates that it is beneficial to have a longer follow-up period for cases of conservative surgery.

There are several studies which state that it is preferable to use a conservative approach when possible on patients who develop recurrence, because there are more problems associated with a second operation, such as mortality, damage to the gallbladder, and a lengthy postoperative stay. In our own study, five cases in which recurrence developed were treated with conservative surgery.

Several studies have been published on the use of different concentrations of scolical agents over different periods of time for the development of complications such as recurrence and postoperative cholangitis. The leading scolical agents that have been the subject of research are hypertonic saline (3%-30% and 5-30 min), povidone-iodine (10%), formalin, cetrimide (0.1%-0.4%), chlorhexidine (0.04%-4%), hydrogen peroxide (3%) and ethyl alcohol (95%).

Hypertonic saline solution and Betadine are the most frequently used scolical agents in Turkey<sup>[2,29,30]</sup>. Besim *et al*<sup>[29]</sup> have compared the effects of different scolical agents at different concentrations and found that the use of Betadine, 20% saline, 3% hydrogen peroxide, 95% ethyl alcohol, and 10% Savlon solutions for 15 min inactivated the protoscoleces. The infectivity was higher using concentrations of  $< 20\%$  and for  $< 5$  min. Sclerosing cholangitis has been reported with the use of 20% and 30% hypertonic saline solutions<sup>[30-32]</sup>. In our study, we used either 20% saline or 10% Betadine solution in the cystic cavity for 15 min. None of our cases developed any metabolic problem or sclerosing cholangitis.

In conclusion, the most effective method for preventing postoperative recurrences is radical surgery. This is indicated for patients meeting the indications in terms of age, medical history, location and size of the cyst, and relation of the cyst to the vasculature and biliary

tree. When  $\geq 250$  mL of biliary leakage occurs in the early postoperative period, performing sphincterotomy with ERCP or a stent within 3-5 d allows early discharge from hospital, decreases the need for repeat surgery, and reduces the costs and psychosocial problems that may occur with prolonged follow-up.

## COMMENTS

### Background

The development of hydatid disease associated with *Echinococcus granulosus* is still an important health problem. Treatment choices are medical, conservative surgery, radical surgery, laparoscopic surgery and PAIR technique. The advantages and disadvantages of these techniques have not as yet been fully clarified.

### Research frontiers

In this study, although the patients who had undergone radical surgery did not experience any recurrence of the disease during postoperative follow-up, the operating time was significantly shorter for conservative surgery.

### Peer review

The overall impression of the article is positive and it might be relevant for publication in *World Journal of Gastroenterology* as it deals with an important and relatively rare disease, which has no clear and unequivocal management guidelines.

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## Colorectal cancer in Guangdong Province of China: A demographic and anatomic survey

An-Gao Xu, Zhi-Jin Yu, Bo Jiang, Xin-Ying Wang, Xu-Hui Zhong, Ji-Hong Liu, Qiu-Yun Lou, Ai-Hua Gan

An-Gao Xu, Zhi-Jin Yu, Xu-Hui Zhong, Ji-Hong Liu, Qiu-Yun Lou, Ai-Hua Gan, Department of Gastroenterology, Huizhou Municipal Central Hospital, Huizhou 516001, Guangdong Province, China

Bo Jiang, Xin-Ying Wang, Department of Gastroenterology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China

Author contributions: Xu AG and Yu ZJ provided vital reagents, designed the study and finished all the statistics analysis; Jiang B and Wang XY collected the data of patients in Nanfang Hospital and wrote the manuscript; Zhong XH collected the data of patients in Meizhou People's Hospital; Liu JH collected the data of patients in North-Guangdong People's Hospital of Shaoguan; Lou QY collected the information of patients in Huizhou Central People's Hospital; Gan AH collected the information of patients in the Affiliated Hospital of Guangdong Medical College.

Correspondence to: Dr. An-Gao Xu, Department of Gastroenterology, Huizhou Municipal Central Hospital, Huizhou 516001, Guangdong Province, China. [angao62@21cn.com](mailto:angao62@21cn.com)

Telephone: +86-752-2288288 Fax: +86-752-2288288

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(2004-2006) and the proportion of young CRC patients decreased from 8.0% to 5.9% over the same period. Of 8172 lesions, 4434 (54.3%) were located in rectum and 3738 (45.7%) in colon. The incidence of rectal cancer decreased significantly from 59.4% (1989-1991) to 51.8% (2004-2006) and right sided colon cancer increased from 40.6% to 48.2%. The mean age, anatomic distribution, histological type and differentiation degree were significantly different among the four geographical areas ( $P < 0.05$ ).

**CONCLUSION:** The hospitalization rate for CRC has increased in Guangdong in recent years. The characteristics of CRC from the five hospitals located in the four different areas of Guangdong Province are also different. Further studies are needed to assess more recent trend in the incidence and prevalence of CRC as well as the respective roles of genetic and environmental factors in CRC.

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**Key words:** Colorectal cancer; Survey; Characteristics; Differentiation

**Peer reviewer:** Dr. Charles P Heise, MD, Associate Professor, Department of Surgery, University of Wisconsin School of Medicine, 600 Highland Avenue, Madison, WI 53792, United States

Xu AG, Yu ZJ, Jiang B, Wang XY, Zhong XH, Liu JH, Lou QY, Gan AH. Colorectal cancer in Guangdong Province of China: A demographic and anatomic survey. *World J Gastroenterol* 2010; 16(8): 960-965 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i8/960.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i8.960>

### Abstract

**AIM:** To determine the basic demographic features of colorectal cancer (CRC) in five hospitals located in four different areas of Guangdong Province, China.

**METHODS:** A review of patient records from 1986 to 2006 from five hospitals was conducted. Patient data was obtained, including age, gender, location of lesions, staging and histological type of CRC. The Chi-square test was used to assess differences in rates and a significance level of 0.05 was used. Univariate comparisons were made *via* Fisher's exact tests.

**RESULTS:** Analysis was carried out on 8172 CRC patients, 6.1% (499/8172) of the patients were aged  $\leq 30$  years. The peak incidence was between the ages 61-70 years (27.8%). The mean age at CRC diagnosis increased from 52 years (1986-1988) to 60 years

### INTRODUCTION

Colorectal cancer (CRC) is one of the most common gastrointestinal tumors and ranks as the third most com-



mon cancer in the world<sup>[1]</sup>. CRC has been thought to be less common in Asian compared with Western countries<sup>[2,3]</sup>. However, recent studies from Japan, Korea and Hong Kong have shown that CRC has not only high incidence rates but also an increasing trend in the population<sup>[4-6]</sup>. Previous studies implicated that cause of CRC was more closely related to dietary habits and geography than race<sup>[7-9]</sup>. The incidence of CRC in China was lower than that in the West, but has increased in recent years<sup>[10,11]</sup> and has become a substantial cancer burden in China, particularly in the more developed provinces. Some studies have reported changes in the characteristics of colorectal cancers in China<sup>[12,13]</sup>. However, due to a lack of an effective nation-wide colorectal cancer surveillance system, there has been little information available on the relationship between colorectal cancer and geographical environment and economic status in China. The basic demographic characteristics of CRC have changed with the changing of lifestyle in Guangdong. The regional characteristics of CRC in Guangdong need to be better defined.

Guangdong Province is divided into four regions according to geographical location, which include: Triangle area, North area, West area and East area, each area is different in economic status and dietary habits. Therefore, to determine the basic demographic features of patients with CRC in different regions in Guangdong and the trends in different year group, five hospitals were selected from these four areas and 8172 patient records were reviewed. Age, gender, anatomic distribution and histological type were characterized and compared in different areas and year group. The aim of this study was to gain a broader picture of CRC in Guangdong Province and provide important information on the changing epidemiology of this disease over a period of 20 years.

## MATERIALS AND METHODS

We developed a registration form to assist in obtaining the clinical characteristics of 8172 CRC cases in the five hospitals [Nanfang Hospital and Huizhou Central People's Hospital (Pearl River Triangle area in Guangdong, highly developed region), North-Guangdong People's Hospital of Shaoguan (North area of Guangdong, a developed region), the Affiliated Hospital of Guangdong Medical College (West area of Guangdong, an under-developed area) and Meizhou People's Hospital (East area of Guangdong, an under-developed area)]. All cases in this study were identified using a series of unified Code for the following review. The data was collected retrospectively over a 20-year period from January 1986 to December 2006. Patient basic demographic data including age and gender were recorded. The location of the tumor was recorded and classified as right sided (caecum, ascending colon, hepatic flexure and transverse colon) or left sided (splenic flexure, descending colon, sigmoid colon and rectum). Staging of the tumor was graded according to Duke's classification and histological

Table 1 Basic demography of study population (*n* = 8172)

Gender	
Male:female	4841:3331
Mean age (yr)	56
Area	
Pear River Triangle area	4327
East area	1583
West area	1480
North area	782

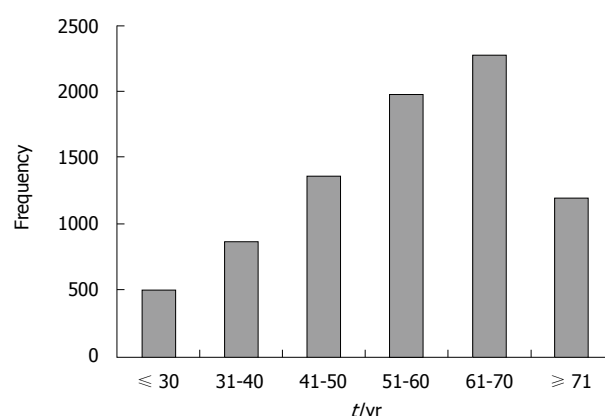


Figure 1 Frequency of colorectal cancer (CRC) cases by age (*n* = 8172).

types were also recorded. The histological type of CRC was determined by two experienced pathologists. The study protocol was approved by the Ethics Committee of Nanfang Hospital.

## Statistical analysis

According to the clinical data, we analysed the clinical characteristics of age, gender and location of tumor, and put all information to a computer. A database was established using EpiData 3.1. The  $\chi^2$  test was used to assess differences in rates, and a significance level of 0.05 was used. Univariate comparisons were made *via* Fisher's exact test.

## RESULTS

### Age and gender

The hospitalization rate for CRC in 2004-2006 was approximately 3.1-fold higher than that in 1986-1988. From 1986 to 2006, 8172 patients aged 5-91 years were investigated and the mean age was 56 years (Table 1). Of the 8172 patients, 4841 (59.2%) were male and 3331 (40.8%) were female, with a male to female ratio of 1.5:1. The highest hospitalization rate for CRC occurred in the Triangle area. Increasing age was associated with a change in the male to female ratio from 1.1:1 to 1.7:1. The overall peak incidence of age was between 61-70 years (Figure 1). Mean age of patients in 2004-2006 increased by 8 years compared with that of patients in 1986-1988 and the ratio of young CRC patients ( $\leq 30$  years) decreased from 7.2% to 5.2%.

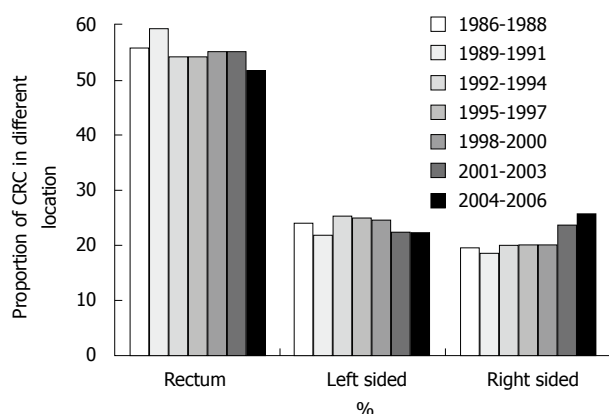


Figure 2 Year group and location of CRC.

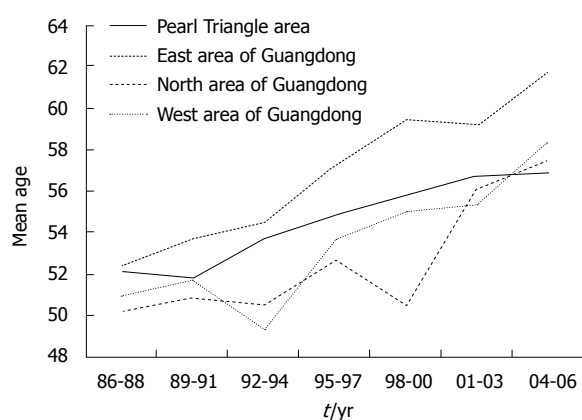


Figure 3 Mean age in different areas in different year group.

### Staging of colorectal cancer

Of 8172 lesions, 5670 lesions could be staged by Duke's staging: Duke's A stage 346 (6.1%), Duke's B stage 2829 (49.9%), Duke's C stage 1922 (33.9%), and Duke's D stage 573 (10.1%). The percentage of Duke's A stage increased from 4.5% to 7.7% and Duke's D stage decreased from 13.4% to 10.0%. The difference was significant ( $\chi^2 = 154.700$ ,  $P = 0.000$ ).

### Anatomic distribution of tumors

Of 8172 lesions, 4434 (54.3 %) were located in the rectum and 3738 (45.7%) in the colon, the ratio of rectum cancer to colon cancer was 1.2:1. The anatomic distribution of the tumors is shown in Table 2 and the distribution between age groups and classification as rectum cancer, right sided or left sided are shown in Table 3. The proportion of right-sided lesions increased, but the difference was not statistically significant ( $P > 0.05$ ). The proportion of CRC in the rectum decreased but that of right sided cancer increased ( $P < 0.05$ ). The relationship between distribution and year group was analyzed in Figure 2. The proportion of each distribution group has no change.

### Histological type

The CRC was classified as well, or moderately differentiated and poorly-differentiated carcinoma. Of 6638 lesions,

Table 2 Anatomic distribution of CRC ( $n = 8172$ )

	<i>n</i>	%
Rectum	4434	54.3
Sigmoid	1505	18.4
Descending	292	3.6
Splenic flexure	113	1.4
Transverse	485	5.9
Hepatic flexure	365	4.5
Ascending	703	8.6
Caecum	275	3.4
Total	8172	100

CRC: Colorectal cancer.

Table 3 Age group and location of CRC *n* (%)

Age group (yr)	Rectum	Left sided	Right sided	Total
≤ 30	301 (60.3)	102 (20.4)	96 (19.3)	499 (100.0)
31-40	496 (57.0)	194 (22.3)	179 (20.6)	869 (100.0)
41-50	786 (57.8)	297 (21.8)	278 (20.4)	1361 (100.0)
51-60	1067 (54.0)	499 (25.2)	411 (20.8)	1977 (100.0)
61-70	1201 (52.9)	534 (23.5)	536 (23.6)	2271 (100.0)
> 71	583 (48.8)	304 (25.4)	308 (25.8)	1195 (100.0)
Total	4434 (54.3)	1930 (23.6)	1808 (22.1)	8172 (100.0)

Significance was determined using Chi-squared tests ( $\chi^2 = 39.223$ ,  $P = 0.000$ ).

the histological type was classified as: tubular adenocarcinoma 4913 (75.6%), polypoid adenocarcinoma 578 (8.9%), mucinous adenocarcinoma 800 (12.3%), signet ring cell carcinoma 51 (0.8%), undifferentiated carcinoma 12 (0.2%), carcinoid 22 (0.3%), squamous carcinoma 21 (0.3%), adenosquamous carcinoma 9 (0.1%), and other types 95 (1.46%). In the young CRC patient group ( $\leq 30$  years), the proportion of undifferentiated cancer was 50.1%, while only 27.0% in the group over 40 years of age. There was a significant difference in the different histological groups ( $\chi^2 = 232.823$ ,  $P < 0.001$ ). In comparison of years 1986-1988 and 2004-2006, the proportion of well- and moderately differentiated tumors increased from 60.5% to 74.7%, while that of poorly-differentiated decreased from 39.5% to 25.3%. The difference was statistically significant ( $\chi^2 = 128.505$ ,  $P < 0.001$ ).

### CRC patients in different areas of Guangdong

As shown in Figure 3 and Table 4, the mean age of CRC in the four geographic areas increased, of which the lowest mean age was found in North Guangdong, and the highest mean age in East Guangdong. There was no significant difference in gender distribution (data not shown). Anatomic distribution in the four different geographic areas is shown in Table 5, with rectal cancer representing the largest proportion of cases, followed by left-sided and right-sided CRC. There was a statistically significant difference in the anatomic distribution among the four geographic regions ( $P < 0.05$ ). Comparison of histological type ( $\chi^2 = 459.561$ ,  $P < 0.001$ ) and degree

Table 4 Mean age and year group in different areas

Yr	n	mean age (yr)	≤ 30 yr	31-40 yr	41-50 yr	51-60 yr	61-70 yr	≥ 71 yr	Male:female
1986-1988	462	52	37	62	101	153	82	47	1.7:1
1989-1991	532	55	41	69	104	159	125	34	1.7:1
1992-1994	866	56	56	116	158	251	208	77	1.6:1
1995-1997	1103	58	68	133	183	270	341	108	1.3:1
1998-2000	1523	59	85	138	267	358	447	228	1.5:1
2001-2003	1793	60	99	174	275	379	520	346	1.4:1
2004-2006	1893	60	113	177	273	407	548	375	1.5:1
	3.1 <sup>1</sup>	8 <sup>2</sup>	2.1 <sup>1</sup>	2.9 <sup>1</sup>	1.7 <sup>1</sup>	1.7 <sup>1</sup>	5.7 <sup>1</sup>	7.0 <sup>1</sup>	<sup>3</sup>

<sup>1</sup>Increasing fold; <sup>2</sup>Increasing years of mean age; <sup>3</sup> $\chi^2 = 12.703$ ,  $P = 0.013$ , the difference was significant.

Table 5 Anatomic distribution, histology type, differentiation degree of CRC in different areas n (%)

Areas	Rectum	Left sided	Right sided	Total	Tubular adenocarcinoma	Papillary adenocarcinoma	Other adenocarcinoma	Total	Well-and moderately-differentiated	Poorly-differentiated	Total
Pearl Triangle area	2349 (54.3)	1039 (24.0)	939 (21.7)	4327 (100.0)	3436 (79.4)	363 (8.4)	528 (1.4)	4327 (100.0)	2914 (81.6)	627 (18.4)	3571 (100.0)
East area	901 (56.9)	363 (22.9)	319 (20.2)	1583 (100.0)	1165 (73.6)	271 (17.1)	147 (9.4)	1583 (100.0)	878 (77.1)	261 (22.9)	1139 (100.0)
North area	415 (53.1)	173 (22.1)	194 (24.8)	782 (100.0)	484 (61.9)	124 (15.9)	174 (22.1)	782 (100.0)	299 (47.8)	326 (52.2)	625 (100.0)
West area	769 (52.0)	355 (24.0)	356 (24.0)	1480 (100.0)	1097 (74.1)	15 (1.0)	368 (24.9)	1480 (100.0)	829 (63.6)	474 (36.4)	1303 (100.0)
Total	4434 (54.3)	1930 (23.6)	1808 (22.1)	8172 (100.0) <sup>1</sup>	6182 (75.6)	773 (9.5)	1217 (2.0)	8172 (100.0) <sup>2</sup>	4920 (74.8)	1718 (25.2)	6638 (100.0) <sup>3</sup>

<sup>1</sup> $\chi^2 = 13.307$ ,  $P = 0.038$ ; <sup>2</sup> $\chi^2 = 459.561$ ,  $P = 0.000$ ; <sup>3</sup> $\chi^2 = 409.296$ ,  $P = 0.000$ .

of differentiation ( $\chi^2 = 409.296$ ,  $P < 0.001$ ) in the four areas is shown in Table 5, there were significant differences in the four geographic areas.

## DISCUSSION

China has experienced a dramatic change in economy and lifestyle over the past two decades and this has led to a substantial increase in the incidence of CRC<sup>[14,15]</sup>, especially in Guangdong Province. It is important to understand the epidemiological characteristics of CRC in Guangdong. Data from the five hospitals in four representative areas of Guangdong were selected as a representative sample of CRC characteristics in Guangdong Province.

Although the hospitalization rate was not equal to the incidence rate, the increasing hospitalization rate in our study provided some information about the characteristics of CRC in recent years. Multiple risk factors increasing the incidence of colorectal cancer include: age, dietary habit, economics status and geographic location<sup>[16]</sup>. Previous studies suggested that the epidemiology of CRC is based on three main characteristics in China<sup>[17]</sup>. First, the peak age of CRC in China was lower than that in Western Countries, and the mean age was 45 years. Second, a high proportion of rectal cancer to colon cancer (1.5:1), and finally, a higher proportion of young CRC patients (10%-19%). Our data showed that the mean age was 56 years and this trend could be found in all the four areas, which was much higher than that in the previous studies. An important attribute of this trend was the increasing proportions of the elderly patients<sup>[6,18]</sup>.

A high proportion of young CRC patients shown by previous studies has been an important characteristic of CRC in China, but it was difficult to compare data regarding age<sup>[19-21]</sup>. Compared with the elderly CRC patients, the young CRC patients had the following characteristics: a low ratio of male to female; a high proportion of rectum cancer; a high proportion of poorly differentiated carcinomas; and a low proportion of right sided lesions. In a recent study, we defined young age as 30 years<sup>[22]</sup>, and using this definition, the proportion of young CRC patients decreased while that of elderly CRC patients ( $\geq 60$  years) increased. But the number of patients hospitalized for CRC was similar, suggesting that the decreasing number of young CRC patients was associated with the increase of elderly CRC patients.

Previous studies have shown that the proportion of female CRC patients has increased in recent years<sup>[23,24]</sup>. One possible explanation for the role of gender may be the effect of female hormones<sup>[25]</sup>. Recently there have been suggestions that hormonal replacement therapy may decrease the incidence of CRC in female<sup>[20]</sup>. However, our data is not in agreement with this trend, but the ratio of male to female increased with age. However, this requires further research.

The prevalence of cancer in the left or right colon was different based on the age, gender, as well as high- and low-incidence nations<sup>[26]</sup>. It is controversial about the anatomic distribution of tumors, particularly about the changes observed with time<sup>[27]</sup>. Previous studies showed the ratio of rectum cancer to colon cancer was 1.5:1, and a left to right sided shift of tumors was reported in China<sup>[13]</sup>. Other studies have shown that Asians and Pacific Islanders

have a higher incidence of distal lesions in older patients ( $\geq 70$  years), compared with proximal cancers<sup>[28]</sup>. Our data clearly showed a decrease of rectal cancer and an increase of right sided lesions, but no significant difference between different age groups. Cancers of proximal and distal colon are different s because of their embryologic origin, genetic factors and biologic identity<sup>[27]</sup>. The shift of tumors could be attributed to the change of life style, environmental factors and the increase of the elderly group<sup>[29,30]</sup>. Our results also suggested that the flexible sigmoidoscopy is not the first choice for CRC screening, even though it is more cost-effective compared with screening colonoscopy. Colonoscopy may be the preferred initial screening test. Most of CRC in present study is tubular adenocarcinoma and the proportion of tubular adenocarcinoma decreased from 79.3% to 67.7% with the shift from rectum to right colon. The proportion of mucinous carcinoma and signet-ring cell carcinoma increased from 9.3% to 19.0% and similar results were reported previously by other studies. This result may be related to different genetic background and different location of tumors.

In the present study, we report the characteristics of CRC between five hospitals located in four different areas in Guangdong. The mean age of CRC in the four areas increased, the lowest mean age was seen in North Guangdong and the highest mean age in East Guangdong. There is also a significant difference in anatomic distribution, histological type and differentiation type in the four areas. The data suggests that the hospitalization rate for CRC has increased over the past 20 years in Guangdong. The characteristics of CRC are different in the five hospitals located in the four different geographic areas. Further studies are needed to assess more recent trends in the incidence and prevalence of CRC as well as the respective roles of genetic and environmental factors of CRC in China.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

The incidence of colorectal cancer (CRC) in China is lower than that in the west countries, but has increased in recent years and become a substantial cancer burden in China, particularly in the more developed areas. Some studies have reported changes in the characteristics of CRC in China. However, due to a lack of an effective nation-wide surveillance system, there has been little information available on the relationship between CRC and geographical environment and economic status in China.

### Research frontiers

In the present study, the authors report a large scale of survey on the characteristics of CRC in five hospitals located in four different areas in Guangdong, China. Their data will benefit the study on prevention and treatment of CRC.

### Innovations and breakthroughs

This study has gained a broader picture of CRC in Guangdong Province and provide important information on the changing epidemiology of this disease over a period of 20 years.

## Applications

Further studies are needed to assess more recent trends in the incidence and prevalence of CRC as well as the respective roles of genetic and environmental factors of CRC in China based on the present study.

## Peer review

The authors present an analysis of colorectal cancer within 4 regions of Guangdong Province of China over a 20-year period. Their case analysis suggests an overall increase in cancer cases as well as a rising mean age at presentation. Rectal cancer was found to be more common than colon cancer though the incidence of rectal cancer appears to have decreased while right-sided colon cancers have increased.

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## Increased liver stiffness in alcoholic liver disease: Differentiating fibrosis from steatohepatitis

Sebastian Mueller, Gunda Millonig, Lucie Sarovska, Stefanie Friedrich, Frank M Reimann, Maria Pritsch, Silke Eisele, Felix Stickel, Thomas Longerich, Peter Schirmacher, Helmut Karl Seitz

Sebastian Mueller, Gunda Millonig, Lucie Sarovska, Stefanie Friedrich, Frank M Reimann, Helmut Karl Seitz, Department of Medicine and Center for Alcohol Research, Salem Medical Center, University of Heidelberg, Zeppelinstraße 11-33, 69121 Heidelberg, Germany

Maria Pritsch, Institute of Medical Biometry and Informatics, University of Heidelberg, 69121 Heidelberg, Germany

Silke Eisele, Felix Stickel, Institute of Clinical Pharmacology, University of Bern, 3010 Bern, Switzerland

Thomas Longerich, Peter Schirmacher, Department of Pathology, University of Heidelberg, 69121 Heidelberg, Germany

**Author contributions:** Mueller S designed and performed the study and wrote the paper; Seitz HK designed the study; Millonig G, Sarovska L, Friedrich S, Eisele S, Stickel F and Reimann FM performed the study; Pritsch M performed statistical analysis; Longerich T and Schirmacher P performed histological analysis.

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**Correspondence to:** Sebastian Mueller, MD, PhD, Department of Medicine and Center for Alcohol Research, Salem Medical Center, University of Heidelberg, Zeppelinstraße 11-33, 69121 Heidelberg, Germany. [sebastian.mueller@urz.uni-heidelberg.de](mailto:sebastian.mueller@urz.uni-heidelberg.de)  
Telephone: +49-6221-483210 Fax: +49-6221-484494

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### Abstract

**AIM:** To test if inflammation also interferes with liver stiffness (LS) assessment in alcoholic liver disease (ALD) and to provide a clinical algorithm for reliable fibrosis assessment in ALD by FibroScan® (FS).

**METHODS:** We first performed sequential LS analysis before and after normalization of serum transaminases in a learning cohort of 50 patients with ALD admitted for alcohol detoxification. LS decreased in almost all patients within a mean observation interval of 5.3 d. Six patients (12%) would have been misdiagnosed with F3

and F4 fibrosis but LS decreased below critical cut-off values of 8 and 12.5 kPa after normalization of transaminases.

**RESULTS:** Of the serum transaminases, the decrease in LS correlated best with the decrease in glutamic oxaloacetic transaminase (GOT). No significant changes in LS were observed below GOT levels of 100 U/L. After establishing the association between LS and GOT levels, we applied the rule of GOT < 100 U/L for reliable LS assessment in a second validation cohort of 101 patients with histologically confirmed ALD. By excluding those patients with GOT > 100 U/L at the time of LS assessment from this cohort, the area under the receiver operating characteristic (AUROC) for cirrhosis detection by FS improved from 0.921 to 0.945 while specificity increased from 80% to 90% at a sensitivity of 96%. A similar AUROC could be obtained for lower F3 fibrosis stage if LS measurements were restricted to patients with GOT < 50 U/L. Histological grading of inflammation did not further improve the diagnostic accuracy of LS.

**CONCLUSION:** Coexisting steatohepatitis markedly increases LS in patients with ALD independent of fibrosis stage. Postponing cirrhosis assessment by FS during alcohol withdrawal until GOT decreases to < 100 U/mL significantly improves the diagnostic accuracy.

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**Key words:** Alcoholic liver disease; Alcoholic steatohepatitis; Liver cirrhosis; Bilirubin; Tissue elasticity imaging; FibroScan

**Peer reviewer:** Dr. Søren Møller, Chief, Physician, Department of Clinical Physiology 239, Hvidovre Hospital, Kettegaard alle 30, DK-2650 Hvidovre, Denmark

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## INTRODUCTION

Alcoholic liver disease (ALD) is the most common cause of liver cirrhosis in the Western world. It typically presents with various histological features ranging from steatosis to steatohepatitis and fibrosis/cirrhosis. Liver biopsy is currently considered the gold standard for assessing hepatic fibrosis or cirrhosis in these patients. However, it is an invasive procedure, with rare but potentially life-threatening complications<sup>[1]</sup>. In addition, the accuracy of liver biopsy in assessing fibrosis is limited owing to sampling error and interobserver variability<sup>[2-6]</sup>.

Transient elastography (FibroScan<sup>®</sup>) is a novel rapid and noninvasive method to assess liver fibrosis *via* liver stiffness (LS)<sup>[7]</sup>. LS measurements can be routinely performed in more than 95% of patients but is limited in those with severe obesity and ascites<sup>[8]</sup>. LS has mainly been studied in patients with viral hepatitis, but also ALD, and LS was shown to be strongly associated with the degree of liver fibrosis in all of these patients<sup>[9-14]</sup>. In these studies, cut-off values have been defined that allow the diagnosis of advanced fibrosis (F3/F4). Despite some variability, cut-off values of 8.0 and 12.5 kPa are widely accepted to identify patients with F3 and F4 fibrosis, respectively. These cut-off values are used in parts of our study for comparison purposes.

Although LS closely correlates with fibrosis stage, it also increases in patients with mild<sup>[15,16]</sup> or acute hepatitis<sup>[17]</sup>, cholestasis<sup>[18]</sup> and liver congestion<sup>[19]</sup>, independent of the degree of fibrosis. High transaminase counts, cholestasis and liver congestion can even cause LS that exceeds cut-off values for F4 fibrosis (cirrhosis)<sup>[17-19]</sup>. Since steatohepatitis often coexists with all stages of fibrosis in patients with ALD it may directly interfere with reliable assessment of fibrosis in these patients. Indeed, unusually high cut-off values for liver cirrhosis (19.5 kPa<sup>[13]</sup> and 22.6 kPa<sup>[14]</sup>) have been reported recently for cirrhosis in patients with ALD compared to cut-off values of around 12-14 kPa in viral cirrhosis<sup>[12]</sup>. In these ALD studies, patients had elevated serum transaminases but the impact of alcoholic steatohepatitis (ASH) on LS was not considered.

The aim of the present sequential FibroScan (FS) study was to determine the impact of ASH on LS and to develop recommendations for non-invasive fibrosis assessment in patients with ALD. We eventually demonstrate that such selection criteria improve the diagnostic value of FS.

## MATERIALS AND METHODS

### Patients

Patients were examined after obtaining informed con-

**Table 1** Patients' characteristics of the learning cohort and validation cohort

Parameter	Learning cohort (n = 50)		Validation cohort (n = 101)	
	mean	SD	mean	SD
Male	37		73	
Female	13		28	
Age (yr)	55.8	10.8	53.2	10.6
BMI (kg/m <sup>2</sup> )	26.0	4.4	25.4	4.2
GOT (U/L)	135.2	99.9	95.0	76.0
GPT (U/L)	104.6	173.4	90.2	133.7
GGT (U/L)	591.1	828.0	568.6	762.8
AP (U/L)	129.8	102.7	131.9	110.4
Bilirubin (mg/dL)	2.1	3.3	2.0	3.9
Albumin (g/dL)	4.0	0.6	4.0	0.7
Protein (g/dL)	7.0	0.7	7.0	0.8
Quick (%)	98.5	19.5	95.3	20.8
INR	1.0	0.2	1.0	0.2
Urea	32.5	42.5	27.1	31.8
Creatinine (mg/dL)	0.9	0.5	0.8	0.4
PTT (s)	34.9	7.6	35.1	7.9
Hb (g/dL)	13.7	1.9	13.5	2.0
Hk (%)	39.9	5.3	39.7	5.6
Erythrocytes (/pL)	4.1	0.6	4.0	0.6
Leukocytes (/nL)	7.0	2.0	7.3	2.9
Thrombocytes (/nL)	202.5	99.2	204.5	112.5
Glucose (mg/dL)	100.1	20.7	97.2	18.9
HbA1C (%)	5.7	1.7	5.5	1.3
Triglycerides (mg/dL)	222.4	259.5	204.1	208.8
Cholesterol (mg/dL)	217.5	51.8	212.5	63.6
Ferritin (ng/mL)	636.3	580.7	671.7	589.4
Transferrin (g/L)	2.2	0.4	2.1	0.5
Iron (µg/dL)	109.0	51.9	107.1	59.6
CRP (mg/dL)	2.2	3.8	2.3	3.1
Alcohol (g/d)	144.1	106.1	146.8	100.8
LS (kPa)	20.1	24.3	20.4	22.4

GOT: Glutamic oxaloacetic transaminase; GGT:  $\gamma$  glutamyl transferase; GPT: Glutamic pyruvic transaminase; AP: Alkaline phosphatase; INR: International normalized ratio; PTT: Prothrombin time; Hb: Hemoglobin; Hk: Hematocrit; HbA1C: Glycosylated Hb; CRP: C-reactive protein; LS: Liver stiffness; BMI: Body mass index.

sent. The study was approved by the Ethical Committee of the University of Heidelberg.

**Learning cohort:** In the first part of the study, sequential analysis of LS was performed in 50 patients with ALD presenting at Salem Medical Center/University of Heidelberg from June 2007 to March 2009 for alcohol detoxification. The cohort consisted of 37 male and 13 female patients with ALD. The mean age was  $55.8 \pm 10.8$  years. Observation intervals ranged from 2-10 d (mean 5.3 d). Longer time intervals were excluded to avoid potential changes in fibrosis stage. Patients showed typical laboratory findings of ASH with glutamic oxaloacetic transaminase (GOT) higher than serum glutamic pyruvic transaminase (GPT), steatosis in abdominal ultrasound and various degrees of LS. Patients' characteristics are detailed in Table 1.

Alcohol detoxification was performed using a chlor-methiazole standard tapering protocol for 3 to 10 d. Measurements of LS were performed on the day of

admission and on the day of discharge from Salem Medical Center. Patients with additional or other liver disease and co-morbidities known to interfere with LS measurements such as liver tumors, congestive heart failure or extrahepatic cholestasis were excluded from the study.

**Validation cohort:** In the second part of our study we included 101 patients with histologically staged ALD, a full set of blood tests and FS examination at the time of liver biopsy. The cohort included 73 men and 28 women with a mean age of  $53.6 \pm 10.6$  years. More patient details are given in Table 1. Importantly, the learning cohort and validation cohort did not differ significantly with regard to clinical parameters, age or gender. Liver biopsies were staged according to the Kleiner Score F0-F4<sup>[20]</sup>. F4 fibrosis was found in 26 patients (25.7%), F3 in 19 patients (18.8%), F2 in 36 patients (36.6%), F1 in 14 patients (13.8%), and F0 in 6 patients (5.9%). Thus, 45% had significant fibrosis stage F3-4.

### LS measured by transient elastography

LS was measured by FS (Echosens, Paris, France) as described recently in detail using the M probe<sup>[7]</sup>. The tip of the probe transducer was placed on the skin between the rib bones and the level of the right lobe of the liver. The measurement depth was between 25 and 65 mm below the skin surface. Ten measurements were performed with success rates of at least 60%. The results were expressed in kilopascals. The median value was taken as the representative value. Based on previous studies<sup>[10]</sup> and a recent meta-analysis<sup>[12]</sup>, cut-off values of 8.0 and 12.5 kPa were considered to be optimal for detecting F3 and F4 fibrosis stages, respectively. LS values from 2.4 to 5.5 kPa are considered as normal<sup>[10]</sup> although no large F0 validated control groups have been studied so far. The mean interquartile range (IQR) was 16% in all measurements. FS measurements with an IQR higher than 40% were excluded. Five of the initial 106 patients (3.2%) were excluded because of invalid LS measurements or high IQR values.

### Ultrasound examination

Ultrasound examination was routinely performed in addition to FS measurements to exclude extrahepatic cholestasis, liver congestion or liver tumors after patients had fasted for at least 6 h. Liver cirrhosis was suspected when 2 of the following criteria were present: (1) nodular aspect of the liver surface; (2) portal vein diameter > 12 mm; (3) collateral circulation; and (4) hypertrophy of segment 4 (quadrate lobe)<sup>[21]</sup>.

### Histology

Liver biopsy specimens were fixed in formalin and embedded in paraffin. All biopsy specimens were analyzed independently by 2 experienced pathologists (TL, PS) blinded to the results of FS and other clinical data. Liver fibrosis and necroinflammatory activity in patients with

ALD were scored according to the recently established Kleiner score<sup>[20]</sup>. The length of each liver biopsy specimen (in millimeters) and the number of fragments were recorded. Only biopsies > 15 mm were included.

### Statistical analysis

Correlations between changes in laboratory findings (serum transaminases, cholestasis parameters), LS and histological scores were calculated using the Spearman correlation for nonparametric variables (regression coefficient  $r$ ,  $P$ ). Differences were considered significant at  $P < 0.05$ . The diagnostic performance of FS was assessed by using receiver operating characteristic (ROC) curves. A patient was assessed as positive according to whether LS was greater than a given cut-off value. The ROC curve is a plot of sensitivity *vs* 1-specificity for all possible cut-off values. The most commonly used index of accuracy is the area under the ROC curve (AUROC), with values close to 1.0 indicating high diagnostic accuracy. As can be seen in the Results section from the box plot, only fibrosis stages F3 and F4 were successfully differentiated by FS. Therefore, AUROC and cut-off values were only determined for these fibrosis stages. All statistical analyses were performed with SPSS, version 12.0.1 (SPSS, Inc., Chicago, USA).

## RESULTS

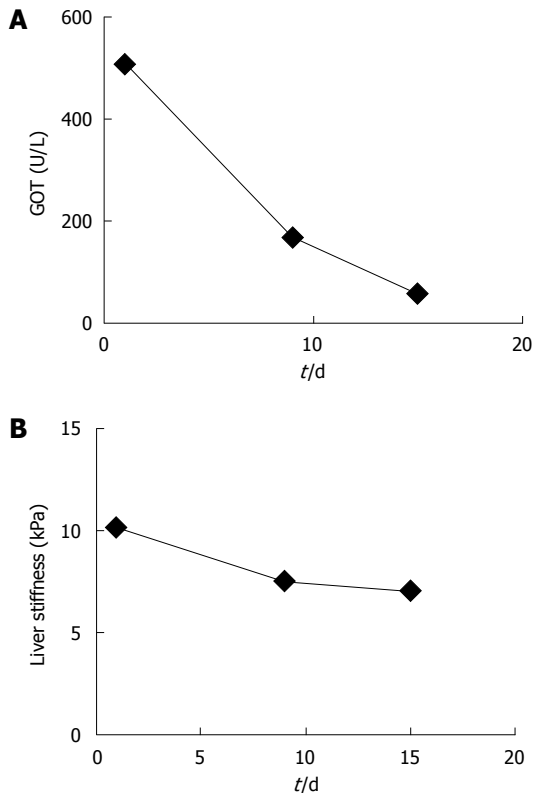
### Dynamics of LS and transaminases in a single patient with acute ASH during alcohol withdrawal

At first presentation, this female patient with chronic alcohol abuse presented with typical clinical and laboratory findings of acute and severe ASH with GOT of 511 U/L, GPT of 255 U/L and bilirubin of 3.5 mg/dL. The initial LS was 10.2 kPa which suggested advanced F3 fibrosis (Figure 1A and B). Within 9 d of alcohol detoxification, GOT decreased to 164 U/L and LS to 7.6 kPa. After another 6 d, GOT had almost normalized (55 U/L) while LS stabilized at 7.1 kPa. At the same time bilirubin had completely normalized. The patient was biopsied and histology confirmed a mild perivenular and periportal fibrosis (F2). This single case clearly demonstrated that LS increased as a result of steatohepatitis, irrespective of liver fibrosis. It also suggested that laboratory parameters such as GOT levels could be valuable bedside criteria to identify patients qualifying for reliable fibrosis assessment by FS.

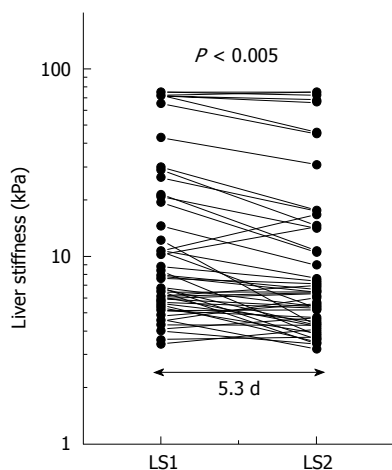
### LS decreases in patients during alcohol detoxification independent of fibrosis stage

The observation above prompted us to sequentially study LS in patients with ALD undergoing alcohol detoxification under clinical surveillance (learning cohort). LS and serum transaminase activities in patients with various stages of fibrosis and steatohepatitis were obtained before and after detoxification from alcohol. Initial LS ranged from 2.5 to 75 kPa with 20 patients exceeding the cut-off value for F4 cirrhosis at 12.5 kPa.



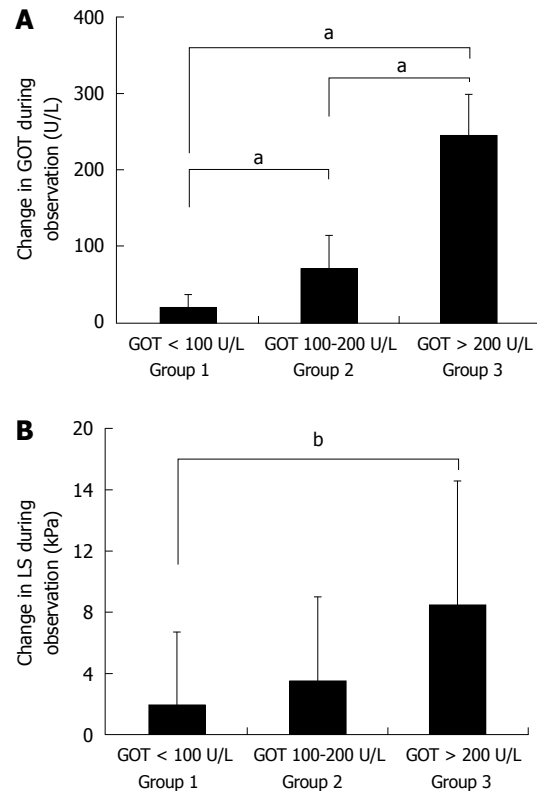


**Figure 1** Changes in liver stiffness (LS) in a single patient with severe alcoholic liver disease (ALD) during alcohol detoxification. Upon alcohol withdrawal, GOT (A) almost normalized and LS (B) decreased by more than 3 kPa. LS eventually remained stable at ca. 7 kPa corresponding well with the histologically confirmed F2 fibrosis stage.



**Figure 2** Sequential analysis of LS in 50 ALD patients following alcohol withdrawal over a mean observation period of 5.3 d. LS decreased in almost all patients during alcohol detoxification. A semilogarithmic plot was chosen to better visualize and compare changes in LS at various initial levels.

During the observation interval LS decreased significantly ( $P < 0.001$ ; mean decrease in LS, 3.5 kPa; maximum decrease, 26.3 kPa). LS decreased in 6 patients (12%) below critical cut-off values of 12.5 kPa for F4 cirrhosis ( $n = 2$ ) and 8.0 kPa for F3 fibrosis ( $n = 4$ ). Figure 2 shows the 2 sequential LS measurements depicted as a semilogarithmic plot. A decrease in LS correlated sig-



**Figure 3** Decrease in LS depends on the mean decrease in GOT levels (steatohepatitis) during alcohol withdrawal. A: Group 1 to 3 differ significantly ( $^aP < 0.05$ ) with regard to the mean decrease in GOT during alcohol withdrawal. Group 1 included patients with severe alcoholic steatohepatitis and GOT levels > 200 U/L; group 2 included patients with GOT values between 100 and 200 U/L; group 3 were patients with GOT values < 100 U/L. B: LS decreased by 8.4, 3.5 and 1.8 kPa, respectively, with a significant difference between groups 1 and 3 ( $^bP < 0.01$ ) (details in text).

nificantly with a decrease in GOT ( $r = 0.30$ ,  $P < 0.05$ ) and bilirubin ( $r = 0.37$ ,  $P < 0.05$ ). In 50% of patients, transaminases completely normalized during alcohol detoxification.

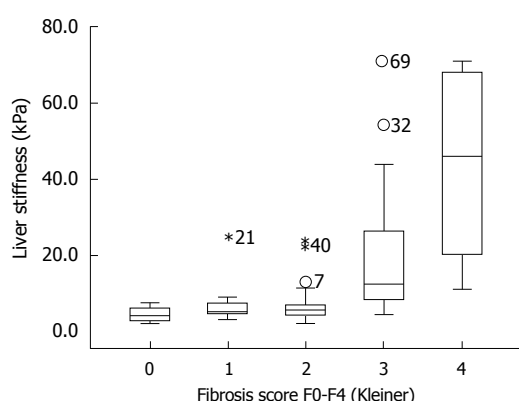
#### ALD patients without significant laboratory signs of steatohepatitis (GOT < 100 U/L) show stable LS

It is known that in ALD patients GOT levels highly correlate with histological degree of ASH<sup>[22,23]</sup>. We therefore divided all 50 patients into 3 groups according to their degree of inflammation (mild, medium, severe) represented by their initial levels of GOT (not shown). Group 1 included patients with severe ASH and GOT levels > 200 U/L, group 2 contained patients with GOT values between 100 and 200 U/L and group 3 patients with GOT values below 100 U/L. There was no significant difference between the 3 groups with respect to LS, observation interval, and IQR. However, as shown in Figure 3A, all groups differed significantly with regard to the mean decrease in GOT during alcohol withdrawal. Group 1 showed a mean GOT decrease of 244.8 U/L, group 2 of 70.1 U/L, and group 3 of 15.0 U/L. At the same time, LS decreased by 8.4, 3.5 and 1.8 kPa in groups 1, 2 and 3, respectively (Figure 3B) with a sig-

**Table 2** Excluding patients with elevated GOT levels indicating steatohepatitis improves the diagnostic value (AUROC, sensitivity and specificity) of FibroScan for the diagnosis of F3/F4 fibrosis

	Area under the curve (AUROC)	Std. error	Cut-off value (kPa)	Sensitivity	Specificity	Number of included patients
F4	0.921	0.03	11.5	1.00	0.77	101
			12.5 <sup>1</sup>	0.96 <sup>1</sup>	0.80 <sup>1</sup>	101 <sup>1</sup>
F4 without GOT > 100 U/L	0.944	0.02	11.5	1.00	0.84	86
			12.5 <sup>1</sup>	0.95 <sup>1</sup>	0.90 <sup>1</sup>	86 <sup>1</sup>
F4 without GOT > 50 U/L	0.945	0.03	10.4	1.00	0.87	66
			12.5 <sup>1</sup>	0.92 <sup>1</sup>	0.91 <sup>1</sup>	66 <sup>1</sup>
F3/4	0.914	0.03	8.0	0.91	0.75	101
F3/4 without GOT > 100 U/L	0.922	0.03	8.0	0.87	0.87	80
F3/4 without GOT > 50 U/L	0.946	0.03	8.0	1.00	0.84	67

<sup>1</sup>Represent commonly used cut-off values (12.5 and 8.0 kPa) for comparison with other studies. Additional cut-off values with 100% sensitivity and different cut-off values for F4 fibrosis are specific to this study.



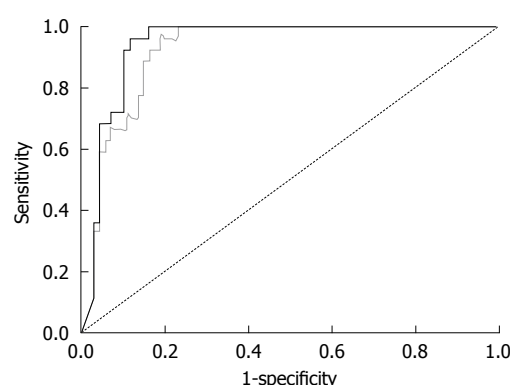
**Figure 4** Box blot showing LS as a function of fibrosis stages F0-F4. A significant increase in LS is seen from fibrosis stage F3 onwards. The length of the box represents the interquartile range within which 50% of the values were located. The line through the middle of each box represents the median. The error bars show the minimum and maximum values (range). Numbered symbols represent out-of-range patients excluded by the box plot.

nificant difference between group 1 and 3 ( $P < 0.01$ ). In group 1, 6 out of 8 patients (75%) showed a decrease in LS below critical cut-off values for F3/F4 fibrosis of 8.0 and 12.5 kPa (not shown). In contrast, only 2 patients crossed the critical cut-off value in group 2 (9%) and none in group 3.

Two patients had an increase in LS from 10.2 to 14.4 kPa and 10.7 to 16.4 kPa during alcohol detoxification therapy. Patient 35 showed an increase in GOT levels and compliance to alcohol abstinence could be questioned in this case while the reason for increased LS in the other patient remained unclear. In conclusion, LS is more likely to decrease in ALD patients with high initial GOT levels but remains stable once GOT levels are below 100 U/L.

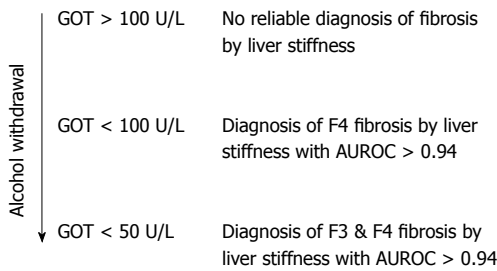
#### **Restriction of LS measurements to patients with GOT < 100 U/L significantly improves assessment of F4 fibrosis**

The finding that LS remained stable once GOT levels were below 100 U/L led us to the hypothesis that the diagnostic accuracy of FS will increase when ALD patients with GOT > 100 U/L are excluded. To test this hypoth-



**Figure 5** Receiver operating characteristic curves (ROC) for LS in patients with F4 fibrosis (liver cirrhosis) before (gray) and after (black) exclusion of patients with GOT > 100 U/L. The areas under the ROC (AUROC) curve are given in Table 2.

esis, we prospectively studied LS in our validation cohort of 101 consecutive patients with biopsy-staged ALD including or excluding patients with GOT > 100 U/L. The patients' characteristics are given in Table 1. Including all patients we were able to show that LS correlated highly significantly with the histological fibrosis score ( $r = 0.72$ ,  $P < 0.001$ ) thus confirming recent studies<sup>[13,14]</sup>. As seen in the box plot (Figure 4), markedly elevated LS was mainly observed in advanced fibrosis (F3 and F4). Figure 5 shows the ROC curve for F4 fibrosis in the cohort of 101 patients including all patients (gray line). AUROC, which represents the diagnostic accuracy of a novel method as compared to the gold/best standard (histology) for fibrosis stage F4 was 0.921 (95% confidence interval, 0.87-0.97) (Table 2). We then identified 15 of these 101 patients (14.8%) with laboratory signs of significant ASH with an elevated GOT > 100 U/L. In 9 of these patients, there was a discrepancy between LS values of > 12.5 kPa but no histological evidence of liver cirrhosis. After exclusion of these patients with GOT > 100 U/L, AUROC for cirrhosis assessment by FS increased from 0.921 to 0.946, while specificity increased significantly from 80% to 90% at a sensitivity of 96%. Exclusion of patients with only a slight elevation of GOT (> 50 U/L)



**Figure 6** Proposed algorithm for the noninvasive assessment of fibrosis by FibroScan in patients with ALD. GOT levels are used as criteria to exclude ongoing steatohepatitis that may falsely increase LS diagnosis independent of fibrosis stage. F4 cirrhosis can be detected by FibroScan with high accuracy if restricted to patients with GOT levels < 100 U/L. F3 fibrosis stage can be assessed with comparable accuracy if restricted to patients with GOT < 50 U/L (details in text).

or histological signs of steatohepatitis did not further improve diagnostic accuracy for F4 fibrosis in our patient cohort. In contrast, diagnostic accuracy for F3 fibrosis (cut-off value 8 kPa) further improved when excluding patients with GOT > 50 U/L (Table 2). In summary, these data demonstrated that excluding patients with laboratory signs of ASH significantly improved the diagnostic accuracy of FS in detecting fibrosis.

## DISCUSSION

LS correlates well with the histological stage of liver fibrosis in various liver diseases including patients with ALD<sup>[7-14]</sup>. However, independent of the degree of fibrosis, LS is also increased in patients with inflammatory hepatitis<sup>[15-17]</sup>, congestive heart failure<sup>[19]</sup> and cholestasis<sup>[18]</sup>. Therefore, refined clinical algorithms are required to exclude patients with increased LS as a result of such confounding factors.

We here found in a learning cohort of 50 patients with ALD that LS decreased significantly in patients with ALD following alcohol detoxification and resolution of steatohepatitis as measured by transaminases. The decrease in LS during alcohol detoxification could not be explained by changes in fibrosis stage given the short observation interval of 5.3 d. Therefore any change in LS must be attributed to other factors, most likely steatohepatitis. Of the serum transaminases, GOT showed the greatest correlation with the decrease in LS. This emphasizes the role of steatohepatitis in reversible elevation of LS. In fact, no significant changes in LS were observed in patients with GOT < 100 U/L, which fits clinical data indicating that at GOT levels < 100 U/L no relevant steatohepatitis has been demonstrated<sup>[22,23]</sup>.

After demonstrating that resolution of steatohepatitis decreased LS, we tested the hypothesis that the accuracy of cirrhosis detection can be increased by excluding patients with clinically relevant steatohepatitis. A validation cohort of 101 patients with histologically confirmed ALD was established and we compared FS accuracy in the whole cohort, and after excluding those patients with relevant clinical steatohepatitis (defined as GOT >

100 U/L). Indeed, including only ALD patients with GOT levels < 100 U/L increased the sensitivity and specificity of LS in diagnosing liver cirrhosis. We therefore suggest that GOT levels should be considered when assessing fibrosis by transient elastography in patients with ALD, and that elevated LS values at GOT > 100 U/L should be interpreted with caution because of the possibility of falsely elevated LS as a result of steatohepatitis.

This improvement in diagnostic accuracy is remarkable since any increase in AUROC above 0.9 is an important improvement given a prevalence of significant disease (F3/F4) of > 40% and an assumed 'best scenario' of liver biopsy accuracy of 90%<sup>[24]</sup>. We would also like to point out that the impact of these novel criteria clearly depends on the percentage of patients with significant ASH, which was rather low (< 15%) in our study cohort. The combined effect of both inflammation and fibrosis on LS in patients with ALD may also explain the unusually high cut-off values for F4 cirrhosis of 19.5 kPa and 22.6 kPa and a rather low AUROC of 0.87 that has recently been reported in patients with ALD<sup>[13,14]</sup>.

Although the rule of GOT < 100 U/L seems to be sufficient to eliminate any inflammation-based interference on F4 fibrosis assessment by FS, even modest signs of inflammation become relevant for lower fibrosis stages such as F3. Thus, our analysis showed that assessment of F3 fibrosis by FS was further improved if LS measurements were restricted to patients with completely normalized GOT levels. This outcome can be explained by the fact that the cut-off value for F3 fibrosis (8 kPa) can be easily reached by even mild steatohepatitis.

We conclude that, in patients with ALD, a more accurate noninvasive assessment of fibrosis stage by transient elastography can be achieved if the degree of steatohepatitis is considered. A clinical algorithm is proposed in Figure 6. It should be mentioned that other known interferences with LS assessment such as liver metastasis, mechanic cholestasis<sup>[18]</sup> or liver congestion<sup>[19]</sup> should also be excluded at the time of transient elastography. Finally, we suggest that these criteria may also be useful in improving the assessment of noninvasive fibrosis by FS in patients with other liver diseases.

## COMMENTS

### Background

Transient elastography (Fibroscan®) is a new ultrasound technology where the liver stiffness (LS) is measured via propagation velocity of an ultrasound wave. LS is low (2-6 kPa) in the soft healthy liver but dramatically increased in liver cirrhosis (up to 75 kPa). Fibroscan is now widely studied since it is a reliable, easy to perform and noninvasive bedside test to screen for liver cirrhosis. However, recent studies showed that LS is also increased under conditions other than cirrhosis namely liver congestion, cholestasis and inflammation (hepatitis). In particular, inflammation is a common confounding factor for chronic liver disease and novel algorithms are required to clearly determine whether LS is increased as a result of cirrhosis or inflammation. Dr. Sebastian Mueller and his colleagues from Heidelberg University identified in their recent study novel bedside criteria that allowed the differentiation of cirrhosis from inflammation in patients with alcoholic liver disease (ALD), the most common liver disease in developed countries.

## Research frontiers

In a cohort of 50 patients who were hospitalized for alcohol withdrawal LS was analyzed for about a week in parallel with liver blood tests. LS decreased in all patients during alcohol withdrawal owing to reduction in liver inflammation and normalization of liver blood tests. In 6 of these 50 patients, the initial LS value had suggested liver cirrhosis but, after alcohol withdrawal, it decreased below the critical cut-off and misdiagnosis of liver cirrhosis could be avoided. Glutamic oxaloacetic transaminase (GOT) values > 100 U/L indicated inflammatory interference, while at lower counts LS was a reliable parameter for determining inflammation-independent liver fibrosis. Therefore the hypothesis was generated that measuring LS after resolution of the inflammation and at GOT levels < 100 U/L would result in more accurate assessment of LS. To test this hypothesis 100 patients with ALD underwent liver biopsy and LS determination at the same time. Comparing the stage of liver fibrosis assessed by liver biopsy with LS showed concordance in 92% of patients. When those patients with elevated liver enzyme counts > 100 U/L were excluded, transient elastography increased in accuracy for diagnosing liver cirrhosis to 94.5%.

## Innovations and breakthroughs

The study defined novel bedside criteria based on liver transaminases that optimized noninvasive diagnosis of liver cirrhosis in patients with ALD. Active inflammation of the liver (steatohepatitis) should be excluded first by blood tests prior to the noninvasive assessment of liver fibrosis by transient elastography. If GOT levels are < 100 U/L, the LS value can identify liver fibrosis and can be used as a diagnostic tool.

## Applications

The novel criteria should be applied to all LS measurements with ongoing steatohepatitis (increased transaminases in the blood test). Thus, a blood test is required for the correct interpretation of LS data. These novel criteria probably also hold true for other liver disease.

## Peer review

This is an interesting paper on a highly relevant topic. The presence of steatohepatitis was evidenced by increased GOT.

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## Catalytic domain of PDC-E2 contains epitopes recognized by antimitochondrial antibodies in primary biliary cirrhosis

Sandra Braun, Christoph Berg, Sandra Buck, Michael Gregor, Reinhild Klein

Sandra Braun, Sandra Buck, Reinhild Klein, Department of Internal Medicine II, University of Tuebingen, 72076 Tuebingen, Germany

Christoph Berg, Michael Gregor, Department of Internal Medicine I, University of Tuebingen, 72076 Tuebingen, Germany

Author contributions: Braun S and Buck S performed the research; Berg C and Gregor M provided patients' serum samples and clinical data; Braun S and Klein R analyzed the data; Klein R designed the research and wrote the paper.

Correspondence to: Reinhild Klein, MD, Associate Professor, Department of Internal Medicine II, University of Tuebingen, Otfried-Muller-Str. 10, 72076 Tuebingen, Germany. [reinhild.klein@med.uni-tuebingen.de](mailto:reinhild.klein@med.uni-tuebingen.de)

Telephone: +49-7071-2984479 Fax: +49-7071-292760

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### Abstract

**AIM:** To search for further immunodominant peptides of the pyruvate dehydrogenase complex E2-component (PDC-E2) recognized by antimitochondrial antibodies (AMA) in primary biliary cirrhosis (PBC).

**METHODS:** Sera from 95 patients with PBC were tested by enzyme-linked immunosorbent assay against 33 synthetic overlapping peptides (25 amino acids; aa) covering the entire length of the E2-subunit of PDC-E2. Furthermore, the inner lipoyl peptide 167-184 was used in an unlipoylated and a lipoylated form as well as coupled to ovalbumin. Sera from 11 AMA negative/ANA positive PBC patients, 63 patients with other liver disorders and 22 healthy blood donors served as controls.

**RESULTS:** Of the 95 PBC-sera, 74% reacted with the peptide 475-499 and 58% with the peptide 407-431 located within the catalytic domain of PDC-E2. Patients with other disorders or healthy controls were positive in only up to 18%. Antibodies to the unlipoylated

and lipoylated peptide 167-184 within the inner lipoyl domain were found in only 5% and 11% of the PBC sera, respectively; using ovalbumin-coupled peptides, the incidence increased up to 57% (unlipoylated form).

**CONCLUSION:** Peptides within the catalytic site of PDC-E2 rather than the previously reported lipoyl binding peptide 167-184 may represent major immunodominant epitopes recognized by AMA in PBC.

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**Key words:** Anti-M2; Epitope mapping; E2-subunit; Pyruvate dehydrogenase complex; Inner lipoyl domain; Active site; Catalytic domain; Primary biliary cirrhosis

**Peer reviewers:** Henning Schulze-Bergkamen, MD, Henning Schulze-Bergkamen, First Medical Department, University of Mainz, Langenbeckstr. 1, 55101 Mainz, Germany; Sun-Lung Tsai, MD, PhD, Professor, Director, Hepatogastroenterology Section, Department of Internal Medicine and Liver Research Unit, Department of Medical Research, Chi Mei Medical Center, 901 Chung Hwa Road, Young-Kang City, Tainan County 710, Taiwan, China

Braun S, Berg C, Buck S, Gregor M, Klein R. Catalytic domain of PDC-E2 contains epitopes recognized by antimitochondrial antibodies in primary biliary cirrhosis. *World J Gastroenterol* 2010; 16(8): 973-981 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v16/i8/973.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i8.973>

### INTRODUCTION

Antimitochondrial antibodies (AMA) are one of the most important criteria for the diagnosis of primary biliary cirrhosis (PBC), a chronic cholestatic liver disease of unknown etiology, which affects mainly middle-aged women and leads to a destruction of small bile ducts. Their target antigen named M2<sup>[1,2]</sup> is attached to the

inner mitochondrial membrane<sup>[3]</sup> and consists of five components<sup>[4]</sup>, which have been identified in the following years on molecular bases as subunits of the 2-oxo acid dehydrogenase complex of the inner mitochondrial membrane: the pyruvate dehydrogenase complex (PDC), the 2-oxoglutarate dehydrogenase complex and the branched-chain 2-oxo acid dehydrogenase complexes<sup>[5-7]</sup>. Each complex comprises multiple copies of three component enzymes termed E1 (a thiamine pyrophosphate-dependent decarboxylase), E2 (a coenzyme A-dependent decarboxylase), and E3 (a dihydrolipoyl dehydrogenase). The major M2-antigen which is recognized by nearly 90% of PBC sera is the E2 component of PDC.

The immunodominant epitope within PDC-E2 has been mapped to the region associated with the inner lipoyl domain wherein a lysine residue binds the lipoic cofactor for the enzyme with a minimum of 75 residues necessary for characteristic antibody recognition<sup>[6,8,9]</sup> suggesting that a conformational autoepitope may be primarily recognized<sup>[9]</sup>. A minor epitope is associated with the outer lipoyl domain<sup>[6,10]</sup>. Hitherto, epitope mapping with PBC sera has linked AMA-reactivity to the highly conserved amino acids surrounding the lipoyl-lysine K173, particularly linear peptides AEIETDKATIGFEVQEEG (corresponding to aa 167-184 within the human PDC-E2 but primarily labeled aa 81-100<sup>[6]</sup> according to its location within a subclone pRMIT-603 used to identify the immunodominant epitope) or LLAEIETDKATIGF (165-178)<sup>[11]</sup>. It was shown that these short peptides absorbed most reactivity with PBC sera by enzyme-linked immunosorbent assay (ELISA), albeit only at a serum dilution of 1:80000<sup>[6]</sup>. Binding of lipoic acid cofactor to K173 has been discussed to be necessary for antibody reaction<sup>[10,12]</sup>.

Until now, the large fragment of the catalytic domain of PDC-E2 (aa 331-560) has been regarded as 'immunologically silent' due to negative results in several studies<sup>[6,9,12-14]</sup>. In the present study we used peptides spanning the whole PDC-E2 enzyme, and it will be shown that sera from PBC patients recognize epitopes within the catalytic domain in an even higher incidence than those in the inner lipoyl domain.

## MATERIALS AND METHODS

### Patients

Sera from 95 patients with clinically well defined PBC were analyzed. All patients had been seen by one of the authors (Berg C), and all were AMA/anti-M2-anti-PDC-E2 positive. Detailed clinical, biochemical and histological data are given in Table 1.

Liver biopsy had been performed in 65 of the patients and was interpreted by Dr. Bianchi L, Professor, University of Basel, Switzerland. All patients had been followed for more than 5 years (range: 5-28 years). At time of first serum analysis, 65 had not yet received any therapy, 30 were already treated with ursodeoxycholic acid (at least 6 mo, doses 1000-1500 mg/d).

**Table 1 Clinical, biochemical, histological and serological data in 95 patients with PBC (mean  $\pm$  SD)**

Parameters	
Females:males	88:7
Age (yr)	
mean $\pm$ SD	50 $\pm$ 9.8
Range	21-70
Biochemical parameters <sup>1</sup>	
AP (U/L) (normal < 120)	453 $\pm$ 451
GT (U/L) (normal < 50)	154 $\pm$ 143
ASAT (U/L) (normal < 35)	39.4 $\pm$ 42.6
ALAT (U/L) (normal < 35)	51 $\pm$ 49.3
Cholinesterase (kU/L) (normal > 5)	4.7 $\pm$ 1.7
Bilirubin (mg%) (normal < 1.5)	1.62 $\pm$ 0.8
Cholesterol (mg/dL) (normal < 200)	251 $\pm$ 69
Eosinophils (%) (normal < 4)	3.61 $\pm$ 2.36
IgG globulins (mg%) (normal < 1800)	1679 $\pm$ 855
IgA globulins (mg%) (normal < 400)	269 $\pm$ 158
IgM globulins (mg%) (normal < 280)	416 $\pm$ 255
Histological data <sup>2</sup>	
PBC stage I / II (n)	45
PBC stage III / IV (n)	20
Serological data	
Antimitochondrial antibodies (number positive)	
AMA (IFT) (titer > 1:160)	95
Anti-M2 (ELISA)	
Total	95
IgG type	87
IgM type	72
Anti-PDC-E2 (ELISA)	
Total	95
Antinuclear antibodies (number positive) (titer > 1:160)	
Nuclear dots (sp 100)	16
Nuclear membrane (gp 210)	10
Centromeres	8

<sup>1</sup>Referring only to the 65 untreated patients; <sup>2</sup>Liver biopsy had been performed in 65 patients; 42 of them were untreated, and of these patients 30 were in stage I / II and 12 in stage III / IV. IFT: Immunofluorescence test; ELISA: Enzyme-linked immunosorbent assay; PBC: Primary biliary cirrhosis; PDC-E2: Pyruvate dehydrogenase complex E2-component.

Furthermore, sera from 11 patients with clinically and histologically proven PBC being AMA negative but ANA positive were investigated. Five had antibodies to nuclear dots (sp 100), three to nuclear membranes (gp 210) and two to centromeres.

The investigations have been approved by the local Ethics committee.

### Control groups

As controls we used sera from 36 patients with untreated autoimmune hepatitis, 27 patients with alcoholic liver disease, and 32 patients with collagen disorders. All patients have been seen and followed either by Berg C or Klein R. Diagnosis was based on clinical, biochemical and serological parameters.

Sera from 22 healthy blood donors (kindly provided by Dr. Wernet D, Professor, Institute for Transfusion-medicine, University of Tübingen, Germany) were used as controls, all being negative for AMA/anti-M2 as shown by immunofluorescence test (IFT), ELISA and Western blotting. All sera were stored at -20°C.



**Figure 1** Amino acid (aa) sequence of the human pyruvate dehydrogenase complex E2-component (PDC-E2). For epitope mapping 25 mer peptides with 8 overlapping amino acids were constructed (see also Table 2). Overlapping amino acids are printed in bold; amino acid sequences of the immunodominant lipoyl binding epitopes in the outer (aa 41-53) and inner lipoyl domain (aa 167-183) are underlined. \*Lipoyl binding lysine in the outer and inner lipoyl domains; \*\*Active sites within the catalytic domain (S480 and H534).

## Antigens

The M2-antigen was released from beef heart submitochondrial particles by chloroform treatment as described<sup>[2]</sup>. Commercial PDC (from porcine heart) was obtained from Sigma-Aldrich (St. Louis, USA).

Thirty-three synthetic overlapping peptides covering the entire length of PDC-E2 were obtained from Biotrend (Cologne, Germany). Peptides were 25 amino acids (aa) long with 8 overlapping aa (Figure 1).

Peptide 11 contained the immunodominant decameric epitope consisting of aa IETDKATIGF as reported by Van de Water *et al.*<sup>[6]</sup>, which had been extended to a 15 amino acid peptide by Amano *et al.*<sup>[15]</sup> (IETDKATIGFEVQEE). All peptides were high-performance liquid chromatography (HPLC)-purified (more than 90% purity). An irrelevant peptide was used as background control. The peptides were reconstituted at 5 mg/mL DMSO and stored at -20°C.

The immunodominant peptide 167-184 (AEIETDKATIGFEVQEEG) was synthesized (Biotrend, Cologne, Germany) in an unlipoilated form and a form in which lipoic acid was coupled to lysine at position 173 (167-184-LA)<sup>[12]</sup>. Purity and conjugation was proven by HPLC (elution time 984.5 m/z *vs* 1076.7 m/z; purity > 95%). Since these peptides may be recognized only when attached to a carrier<sup>[12]</sup>, both forms of peptides were also coupled to ovalbumin (Biotrend, Cologne, Germany). For this purpose, at the C-terminal end a cysteine had to be added (AEIETDKATIGFEVQEEGC-OVA) which had to be substituted by a lysine for coupling the LA-

conjugated [AEIETDK(α-lipoic)ATIGFEVQEEGK-OVA]peptide. The presence of the hydrophobic lipoic acid moiety covalently bound to the peptide was again confirmed by the significant differences in HPLC-elution profiles when peptides without or with LA were analyzed (elution time 1.067.8 m/z *vs* 1.163.3 m/z; purity > 93%).

## IFT

In the IFT cryostat sections from rat liver, kidney, heart, stomach and human thyroid were used to detect AMA and other autoantibodies<sup>[16]</sup>.

## ELISA

The ELISA for the detection of anti-M2/PDC-E2 antibodies was performed as described<sup>[17]</sup>.

Antibody reactivity with PDC-E2 peptides was determined using microtiter plates (Maxisorp, Nunc, Denmark) coated with 100 μL of each peptide at a concentration of 25 μg/mL in coating buffer (hydrogen bicarbonate buffer, 0.2 mol/L, pH 9.6) overnight at 4°C. After extensive washing and blocking with phosphate buffered saline (PBS) (60 mmol/L, pH 7.4) containing 1% bovine serum albumin (BSA) for 60 min they were incubated with 100 μL of patients' sera at a dilution of 1:500 for 90 min at room temperature. After washing with PBS containing 0.2% Triton X 100 and 0.5% BSA, the wells were incubated with peroxidase-conjugated monovalent anti-human IgG- and IgM-antibodies from goat (Dianova, Hamburg, Germany; dilution 1:3000) for 60 min at room temperature, washed as above, and AMA reactivity detected using orthophenyldiamine as substrate. Antibody reactivity was given as absorbance × 1000.

Optimal peptide concentrations (25 μg/mL) and serum dilutions (1:500) had been determined by serial dilutions prior to the study.

Normal ranges for antibody reactivities with all antigens/peptides were determined by analysis of 22 healthy donors. Mean value of their absorbance plus double the standard deviation was defined as cut off value.

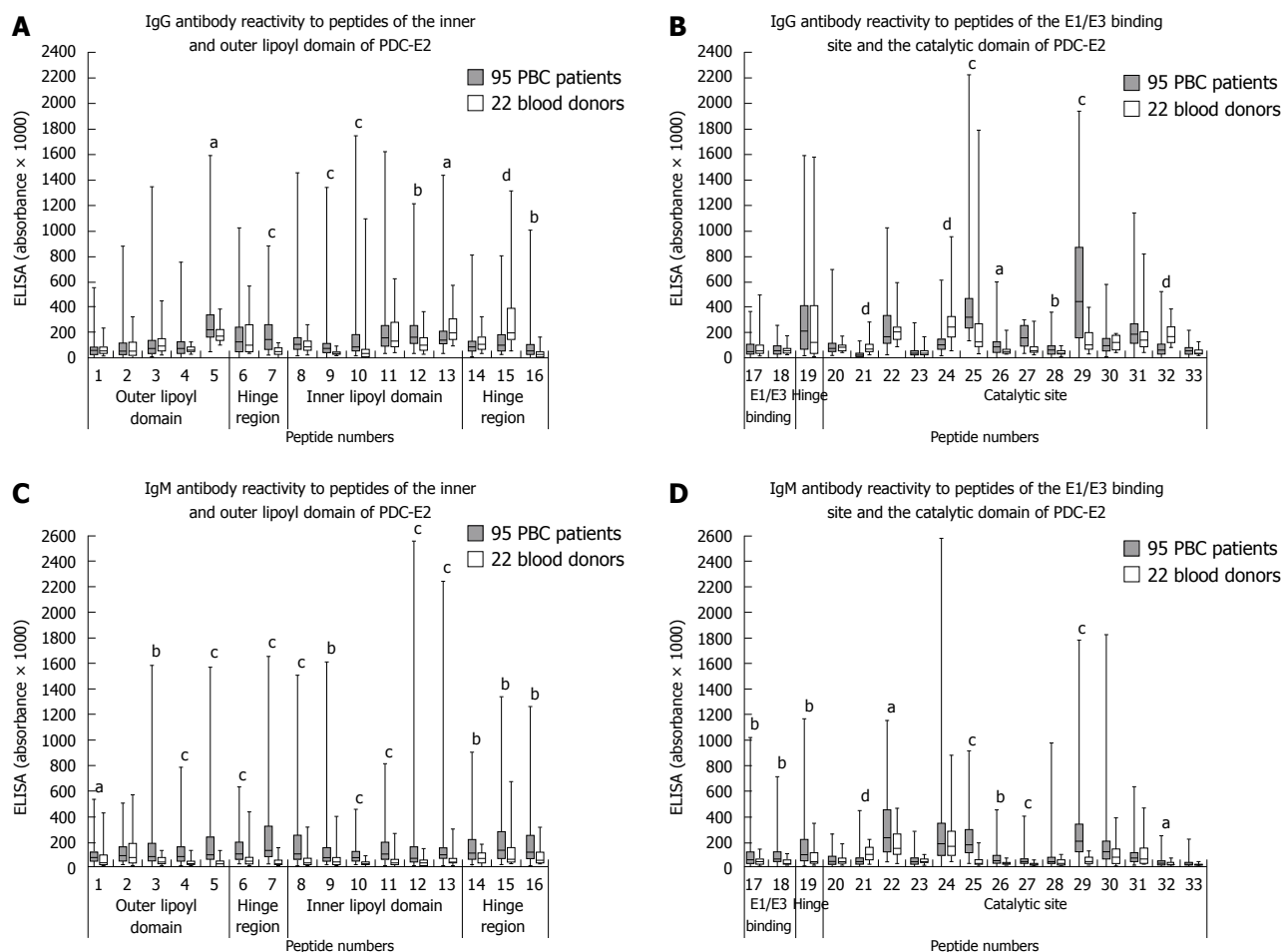
## Statistical analysis

For the comparison of antibody reactivity in different groups of patients, SPSS version 15.0 was used applying the non parametric Mann-Whitney test. For analysis of paired data the Wilcoxon signed rank test was used. Differences with *P* < 0.05 were considered statistically significant.

## RESULTS

### High incidence of antibodies to epitopes within the catalytic site of PDC-E2 in PBC sera

Sera from 95 patients with PBC all being anti-M2/PDC-E2 positive in the ELISA using the purified M2-antigen and the commercially available PDC were tested



**Figure 2** Box plots showing the reactivity of sera from 95 primary biliary cirrhosis (PBC) patients (grey bars) and 22 blood donors (white bars) with 33 overlapping peptides spanning the whole PDC-E2 sequence. IgG antibody reactivities. A: Peptide 1-16; B: Peptide 17-33; IgM antibody reactivities. C: Peptide 1-16; D: Peptide 17-33. Solid bars extend from the 25th to 75th percentile. The line in the middle is the median. The whiskers extend down to the lowest value and up to the highest value. Significantly higher antibody reactivity with PBC sera than with sera from healthy individuals: <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>c</sup> $P < 0.001$ . Significantly lower antibody reactivity with PBC sera than with sera from healthy individuals: <sup>d</sup> $P < 0.001$ . ELISA: Enzyme-linked immunosorbent assay.

against 33 peptides (25 mers) spanning the whole sequence of PDC-E2.

Surprisingly, the reactivity of the PBC sera with the peptides 3, 10 and 11 containing the immunodominant lipoyl binding epitopes in the outer (aa 41-53) and inner lipoyl domain (aa 167-183)<sup>[6,10]</sup> was rather low although significantly higher than that of healthy controls (Figure 2A and C). Only 11% had IgG-antibodies to peptide 3, 29% to peptide 10, and 12% to peptide 11; IgM-antibodies were observed in 28%, 37%, and 39%, respectively. In contrast, up to 74% of the PBC sera reacted with the two peptides 25 and 29 within the catalytic domain and the peptide 7 (aa 101-125) in the first hinge region (Tables 2 and 3), and IgG and IgM reactivity towards these three peptides was significantly higher in PBC patients than in controls (Figure 2).

The peptides 15, 21 and 24 were the only ones whose reactivity was lower with PBC sera as compared to control sera (Figure 2A, B and D).

Most PBC sera recognized several peptides in parallel. IgM-antibodies generally reacted with a higher diversity of peptides than IgG-antibodies (IgG: mean  $\pm$  SD:  $5.3 \pm 7.7$  peptides, median: 3 peptides, range: 0-30

peptides; IgM: mean  $\pm$  SD:  $9 \pm 7.1$  peptides, median: 7 peptides, range: 0-28 peptides). Two PBC sera had neither IgG- nor IgM-antibodies to any of the 33 peptides although both sera showed high antibody reactivities towards PDC and M2 in the ELISA.

Sera from patients with AMA negative/ANA positive PBC hardly reacted with any of the peptides (Tables 2 and 3).

Incidence and reactivity of antibodies to the different peptides in sera from patients with autoimmune hepatitis, alcoholic liver disease and collagen disorders resembled that in healthy controls (data not shown).

#### Reactivity of PBC-sera with the inner lipoyl domain epitope aa 167-184 using different conjugates

In view of the fact that the 25 mers peptide 10 (aa 152-176) and 11 (aa 169-193) used in the epitope mapping above did not completely respond to the published sequence 167-184 (AEIETDKATIGFEVQEEG), we also synthesized the latter peptide in an unlipoylated form and a form containing lipoic acid (LA) at aa K173 (peptide 167-184-LA) and applied both forms in the ELISA. In accordance to a previous study<sup>[12]</sup>, also with



**Table 2** Reactivity and incidence of IgG-antibodies to 33 PDC-E2 peptides in PBC patients as compared to healthy individuals *n* (%)

	Peptide No.	Amino acid	PBC patients		Healthy individuals ( <i>n</i> = 22)
			Anti-M2+ ( <i>n</i> = 95)	AMA-/ANA+ ( <i>n</i> = 11)	
Outer lipoyl domain (aa 1-80)	1	-2-23	4 (4)	0	1 (5)
	2	16-40	6 (6)	0	2 (9)
	3	33-57	10 (11)	0	2 (9)
	4	50-74	15 (16)	0	0
	5	67-91	26 (27)	0	2 (9)
Hinge	6	84-108	17 (18)	0	3 (14)
	7	101-125	42 (44) <sup>1</sup>	0	0
Inner lipoyl domain (aa 133-217)	8	118-142	14 (15)	0	1 (5)
	9	135-159	32 (34)	0	0
	10	152-176	28 (29)	0	2 (9)
	11	169-193	11 (12) <sup>2</sup>	0	2 (9)
	12	186-210	16 (17)	0	1 (5)
Hinge	13	203-227	6 (6)	0	3 (14)
	14	220-244	4 (4)	0	2 (9)
	15	237-261	3 (3)	0	4 (18)
	16	254-278	20 (21)	0	1 (5)
E1/E3-binding site (aa 272-303)	17	271-295	3 (3)	0	1 (5)
	18	288-312	7 (7)	0	1 (5)
Hinge	19	305-329	19 (20)	0	3 (14)
Catalytic site (aa 331-561)	20	322-346	12 (13)	2 (18)	1 (5)
	21	339-363	0	0	1 (5)
	22	356-380	19 (20)	0	1 (5)
	23	373-397	2 (2)	1 (9)	1 (5)
	24	390-414	1 (1)	1 (9)	2 (9)
	25	407-431	53 (56) <sup>3</sup>	0	4 (18)
	26	424-448	10 (11)	0	1 (5)
	27	441-465	5 (5)	0	2 (9)
	28	458-482	18 (19)	0	0
	29	475-499	55 (58) <sup>4</sup>	0	1 (5)
	30	492-516	9 (9)	0	0
	31	509-533	17 (18)	0	2 (9)
	32	526-550	2 (2)	0	1 (5)
	33	543-561	11 (12)	0	1 (5)

<sup>1</sup>Twelve patients had antibodies only of the IgG-, 28 only of the IgM-, and 30 of the IgG- and IgM-type; in total, 70 (71%) reacted with peptide 7; <sup>2</sup>Four patients had antibodies only of the IgG-, 30 only of the IgM-, and 7 of the IgG- and IgM-type; in total, 41 (43%) reacted with peptide 11; <sup>3</sup>Seventeen patients had antibodies only of the IgG-, 18 only of the IgM-, and 37 of the IgG- and IgM-type; in total, 72 (76%) reacted with peptide 25; <sup>4</sup>Two had antibodies only of the IgG-, 16 only of the IgM-, and 53 of the IgG- and IgM-type; in total, 71 (75%) reacted with peptide 29. Similar data as for healthy controls were obtained with sera from patients with autoimmune hepatitis, alcoholic liver disease and collagen disorders. Peptides 3, 10, 11, 25 and 29 are previous and novel immunodominant epitopes. +: Positive; -: Negative.

these peptides reactivity of the 95 PBC sera was low (Table 4).

In order to find out whether the antibodies may recognize a conformational epitope the peptides were coupled to ovalbumin (OVA-peptide 167-184 and OVA-peptide 167-184-LA)<sup>[12]</sup>. With these conjugated peptides reactivity was stronger, but most sera showed a high reactivity already with OVA alone (Figure 3). IgG- and IgM-reactivity towards the unlipoylated OVA 167-184 was significantly higher than that towards the lipoylated form (OVA 167-184-LA), reactivity towards both forms of peptides was significantly higher than that against OVA alone.

**Table 3** Reactivity and incidence of IgM-antibodies to 33 PDC-E2 peptides in PBC patients as compared to healthy individuals *n* (%)

	Peptide No.	Amino acid	PBC patients		Healthy individuals ( <i>n</i> = 22)
			Anti-M2+ ( <i>n</i> = 95)	AMA-/ANA+ ( <i>n</i> = 11)	
Outer lipoyl domain (aa 1-80)	1	-2-23	7 (7)	0	1 (5)
	2	16-40	10 (11)	0	2 (9)
	3	33-57	27 (28)	0	0
	4	50-74	33 (35)	1 (9)	1 (5)
	5	67-91	42 (44)	0	1 (5)
Hinge	6	84-108	31 (33)	0	1 (5)
	7	101-125	52 (55) <sup>1</sup>	1 (9)	2 (9)
Inner lipoyl domain (aa 133-217)	8	118-142	34 (36)	0	1 (5)
	9	135-159	35 (37)	0	2 (9)
	10	152-176	35 (37)	0	1 (5)
	11	169-193	37 (39) <sup>2</sup>	0	2 (9)
	12	186-210	27 (28)	0	1 (5)
Hinge	13	203-227	23 (24)	0	2 (9)
	14	220-244	26 (27)	0	0
	15	237-261	43 (45)	0	3 (14)
	16	254-278	33 (35)	0	1 (5)
E1/E3-binding site (aa 272-303)	17	271-295	15 (16)	0	0
	18	288-312	24 (25)	0	1 (5)
Hinge	19	305-329	36 (38)	0	3 (14)
Catalytic site (aa 331-561)	20	322-346	1 (1)	0	0
	21	339-363	4 (4)	0	0
	22	356-380	24 (25)	0	1 (5)
	23	373-397	10 (11)	0	0
	24	390-414	23 (24)	1 (9)	3 (14)
	25	407-431	55 (58) <sup>3</sup>	0	1 (5)
	26	424-448	21 (22)	0	0
	27	441-465	16 (17)	0	0
	28	458-482	15 (16)	0	0
	29	475-499	70 (74) <sup>4</sup>	0	1 (5)
	30	492-516	14 (15)	0	2 (9)
	31	509-533	6 (6)	0	2 (9)
	32	526-550	4 (4)	0	0
	33	543-561	9 (9)	0	1 (5)

<sup>1,2,3,4</sup>Same as Table 2. Peptides 3, 7, 10, 11, 25 and 29 are previous and novel immunodominant epitopes.

After subtracting the reactivities with OVA from that with the OVA-coupled peptides, 42 (44%) of the 95 PBC sera had IgG-antibodies to the unlipoylated (OVA 167-184) and 21 (22%) to the lipoylated peptide (OVA 167-184-LA) (Table 4). IgM antibodies were found in 57% and 31%, respectively.

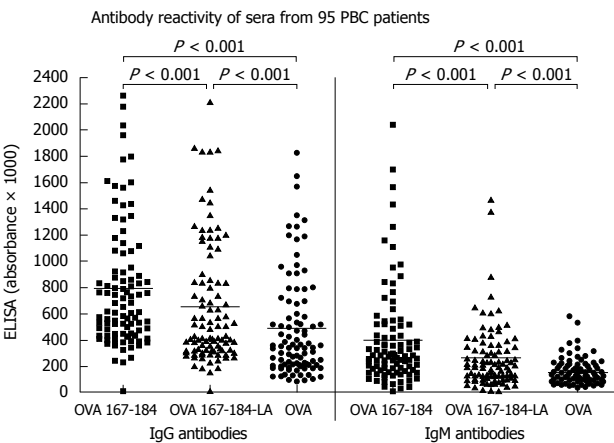
### Influence of UDCA-treatment on anti-peptide reactivities

In order to see whether UDCA-treatment may influence the reactivity with distinct peptides we compared the reaction of 65 patients who were without any therapy at the time of serological analysis with that of 30 patients who received UDCA for at least 6 mo. However, no significant differences in antibody titers were observed for any of these antigens/peptides between the two groups (data not shown), and also mean number of peptides recognized by each patient did not differ [IgG: untreated group: mean  $\pm$  SD  $5.7 \pm 6$  peptides, median 4 peptides, treated group:  $4.5 \pm 5.1$  peptides, median 2 peptides ( $P = 0.11$ ); IgM: untreated group:  $9.2 \pm 7$  peptides, median

**Table 4** Reactivity of sera from 95 anti-M2 positive PBC patients with different peptide conjugates containing the reported immunodominant epitope of PDC-E2<sup>[6,12]</sup>

Peptides of the inner lipoyl domain	IgG-antibodies			IgM-antibodies		
	Positive <i>n</i> (%)	mean absorbance × 1000 ± SD (median)	Cut off value	Positive <i>n</i> (%)	mean absorbance × 1000 ± SD (median)	Cut off value
152-176 (present study peptide 10)	28 (29)	153 ± 243 (87)	150	35 (37)	95 ± 77 (73)	80
169-193 (present study peptide 11)	11 (12)	210 ± 219 (157)	300	37 (39)	143 ± 120 (101)	120
167-184	0	113 ± 63 (93)	100	5 (5)	53 ± 79 (20)	100
167-184-LA	3 (3)	135 ± 71 (111)	100	10 (11)	78 ± 107 (40)	100
OVA-167-184 <sup>1</sup>	42 (44)	316 ± 260 (271)	100	54 (57)	254 ± 318 (117)	100
OVA-167-184-LA <sup>1</sup>	21 (22)	175 ± 280 (148)	100	29 (31)	113 ± 217 (52)	100

<sup>1</sup>After subtraction of reactivity to OVA alone.



**Figure 3** IgG- and IgM-reactivity of sera from 95 PBC patients with the OVA coupled unlipoylated and lipoylated peptide 167-184 (OVA-167-184 and OVC-167-184-LA) (without subtraction of the OVA-values) and OVA alone.

7 peptides, treated group:  $8.5 \pm 7$  peptides, median 6 peptides ( $P = 0.65$ ).

**DISCUSSION**

The inner lipoyl domain of PDC-E2 and especially the peptide aa167-184 has been previously considered the prominent immunodominant epitope recognized by sera from PBC-patients, although there is no general consensus<sup>[6,10,11,15,18-20]</sup>. In these analyses sera from PBC patients were incubated with the peptide, and it was shown that it absorbed the anti-M2 activity, albeit only at high serum dilutions. In other studies fusion proteins were used containing the peptide and tested by ELISA against PBC sera revealing positive results. However, using this peptide in the ELISA, no significant reactivity with PBC sera was observed<sup>[5,12]</sup>. There was evidence that antigenicity could be improved after coupling the peptide to ovalbumin and/or lipoylation of K173<sup>[12]</sup>, but this is also still controversially discussed. Accordingly, the uncertainty on reactivity with the linear epitope and an absolute requirement for lipoylation of PDC-E2 for antibody reactivity strongly suggests that rather a conformational epitope within the inner lipoyl domain may

represent the immunodominant epitope as outlined by several authors<sup>[9,11]</sup>.

In the present study analyzing a large group of 95 patients with clinically, serologically and histologically defined PBC we could largely confirm these data. Thus, testing the sera by ELISA against 25 mer peptides spanning the whole sequence of PDC-E2, we found only a weak antibody reactivity to peptide 10 (aa 152-176) or peptide 11 (aa 169-193) containing the lipoyl binding K173 and a low incidence of positive results. Since these peptides derived from the peptide scan did not completely correspond to the published peptide 167-184, we also synthesized this epitope but again did not obtain significant reactions. In accordance with a previous report, antigenicity could be increased by coupling with OVA<sup>[12]</sup> while lipoylation had no additional effect. However, already antibody reactivity to OVA itself was rather high indicating that this protein is not suitable for coupling autoantigens to detect conformational autoantibodies in sera from patients with autoimmune disorders. The wide distribution of antibodies to OVA in the general population is a well known phenomenon<sup>[21]</sup>.

In contrast, we found strongly reactive linear epitopes in the first hinge region (peptide 7, aa 101-125) and in the catalytic domain of PDC-E2 - until now regarded as ‘immunologically silent’<sup>[9,14]</sup>. Thus, up to 55% of the 95 PBC sera had antibodies of the IgG- or IgM-type to peptide 7 and up to 74% to the peptides aa 407-431 (peptide 25) or aa 475-499 (peptide 29) in the catalytic domain. The specificity of this reaction was underlined by the finding that sera from patients with AMA negative PBC or other disorders did not react with these peptides. Although there have been several studies analyzing the catalytic domain they failed to detect relevant antibody reactivities<sup>[6,9,12]</sup>, but most of them used larger peptide sequences indicating that - in contrast to antibodies to the inner lipoyl domain - antibodies to the catalytic domain may be rather directed against linear and not against conformational peptides.

The reaction of PBC sera with the catalytic domain is of interest since it has been reported by several authors that anti-PDC-E2 antibodies inhibit the PDC-E2 enzyme activity, which has been attributed until now to their binding to the inner lipoyl domain<sup>[22-25]</sup>. However,

since the catalytic domain contains the active site an enzyme inhibition by interference with this region seems even more likely. Thus, the catalytic centre is formed by a long channel across the interface between the catalytic domains of two neighbored E2-subunits. The opening of the channel pointing towards the outer face of the molecule forms the lipoamide binding site, whereas the opposite entrance corresponds to the coenzyme A (CoA) binding site. In the middle of the catalytic centre there is a pair formed by H534 and Ser480 from neighboring E2-subunits (nomenclature according to human PDC) which separates these two substrate binding sites and is directly involved in the transacetylase reaction<sup>[26-32]</sup>. Our observation in the present study that highest antibody reactivity in PBC sera was obtained with peptide 29 (aa 475-499) which contains Ser480, might, therefore, also explain the inhibitory potency of PBC sera on PDC-E2-enzyme activity. The inner catalytic domain as a whole shares less than 35% sequence identity in prokaryote and human PDC. In contrast, the structures at the active sites are highly conserved in pro- and eukaryotes. The reactivity of autoantibodies with evolutionary highly conserved enzymes and their interaction with enzyme function *in vitro* is a quite frequently observed phenomenon in autoimmune disorders in general<sup>[33,34]</sup>.

The etiopathogenetic role of anti-M2/PDC-E2 antibodies for PBC is still a matter of debate. Coupling of the inner lipoyl domain with 2-octynoic acid instead of lipoic acid has been discussed to increase antigenicity of the enzyme PDC-E2, and common environmental, cosmetic and food additives containing this 2-octynoic acid have, therefore, be postulated to play a role in their induction by formation of an altered PDC-E2 in the sense of a neoantigen<sup>[15,35,36]</sup>. Our observation that anti-PDC-E2 antibodies react preferentially with the active site of the catalytic domain of PDC-E2 is strongly suggestive for a further mechanisms including molecular mimicry or defects in apoptosis<sup>[5,37,38]</sup>. Interestingly, similarity alignment (<http://www.expasy.org>) revealed that a sequence consisting of the five aa TFTIS within peptide 29 containing the active site S480 shares an identity of 80% with a five-aa-peptide within an envelope protein of human  $\beta$ -retrovirus previously cloned from patients with PBC<sup>[39]</sup>. The relevance of this observation is, of course, still rather speculative.

Another peptide strongly recognized by PBC sera (up to 58%) was the peptide 25. However, 18% of healthy controls also reacted with this peptide indicating that probably natural autoantibodies towards this region exist. Also the fact that antibody reactivity to some peptides within the catalytic domain did not differ significantly between healthy individuals and PBC-patients (i.e. peptide 22, 30, 31) or were even lower in the PBC sera than in sera of the controls (peptides 15, 21 and 24) may point towards the existence of natural autoantibodies to several PDC-E2 epitopes as also outlined by others<sup>[5,13]</sup>. It is well known that natural autoantibodies, which play an important role in the 'first line defense' react preferentially with archaic enzymes, and this would fit with our observation. Interestingly, we

also observed antibodies to the PDC-E2 complex in sera from healthy family members of PBC-patients, but these seem to recognize other epitopes than the patients' sera (manuscript in preparation).

In general, most sera recognized several epitopes on the entire PDC-E2 molecule, and diversity of IgM antibodies was even higher than that of IgG antibodies. Using overlapping octameric peptides representing the inner lipoyl domain of PDC-E2 similar observations were made by Mackay *et al.*<sup>[5]</sup>, and they also noted that normal human sera reacted with multiple peptides, although levels of reactivity were generally lower than those observed using PBC sera<sup>[5,40]</sup>.

Two of the 95 PBC sera did not react with any of the 33 peptides although they were strongly anti-M2/PDC-E2 positive in ELISA and Western blotting again underlining the importance of conformational epitopes. Our preliminary findings that anti-M2/PDC-activity could not be significantly absorbed with any of the linear 167-184 peptides or with peptide 25 or 29 (data not shown), fit to this concept. Based upon molecular modeling of antibodies reacting with antigens, over 90% of B-cell epitopes are thought to be conformational<sup>[41,42]</sup>. Attempts to map B-cell epitopes have, therefore, met with mixed success depending largely on the system under investigation. Since there are so many different types of autoepitopes, linear, conformational, cryptic, *etc.*<sup>[43]</sup>, no universally applicable method is available that allows for the identification of all autoepitopes. Performing those analyses one has, therefore, to be aware of the techniques' advantages and disadvantages. Peptide scan as used in the present study does not allow determining conformational epitopes but it has the advantage that even cryptic epitopes can be detected. This method has been proven useful in the identification of immunodominant epitopes of several autoantigens for instance in collagen disorders<sup>[43]</sup>; and considering the concept of "epitope-spreading" it is not unlikely that the multiple immunoreactivity towards several linear and conformational epitopes in an autoimmune disease starts with cross-reactivity to a single (linear) peptide which may lead us to the initiating agent.

In conclusion, we have firmly documented that PBC sera react preferentially with peptides of the catalytic domain of PDC-E2. However, it still has to be proven whether this catalytic domain is also targeted by T-cells. Furthermore, the data again underline the importance of conformational epitopes for AMA-reactivity in PBC.

## COMMENTS

### Background

Antimitochondrial antibodies (AMA) reacting with the 2-oxo acid dehydrogenase complex of the inner mitochondrial membrane are highly specific for the serological diagnosis of primary biliary cirrhosis (PBC), a chronic cholestatic liver disorder of unknown etiology.

### Research frontiers

The M2-antigen consists of five components which have been identified as subunits of the 2-oxo acid dehydrogenase complex: the pyruvate



dehydrogenase complex (PDC), the 2-oxoglutarate dehydrogenase complex and the branched-chain 2-oxo acid dehydrogenase complexes. Each complex comprises multiple copies of three component enzymes termed E1, E2, and E3. The major M2-antigen which is recognized by nearly 90% of PBC sera is the E2 component of PDC.

### Innovations and breakthroughs

Within PDC-E2 an immunodominant epitope has been identified which is associated with the inner lipoyl domain (aa 167-184) wherein a lysine residue (K173) binds the lipoyl cofactor for the enzyme. Binding of lipoyl cofactor to K173 has been discussed to be necessary for antibody reaction. Replacement of this lipoyl acid by 2-octynoic acid seemed even to enhance antibody binding. It was, therefore, speculated that xenobiotics may lead to an accumulation of 2-octynoic acid hereby preventing binding of lipoyl acid to the PDC-E2 and generation of neoantigen with the consequence of generation of autoreactive B- and T-cells. The present study contradicts this concept to some extent indicating that epitopes within the catalytic site of PDC-E2 are even stronger reactive with AMA.

### Applications

The identification of immunodominant epitopes recognized by autoreactive T- or B-cells may improve our understanding with respect to their generation (for instance as consequence of cross reactivity with infectious agents or induction by neoantigens) and their possible pathogenetic role in PBC.

### Terminology

The 2-oxoacid dehydrogenase complex is a multi enzyme complex located at the inner membrane of mitochondria which catalyzes the oxidative decarboxylation of 2-oxo acids to the corresponding acyl-coenzyme A. It is not yet known why this non-organ specific protein complex becomes an autoantigen recognized by autoantibodies in a disease affecting mainly bile ducts such as PBC.

### Peer review

The authors analyse immunodominant targets of PDC-E2 in sera of about 100 patients with PBC. Interestingly, they show that the catalytic site of PDC-E2 contains several immunodominant epitopes. The group has an outstanding expertise in the field of clinical immunology. The data presented in the manuscript add to the understanding of immunological events in PBC.

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## Clinical and cost impact of intravenous proton pump inhibitor use in non-ICU patients

Soumana C Nasser, Jeanette G Nassif, Hani I Dimassi

Soumana C Nasser, Jeanette G Nassif, Hani I Dimassi, Department of Pharmacy Practice, School of Pharmacy, Lebanese American University, Byblos Campus, PO Box 36, 13-5053 Beirut, Lebanon

**Author contributions:** Nasser SC designed the trial, contributed in data collection and entry, analyzed and interpreted the data, wrote and critically revised the manuscript; Nassif JG helped in designing the trial, reviewed the literature, wrote and critically revised the manuscript; Dimassi HI conducted the statistical analysis, interpreted the data and reviewed the manuscript.

**Correspondence to:** Soumana C Nasser, Pharm. D, Clinical Assistant Professor, Department of Pharmacy Practice, School of Pharmacy, Lebanese American University, Byblos Campus, PO Box 36, 13-5053 Beirut,

Lebanon. [soumana.nasser@lau.edu.lb](mailto:soumana.nasser@lau.edu.lb)

Telephone: +961-3-489860 Fax: +961-9-547256

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### Abstract

**AIM:** To assess the appropriateness of the indication and route of administration of proton-pump-inhibitors (PPIs) and their associated cost impact.

**METHODS:** Data collection was performed prospectively during a 6-mo period on 340 patients who received omeprazole intravenously during their hospital stay in non-intensive care floors. Updated guidelines were used to assess the appropriateness of the indication and route of administration.

**RESULTS:** Complete data collection was available for 286 patients which were used to assess intravenous (IV) PPIs utilization. Around 88% of patients were receiving PPIs for claimed stress ulcer prophylaxis (SUP) indication; of which, only 17% met the guideline criteria for SUP indication, 14% met the criteria for non-steroidal-anti-inflammatory drugs-induced ulcer prophylaxis, while the remaining 69% were identified as having an unjustified indication for PPI use. The

initiation of IV PPIs was appropriate in 55% of patients. Half of these patients were candidates for switching to the oral dosage form during their hospitalization, while only 36.7% of these patients were actually switched. The inappropriate initiation of PPIs *via* the IV route was more likely to take place on the medical floor than the surgical floor (53% *vs* 36%,  $P = 0.003$ ). The cost analysis associated with the appropriateness of the indication for PPI use as well as the route of administration of PPI revealed a possible saving of up to \$17732.5 and \$14571, respectively.

**CONCLUSION:** This study highlights the over-utilization of IV PPIs in non-intensive care unit patients. Restriction of IV PPI use for justified indications and route of administration is recommended.

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**Key words:** Cost saving; Lebanon; Non-intensive care unit patients; Omeprazole; Over-utilization; Proton-pump-inhibitors; Stress ulcer prophylaxis

**Peer reviewer:** Jorgen Rask-Madsen, MD, FRCP, Professor of Gastroenterology, Department of Gastroenterology, Herlev Hospital, Skodsborg Strandvej 280A, 2942, DK-2730, Herlev, Denmark

Nasser SC, Nassif JG, Dimassi HI. Clinical and cost impact of intravenous proton pump inhibitor use in non-ICU patients. *World J Gastroenterol* 2010; 16(8): 982-986 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i8/982.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i8.982>

### INTRODUCTION

Proton-pump-inhibitors (PPIs) are the most effective agents for reducing gastric acid secretion and are commonly used in a variety of gastrointestinal (GI) related disorders<sup>[1]</sup>. The dramatic increase in PPI prescribing patterns over the past several years has raised concerns

relating to their appropriate utilization and associated cost<sup>[2,3]</sup>. Many health care centers have raised concerns related to the inappropriate use of the intravenous (IV) route of administration and unsuitable indications and to a lesser extent incorrect doses and length of therapy. Furthermore, many patients are being inappropriately discharged on PPIs which could potentially increase treatment costs, and the risk of pneumonia and *Clostridium difficile* associated disease<sup>[4]</sup>. Part of the over-utilization of IV PPIs can be explained by their safety profile and the tendency of physicians to manage ill in-patients aggressively<sup>[5]</sup>. Approved indications for IV PPIs are limited to erosive esophagitis in patients unable to tolerate oral medications and patients with pathologic hypersecretory state including Zollinger-Ellison syndrome<sup>[6]</sup>. Oral PPIs are associated with several advantages compared to the IV formulation including lower cost, reduced utilization of hospital resources, and fewer IV related complications. Guda *et al*<sup>[6]</sup> in 2004 reporting on the use of IV PPIs in two hospital settings observed that 56% of patients receiving IV PPIs had inappropriate indications and the majority of these claimed to have stress ulcer prophylaxis (SUP) indications. Prophylaxis against stress ulcer is not routinely recommended in general medicine patients<sup>[4]</sup>. Inappropriate dosing of IV PPIs has attributed to a 292% increase in the cost related to these medications and hence has resulted in an additional expenditure of \$7766 for 64 patients<sup>[7]</sup>. There is a need to assess the prescribing pattern of IV PPIs in the Middle East and Arab countries. In response to the inadequate literature available in the aforementioned region, a drug use evaluation was conducted to assess the appropriateness of the indication and route of administration of PPIs and their associated cost impact in a university hospital in Lebanon.

## MATERIALS AND METHODS

Data collection was performed prospectively from October 15, 2008 to April 15, 2009 on 340 adult patients who received a PPI after being admitted to the medical or surgical floors of a 200 beds university hospital in the Beirut area that attracts patients from all over the country. Patients were identified from the pharmacy computer system prior to and during the dispensing process. Data collection forms were filled for 340 patients, out of which 286 were determined to be complete and accurate. The form included data on patient demographics, medical problems, list of medications used, pertinent laboratory data, and criteria for PPI IV indication, dose, frequency, duration, and indication for switching to oral formulation. Assessment of the appropriateness of IV PPI use was then performed based on the American Society of Health-System Pharmacists guidelines<sup>[8]</sup> and the Eastern Association for the Surgery of Trauma for SUP<sup>[9]</sup>, non-steroidal-anti-inflammatory drugs (NSAIDs)-induced ulcer prophylaxis<sup>[10]</sup>, and criteria for IV to *po* conversion<sup>[11]</sup>.

Patients assessed for PPI indication were divided into two groups, one group of patients using the drug for treatment indication and another group using the drug

Table 1 Patient characteristics (*n* = 286)

	<i>n</i> (%)
Age, yr (median ± IQR)	64 ± 30
Weight, kg (median ± IQR)	72 ± 23
Creatinine clearance, mL/min (median ± IQR)	71.5 ± 57.6
Age ≥ 65 yr	143 (51)
Male	149 (52)
Medical floor	146 (51)
Surgical floor	140 (49)
Dose 40 mg omeprazole	272 (95)
Frequency as once daily	251 (88)
Medications	
Heparins	133 (47)
NSAIDs	74 (26)
Corticosteroids	74 (26)
Aspirin (100 mg)	42 (15)
Clopidogrel	15 (5)
Acenocoumarol	10 (4)

IQR: Interquartile range; NSAIDs: Non-steroidal-anti-inflammatory drugs.

for claimed SUP indication. The claimed SUP indication group was then sub-classified into (1) meeting criteria for SUP indication; (2) meeting criteria for NSAIDs-induced ulcer prophylaxis; and (3) unjustified prophylactic use.

Another analysis, focusing on the route of administration, divided patients into two groups, one group representing those with appropriate initial IV use and the other group representing inappropriate initial IV use.

Cost analysis was performed comparing the cost associated with appropriate initial IV PPI with that of inappropriate initial IV PPI use. The cost included 100 mL solution bag, 5 mL syringe, drug vial, IV line and related materials. Cost analysis was performed on the unnecessary duration of IV use, in cases where oral PPI was used instead of IV and inappropriate utilization. It was assumed that switching from IV to PO was done 1 d earlier in the group of appropriate IV users and prior to initial dispensing in the group of inappropriate IV users.

## Statistical analysis

Statistical analysis was performed using SPSS software. Data were entered into the computer and frequencies, percentages, means, and standard deviations were calculated. Differences in percentages were assessed using the  $\chi^2$  test, and differences in means were assessed using the student *t* test. *P*-values were assessed at the 5% level.

## RESULTS

### Patient characteristics

The study included a similar number of males and females. Half of the patients were 65 years or older, and most of them were receiving omeprazole 40 mg IV once daily. The number of patients from the medical floor and the surgical floor was equal (Table 1).

The reasons for hospital admission were various and included surgery, pain, infection, cancer treatment, dyspnea, trauma or fracture.

Table 2 Justification of PPI indications

	<i>n</i> (%)
Out of the total sample ( <i>n</i> = 286)	
Treatment indications	35 (12)
Claimed as using PPI for SUP indication	251 (88)
Classification of patients claimed as using PPI for SUP indication ( <i>n</i> = 251)	
Meeting criteria for SUP indication	43 (17)
Meeting criteria for NSAIDs-induced prophylaxis	35 (14)
Age ≥ 65 yr + NSAIDs	12 (5)
NSAIDs + antithrombotics	23 (9)
NSAIDs + corticosteroids	17 (7)
Unjustified prophylactic use	173 (69)
Age ≥ 65 yr	81 (32)
Age ≥ 65 yr + LMWH	55 (22)
Age ≥ 65 yr + antiplatelets	60 (24)
Age ≥ 65 yr + corticosteroids	25 (10)
Others	90 (36)

PPI: Proton-pump-inhibitor; SUP: Stress ulcer prophylaxis.

Table 3 Correlation of initial IV administration with indication and eligibility for oral conversion *n* (%)

	Appropriate initial IV use	Inappropriate initial IV use	<i>P</i> value
Total IV use ( <i>n</i> = 286)	158 (55)	128 (45)	
Switched	58 (37)	49 (38)	0.785
Candidate for Switch	82 (52)	127 (99)	< 0.001
Indication			
Treatment (35)	22 (14)	13 (10)	0.556
SUP justified (43)	26 (17)	17 (13)	0.556
NSAIDs-induced (justified) (35)	17 (11)	18 (14)	0.556
Other unjustified prophylaxis (173)	93 (59)	80 (63)	0.556
Frequency as once daily	138 (87)	113 (88)	0.81
Dose 40 mg	152 (96)	120 (95)	0.491
Medical floor	68 (47)	78 (53)	0.003
Surgical floor	90 (64)	50 (36)	0.003
Duration of IV use (median ± IQR)	4.0 ± 4.0	4.0 ± 4.0	0.422

IV: Intravenous.

The most common criteria for using the IV route of administration were an order for nothing by mouth (*n* = 95, 33.2%), unable to swallow (*n* = 18, 6.3%), severe nausea and vomiting (*n* = 17, 5.9%), acute GI bleeding (*n* = 12, 4.2%), and others (*n* = 15, 5.2%) such as gastric obstruction, ileus, severe diarrhea, and malabsorption. Many patients were on antithrombotics, NSAIDs and corticosteroids during hospitalization (Table 1). Other medications included antibiotics (*n* = 205, 71.7%), analgesics (*n* = 171, 59%), metoclopramide (*n* = 98, 34.3%), antihypertensive drugs (*n* = 101, 35.3%) and insulin (*n* = 32, 11.2%).

### Justification of PPI use

The use of IV PPIs was assessed in 286 patients. The indication for claimed SUP was used in 88% of patients, of which only 17% met the guideline criteria for SUP indication, 14% met the criteria for NSAIDs-induced ul-

Table 4 Cost analysis for inappropriate route of administration and indication

	Appropriate initial IV PPI use	Inappropriate initial IV PPI use
Candidate for switch	<i>n</i> = 82	<i>n</i> = 128
Cost of 40 mg IV PPI daily dose that could be avoided	\$1681	\$13120
Cost of 40 mg PPI oral daily dose (if used instead of IV)	\$184	\$1434
Cost that could be avoided with the oral use	\$1497	\$11686
Total cost that could be avoided with IV to po conversion	\$13183	
Cost that could be avoided from Unjustified prophylactic use of IV PPI ( <i>n</i> = 173)	\$17732.5	

cer prophylaxis while the majority (69%) were receiving PPI for an unjustified indication. These indications for PPI treatment were peptic ulcer (4.5%), GERD (1.4%), and stress ulcer treatment (2.4%). Stratification of the unjustified prophylactic use was correlated with age ≥ 65 years and whether or not the patient was receiving antithrombotics or corticosteroid therapy (Table 2). Antithrombotics mainly consisted of low molecular-weight heparins.

### Appropriateness of initial iv use and eligibility for oral conversion

Only about 50% of patients who were initiated on PPIs *via* the IV route were deemed appropriate, of which half were candidates for switching to the oral form during their hospitalization. Only one third of the latter were switched to the oral form. The inappropriate initiation of PPIs *via* the IV route was more likely to take place on the medical floor compared with the surgical floor (53% *vs* 36%, *P* = 0.003).

Regardless of the type of indication, the rate of inappropriate initiation of PPIs *via* the IV route was similar to the rate of appropriate initiation (*P* = 0.556). The likelihood of switching patients to the oral form was independent of the appropriateness of initial IV use (36.7% *vs* 38.3%, *P* = 0.785) (Table 3). Most patients received a 40 mg IV dose once daily for a period of approximately 5 d.

### Cost analysis

The cost analysis was based on the mean of 5-d of IV administration which could be avoided in the group with inappropriate IV use, and the assumption that switching could be done at least 1 d earlier in the group with appropriate IV use.

If PPIs were used for the appropriate indication and by the correct route of administration in the 286 patients assessed during the 6-mo period, at least \$17732.5 and \$13183, could have been saved, respectively (Table 4).

## DISCUSSION

The high prescribing pattern of IV PPIs has been dem-



onstrated in this study. The majority of patients were identified as using IV PPIs for claimed SUP indication with a relatively small percentage of patients (17%) meeting the criteria for SUP, which is expected since only non-intensive care unit (ICU) patients were included in the trial. The inappropriateness of use was encountered more on the medical floor compared with the surgical floor. This highlights the need for a clinical pharmacist in that area. Among other patients claimed to be treated for SUP, 14% met the criteria for other indications such as prophylaxis of NSAIDs-induced ulcer. This left a large number of patients using PPIs for unjustified indications. Thus, to minimize over-utilization of PPIs for SUP, prescribers should specify the indication and the reason for the PPI on the medication orders, which would then facilitate medical order screening by the dispensing pharmacist. Around 36% of the patients who claimed to receive IV PPIs for SUP had no risk factors or any known justification for PPI use. It seems that age  $\geq 65$  years old was used as a criterion for PPI use; however, these patients had several medical illnesses and were on multiple drug regimens that may have increased the risk of adverse effects. A retrospective review of the medical charts of elderly patients revealed that around 30% of geriatric patients with a prescription for a PPI from a geriatric ambulatory care practice within an urban academic medical center had no documented indication<sup>[12]</sup>.

These findings highlight the role of the clinical pharmacist in the selection of appropriate candidates for switching.

The cost burden associated with inappropriate IV PPI use should be minimized. An annual projection of cost could reflect a saving of \$26 366 and \$35 465 with the appropriate route of administration and indication, respectively. It is important to note that this cost saving was underestimated since the cost of health care staff time was not included in the calculations and the delay in switching was assumed to be only 1 d. This highlighted the crucial role of a clinical pharmacist to review orders for proper indications, route of administration, and appropriate timing for switching.

The results of this study are comparable with several trials in the USA and UK that discussed the inappropriate use of IV PPIs in several institutions<sup>[7,13-15]</sup>. SUP was the most commonly encountered claimed indication for IV PPIs. A 14-mo observational study assessing the use of IV PPIs in non-ICU patients revealed that 22% of patients were receiving the drug according to their established indications. SUP was the presumed indication in 70% of patients receiving IV PPIs, of which only 13% were considered appropriate<sup>[7]</sup>. These findings were also supported by Qadeer *et al.*<sup>[16]</sup> who addressed the concern of SUP in non-ICU patients, stating that such an intervention is often unnecessary and not recommended.

With regard to the limitations of this study, the study was observational and conducted in a single academic medical center. In addition, there are no current established guidelines for the appropriate use of IV PPIs in

the hospital to evaluate their actual use. Moreover, we assumed that patients with no clear documented indication for PPI use received the drug for SUP. An additional step could have been to investigate the seniority and/or the specialty of the physicians over-prescribing IV PPIs.

As for the strengths of this study, the number of patients involved was adequate ( $n = 286$ ) over a sufficient period of time (6 mo). Non-ICU adult patients were selected because these patients may be more prone to inappropriate SUP treatment. Patients were monitored and followed during their hospital stay until discharge, PPI discontinuation or switching to oral PPI. In addition, there are limited data on the utilization pattern of an IV PPI in an institutional setting.

This study highlights the over-utilization of PPIs *via* the IV route of administration and for a claimed SUP indication in non-ICU patients, which results in increased cost to the patients, institution and payers.

Improving the prescribing patterns requires the Hospital Pharmacy and Therapeutics Committee to establish guidelines with input from gastroenterologists on the proper indications for IV PPIs, criteria for switching, dosing, and duration of therapy. Implementation of these guidelines requires multidisciplinary involvement and education of health care professionals concerning appropriate use of this class of medication. Other approaches include creating an IV order template, pharmacists reviewing orders before dispensing to patients, and automatic switching to an H<sub>2</sub> blocker if a PPI was ordered for SUP until more robust trials on PPI become available<sup>[17,18]</sup>.

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## COMMENTS

### Background

Proton pump inhibitors (PPIs) are the most effective agents for treating acid related gastrointestinal (GI) disorders. The utilization of intravenous (IV) formulations of PPIs has dramatically increased in health care institutions for inappropriate indications, route of administration and length of treatment. This is associated with an increased cost burden, increased risk of IV related infections, and utilization of hospital resources.

### Research frontiers

Utilization of IV PPIs in a hospital setting in a Middle East country was evaluated and compared to data from European and USA hospitals. The evaluation was carried out in non-intensive care units where patients do not meet the indication and/or route of administration criteria for IV PPIs, especially those receiving the drug for stress ulcer prophylaxis (SUP) where little or no evidence supports their use.

### Innovations and breakthroughs

Reports of the over-utilization of IV PPIs have been published in different international journals mainly involving European and American hospital settings. Available literature from the Middle East (especially prospective in nature) addressing this overuse pattern in non-intensive care units and emphasizing the role of a clinical and hospital pharmacist in health care systems is inadequate. In addition, a detailed cost analysis projection was performed for inappropriate indications and route of administration of PPIs.

## Applications

The study highlights the essential role of the clinical pharmacist in defining candidates for IV PPIs, especially for SUP, and fosters the role of the Pharmacy and Therapeutics Committee in implementing restriction guidelines for IV PPIs in patients who are not candidates for oral treatment or when the IV route shows better efficacy. A multifaceted approach is needed to improve the prescribing pattern which would involve education of health care professions regarding the appropriate prescribing pattern of IV PPIs.

## Terminology

Proton pump inhibitors are the most effective anti-secretory agents which inhibit the final step in gastric acid secretion and are used to treat several GI related disorders. Stress ulcers also called stress mucosal related disease is a form of hemorrhagic gastritis that occurs in patients after major stressful events such as trauma, surgery, or severe head injury.

## Peer review

The paper is well written. The observations are comparable to those made in Europe, USA, and Asia, as acknowledged by the authors in Discussion. The appropriateness of the statistics applied to analyze the data obtained may be debated, but the use of non-parametric tests is considered preferable in this type of studies.

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## Effects of anti-hypertensive drugs on esophageal body contraction

Koichi Yoshida, Kenji Furuta, Kyoichi Adachi, Shunji Ohara, Terumi Morita, Takashi Tanimura, Shuji Nakata, Masaharu Miki, Kenji Koshino, Yoshikazu Kinoshita

Koichi Yoshida, Kenji Furuta, Shunji Ohara, Terumi Morita, Takashi Tanimura, Shuji Nakata, Masaharu Miki, Kenji Koshino, Yoshikazu Kinoshita, Second Department of Internal Medicine, Shimane University Faculty of Medicine, 89-1 Enya-cho, Izumo-shi, Shimane 693-8501, Japan

Kyoichi Adachi, Department of Clinical Nursing, Shimane University Faculty of Medicine, Enya-cho, Izumo-shi, Shimane 693-8501, Japan

Author contributions: Yoshida K and Furuta K contributed equally to this work; Yoshida K, Furuta K, Adachi K and Kinoshita Y designed the research; Yoshida K, Furuta K, Adachi K, Ohara S, Morita T, Tanimura T, Nakata S, Miki M, Koshino K and Kinoshita Y performed the research; Yoshida K, Furuta K, Adachi K and Kinoshita Y analyzed the data and wrote the manuscript.

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Correspondence to: Kenji Furuta, MD, PhD, Second Department of Internal Medicine, Shimane University Faculty of Medicine, 89-1 Enya-cho, Izumo-shi, Shimane 693-8501, Japan. [kfuruta@med.shimane-u.ac.jp](mailto:kfuruta@med.shimane-u.ac.jp)

Telephone: +81-853-202190 Fax: +81-853-202187

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### Abstract

**AIM:** To clarify the effects of anti-hypertensive drugs on esophageal contraction and determine their possible relationship with gastro-esophageal reflux disease.

**METHODS:** Thirteen healthy male volunteers were enrolled. Esophageal body peristaltic contractions and lower esophageal sphincter (LES) pressure were measured using high resolution manometry. All subjects were randomly examined on four separate occasions following administrations of nifedipine, losartan, and atenolol, as well as without any drug administration.

**RESULTS:** Peristaltic contractions by the esophageal body were separated into three segments by two troughs. The peak peristaltic pressures in the mid and lower segments of the esophageal body under atenolol administration were significantly higher than those without medication in a supine position. On the other hand, peristaltic pressures under nifedipine administration were lower than those observed without drug administration. Losartan did not change esophageal body peristalsis. Atenolol elevated LES pressure and slowed peristaltic wave transition, while the effects of nifedipine were the opposite.

**CONCLUSION:** Among the anti-hypertensive drugs tested, atenolol enhanced esophageal motor activity, which was in contrast to nifedipine.

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**Key words:** Anti-hypertensive drug; High-resolution manometry; Lower esophageal sphincter; Esophageal body contraction; Calcium-channel blocker;  $\beta$ 1 blocker

**Peer reviewer:** Andrew Ukleja, MD, Assistant Professor, Clinical Assistant Professor of Medicine, Director of Nutrition Support Team, Director of Esophageal Motility Laboratory, Cleveland Clinic Florida, Department of Gastroenterology, 2950 Cleveland Clinic Blvd., Weston, FL 33331, United States

Yoshida K, Furuta K, Adachi K, Ohara S, Morita T, Tanimura T, Nakata S, Miki M, Koshino K, Kinoshita Y. Effects of anti-hypertensive drugs on esophageal body contraction. *World J Gastroenterol* 2010; 16(8): 987-991 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i8/987.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i8.987>

### INTRODUCTION

Impaired esophageal motor functions, such as decreased

lower esophageal sphincter (LES) pressure and weak esophageal body peristalsis, are the main causes of gastro-esophageal reflux disease (GERD)<sup>[1,2]</sup>. Conventional manometry has demonstrated that several different drugs impair esophageal motor function. It has also been speculated that they increase esophageal acid exposure<sup>[3]</sup>. Nifedipine, a calcium-channel blocker, was shown to reduce LES pressure, as well as the amplitude and duration of esophageal peristaltic contractions, in healthy subjects. It also increased esophageal acid exposure time<sup>[4-6]</sup>. In addition, candesartan, an angiotensin II (Ang II) receptor antagonist, was reported to reduce the amplitude of swallow-induced peristaltic contractions and LES pressure<sup>[7]</sup>. In contrast, atenolol, a catecholamine  $\beta$  receptor antagonist, was found to inhibit the relaxation of esophageal smooth muscle induced by catecholamine  $\beta$  stimulation<sup>[8]</sup>.

Recent studies using high-resolution manometry revealed that esophageal body peristalsis was composed of a chain of three contraction segments separated by two troughs<sup>[9-11]</sup>. Conventional esophageal manometry techniques are not sensitive enough to detect those three separate contraction segments<sup>[12-14]</sup>. The uppermost (first) peristaltic segment represents the skeletal muscle component of the esophageal body, while the lowest (third) segment represents the smooth muscle component. In the middle (second) segment, the muscle tissue is considered to shift from skeletal to smooth type<sup>[9-13]</sup>. Therefore, neuromuscular contraction control might be different between the three segments. In addition, the second segment was reported to be more responsive to cholinergic stimulation, whereas the third has been demonstrated to be under stronger control of non-cholinergic and non-adrenergic neurons<sup>[15-17]</sup>. Therefore, the various drugs reported to have an influence on esophageal motor function might have different effects on each of the segments of the esophageal body, though the effects of anti-hypertensive drugs on these segments have not been investigated.

The aim of this study was to clarify the effects of three different types of anti-hypertensive drugs on the three different segments of esophageal body contractions using a recently developed high-resolution manometric system.

## MATERIALS AND METHODS

### Subjects

Thirteen male volunteers were recruited for this study (mean age: 34.7 years). None of the subjects had upper gastrointestinal symptoms, a history of upper gastrointestinal surgery, or were taking medications known to influence esophageal motor function. Written informed consent was obtained from each volunteer before starting the study, which was carried out in accordance with the Declaration of Helsinki. This study was approved by the Ethics Committee of Shimane University.

### Study protocol

We evaluated esophageal motor function under sepa-

rate administrations of nifedipine, a calcium-channel blocker, atenolol, a catecholamine  $\beta$  receptor antagonist, and losartan, an angiotensin II receptor antagonist. All subjects were examined four separate times after an overnight fast, with the following administrations; (1) without medication, (2) 10 mg of nifedipine, (3) 50 mg of losartan, and (4) 50 mg of atenolol, which were performed randomly in each case. The administrations of nifedipine, losartan, and atenolol were given at 1, 4, and 4 h, respectively, before performing the esophageal motor function test, to match the maximal blood concentration of each drug with the examination period. In addition, all subjects were instructed to drink 200 mL of water at 4 h and 1 h before each esophageal motor function test.

### Esophageal manometry

We performed high-resolution manometric tests using a ManoScan 360™ (Sierra Scientific Instruments, Inc., California)<sup>[18]</sup>. The manometric catheter used with this system is 4.2 mm in diameter and has 36 intraluminal pressure transducers at 1-cm intervals, which are used to measure peristaltic pressure in the upper esophageal sphincter (UES) to LES simultaneously and continuously. Before performing esophageal pressure measurements, transducers were calibrated at 0 and 100 mmHg using externally applied pressure, according to the manufacturer's instructions. The manometric catheter was inserted in a transnasal manner using 2% lidocaine jelly (Xylocaine jelly; AstraZeneca Co., Osaka, Japan), LES pressure (LESP) was then measured in a sitting position during a 5-min resting period. Next, esophageal body peristaltic function in the sitting position was examined by swallowing 5 mL of room temperature water, which was repeated at 2-min intervals until five recordings of complete esophageal peristalsis were obtained. After finishing the tests in the sitting position, they were repeated in a supine position. The peristaltic contractions in the esophageal body were divided into three different segments (segments 1, 2, and 3 from oral to anal) separated by two troughs, as shown in Figure 1. LESP and peak intraesophageal contraction pressure in the three segments of the esophageal body were analyzed using ManoView™ analysis software (Sierra Scientific Instruments, Los Angeles, CA). Peristaltic contraction velocity between 5 and 15 cm above LES was also determined.

### Statistical analysis

Statistical analysis of paired data was performed using a Wilcoxon signed rank test. All calculations were done using the Stat View 5.0 software package (Abacus Concepts Inc., Berkeley, CA, USA) for Macintosh. Differences at  $P < 0.05$  were considered to be statistically significant.

## RESULTS

All 13 subjects completed the study protocol without any adverse events. LESP values in the supine position were significantly higher than those in the sitting position, both with and without administration of the drugs investigated in this study. Furthermore, LESP with the



**Table 1** Resting LES pressure with and without administration of anti-hypertensive drugs (mean  $\pm$  SE)

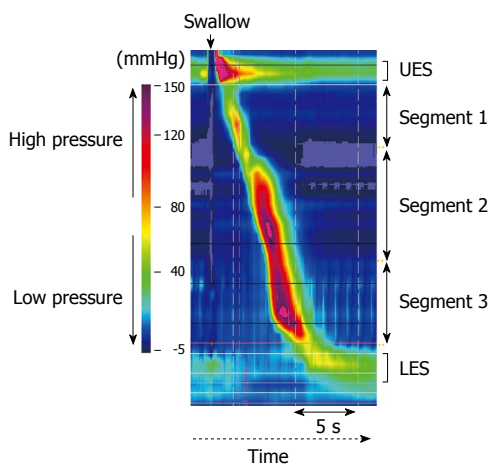
	No medication		Nifedipine		Losartan		Atenolol	
	Sitting	Supine	Sitting	Supine	Sitting	Supine	Sitting	Supine
LESP (mmHg)	12.8 $\pm$ 2.4	19.8 $\pm$ 1.5 <sup>a</sup>	10.2 $\pm$ 1.9	16.7 $\pm$ 1.7 <sup>a</sup>	12.4 $\pm$ 2.7	20.8 $\pm$ 2.1 <sup>a</sup>	15.8 $\pm$ 2.0	21.8 $\pm$ 2.4 <sup>a</sup>

<sup>a</sup>Significantly different in comparison with sitting ( $P < 0.05$ ). LESP: Lower esophageal sphincter pressure.**Table 2** Peak peristaltic pressure in three segments during esophageal body contractions with and without administration of antihypertensive drugs (mean  $\pm$  SE)

	No medication		Nifedipine		Losartan		Atenolol	
	Sitting	Supine	Sitting	Supine	Sitting	Supine	Sitting	Supine
Segment 1 (mmHg)	45.3 $\pm$ 12.9	72.4 $\pm$ 14.4	42.1 $\pm$ 10.1	77.4 $\pm$ 12.6	40.5 $\pm$ 10.5	77.5 $\pm$ 12.0	42.8 $\pm$ 8.2	79.3 $\pm$ 12.2
Segment 2 (mmHg)	105.5 $\pm$ 10.7	117.0 $\pm$ 12.3	83.7 $\pm$ 9.7	109.5 $\pm$ 15.4	96.4 $\pm$ 17.3	120.5 $\pm$ 34.9	122.0 $\pm$ 15.6	153.8 $\pm$ 12.4 <sup>a</sup>
Segment 3 (mmHg)	141.7 $\pm$ 15.2	148.9 $\pm$ 31.6	115.5 $\pm$ 20.6	140.4 $\pm$ 9.8	152.2 $\pm$ 27.9	181.1 $\pm$ 31.9	216.9 $\pm$ 39.4 <sup>a</sup>	246.5 $\pm$ 45.1 <sup>a</sup>

<sup>a</sup>Significantly different in comparison with no medication ( $P < 0.05$ ).**Table 3** Velocity of esophageal peristalsis with and without administration of anti-hypertensive drugs (mean  $\pm$  SE)

	No medication		Nifedipine		Losartan		Atenolol	
	Sitting	Supine	Sitting	Supine	Sitting	Supine	Sitting	Supine
Velocity (mm/s)	4.9 $\pm$ 0.4	4.6 $\pm$ 0.3	5.1 $\pm$ 0.4	4.8 $\pm$ 0.2	4.8 $\pm$ 0.5	4.6 $\pm$ 0.4	4.7 $\pm$ 0.3	4.4 $\pm$ 0.2 <sup>a</sup>

<sup>a</sup>Significantly different in comparison with no medication ( $P < 0.05$ ).**Figure 1** Peristaltic contractions in the esophageal body were divided into three different segments (segments 1, 2, and 3, from oral to anal) by two troughs. UES: Upper esophageal sphincter; LES: Lower esophageal sphincter.

administration of nifedipine tended to be lower, while that under the administration of atenolol tended to be higher, as compared to the control (Table 1).

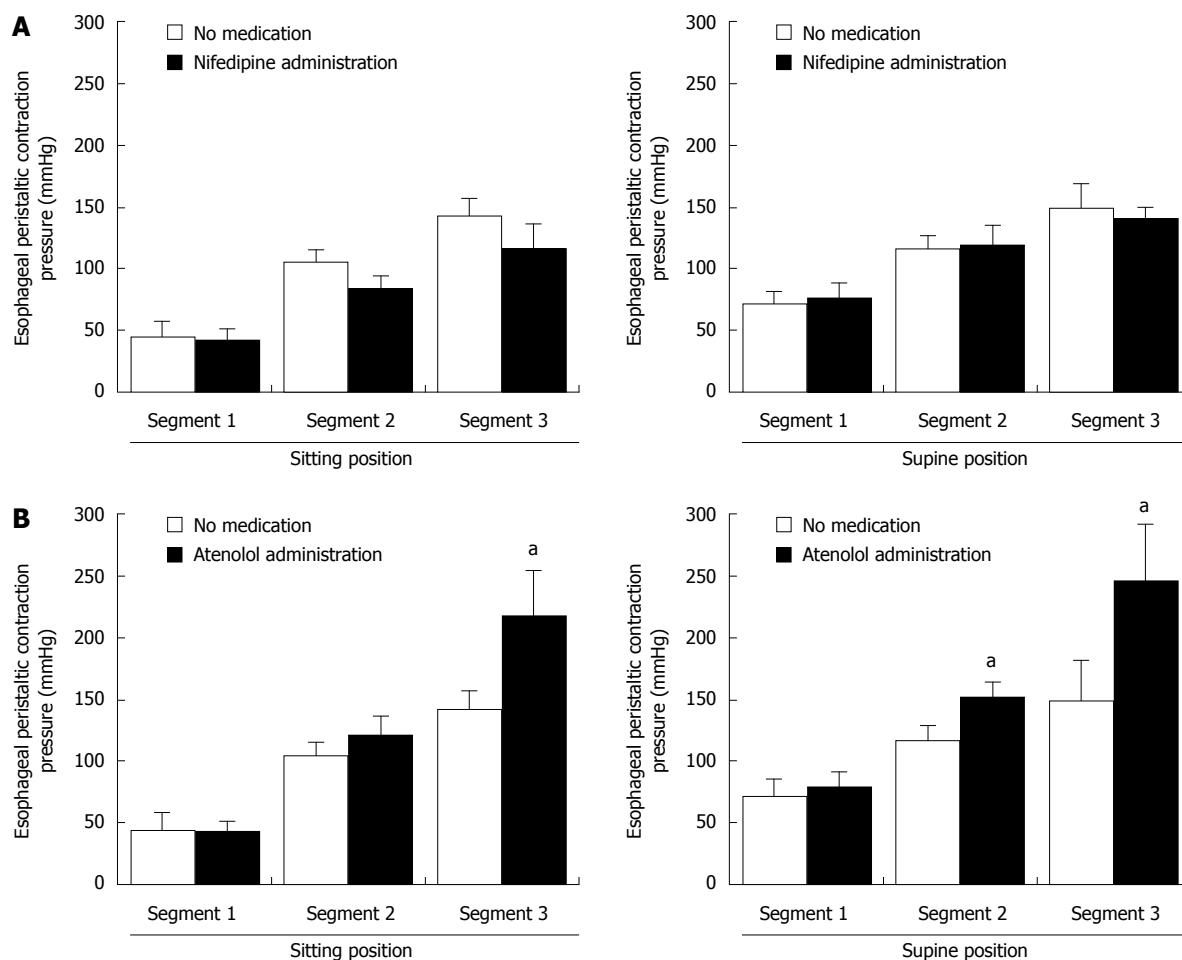
The peak peristaltic pressures in the three segments of the esophageal body in the supine position were significantly higher than those in the sitting position, and also significantly increased from segment 1 to 3 (Table 2). However, there was no difference in peak contraction pressure in segment 1 of the esophageal body with and

without administration of the drugs. In contrast, peak contraction pressure in segments 2 and 3 with nifedipine administration tended to be lower than those without medication (Table 2 and Figure 2). On the other hand, peak pressures in those segments under atenolol administration were higher than without medication (Table 2 and Figure 2). Losartan did not significantly affect peak contraction pressures in the second and third segments (Table 2).

Esophageal peristaltic velocity in the supine position tended to be lower than that in the sitting position. Furthermore, under the administration of nifedipine, it tended to be faster than that without medication. On the other hand, velocity with atenolol was significantly slower than that without medication (Table 3).

## DISCUSSION

Calcium-channel blockers, such as nifedipine, inhibit the entry of calcium into smooth muscle cells of the arterial wall, and are widely used for treatment of patients with ischemic heart disease and hypertension<sup>[5]</sup>. Orally administered nifedipine was shown not only to decrease LESP in healthy subjects and patients with achalasia, but also reduced the amplitude and duration of esophageal peristaltic contractions in healthy subjects<sup>[4,6]</sup>. Ang II is the key mediator of the rennin-angiotensin system, which maintains extracellular fluid volume and electrolyte homeostasis, and also regulates vascular tone and blood pressure<sup>[19]</sup>. The



**Figure 2** Peak esophageal peristaltic pressures in the three segments with and without administration of drugs. A: Nifedipine; B: Atenolol. <sup>a</sup> $P < 0.05$  vs no medication.

physiological functions of Ang II are mediated specifically by the Ang II type 1 (AT1) and type 2 (AT2) receptors. Losartan, an AT1 receptor antagonist, was previously reported to reduce the amplitude of swallow-induced peristaltic esophageal contractions and LESP<sup>[7]</sup>. The catecholamine  $\beta$ -adrenoceptor is currently classified into  $\beta_1$ ,  $\beta_2$ , and  $\beta_3$  subtypes, all of which are expressed in smooth muscle cells. Smooth muscle in the gastrointestinal tract is known to have the  $\beta_1$ -adrenoceptor subtype and it has been shown that smooth muscle relaxes in response to  $\beta$ -adrenoceptor stimulation. In addition, atenolol, a  $\beta_1$  blocker, was reported to inhibit the relaxation of esophageal smooth muscle induced by  $\beta_1$ <sup>[8]</sup>. These observations of the effects of nifedipine, losartan, and atenolol were obtained in studies that used conventional intraesophageal pressure monitoring systems. Those are able to determine intraesophageal pressure at some sites, but they are not sensitive enough to measure peristaltic contractions in the three different esophageal body segments<sup>[8,9]</sup>.

Peristaltic contraction in segment 3 of the esophageal body is the strongest in amplitude and the most important factor for volume clearance of acidic refluxant from the stomach<sup>[20]</sup>. Therefore, to clarify the effects of the three anti-hypertensive drugs on the development of GERD, their effects on the three different segments,

especially the lowest segment (segment 3) were investigated in the present study.

We used high-resolution manometry to measure intraesophageal pressure in 36 different sites at the same time, while contractions in each of the three esophageal segments were separately measured<sup>[14]</sup>. As reported by other investigators using conventional manometry, nifedipine tended to decrease LESP, while atenolol increased it. Similarly, nifedipine tended to decrease the amplitude of peristaltic contractions in segment 3, while atenolol significantly increased it in both the sitting and supine positions. These observations fit well with previous reports that a regular administration of calcium antagonists for treatment of hypertension is a risk factor for the future occurrence of GERD. Interestingly, atenolol significantly elevated LESP and the peristaltic amplitude in the lower esophageal body (segment 3). Such atenolol-induced alterations of esophageal motor activity may prevent the development of GERD.

An interesting finding in our study was the reciprocal relationship between contraction amplitude and peristaltic velocity of the esophageal body. Factors that induce augmentation of peristaltic amplitude, such as atenolol administration and the supine position, were found to delay peristaltic velocity<sup>[21,22]</sup>. On the other hand, factors

that caused a decrease in contraction amplitude, such as nifedipine administration and the sitting position, accelerated peristaltic contraction velocity<sup>[21,22]</sup>. Although the mechanisms by which these reciprocal phenomena occur are not clear, slowly progressing high amplitude peristalsis might be a more efficient peristaltic wave for propelling esophageal contents down to the stomach.

Another interesting finding was the lack of significant effect by losartan on esophageal motor activity, which differs from previous reports, though the reason is not clear. We used modern high-resolution measurements in the present study. Therefore, at least in healthy volunteers, we believe that the inhibiting effect of losartan on esophageal motor function is not clinically important.

In conclusion, of the anti-hypertensive drugs tested, atenolol enhanced esophageal motor activity, which was in contrast to a calcium antagonist.

## COMMENTS

### Background

Nifedipine, a calcium-channel blocker, was shown to decrease lower esophageal sphincter pressure and increase esophageal acid exposure time, while atenolol, a  $\beta_1$  blocker, was shown to inhibit relaxation of the smooth muscle of the esophagus. However, the influence of these anti-hypertensive drugs on the segment of esophageal body contraction using high-resolution manometry was not fully investigated.

### Research frontiers

Several reports have demonstrated that anti-hypertensive drugs affect the esophageal motor function and might facilitate gastroesophageal reflux. However, the details of the drug-induced impairment of esophageal motor function are not clear. This is the first published data concerning the use of high-resolution manometry to study the effects of anti-hypertensive drugs on the motor activity of the different segments of the esophageal body.

### Innovations and breakthroughs

Previous data on the effect of anti-hypertensive drugs were obtained in studies that used conventional intraesophageal pressure monitoring systems. In this study, the authors observed esophageal body contraction using high-resolution manometry with 36 intraluminal transducers. The esophageal body was divided into three segments manometrically. Nifedipine and atenolol were found to affect motor activity only in the middle and lower segments of the esophagus. Anatomical differences in the contraction of the esophageal body during the administration of anti-hypertensive drugs were clarified for the first time.

### Applications

By understanding the anatomical responses of the esophageal body motor activity to the administration of anti-hypertensive drugs, more appropriate selection of anti-hypertensive drugs for patients with gastro-esophageal reflux disease (GERD) will be facilitated.

### Terminology

Nifedipine, atenolol, and losartan are all drugs widely used for the treatment of hypertension. As both of hypertension and GERD are common diseases, the effects of anti-hypertensive drugs on the esophageal motor activity are important.

### Peer review

This is a well-designed study with limited new information about drug effects on esophageal motility. The references are well selected and the discussion is good.

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## Endoscopy-based early enterostomy closure for superior mesenteric arterial occlusion

Takatsugu Oida, Hisao Kano, Kenji Mimatsu, Atsushi Kawasaki, Youichi Kuboi, Nobutada Fukino, Sadao Amano

Takatsugu Oida, Hisao Kano, Kenji Mimatsu, Atsushi Kawasaki, Youichi Kuboi, Nobutada Fukino, Department of Surgery, Social Insurance Yokohama Central Hospital, 268 Yamashita-cho, Naka-ku, Yokohama 231-8553, Japan  
 Sadao Amano, Department of Surgery, Nihon University School of Medicine, 30-1, Kami-cho, Itabashi-ku, Tokyo 177-0023, Japan

Author contributions: Oida T, Mimatsu K, Kano H, Kawasaki A, Kuboi Y and Fukino N carried out the surgery and were consultants overseeing the patients' care; Oida T wrote the manuscript; Amano S drafted the manuscript and revised it critically.

Correspondence to: Takatsugu Oida, MD, PhD, Department of Surgery, Social Insurance Yokohama Central Hospital, 268 Yamashita-cho, Naka-ku, Yokohama 231-8553, Japan. [ooida.takatsugu@yokochu.jp](mailto:ooida.takatsugu@yokochu.jp)

Telephone: +81-45-6411921 Fax: +81-45-6719872

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**CONCLUSION:** Endoscopic examination of blood flow and edema in the remnant bowel is useful to assess the feasibility of early closure of enterostomy in SMAO cases.

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**Key words:** Superior mesenteric arterial occlusion; Closure of enterostomy; Endoscopic inspection

**Peer reviewer:** Dr. Marek Bebenek, MD, PhD, Department of Surgical Oncology, Regional Comprehensive Cancer Center, pl. Hirszfelda 12, 53-413 Wroclaw, Poland

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### Abstract

**AIM:** To evaluate the efficacy of endoscopic examination of blood flow and edema in the remnant bowel.

**METHODS:** We retrospectively studied 15 patients who underwent massive bowel resection with enterostomy for superior mesenteric arterial occlusion (SMAO); the patients were divided into a delayed closure group (D group) and an early closure group (E group).

**RESULTS:** The mean duration from initial operation to enterostomy closure was significantly shorter in the E group ( $18.3 \pm 2.1$  d) than in the D group ( $34.3 \pm 5.9$  d) ( $P < 0.0001$ ). The duration of hospitalization after surgery was significantly shorter in the E group ( $33 \pm 2.2$  d) than in the D group ( $51 \pm 8.9$  d) ( $P < 0.0002$ ).

### INTRODUCTION

Construction of a temporary stoma is a relatively common surgical procedure. A transient stoma is known to lower the operative risk, but it should be closed at the earliest opportunity; however, in the literature, the morbidity and mortality rates after ileostomy or colostomy closure are rather high<sup>[1-8]</sup>. Several studies have compared colostomy closure and ileostomy closure, and found that a multitude of factors influence the development of complications after stoma closure, such as perioperative treatment, time of operation, and surgical technique<sup>[9-12]</sup>. Patients with superior mesenteric arterial occlusion (SMAO) often require massive bowel resection because of considerable intestinal necrosis. Massive intestinal resection often causes short-bowel syndrome and necessitates parenteral nutrition. In order to avoid



the development of this syndrome, it is important to retain as much of the remnant bowel as possible. SMAO is known to frequently occur in patients with atrial fibrillation; hence, even if the patients survive after the initial operation, the risk of disease recurrence remains considerably high. Moreover, reperfusion injury may occur in SMAO patients, thereby causing unstable hemodynamics and multiple organ failure<sup>[13,14]</sup>. Calvin *et al.*<sup>[15]</sup> reported that bowel infarction recurred in 32% of patients early after the resection of the necrotic bowel. In the case of enterostomy, it is difficult to determine the timing of enterostomy closure after the initial operation. In order to assess the feasibility of early closure and its outcome, we endoscopically inspected blood flow and edema in the remnant bowel of stoma patients and defined a minimal delay as optimal for closing small bowel stomas. In this study, we evaluated the efficacy of endoscopic examination of blood flow and edema in the remnant bowel.

## MATERIALS AND METHODS

### Patients

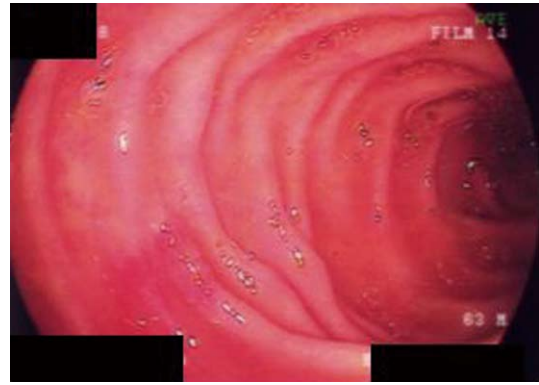
We retrospectively studied 15 patients (12 men and 3 women; age range: 57-74 years; mean age: 68 years) who had undergone massive bowel resection with enterostomy for SMAO between April 1990 and March 2009 at the Department of Surgery, Social Insurance Yokohama Central Hospital, Yokohama, Japan. The patients were divided into 2 groups according to the timing of enterostomy closure: delayed closure group (D group) and early closure group (E group). All patients gave written informed consent to this study.

### Surgical technique

The technique used for stoma closure did not differ between the E and D groups. In brief, after thorough mobilization of the bowel *via* a parastomal incision, a stoma was excised and the adhesions between the bowel and the peritoneum and omentum were cleared. Both the bowel ends were resected, and sutured manually with 2-layered end-to-end anastomosis.

### Assessment of the enterostomy closure on the basis of the clinical findings

After initial surgery, when there was an improvement in the small intestinal dilatation and sufficient bowel movement was observed, the patients were allowed to resume a normal diet. Subsequently, parenteral nutritional support was initiated for the patients in whom oral intake was not sufficient, or for those with severe diarrhea. The clinical findings were confirmed as positive when (1) oral intake was sufficient; (2) diarrhea was controlled by medications; and (3) initial disease was sufficiently controlled without recurrence. When these criteria were satisfied, enterostomy closure was performed. These patients were classified as group D.



**Figure 1** Endoscopic inspection of remnant bowel. Endoscopic examination revealed that sufficient blood flow had been retained and edema had subsided in the remnant bowel.

### Endoscopic inspection

After initial surgery, when there was an improvement in the small intestinal dilatation and sufficient bowel movement was observed, endoscopic examination was performed. We used a cholangioendoscope (Olympus CHF Type P20Q; Olympus Medical Systems Co., Tokyo, Japan) for endoscopy, and it was inserted from the opening of the enterostomy.

### Assessment of enterostomy closure on the basis of the endoscopic findings

The endoscopic findings were confirmed as positive when (1) sufficient blood flow resumed in the remnant bowel; and (2) the bowel edema subsided. Enterostomy closure was performed when these criteria were satisfied (Figure 1). However, if the criteria were not met, endoscopic examination was performed again after 3 d. These patients were classified as group E.

### Statistical analysis

Results are presented as the mean  $\pm$  SD unless otherwise stated. Univariate analysis was performed using Student's *t* test for continuous variables and Fisher's exact test and chi-square test for categorical variables. A *P* value of less than 0.05 was considered statistically significant.

## RESULTS

Delayed closure of enterostomy (D group) was performed in 8 patients and early closure of enterostomy (E group) was performed in 7 patients. Table 1 shows the patient characteristics and initial operative variables. No differences were observed in the mean age and sex ratio between the patient groups. Risk factors and initial diseases for the SMAO patients in the E group were: hypertension in 85.7%, diabetes mellitus in 57.1%, atrial fibrillation in 57.1%, and apoplexy in 14.3%; while those for the patients in the D group were: hypertension in 65.2%, diabetes mellitus in 62.5%, atrial fibrillation in 37.5%, and apoplexy in 25%. There was no significant difference between the groups with regard to the pro-

Table 1 Characteristics of patients *n* (%)

	Early closure group ( <i>n</i> = 7)	Delayed closure group ( <i>n</i> = 8)	<i>P</i> value
Age (yr)	67.0 ± 4.6	69.4 ± 5.6	< 0.3885
Sex ratio (male:female)	6:1	6:2	1
Initial disease			
HT	6 (85.7)	5 (62.5)	< 0.5692
DM	4 (57.1)	5 (62.5)	1
Af	4 (57.1)	3 (37.5)	< 0.6193
Apo	1 (14.3)	2 (25)	1
Length of resected bowel (cm)	265 ± 38	239 ± 32	< 0.168
Initial enterostomy complication			
Skin erosion	1 (14.3)	3 (37.5)	< 0.5692

HT: Hypertension; DM: Diabetes mellitus; Af: Atrial fibrillation; Apo: Apoplexy.

portion of patients with risk factors and initial diseases. The mean length of the resected bowel at the initial operation was 265 ± 38 cm in the E group and 239 ± 32 cm in the D group; there was no significant difference between the groups in this regard. All the patients in the E and D groups underwent jejunostomy. No deaths occurred in either of the groups. The most common initial postoperative complication observed was skin erosion, with an incidence rate of 14.3% in the E group, and 37.5% in the D group.

Table 2 shows the postoperative variables. The mean duration from initial operation to enterostomy closure was significantly shorter in the E group (18.3 ± 2.1 d) than in the D group (34.3 ± 5.9 d) (*P* < 0.0001). The postoperative complications observed were wound infection and pneumonia, with an incidence rate of 14.3% each in the E group, and 25% each in the D group. There was no significant difference between the 2 groups with regard to postoperative complications (*P* = 1). The duration of hospitalization after surgery was significantly shorter in the E group (33 ± 2.2 d) than in the D group (51 ± 8.9 d) (*P* < 0.0002).

## DISCUSSION

Under favorable local or general conditions, a transient small bowel stoma creation may be required to protect a distal anastomosis or to avoid intraperitoneal intestinal anastomosis. It is generally recommended that the temporary stoma be closed within 9–12 wk after its construction<sup>[16]</sup>. However, because some patients poorly tolerate the temporary stoma owing to extracellular dehydration, difficult pouch fitting, parenteral nutrition requirement in the cases when the stoma is very proximal, and psychological or social impact, it might be advisable to opt for early closure<sup>[17]</sup>.

On the other hand, patients with SMAO often require massive bowel resection because of considerable intestinal necrosis. Massive intestinal resection often causes short-bowel syndrome and necessitates parenter-

Table 2 Outcomes *n* (%)

	Early closure group ( <i>n</i> = 7)	Delayed closure group ( <i>n</i> = 8)	<i>P</i> value
Time of closure of enterostomy after initial operation (d)	18.3 ± 2.1	34.3 ± 5.9	< 0.0001 <sup>1</sup>
Complications			
Wound infection	1 (14.3)	2 (25)	1
Pneumonia	1 (14.3)	2 (25)	1
Hospital stay (d)	33.0 ± 2.2	51.1 ± 8.9	< 0.0002 <sup>1</sup>

<sup>1</sup>Significant difference.

al nutrition. To avoid this syndrome, it is important to retain as much of the remnant bowel as possible. However, SMAO is frequently observed in patients with atrial fibrillation; hence, even if the patients survive after initial operation, the risk of disease recurrence is considered high. Moreover, reperfusion injury may occur in SMAO patients and may cause unstable hemodynamics and multiple organ failure<sup>[10,11]</sup>. Calvien *et al*<sup>[12]</sup> reported that bowel infarction recurred in 32% of the patients early after the resection of necrotic bowel. If we deem primary anastomosis as risky owing to the presence of ischemic changes along the bowel, we perform the bowel resection without anastomosis, and perform an enterostomy. However, a bowel stoma or enterostomy is also a major psychological handicap (altered body schema, odor, uncontrolled emissions, *etc.*) and causes significant physical stress (risk of severe dehydration and electrolyte imbalance). Local care in an intensive care unit needs to be prolonged sometimes, to avoid secondary skin erosions caused by the highly corrosive digestive enzymes. Parenteral nutritional support may also be required if the enterostomy is very proximal, with associated risk of infection caused by the insertion of a central venous catheter. Moreover, it is important to perform early closure of enterostomy to avoid adverse effects on the quality of life of patients. In order to assess the feasibility of early closure of enterostomy, it is necessary to adequately examine the blood flow and edema in the remnant. Thus, we directly examined the blood flow and edema in the remnant bowel of these patients by endoscopy.

Generally, endoscopic examination of the upper digestive tract is performed using esophagogastroduodenoscope, and that of the lower digestive tract (colon and rectum) is performed using colon fiberscope. However, because the opening of the jejunostomy was small in our cases, it would have been difficult to smoothly insert the esophagogastroduodenoscope or colon fiberscope into the small intestine *via* the jejunostomy. Moreover, these procedures are painful for the patients because the diameters of these endoscopes are relatively large. Hence, we used the cholangioendoscope that has a relatively smaller diameter and can be smoothly inserted into the intestine without causing any pain. A

trans-nasal esophagogastroduodenoscope with a small diameter can also be used.

In the case of enterostomy created due to SMAO, it is difficult to determine the timing of enterostomy closure after initial operation. The time chosen for enterostomy closure should be accurately determined taking the following two factors into account: the risk of anastomotic leak that usually occurs between 5 and 7 d<sup>[18]</sup> postoperatively; the development of dense adhesions due to acute inflammation that appear 2 wk after the creation of the enterostomy<sup>[19]</sup>. Moreover, the risk of bowel infarction recurrence should be carefully assessed, as high rates have been reported after necrotic bowel resection<sup>[15]</sup>. Megengaux *et al*<sup>[19]</sup> reported that small bowel stomas can be closed on the 10th d after initial surgery, without major complications. Their concept of choosing postoperative day 10 for the closure of stoma was based on the fact that this time point comes after the days when anastomotic leakages are frequently observed, i.e. postoperative days 5-7<sup>[18]</sup>, and before postoperative day 14, thereby ensuring that acute inflammation does not develop. However, because immediate anastomosis is associated with a high risk of dehiscence, Hanish *et al*<sup>[20]</sup> suggested that it is preferable to avoid this procedure in stoma patients who present with perforation associated with peritonitis or ischemia due to extensive mesenteric infarction. Because our patients required stoma creation due to SMAO, we considered that it was necessary to precisely investigate the blood flow in the remnant bowel before early closure of the stoma. In the patients of the early closure group, enterostomy closure was performed after  $18.3 \pm 2.1$  d, and no complications associated with anastomosis developed thereafter. This time is significantly shorter compared to the recommended time of 9-12 wk after the construction of enterostomy<sup>[16]</sup>.

Hospitalization in the patients of the early closure group was significantly shorter than that in the patients of the delayed closure group. The incidence rate of complications such as wound infection and pneumonia in the patients of the early closure group was 14.3%, while that of the patients of the delayed closure group was 25%. Although there was no significant difference between groups, the incidence rate of complications was lower in the early closure group than in the delayed closure group.

To the best of our knowledge, early closure of jejunostomy based on endoscopic findings *via* the stoma has never been studied in patients with SMAO. On the basis of these results, we conclude that endoscopic examination of blood flow and edema in the remnant bowel is a useful predictor to determine the time of enterostomy closure in SMAO cases.

## COMMENTS

### Background

A transient stoma is known to lower the operative risk, but it should be closed

at the earliest opportunity; however, the morbidity and mortality rates after ileostomy or colostomy closure are rather high. It is generally recommended that the temporary stoma be closed within 9-12 wk after its construction. Fifteen patients who underwent massive bowel resection with enterostomy for superior mesenteric arterial occlusion (SMAO) were divided into a delayed closure group (D group) and an early closure group (E group).

### Research frontiers

The mean duration from initial operation to enterostomy closure was significantly shorter in the E group ( $18.3 \pm 2.1$  d) than in the D group ( $34.3 \pm 5.9$  d) ( $P < 0.0001$ ). The duration of hospitalization after surgery was significantly shorter in the E group ( $33 \pm 2.2$  d) than in the D group ( $51 \pm 8.9$  d) ( $P < 0.0002$ ).

### Innovations and breakthroughs

Endoscopic examination of the blood flow and edema in the remnant bowel was found to be useful for assessing the feasibility of early closure of enterostomy in SMAO cases.

### Applications

In the authors' study, the number of patients with endoscopic examination of the blood flow and edema in the remnant bowel was very small, but they can propose that endoscopic examination of blood flow and edema in the remnant bowel is a useful predictor to determine the time of enterostomy closure in SMAO cases.

### Peer review

This manuscript is well constructed and the comparison of two methods of the preoperative evaluation clearly delivered. The conclusions were supported by the data. The topic is particularly interesting and up-to-date, because of the lack of surgical procedures to determine the timing of enterostomy closure. The work is written in good English. As a result, the work meets the standards of the *World Journal of Gastroenterology*. In conclusion the manuscript can be published.

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## Prognosis and feasibility of *en-bloc* vascular resection in stage II pancreatic adenocarcinoma

K Dilip Chakravarty, Jun-Te Hsu, Keng-Hao Liu, Chun-Nan Yeh, Ta-Sen Yeh, Tsann-Long Hwang, Yi-Yin Jan, Miin-Fu Chen

K Dilip Chakravarty, Jun-Te Hsu, Keng-Hao Liu, Chun-Nan Yeh, Ta-Sen Yeh, Tsann-Long Hwang, Yi-Yin Jan, Miin-Fu Chen, Department of General Surgery, Chang Gung Memorial Hospital, 5, Fu-Hsing Street, Kwei-Shan Shiang, Taoyuan 333, Taiwan, China

Author contributions: Hwang TL and Chakravarty KD contributed equally to this work; Chakravarty KD wrote the paper; Hsu JT, Liu KH, Yeh CN, Yeh TS, Hwang TL, Jan YY and Chen MF provided the cases and suggestions.

Correspondence to: Tsann-Long Hwang, MD, Department of General Surgery, Chang Gung Memorial Hospital, 5, Fu-Hsing Street, Kwei-Shan Shiang, Taoyuan 333, Taiwan, China. [hwangtl@adm.cgmh.org.tw](mailto:hwangtl@adm.cgmh.org.tw)

Telephone: +886-3-3281200-3219 Fax: +886-3-3285818

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### Abstract

**AIM:** To establish the prognosis and feasibility of *en-bloc* vascular resection of stage II pancreatic adenocarcinoma of the head and uncinate process.

**METHODS:** We retrospectively analyzed 87 patients with stage II pancreatic adenocarcinoma, who were subjected to pancreaticoduodenectomy (PD) and pylorus-preserving PD (PPPD) between 1996 and 2006 in Chang Gung Memorial Hospital, Taiwan. Twelve and 75 patients underwent PD/PPPD with and without resection of portal vein/superior mesenteric vein (PV/SMV), respectively.

**RESULTS:** The overall 1- and 3-year survival rates of patients undergoing PD/PPPD with and without vascular resection were 50.0% and 16.7%, and 44.4% and 12.2%, respectively. Morbidity and mortality rates in the PV/SMV resection vs non-resection group were 50.0% and 0.0%, and 40.0% and 2.7%, respectively. In multivariate analysis, serum bilirubin, histological

differentiation and adjuvant chemotherapy were independent prognostic factors that influenced survival.

**CONCLUSION:** In stage II adenocarcinoma of the pancreatic head and uncinate process, serum bilirubin, histological differentiation and adjuvant chemotherapy were independent prognostic factors, and *en-bloc* vascular resection is a feasible option in carefully selected patients.

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**Key words:** Pancreatic neoplasms; Adenocarcinoma; Portal vein; Superior mesenteric vein; Pancreaticoduodenectomy; Chemotherapy

**Peer reviewers:** Hiroshi Yoshida, MD, First Department of Surgery, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan; Salvatore Gruttaduria, MD, Assistant Professor, Abdominal Transplant Surgery, ISMETT, Via E. Tricomi, 190127 Palermo, Italy

Chakravarty KD, Hsu JT, Liu KH, Yeh CN, Yeh TS, Hwang TL, Jan YY, Chen MF. Prognosis and feasibility of *en-bloc* vascular resection in stage II pancreatic adenocarcinoma. *World J Gastroenterol* 2010; 16(8): 997-1002 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i8/997.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i8.997>

### INTRODUCTION

Pancreaticoduodenectomy (PD) combined with vascular resection in locally advanced pancreatic malignancies was initially associated with high morbidity and mortality rates<sup>[1]</sup>. However, with improvement in surgical techniques and postoperative care, more aggressive *en-bloc* resection of pancreatic malignancies, along with portal vein/superior mesenteric vein (PV/SMV) is being

carried out at present, without any increase in surgical complications<sup>[2]</sup>. Unexpected tumor invasion to the lateral or posterolateral wall of the confluence of the PV/SMV is a common finding during PD for pancreatic malignancies<sup>[3]</sup>. The 5-year survival rate following PD or pylorus-preserving PD (PPPD) is 10%-15%, and the reported survival for > 5 years is less<sup>[4-6]</sup>. Mortality associated with PD has decreased dramatically to 0%-5% over the past two decades, but morbidity remains as high as 35%-60%<sup>[7-11]</sup>. Few studies are available regarding stage II pancreatic adenocarcinoma with special attention to adenocarcinoma located in the pancreatic head and uncinate process, which is more likely to invade the PV/SMV because of its close proximity to these vessels. The aim of the present study was to establish the prognostic factors and feasibility of *en-bloc* vascular resection in patients with stage II adenocarcinoma of the pancreatic head and uncinate process following PD/PPPD.

## MATERIALS AND METHODS

### Patient population

From January 1996 to December 2006, 129 consecutive patients with stage I to III adenocarcinoma of the pancreatic head and uncinate process were subjected to surgery at Chang Gung Memorial Hospital, Taipei, Taiwan. We included only stage II adenocarcinoma of the pancreatic head and uncinate process [87 patients; stage II A ( $n = 14$ ) and II B ( $n = 73$ )], as there is more possibility of vascular encasement in stage II pancreatic cancers located in the head and uncinate process. The tumors were staged according to the 6th edition of the American Joint Committee on Cancer Staging System (2002). Survival duration was calculated from the time of surgery to death or the last follow-up date (December 31, 2007), irrespective of the cause of death.

### Surgical procedure

PD and PPPD were considered as standard procedures. Resection margins from the common bile duct, pancreatic neck, retropancreatic tissue, and from the PV or SMV (in PV/SMV resection) were sent routinely for frozen sectioning, and in cases with positive resection margins, wider resection was performed until a negative resection margin was achieved. Lymph nodes around the hepaticoduodenal ligament, common hepatic artery, celiac trunk, PV, SMV and retropancreatic area were routinely dissected and removed. Reconstruction was performed by pancreaticojejunostomy. In case of PV/SMV encasement, segmental resection and reconstruction by end-to-end anastomosis ( $n = 9$ ) or a vascular graft ( $n = 3$ ; one autologous and two ePTFE grafts) were performed. Ten patients had segmental resection of the PV and two had combined PV/SMV segmental resection, and the splenic vein was anastomosed to the main portal trunk in both cases. All the patients who underwent vascular reconstruction were treated with a single dose

of heparin intraoperatively and postoperatively; heparin was not used routinely. These patients were monitored by Doppler study for vascular graft patency in the early postoperative period.

### Statistical analysis

Clinical records were compared by either Fischer's exact test or Pearson's  $\chi^2$  test, as appropriate. Age was analyzed using Student's *t* test. Patient survival rate was calculated by the methods of Kaplan-Meier and log-rank test to determine univariate significance. Factors that were deemed of potential importance on the univariate analysis ( $P < 0.05$ ) were included in the multivariate analysis. Cox's regression was used for the multivariate analysis of these factors.  $P < 0.05$  was regarded as significant. Statistical analyses were performed with SPSS for windows, version 11.5 (SPSS, Inc., Chicago, IL, USA).

## RESULTS

### Patient demographics

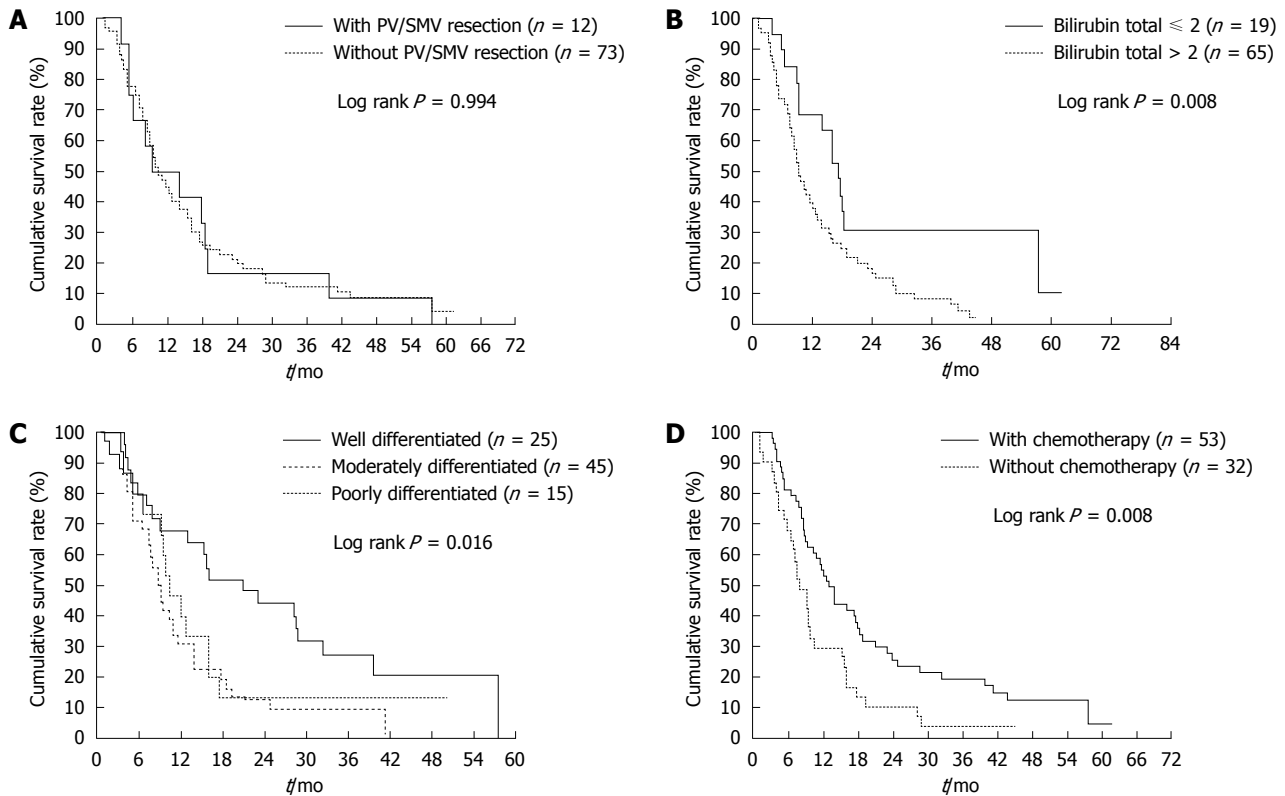
Twelve of 87 (13.8%) patients underwent PD ( $n = 8$ ) or PPPD ( $n = 4$ ) with PV/SMV resection (group I) and 75/87 (86.2%) patients underwent PD ( $n = 57$ ) or PPPD ( $n = 18$ ) without vascular resection (group II). Tumor location was in the pancreatic head ( $n = 80$ ), uncinate process ( $n = 1$ ), and in both ( $n = 6$ ).

### Patient outcome

Analysis of clinicopathological features (Table 1) revealed predominantly male patients aged > 60 years old, and common symptoms were jaundice, abdominal pain and weight loss. Most common findings were jaundice and anemia. Preoperative biliary drainage was performed (in case of total bilirubin levels > 15 mg/dL) in six (50.0%) and 44 (58.7%) patients in group I and II, respectively. Curative resection was possible in all 12 (100.0%) patients in group I and in only 48 (64.0%) in group II. Adjuvant chemotherapy (5-fluorouracil, gemcitabine and cisplatin) was given to eight (66.7%) and 41 (54.7%) patients in group I and II, respectively. Surgical mortality rate (within 1 mo) was 0.0% and 2.7% in group I and II, respectively. The two deaths in group II were due to sepsis and multiorgan failure. The overall surgical complication rate was 41.4% and the complication rate was higher in group I (50.0% *vs* 40.0%) (Table 2).

### Survival period

The median period of follow-up was 10.36 mo (range: 1.18-61.68 mo), and the last follow up date was December 31, 2007. The overall survival rate at 1 year and 3 years for stage II adenocarcinoma of the pancreatic head and uncinate process, with PV/SMV resection, was 50.0% and 16.7%, respectively in group I and 44.4% and 12.2% in group II (Figure 1A). Two patients in group I survived for > 3 years; one patient died after 4 years and the other survived for 4.5 years and is still



**Figure 1** Overall survival in stage II adenocarcinoma of pancreatic head and uncinate process after pancreaticoduodenectomy (PD)/pylorus-preserving PD (PPPD). A: With and without PV/SMV resection; B: With preoperative total bilirubin level  $\leq$  or  $>$  2 mg/dL; C: In terms of histological differentiation; D: Treated with and without adjuvant chemotherapy.

under follow-up. Univariate (Table 3) and multivariate (Table 4) analyses revealed that serum bilirubin, histological differentiation and adjuvant chemotherapy were significant prognostic factors ( $P < 0.05$ ).

The 1- and 3-year survival rate in patients with and without elevation of serum bilirubin ( $>$  or  $\leq$  2 mg/dL) was 39.1% and 8.2%, and 68.4% and 30.7%, respectively (Figure 1B). In analysis of tumor factors, that is, histological differentiation, the 1-year survival rates in well, moderately well and poorly differentiated groups were 68.0%, 31.8% and 46.7%, respectively. The 3-year survival rates in these three categories were 24.0%, 8.8% and 6.7%, respectively. Better survival was found in the well-differentiated group (Figure 1C). The 1- and 3-year survival in patients treated with or without adjuvant chemotherapy was 54.7% and 18.8%, and 29.0% and 3.2%, respectively (Figure 1D).

## DISCUSSION

In our study, the overall 1- and 3-year survival was comparable to that in the study by Fukuda *et al.*<sup>[12]</sup>, which had a survival rate of 47% and 26.8%, respectively, in group I, and 63.4% and 28.4% in group II patients. However, van Geenen *et al.*<sup>[13]</sup> have reported 55% and 6%, respectively, Ye *et al.*<sup>[14]</sup> have reported 37.7% and 5.6%, respectively, and Launois *et al.*<sup>[15]</sup> have reported 42.4% and 11.9% in group I, which is slightly less than the survival rates in our study. The 3-year survival in the

studies of Aramaki *et al.*<sup>[16]</sup> and Carrère *et al.*<sup>[17]</sup> was 21.3% and 20.0%, and 22.0% and 25.0%, in group I and II, respectively. All the above studies included all stages of pancreatic cancer, irrespective of location in the pancreas. This differs from our study, in which we focused only on stage II adenocarcinoma localized in the pancreatic head and uncinate process, where there is more probability of vascular encasement.

In our study, the mean survival time in patients undergoing curative PV/SMV resection was 16.28 mo. There was not much difference in the mortality rate in PD/PPPD with or without vascular resection, but the associated morbidity was higher in the vascular resection group. This is in contrast to earlier studies that have found that PV/SMV resection does not greatly influence morbidity and mortality in PD<sup>[12,18]</sup>. In our study, all 12 patients in group I had negative resection margins. Previous studies have reported that the resectability rate is high in PD with vascular resection<sup>[14]</sup>. PD/PPD with *en-bloc* vascular resection potentially provides an opportunity to achieve negative resection margins, and thus might be beneficial in achieving better survival rates in carefully selected patients with pancreatic adenocarcinoma<sup>[19,20]</sup>. Hence, in patients who were subjected to palliative treatment alone, based on their preoperative evaluation that showed PV/SMV encasement, some carefully selected patients, as determined by preoperative CT [length and severity (complete *vs* partial circumference) of vascular involvement], may be suitable candidates for *en-bloc* resection

**Table 1** Clinicopathological features of patients with stage II pancreatic adenocarcinoma (mean  $\pm$  SD) *n* (%)

Parameters	With vascular resection ( <i>n</i> = 12)	Without vascular resection ( <i>n</i> = 75)	<i>P</i> value
Age (yr)	62.9 $\pm$ 11.0	62.9 $\pm$ 9.8	0.994
Gender			0.745
Male	7 (58.3)	50 (66.7)	
Female	5 (41.7)	25 (33.3)	
Symptoms			
Jaundice	6 (50.0)	55 (73.3)	0.171
Abdominal pain	6 (50.0)	35 (46.7)	0.830
Body weight loss	6 (50.0)	31 (41.3)	0.573
Anorexia	3 (25.0)	17 (22.7)	1.000
Signs			
Anemic	4 (33.3)	38 (50.7)	0.265
Icterus	6 (50.0)	54 (72.0)	0.178
Abdominal tenderness	3 (25.0)	15 (20.0)	0.707
Albumin (g/dL)	3.9 $\pm$ 0.6	3.7 $\pm$ 0.5	0.205
Total bilirubin (mg/dL)	6.8 $\pm$ 7.0	8.9 $\pm$ 7.1	0.333
Pre-op biliary drainage			0.573
Yes	6 (50.0)	44 (58.7)	
No	6 (50.0)	31 (41.3)	
CEA (ng/mL)			1.000
$\leq$ 5	5 (62.5)	36 (62.1)	
$>$ 5	3 (37.5)	22 (37.9)	
CA19-9 (U/mL)			0.411
$\leq$ 37	3 (33.3)	13 (21.0)	
$>$ 37	6 (66.7)	49 (79.0)	
Operation time (min)	473.9 $\pm$ 185.2	461.6 $\pm$ 110.4	0.826
Blood transfusion (mL)	396 $\pm$ 588	304 $\pm$ 590	0.618
Tumor size (cm)	3.6 $\pm$ 1.6	3.3 $\pm$ 1.4	0.526
Lymph node metastases			0.097
Yes	8 (66.7)	65 (86.7)	
No	4 (33.3)	10 (13.3)	
Curability			0.015
Yes	12 (100.0)	48 (64.0)	
No	0 (0.0)	27 (36.0)	
Postoperative chemotherapy			0.436
Yes	8 (66.7)	41 (54.7)	
No	4 (33.3)	34 (45.3)	

CA19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen.

**Table 2** Morbidity and mortality in patients with stage II pancreatic adenocarcinoma *n* (%)

Parameters	With vascular resection ( <i>n</i> = 12)	Without vascular resection ( <i>n</i> = 75)
Morbidity	6/12 (50.0)	30/75 (40.0)
Pancreatic leakage	2 (16.7)	9 (12.0)
Pancreatic fistula	0 (0.0)	8 (10.7)
Gastrointestinal bleeding	1 (8.3)	6 (8.0)
Pleural effusion	2 (8.3)	4 (5.3)
Delayed gastric emptying	1 (8.3)	3 (4.0)
Wound infection	0 (0.0)	4 (4.0)
Intra-abdominal abscess	0 (0.0)	4 (5.3)
Bile leakage	1 (8.3)	3 (4.0)
Sepsis	0 (0.0)	3 (4.0)
Intra-abdominal bleeding	1 (8.3)	2 (2.7)
Mortality	0 (0.0)	2 (2.7)

of PV/SMV, thus achieving better survival rates. Earlier studies have suggested that encasement of PV or SMV is a function of tumor location rather than more aggressive

**Table 3** Univariate analyses of predictive factors for survival of patients with stage II adenocarcinoma of the pancreas after PD or PPPD, with or without vascular resection

Parameters	Median (mo)	95% CI of median	3-yr survival (%)	<i>P</i> value
Age (yr)				0.852
$\leq$ 70 ( <i>n</i> = 62)	11.51	7.78-15.25	12.0	
$>$ 70 ( <i>n</i> = 23)	9.40	9.15-9.65	16.3	
Sex				0.191
Male ( <i>n</i> = 55)	9.76	7.61-11.91	9.6	
Female ( <i>n</i> = 30)	14.01	3.20-24.82	19.4	
Albumin (g/dL)				0.107
$\leq$ 3.5 ( <i>n</i> = 21)	7.40	3.86-10.94	12.7	
$>$ 3.5 ( <i>n</i> = 47)	11.67	5.96-17.38	12.8	
Total bilirubin (mg/dL)				0.008
$\leq$ 2 ( <i>n</i> = 19)	17.39	15.23-19.55	30.7	
$>$ 2 ( <i>n</i> = 65)	9.40	7.66-11.14	3.5	
Serum CA 19-9 (U/L)				0.167
$\leq$ 37 ( <i>n</i> = 16)	16.11	1.47-30.75	25.0	
$>$ 37 ( <i>n</i> = 54)	11.51	8.17-14.85	12.3	
Serum CEA (ng/mL)				0.455
$\leq$ 5 ( <i>n</i> = 40)	16.08	10.78-21.38	13.4	
$>$ 5 ( <i>n</i> = 24)	7.82	5.18-10.46	16.7	
Blood transfusion				0.491
Yes ( <i>n</i> = 47)	10.88	7.17-14.59	14.9	
No ( <i>n</i> = 38)	9.24	5.59-12.89	9.8	
Tumor size (cm)				0.508
$\leq$ 3 ( <i>n</i> = 43)	13.97	10.77-17.18	13.3	
$>$ 3 ( <i>n</i> = 41)	9.27	8.82-9.72	12.5	
Nodal metastases				0.557
Yes ( <i>n</i> = 71)	9.76	7.69-11.83	13.6	
No ( <i>n</i> = 14)	12.16	0.00-28.36	8.3	
Pre-op biliary drainage				0.262
Yes ( <i>n</i> = 49)	9.24	7.80-10.68	12.0	
No ( <i>n</i> = 36)	13.97	9.44-18.50	14.3	
PV/SMV resection				0.994
Yes ( <i>n</i> = 12)	9.27	0.00-19.54	16.7	
No ( <i>n</i> = 73)	10.36	7.87-12.86	12.2	
Resection margin <sup>1</sup>				0.071
Negative ( <i>n</i> = 58)	11.51	6.46-16.56	16.1	
Positive ( <i>n</i> = 27)	9.27	7.46-11.08	5.1	
Differentiation				0.016
Well ( <i>n</i> = 25)	20.98	9.02-32.94	24.0	
Moderately ( <i>n</i> = 45)	8.81	7.81-9.81	8.8	
Poorly ( <i>n</i> = 15)	10.49	7.01-13.97	6.7	
Adjuvant chemotherapy				0.008
Yes ( <i>n</i> = 53)	12.99	10.44-12.74	18.8	
No ( <i>n</i> = 32)	7.96	5.74-10.19	3.2	

Two cases of mortality were not included in survival analysis. <sup>1</sup>Resection margin was negative in all 12 patients who underwent vascular resection.

behavior, and almost equal or even better survival rates can be achieved by *en-bloc* resection of PV/SMV<sup>[12,19]</sup>. Our study shows that *en-bloc* vascular resection in stage II pancreatic adenocarcinoma is a feasible option in carefully selected patients. Hence, vascular encasement should not be considered as a contraindication for surgery; risk must be balanced against the benefit by case to case evaluation.

Serum bilirubin, histological differentiation and adjuvant chemotherapy were significant prognostic factors in our series. Previous studies have focused on the significance of depth of PV invasion<sup>[18]</sup>, lymph node metastasis<sup>[21]</sup>, tumor size<sup>[22]</sup>, negative resection margin<sup>[23]</sup>, and adjuvant chemotherapy<sup>[24]</sup> in pancreatic adenocarcinoma. These preoperative and intraoperative factors



**Table 4** Multivariate analysis in stage II pancreatic adenocarcinoma of the head and uncinate process

Parameters	Hazard ratio (95% CI)	P value
Bilirubin (mg/dL)		
≤ 2/> 2	2.024 (1.613-3.774)	0.026
Differentiation		0.005
Moderately/well	2.412 (1.379-4.217)	0.002
Poorly/well	2.091 (1.034-4.225)	0.040
Adjuvant chemotherapy		
No/yes	2.068 (1.270-3.366)	0.003
PV/SMV resection		
Yes/no		0.591

help in deciding the extent of resection, proper planning of adjuvant therapy, and predicting the survival outcome in these patients. In our study, preoperative biliary drainage has no statistical significance in the outcome of stage II pancreatic adenocarcinoma, similar to an earlier study<sup>[25]</sup>. Few studies have reported the potential advantages of preoperative biliary drainage, which include improved nutritional, metabolic and immune function, and the possibility of reduced postoperative morbidity and mortality<sup>[26,27]</sup>. In contrast, one study has reported that the biliary stents induce bacterial contamination and enhance the risk of cholangitis because of clogging. Biliary stenting also generates a severe inflammatory response adjacent to the wall of the bile duct, which is probably a factor that is responsible for increased risk of bile leakage and infection after biliodigestive reconstruction<sup>[28]</sup>. An experimental study has indicated that a period of 4-6 wk is necessary to recover metabolic and immune functions so that some benefit may be achieved by preoperative biliary drainage<sup>[29]</sup>.

Histological differentiation was found to be significant in our study in determining survival outcome. Patients with well-differentiated adenocarcinoma had better survival than those with moderately well and poorly differentiated adenocarcinoma. Earlier studies by Sohn *et al*<sup>[22]</sup>, Riediger *et al*<sup>[23]</sup> and Yamaguchi *et al*<sup>[30]</sup> have highlighted the significance of histological differentiation as a prognostic factor in pancreatic adenocarcinoma.

Adjuvant chemotherapy was also found to be statistically significant. In our study, patients who received adjuvant chemotherapy had better survival than those without chemotherapy. Adjuvant chemotherapy in pancreatic cancer substantially improved the disease-free survival and overall increase in survival rate, as shown by our study and an earlier one<sup>[31]</sup>. The drawback of our study is that it was a retrospective analysis. However, it still gives information about the prognostic factors and feasibility of *en-bloc* vascular resection in stage II adenocarcinoma of the pancreatic head and uncinate process.

In summary, our study concludes that serum bilirubin, histological differentiation and adjuvant chemotherapy are independent prognostic factors that influence survival in patients with stage II adenocarcinoma of the pancreatic head and uncinate process. PD/PPPD along with *en-bloc* vascular resection is a technically feasible option in carefully selected patients.

## COMMENTS

### Background

Unexpected tumor invasion to the lateral or posterolateral wall of the confluence of the portal vein/superior mesenteric vein (PV/SMV) is a common finding during pancreaticoduodenectomy for pancreatic malignancies. This study was designed to establish the prognostic factors and feasibility of *en-bloc* vascular resection in stage II adenocarcinoma of the pancreatic head and uncinate process.

### Research frontiers

With improvement in surgical techniques and postoperative care, more aggressive *en-bloc* resection of pancreatic malignancies, along with PV/SMV is being carried out at present, without any increase in surgical complications.

### Innovations and breakthroughs

Only a few studies have investigated stage II pancreatic adenocarcinoma, with special attention to adenocarcinoma in the head and uncinate process, which is more likely to invade the PV/SMV because of its close proximity to these vessels.

### Applications

*En-bloc* vascular resection is a feasible option in carefully selected patients with stage II adenocarcinoma of the pancreatic head and uncinate process. Serum bilirubin, histological differentiation and adjuvant chemotherapy are the independent prognostic factors.

### Peer review

This is a very well-focused paper that reports a single-center experience with *en-bloc* venous resection of stage II pancreatic adenocarcinoma.

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## 64-row multidetector computed tomography portal venography of gastric variceal collateral circulation

Li-Qin Zhao, Wen He, Ming Ji, Peng Liu, Peng Li

Li-Qin Zhao, Wen He, Peng Liu, Peng Li, Department of Radiology, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China

Ming Ji, Department of Gastroenterology, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China

**Author contributions:** He W and Zhao LQ designed the research; Zhao LQ, Liu P and Li P performed the data collection; He W, Zhao LQ and Liu P performed the post-processing of the images; Ji M analyzed the clinical data; Zhao LQ wrote the manuscript; He W revised the paper.

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**Correspondence to:** Wen He, MD, Department of Radiology, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China. [hewen1724@sina.com](mailto:hewen1724@sina.com)

Telephone: +86-10-63137745 Fax: +86-10-63134411

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### Abstract

**AIM:** To study characteristics of collateral circulation of gastric varices (GVs) with 64-row multidetector computer tomography portal venography (MDCTPV).

**METHODS:** 64-row MDCTPV with a slice thickness of 0.625 mm and a scanning field from 2 cm above the tracheal bifurcation to the lower edge of the kidney was performed in 86 patients with GVS diagnosed by endoscopy. The computed tomography protocol included unenhanced, arterial and portal vein phases. The MDCTPV was performed on an AW4.3 workstation. GV were classified into three types according to Sarin's Classification. The afferent and efferent veins of each type of GV were observed.

**RESULTS:** The afferent venous drainage originated mostly from the left gastric vein alone (LGV) (28/86, 32.59%), or the LGV more than the posterior gastric vein/short gastric vein [LGV > posterior gastric vein/short gastric vein (PGV/SGV)] (22/86, 25.58%), as seen by MDCTPV. The most common efferent venous

drainage was *via* the azygos vein to the superior vena cava (53/86, 61.63%), or *via* the gastric/splenorenal shunt (37/86, 43.02%) or inferior phrenic vein (8/86, 9.30%) to the inferior vena cava. In patients with gastroesophageal varices type 1, the afferent venous drainage of GV mainly originated from the LGV or LGV > PGV/SGV (43/48, 89.58%), and the efferent venous drainage was mainly *via* the azygos vein to the superior vena cava (43/48, 89.58%), as well as *via* the gastric/splenorenal shunt (8/48, 16.67%) or inferior phrenic vein (3/48, 6.25%) to the inferior vena cava. In patients with gastroesophageal varices type 2, the afferent venous drainage of the GV mostly came from the PGV/SGV more than the LGV (PGV/SGV > LGV) (8/16, 50%), and the efferent venous drainage was *via* the azygos vein (10/16, 62.50%) and gastric/splenorenal shunt (9/16, 56.25%). In patients with isolated gastric varices, the main afferent venous drainage was *via* the PGV/SGV alone (16/22, 72.73%), and the efferent venous drainage was mainly *via* the gastric/splenorenal shunt (20/22, 90.91%), as well as the inferior phrenic vein (3/23) to the inferior vena cava.

**CONCLUSION:** MDCTPV can clearly display the afferent and efferent veins of all types of GV, and it could provide useful reference information for the clinical management of GV bleeding.

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**Key words:** Computed tomography; Portal venography; Gastric varices; Portal hypertension; Collateral circulation

**Peer reviewer:** Heitor Rosa, Professor, Department of Gastroenterology and Hepatology, Federal University School of Medicine, Rua 126 n.21, Goiania - GO 74093-080, Brazil

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## INTRODUCTION

Liver cirrhosis can result in portal hypertension. Gastric fundic and/or esophageal varices are one of the severe complications. It may cause massive hemorrhage of the upper gastrointestinal tract<sup>[1]</sup>. The clinical management of gastric varices (GV) is related to their hemodynamics and locations. GV were classified into three types according to Sarin's Classification that is based on varices location by endoscopy<sup>[2]</sup>. It is essential to identify the hemodynamics of different types of GV before treatment. The conventional portal vein catheterization provides useful information regarding portal hemodynamics including GV, but it is an invasive method<sup>[3]</sup>. As an almost atraumatic method, computed tomography angiography has been adopted widely to display the portal vein system<sup>[4-7]</sup>. Multidetector computer tomography portal venography (MDCTPV) can display esophageal varices (EV), GV and related bypass circuits more specifically because of the thinner slice and better spatial resolution of the computed tomography scanner<sup>[8,9]</sup>. In our study, 86 GV patients diagnosed by endoscopy were selected and classified into three types according to Sarin's Classification<sup>[2]</sup>, and the afferent and efferent veins of different types of GV were studied by MDCTPV.

## MATERIALS AND METHODS

### Patients

Eighty-six consecutive patients with portal hypertension confirmed by endoscopy were enrolled in this study from April 2007 to December 2008, including 52 men and 34 women, aged 38-76 years (mean: 62.6 years). The etiology of portal hypertension for these 86 patients was post-hepatic cirrhosis ( $n = 45$ ), alcoholic cirrhosis ( $n = 23$ ), primary biliary cirrhosis ( $n = 7$ ), autoimmune hepatic cirrhosis ( $n = 3$ ), cryptogenic cirrhosis ( $n = 1$ ), pancreatic carcinoma ( $n = 4$ ) and chronic pancreatitis ( $n = 3$ ). There were 35 patients with a coexistent diagnosis of hepatoma. According to Child-Pugh grading, 15 cases were grade A, 50 grade B and 21 grade C.

### Examination methods

A GE 64-row MDCT scanner was applied to perform non-enhanced, arterial and portal vein phase vein enhanced scans in all patients. The scanning range was from 2 cm above the tracheal bifurcation to the lower edge of the kidney. One hundred milliliters of non-ionic contrast medium (Omnipaque 350, Nycomed Inc., Princeton, NJ, USA) was injected with a power injector at a rate of 4.0 mL/s. The arterial phase scanning started about 20-30 s after the beginning of injection, and portal phase scanning was initiated 25 s after the beginning of the arterial phase. The reconstitution thickness was set at 0.625 mm.

Fujinon EG 485 (Fujinon, Saitama, Japan) and Olympus CV240 electronic endoscope (Olympus Optical Co. Ltd., Tokyo, Japan) were used for the observation of EV and GV. The location of the varices was recorded according to endoscopic reports and imaging data.

### Image analysis

CTPV images were processed on a GE AW4.3 workstation (GE Medical Systems, Milwaukee, WI, USA). GV and collateral circulation in all cases were analyzed using maximum-intensity projection (MIP) and multi-planer reformation (MPR) techniques. All images were reviewed by three independent experienced radiologists, who were blind to the patients' clinical data. A consecutive conclusion was obtained if their diagnosis was different.

### Classification of GV

According to the varices location under endoscopy<sup>[2]</sup>, GVs were classified into three types: (1) gastroesophageal varices type 1 (GEV1): EV combined with lesser curve GV; (2) gastroesophageal varices type 2 (GEV2): EV combined with GV that extend to the greater curvature; and (3) isolated gastric varices (IGV): varices located on the body and fundus of the stomach, without EV.

Based on CTPV, the afferent venous drainage of GV could be divided into four types<sup>[10,11]</sup>: left gastric vein (LGV) alone; posterior gastric vein/short gastric vein (PGV/SGV) alone; LGV dominant (LGV > PGV/SGV; drainage from both the LGV and PGV/SGV, with the former predominating); and PGV/SGV dominant (PGV/SGV > LGV; drainage from both the LGV and PGV/SGV, with the latter predominating).

## RESULTS

According to endoscopy, we found 48 cases of GEV1 (55.8%), 16 of GEV2 (18.6%) and 22 of IGV (25.6%) (Table 1).

MDCTPV showed that the afferent venous drainage of GV (Table 1) originated from LGV (28/86, 32.59%), PGV/SGV > LGV (22/86, 25.58%), followed by PGV/SGV (20/86, 23.26%) and PGV/SGV > LGV (16/86, 18.60%). Among the GEV1 type, the afferent vein of the GV mostly originated from the LGV (27/48, 56.25%) (Figure 1A) or LGV > PGV/SGV (16/48, 33.33%). Most cases of GEV2 type originated from PGV/SGV > LGV (8/16, 50%) (Figure 2A) or PGV/SGV (4/16, 25.00%), and then LGV > PGV/SGV (3/16, 18.75%). Most IGV cases originated from PGV/SGV (16/22, 72.73%) (Figure 3A and B) or PGV/SGV > LGV (5/22, 22.73%).

The efferent venous drainage of GV differed between patients, and several drainage veins might be observed in one patient as well (Table 1). In 53 cases, the efferent venous drainage was from the azygos vein to the superior vena cava (53/86, 61.63%); eight cases from the inferior phrenic vein to the inferior vena cava (8/86, 9.30%); and 37 cases from the gastric/splenorenal shunt to the inferior vena cava (G/S-R) (37/86, 43.02%). The main efferent venous drainage of GEV1 was *via* the azygos vein into the superior vena cava (43/48, 89.58%) (Figure 1B), whereas the efferent drainage of GEV2 was mostly *via* the azygos vein (10/16, 62.5%) to the superior vena cava, and *via* the gastric/splenorenal shunt to the inferior vena cava (9/16, 56.25%) (Figure 2B). The efferent venous drainage of IGV was mainly *via*



Table 1 Collateral circulation of GV in CTPV

	GEV1	GEV2	IGV	Total
Afferent vein of GV				
LGV	27	1	0	28
LGV > PGV/SGV	16	3	1	22
PGV/SGV > LGV	3	8	5	16
PGV/SGV	2	4	16	20
Efferent vein of GV				
Azygos vein	43	10	0	53
Inferior phrenic vein	3	2	3	8
Gastric/splenorenal shunt	8	9	20	37

GV: Gastric varice; CTPV: Computer tomography portal venography; GEV1: Gastroesophageal varices type 1; GEV2: Gastroesophageal varices type 2; IGV: Isolated gastric varices; LGV: Left gastric vein; PGV: Posterior gastric vein; SGV: Short gastric vein.



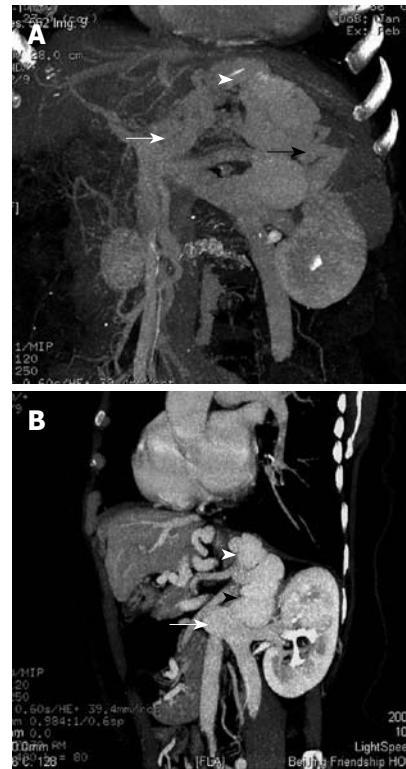
**Figure 1 Gastroesophageal varices type 1.** A: Gastric varice (GV) (black arrow) originated from the left gastric vein (LGV) (white arrow); B: Venous drainage of the GV was via the azygos vein to the superior vena cava (arrow).

the gastric/splenorenal shunt to the inferior vena cava (20/22, 90.91%) (Figure 3A and B).

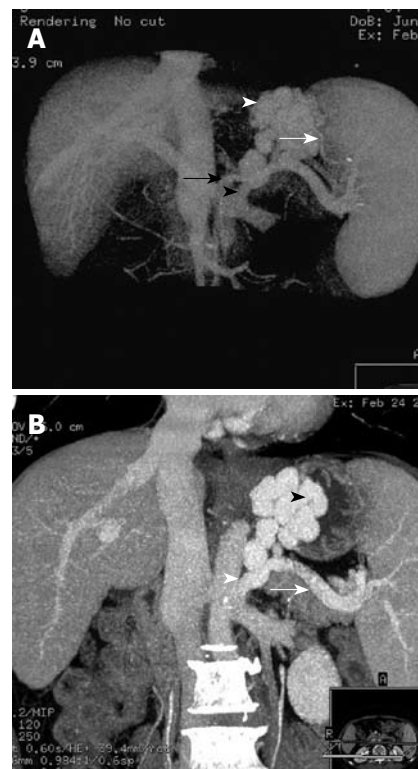
## DISCUSSION

GV are an ominous consequence of portal hypertension<sup>[9]</sup>. Sarin and Ryan have reported that GV are detected in 20% of portal hypertension patients<sup>[12,13]</sup>. Compared to endoscopic findings reported by Sarin *et al*<sup>[12]</sup>, our study with CTPV revealed that the IGV type was more common (25.6%), GEV1 was less common (55.8%) and GEV2 was similar to that report. It was probably because there were more cases of pancreas disease in our study<sup>[14]</sup>.

The afferent venous drainage of GV was mainly from



**Figure 2 Gastroesophageal varices type 2.** A: GV (arrowhead) originated from the posterior gastric vein/short gastric vein (PGV/SGV) (black arrow) and LGV (white arrow), but the former was dominant; B: GV (white arrowhead) draining to the inferior vena cava (white arrow) via the gastric/splenorenal shunt (black arrowhead).



**Figure 3 Isolated gastric varices.** A: GV (white arrowhead) originated from the PGV/SGV (white arrow) and drained to the inferior vena cava (black arrow) via the gastric/splenorenal shunt (black arrowhead); B: GV (black arrowhead) originated from the PGV/SGV and drained to the inferior vena cava via the gastric/splenorenal shunt (white arrowhead), white arrow indicates splenic vein.

three blood supplies: LGV, PGV and SGV. We showed that the afferent venous drainage of GV in GEV1 mostly originated from the LGV or LGV > PGV/SGV. In GEV2, LGV and PGV/SGV participated in the blood supply of GV. As for type IGV, the afferent venous drainage was mainly from the PGV/SGV. Our study revealed that PGV/SGV participated in the blood supply of the three types of GV, which was more than in the former study<sup>[10]</sup>. This may be because more cases of pancreatic cancer and pancreatitis were involved in this study. The latter was called left-sided portal hypertension, because the pancreas diseases involved the splenic vein, which showed PGV/SGV leading to GV, and often not accompanied by EV<sup>[15,16]</sup>.

The blood supply pattern of GV is related closely to the anatomy of the LGV, PGV and SGV. The LGV originates from the portal vein, splenic vein and portosplenic angle. The stem of the LGV gives off anterior and posterior branches above the body of stomach. The anterior branch enters the wall of the fundus and forms the varices, which are continuous with the peri-esophageal varices in the esophagogastric junction. The posterior branch, from which the paraesophageal varices are formed directly, collects blood from the lesser curvature, cardia and lower segment of the esophagus<sup>[17]</sup>. As a result, when the LGV is predominant (GEV1), the location of GV is closer to the cardia, and generally accompanied by EV. The PGV is not present in normal conditions; it only emerges when portal hypertension occurs. It originates from the splenic vein and collects blood from the greater gastric curvature. As a result, in GEV2 and IGV patients, the PGV is one of the main blood vessels. This is the same as reported by Watanabe *et al*<sup>[3]</sup> through portal vein catheterization. In GEV1, the PGV is part of the GV blood supply. The SGV originates from the splenic vein and mainly drains blood from the fundus and greater curvature, thus contributing mainly to the fundus varices.

The para-esophageal varices may connect with the peri-esophageal varices, or join the superior vena cava *via* the azygos vein system, or *via* the inferior phrenic vein to the inferior vena cava<sup>[18]</sup>. Therefore, venous drainage of GEV1 is mostly *via* the azygos vein to the superior vena cava. EV can also exist in GEV2, but the GV blood supply seldom comes from the LGV, thus, in GEV2, only a small part of the GV drainage goes *via* the azygos vein to the superior vena cava or *via* the inferior phrenic vein to the inferior vena cava.

The gastric/splenorenal shunt is the spontaneous portosystemic shunt that runs from the branches of the PGV/SGV *via* the left adrenal vein, retroperitoneal veins and other branches of the left renal vein<sup>[8,19]</sup>. Thus it is likely to be found in GEV2 and IGV, as indicated in our study.

The CTPV view of the blood supply and drainage of GV could provide clinicians with a valuable reference for the endoscopic treatment of GV bleeding<sup>[20,21]</sup>. GV collateral vessels should be given special attention during treatment. GEV2 and IGV are supplied by multiple vessels, therefore, the simple treatment of endoscopic variceal

ligation is not ideal, and has a high rate of recurrence and postoperative hemorrhage. With regard to the gastric/splenorenal shunt that is commonly found with IGV, more attention should be paid to the dosage and injection rate of sclerosant during endoscopic sclerotherapy, so as to prevent the sclerosant from flowing into the systemic circulation *via* the shunt<sup>[22]</sup>. In some GEV1 cases, the incidence of secondary GV increases after treatment of EV<sup>[11]</sup>. On the other hand, after treatment of GV, EV are aggravated because of the special drainage pattern, which can be displayed clearly on CTPV<sup>[18]</sup>. Therefore, CTPV has its value in the follow-up after endoscopic sclerotherapy.

## COMMENTS

### Background

The collateral circulation, location, and hemodynamics of gastric varices (GV) can affect the treatment of GV bleeding.

### Research frontiers

Computed tomography angiography has been used widely to visualize the portal vein system, and it can reveal esophageal varices, GV and related bypass circuits.

### Innovations and breakthroughs

In this study, the afferent and efferent veins of different types of GV, which were classified according to their location, were revealed by the high spatial resolution imaging of multidetector computed tomography (MDCT) and the appropriate post-processing methods.

### Applications

As a noninvasive method, MDCT portal venography could provide clinicians with a valuable reference in the endoscopic and surgical treatment of GV bleeding.

### Peer review

This seems a very interesting new semiological approach to GV.

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## Preparation, physicochemical characterization and cytotoxicity *in vitro* of gemcitabine-loaded PEG-PDLLA nanovesicles

Lin Jia, Jian-Jun Zheng, Shu-Man Jiang, Kai-Hong Huang

Lin Jia, Jian-Jun Zheng, Shu-Man Jiang, Department of Gastroenterology, Guangzhou First Municipal People's Hospital Affiliated to Guangzhou Medical College, Guangzhou 510180, Guangdong Province, China

Kai-Hong Huang, Department of Gastroenterology, Second Affiliated Hospital of Zhongshan University, Guangzhou 510000, Guangdong Province, China

Author contributions: Jia L and Zheng JJ designed the study; Zheng JJ performed the experiment; Jia L, Zheng JJ and Huang KH analyzed the data; Jia L, Jiang SM and Zheng JJ wrote the paper.

Correspondence to: Dr. Lin Jia, Department of Gastroenterology, Guangzhou First Municipal People's Hospital Affiliated to Guangzhou Medical College, No. 1 Panfu Road, Guangzhou 510180, Guangdong Province, China. [jialin@medmail.com.cn](mailto:jialin@medmail.com.cn)  
Telephone: +86-20-81628678 Fax: +86-20-81628809

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### Abstract

**AIM:** To investigate the preparation, physicochemical characterization and cytotoxicity *in vitro* of Gemcitabine-loaded poly(ethylene glycol)-block-poly(D,L-lactide) (PEG-PDLLA) nanovesicles.

**METHODS:** The nanovesicle carriers were prepared from the amphiphilic block copolymer of PEG-PDLLA by a double emulsion technique, and gemcitabine was used as the model drug. The morphology of the nanovesicles was determined by scanning and transmission electron microscopy, and the drug content, drug entrapment and drug-release curve *in vitro* were detected by UV-Vis-NIR spectrophotometry. Cytotoxicity in the human pancreatic cancer cell line SW1990 was tested by 3-(4,5-dimethyl) ethiazole (MTT) assay.

**RESULTS:** The gemcitabine-loaded nanovesicles were hollow nanospheres with a mean size of 200.6 nm, drug

loading of 4.14% and drug embedding ratio of 20.54%. The nanovesicles showed excellent controlled release that was characterized by a fast initial release during the first 72 h, followed by a slower and continuous release. The MTT assay demonstrated that gemcitabine-loaded nanovesicles exhibited dose-dependent and time-delayed cytotoxicity in the human pancreatic cancer cell line SW1990.

**CONCLUSION:** Gemcitabine-loaded PEG-PDLLA nanovesicles prepared by a double emulsion technique exhibited good performance for controlled drug release, and had similar cytotoxic activity to free gemcitabine.

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**Key words:** Copolymer; Cytotoxicity; Gemcitabine; Nanovesicles

**Peer reviewer:** Jamie Murphy, MRCS (Eng), MA, Lecturer in Colorectal Surgery, Centre for Academic Surgery, Royal London Hospital, 3rd Floor Alexandra Wing, London, E1 1BB, United Kingdom

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### INTRODUCTION

Nanovesicles are a new type of drug carrier, which can protect incorporated drugs from *in vivo* metabolism while enhancing their therapeutic effect and reducing their toxicity. Compared with other drug delivery systems, nanovesicles are more suitable for hydrophilic drugs



because of their hollow structure and internal aqueous phase, which can burden high drug loading.

Gemcitabine has been demonstrated to display anti-tumor activity against a wide variety of cancers, including pancreatic, colon, lung, breast, bladder and ovarian cancer<sup>[1-3]</sup>. However, gemcitabine is metabolized rapidly in the blood. Thus, a major limitation of this antitumor drug is that gemcitabine has a very short plasma half-life and strong side effects when administered intravenously<sup>[4]</sup>. As a drug carrier, nanovesicles may promote the efficacy of gemcitabine and reduce its side effects.

Poly(lactic acid) (PLA) is the most widely used synthetic polymer, which is known to be biocompatible and degradable to give the natural product lactic acid<sup>[5]</sup>. However, nanoparticles based on PLA accumulate blood proteins on their surface as they circulate through the body<sup>[6,7]</sup>. This nonspecific absorption of proteins attracts attention from immune cells, with the result that nanoparticles are often removed from circulation before reaching their tumor targets. Modification with poly(ethylene glycol) (PEG) chains immobilized on the surface forms a hydrophilic palisade, which creates repulsion between the nanovesicles, and this repulsion can stop the nanovesicles from agglomerating, thus increasing their dispersion stability in aqueous media<sup>[8-10]</sup>. Furthermore, PEG is able to prevent proteins from adhering to the surface and thus avoids nanovesicles being recognized by macrophages<sup>[11,12]</sup>, which prolongs the circulation time and facilitates nanoparticle uptake by specific cancer cells for cancer therapy<sup>[13,14]</sup>.

However, there have only been a few studies on the incorporation of gemcitabine into PEG-block-poly(D,L-lactide) (PEG-PDLLA) nanovesicles. Therefore, we prepared gemcitabine-loaded nanovesicles, observed their size distribution, morphology and drug-release performance, and carried out a preliminary investigation of their cytotoxicity *in vitro*.

## MATERIALS AND METHODS

### Materials

The amphiphilic block copolymer of PEG-PDLLA was synthesized by Sun Yat-Sen University. Gemcitabine was purchased from Eli Lilly & Co (Indianapolis, IN, United States). RPMI1640 and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, United States).

### Cell culture

The human pancreatic cancer cell line SW1990 was obtained from the Second Affiliated Hospital of Sun Yat-Sen University. All cells were cultured as monolayers at 37°C in a 5% CO<sub>2</sub>/95% humidified atmosphere with RPMI1640 supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mmol/L L-glutamine.

### Preparation of gemcitabine-loaded nanovesicles

Gemcitabine-loaded nanovesicles were prepared by a double emulsion (w/o/w) technique. The preparation was performed as follows: (1) An aqueous solution (2 mg

gemcitabine in 0.2 mL pure water) was added dropwise into an organic solution of the polymer (10 mg PEG-PDLLA in 2 mL trichloromethane), and the first emulsion (w/o) was formed by sonication; (2) The emulsion was added dropwise into an organic solution that consisted of 40 mL polyvinyl alcohol (PVA) solution at 0.5% (w/v) and the double emulsion (w/o/w) was obtained by sonication; (3) The residual trichloromethane of the double emulsion was removed completely by vacuum distillation with a rotary evaporator, and free PVA and non-incorporated gemcitabine was removed by dialysis in the pure water overnight; and (4) Nanovesicles obtained as a suspension were purified by filtration through a syringe filter (pore size 0.45 µm), and then subjected to lyophilization to yield the solid nanovesicle samples. We repeatedly prepared three groups of gemcitabine-loaded nanovesicle samples according to the above processes.

### Determination of gemcitabine incorporation efficiency

Three groups of prepared nanovesicles were redissolved in DMSO. Gemcitabine in the solution was measured by ultraviolet spectroscopy at 275 nm (Perkin-Elmer Lambda 20 UV-Vis spectrophotometer, Beijing, China), and its incorporation efficiency was evaluated by drug loading (DL) and drug embedding ratio (ER). DL (%) = mass of drug in nanovesicles × 100/mass of nanovesicles recovered. ER (%) = mass of drug in nanovesicles × 100/mass of drug used in formulation.

### Nanovesicle size and morphology

After dilution with purified water, nanovesicle size was determined by photon correlation spectroscopy (PCS) (Malvern Autosizer, United States), at a scattering angle of 90° and a temperature of 25°C. Values are reported as the mean diameter (MD). The morphology of the nanovesicles was observed by transmission electron microscopy (TEM) (JEOL-100CXII, Japan) and scanning electron microscopy (SEM) (XL-30, Holland). A drop of the nanovesicles suspension (10 µL) was placed on copper electron microscopy grids (Formvar coated) and stained with 2% (w/v) phosphotungstic acid solution. After 30 s, the sample was washed with purified water and the excess fluid was removed with a piece of filter paper. The dried sample was then examined.

### In vitro release of gemcitabine from nanovesicles

Phosphate buffer solution (PBS) at pH 7.4 and pH 5 was selected for the release medium. Nanovesicle samples (60 mg each) were resuspended in 5 mL PBS (pH 7.4 or 5) and transferred into a dialysis tubing (MW cut-off: 14000 Da). The tubing was placed in 45 mL PBS (pH 7.4 or 5). The release study was performed at 37°C in a Shanghai Yiheng Scientific DKZ Incubator Shaker. At selected time intervals, 5 mL buffered solution outside the dialysis tubing was removed for UV-Vis analysis and replaced with 5 mL fresh buffer solution. Gemcitabine concentration was calculated based on the absorbance intensity at 268 nm. The equations were as follows:  $C_{pH\ 7.4} = 34.724A - 0.2971$ ,  $r^2 = 0.9999$ ;  $C_{pH\ 7.4} = 38.317A - 0.3289$ ,  $r^2 = 0.9999$  (C:

drug concentration; A: absorbance at 268 nm). The cumulative percentage of released gemcitabine (%) could be calculated by the equation:  $Q(\%) = (V_0 \times C_t + V \times \sum C) \times 100\% / (W \times X)$  (Q: cumulative percentage of released gemcitabine;  $C_t$ : gemcitabine concentration in release medium at each time point;  $V_0$ : gross removed volume of release medium; V: volume of sample; W: gross mass of nanovesicles; X: drug content of gemcitabine).

### ***In vitro* cytotoxicity study against human SW1990 pancreatic cancer cells**

The inhibition of cell growth was evaluated by the 3-(4,5-dimethyl) ethiazole (MTT) method using triplicate assays. Gemcitabine and gemcitabine-loaded nanovesicles were diluted in RPMI1640 at  $1.0 \times 10^{-3}$  mol/L, respectively. Then, 100  $\mu$ L cell solution with  $5 \times 10^4$  cells/mL was seeded in 96-well plates (Costar, Cambridge, MA, United States), and allowed to attach overnight. After 24 h incubation, gemcitabine-loaded nanovesicles with concentrations of  $10^{-7}$ ,  $10^{-6}$ ,  $10^{-5}$  and  $10^{-4}$  mol/L were added to the cell cultures. Gemcitabine added at the same concentration acted as a positive control. PBS was added as a negative control. At 24, 48, 72 and 96 h, 20  $\mu$ L MTT and 200  $\mu$ L RPMI culture medium were added to each well and incubated at 37°C for 4 h. Then MTT and culture medium were removed and 200  $\mu$ L DMSO was added. The absorbance of the converted dye, which correlated with the number of viable cells, was determined at 490 nm. Cell viability was determined by the following equation: Cell viability (%) =  $(\text{Abs}_{\text{test cell}} / \text{Abs}_{\text{control cells}}) \times 100\%$ . Cell inhibitory rate was determined by the following formula: Cell inhibitory rate (%) =  $(1 - \text{Abs}_{\text{test cell}} / \text{Abs}_{\text{control cells}}) \times 100\%$ .

### **Statistical analysis**

Statistical analysis was performed with SPSS for Windows version 13.0. Data were expressed as mean  $\pm$  SD, and were compared using one-way ANOVA.  $P < 0.05$  was considered statistically significant based on a two-tailed test.

## **RESULTS**

### **Nanovesicle characteristics**

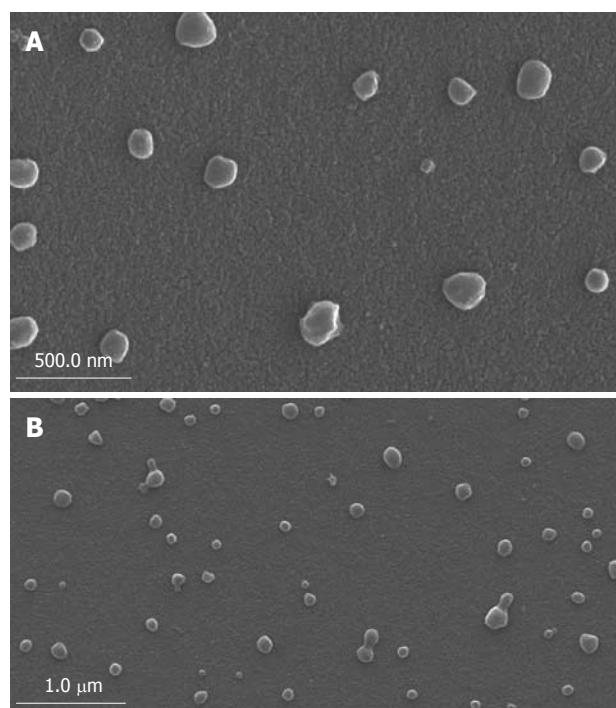
The mean diameter of prepared nanovesicles was 200.6 nm (range: 70-250 nm). As shown by SEM (Figure 1) and TEM (Figure 2), it was clear that the nanovesicles were spherical in shape, and hollow in structure, with a large central cavity in which gemcitabine was loaded.

### **Vesicle loading capacity**

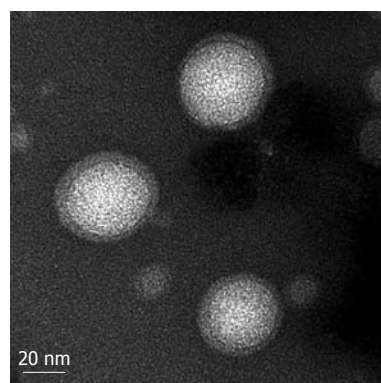
The DL and ER in three groups of gemcitabine-loaded nanovesicles are shown in Table 1. The mean value of DL was  $4.14\% \pm 0.13\%$ , and ER was  $20.54\% \pm 0.92\%$ , which indicated good duplication of the nanovesicle preparation.

### ***In vitro* gemcitabine release study**

The *in vitro* release of gemcitabine-loaded nanovesicles



**Figure 1** Scanning electronic microphotographs of poly (ethylene glycol)-block-poly (D,L-lactide) (PEG-PDLLA) nanovesicles. A:  $\times 40\,000$ ; B:  $\times 20\,000$ .

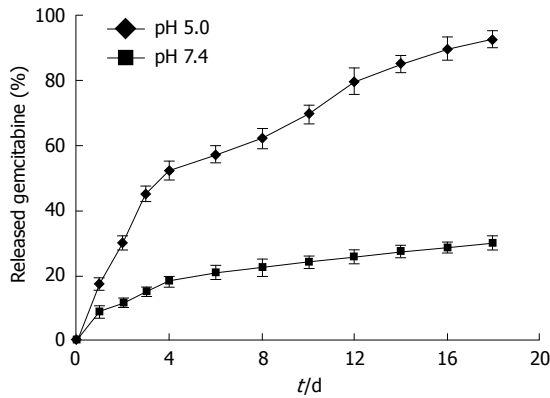


**Figure 2** Transmission electron micrographs of PEG-PDLLA nanovesicles.

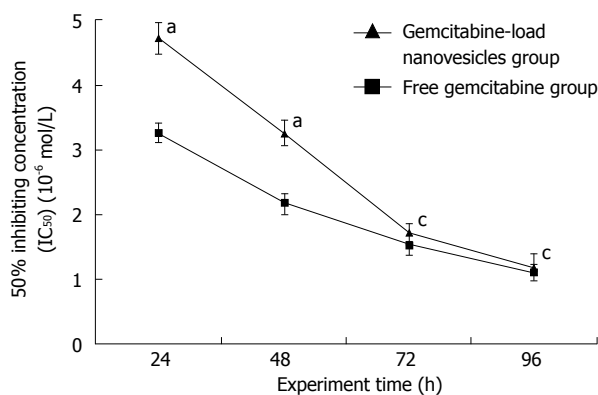
**Table 1** Drug incorporation efficiency of Gemcitabine-load nanovesicles (%)

Sample	1	2	3	mean $\pm$ SD
Drug content	4.23	3.99	4.19	$4.14 \pm 0.13$
Drug encapsulation efficiency	21.34	19.54	20.75	$20.54 \pm 0.92$

in two different buffered solutions (pH 7.4 and 5.0) is shown in Figure 3. A typical two-phase-release was observed in both solutions. A rapid release was observed from gemcitabine-loaded nanovesicles in the first 4 d, and a relatively slower and sustained release was observed in the following days. By comparison with the release at pH 5.0, gemcitabine-release from nanovesicles at pH 7.4 was much slower. In the first 18 d, the cumulative percentage of released gemcitabine at pH 7.4 was



**Figure 3** Release of gemcitabine from nanovesicles at pH 7.4 and 5.0. Data are presented as mean  $\pm$  SD.  $P < 0.05$  at every point of pH 5.0 vs pH 7.4.



**Figure 4** IC<sub>50</sub> of gemcitabine-loaded nanovesicles and free gemcitabine in human SW1990 pancreatic cancer cells. Data are presented as mean  $\pm$  SD. <sup>a</sup> $P < 0.05$ , <sup>c</sup> $P > 0.05$  vs free gemcitabine group.

< 30%, whereas released gemcitabine at pH 5.0 was 92.70%.

### In vitro cytotoxicity study

Table 2 shows the cell inhibitory rate of gemcitabine-loaded nanovesicles and free gemcitabine at a concentration range of  $10^{-7}$  to  $10^{-4}$  mol/L. With increasing drug concentration, pancreatic cancer cells were decreased in the gemcitabine-loaded nanovesicles and free gemcitabine groups. At each drug concentration, the cell inhibitory rate in the gemcitabine-loaded nanovesicles group was lower than that in the free gemcitabine group, however, no statistically significant difference was observed (all  $P > 0.05$ ).

The IC<sub>50</sub> (50% inhibiting concentration) of gemcitabine-loaded nanovesicles and free gemcitabine in human SW1990 pancreatic cancer cells at four time points is shown in Figure 4. IC<sub>50</sub> gradually decreased with time in both groups. In the first 48 h, IC<sub>50</sub> in the gemcitabine-loaded nanovesicles group was significantly higher than that in the free gemcitabine group ( $P < 0.05$ ). At 72 h, IC<sub>50</sub> was also higher in the gemcitabine-loaded nanovesicles group than in the free gemcitabine group, but this difference was not statistically significant ( $P > 0.05$ ). Up to the end point at 96 h, IC<sub>50</sub> was very similar in the two groups.

**Table 2** The inhibitory effect of Gemcitabine-loaded nanovesicles and free gemcitabine on human SW1990 pancreatic cancer cells

Drug concentration (mol/L)	Gemcitabine-loaded nanovesicles	Free gemcitabine	P value
	Cell inhibitory rate (%)	Cell inhibitory rate (%)	
$10^{-7}$	18.3 $\pm$ 1.6	21.7 $\pm$ 2.5	0.480
$10^{-6}$	46.2 $\pm$ 2.7	49.5 $\pm$ 4.1	0.619
$10^{-5}$	82.3 $\pm$ 5.4	78.8 $\pm$ 4.2	0.592
$10^{-4}$	93.2 $\pm$ 5.3	95.2 $\pm$ 3.7	0.552

## DISCUSSION

Gemcitabine is an anticancer nucleoside analogue that is active against various solid tumors. However, with intravenous administration, this drug is inactivated rapidly by enzymatic deamination, and it has a short biological half-life that necessitates the administration of high doses, which leads to unwanted side effects<sup>[15-17]</sup>. Therefore, as a carrier for gemcitabine delivery, nanovesicles, which are superior to other drug delivery systems for encapsulating hydrophilic drugs and can be subjected to surface modification, are used to overcome the above problems. Many advantages can be found in gemcitabine-loaded nanovesicles, including the possibility of targeting specific sites, controlled drug release, and protection of the encapsulated drug<sup>[18-24]</sup>.

Our prepared nanovesicles were spherical in shape with a hollow structure, and gemcitabine was incorporated in the core of the nanovesicles. Generally, nanovesicles < 500 nm are considered ideal for drug delivery systems, because this size allows more effective systemic circulation than smaller molecules, and they can access places in the human body that larger particles cannot reach. At the same time, nanoparticles of this size can penetrate tumors because of the leaky nature of their microvasculature. This classic effect, which is referred to as the “enhanced permeation and retention effect”, results in prolonged circulation and accumulation of a therapeutic agent within the tumor<sup>[25]</sup>. Meanwhile, the modification by PEG allows nanoparticles to escape from the vasculature through the leaky endothelial tissue that surrounds the tumor, and therefore accumulate in certain solid tumors<sup>[26,27]</sup>. As a result, nanoparticles can target the tumor through blood vessels and enhance the anticancer activity of the drug.

As shown in Figure 3, these nanoparticles have a stable release profile. The release of gemcitabine from nanovesicles exhibited a biphasic pattern that was characterized by a fast initial release during the first 72 h, followed by a slower continuous release. The fast release of gemcitabine was probably the result of rapid desorption of gemcitabine from the external layer of the nanovesicles and diffusion of the drug located near the surface, whereas the slower and continuous release may be attributed to slow trans-layer permeation kinetics and diffusion from the interior, together with erosion of the polymer. It has been shown that the aqueous medium



slowly penetrates the internal structure of the particles and causes progressive degradation of the polymer chains.

Another phenomenon was that gemcitabine release from nanovesicles at pH 5.0 was much faster than that at pH 7.4, which indicated that faster degradation of nanovesicles appeared at lower pH. This pH-dependent release is of particular interest in achieving tumor-targeted gemcitabine delivery with nanovesicles. It is expected that most of the gemcitabine encapsulated in nanovesicles will remain in the nanovesicle core for a considerable time period, when the injected nanovesicles remain in the plasma at normal physiological conditions (pH 7.4). However, faster release occurs once the nanovesicles reach the solid tumor site, where the pH value is lower than that in normal tissue<sup>[28]</sup>. In addition, particles are usually internalized inside the cells by endocytosis<sup>[22]</sup>. Therefore, further accelerated release inside the endosome/lysosome of tumor cells may occur as a result of the decreased pH values.

Moreover, in our study, gemcitabine-loaded nanovesicles were similar to free gemcitabine in terms of their cell inhibitory effect at different drug concentrations. Besides, time-delayed cytotoxicity was exhibited by gemcitabine-loaded nanovesicles in the human pancreatic cancer cell line SW1990. In the first 48 h, IC<sub>50</sub> of gemcitabine-loaded nanovesicles was significantly higher than that of free gemcitabine. However, in the following 48 h, IC<sub>50</sub> of gemcitabine-loaded nanovesicles gradually approached that of free gemcitabine. Up to the end of our experiment, IC<sub>50</sub> was very similar in the two groups. It is estimated that the cell inhibitory effect of gemcitabine-loaded nanovesicles is more obvious during the period of sustained release because of its time-dependent release and delayed nuclear uptake in human SW1990 pancreatic cancer cells, which is consistent with *in vitro* gemcitabine release studies. Therefore, our future studies will involve *in vivo* experiments and related research on gemcitabine-loaded nanovesicles.

In summary, using the double emulsion technique, small, spherical and submicron sized (< 210 nm) PEG-PDLLA nanovesicles loaded with gemcitabine were prepared which did not change the drug structure or cytotoxicity. Compared with free gemcitabine, the gemcitabine-loaded nanovesicles elicited a time-dependent improvement in cytotoxic effect that was related to delayed release. This may possibly improve the administration of gemcitabine to pancreatic cancer patients.

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## COMMENTS

### Background

Gemcitabine is used widely for the treatment of pancreatic cancer. However,

because of its rapid metabolism in the blood, gemcitabine has a very short plasma half-life and strong side effects when administered intravenously. As a drug carrier, nanovesicles may promote the efficacy of gemcitabine and reduce its side effects.

### Research frontiers

Among the nanoparticle drug delivery systems, nanovesicles are recommended as drug delivery devices for anticancer agents. The double emulsion technique is suitable for the preparation of poly(ethylene glycol)-block-poly(D,L-lactide) (PEG-PDLLA) gemcitabine-loaded nanovesicles.

### Innovations and breakthroughs

On the basis of previous data, this is the first study of the preparation, physicochemical characterization and *in vitro* cytotoxicity of gemcitabine-loaded nanovesicles. The results of the present study showed that the small, spherical and submicron sized gemcitabine-loaded nanovesicles, which were prepared by a double emulsion technique, did not change the drug structure or cytotoxicity. The gemcitabine-loaded nanovesicles elicited a time-dependent improvement in cytotoxic effect that was related to delayed drug release, compared with free gemcitabine.

### Applications

Gemcitabine-loaded PEG-PDLLA nanovesicles exhibited good controlled drug release, and similar cytotoxic activity to free gemcitabine. This may provide a way of improving the administration of gemcitabine to pancreatic cancer patients.

### Terminology

The double emulsion (w/o/w) technique is a biochemical engineering method. It is performed as follows: an aqueous solution is added to an organic solution of the polymer, and the first emulsion (w/o) is formed by sonication. The emulsion is added to an organic solution, and the double emulsion (w/o/w) is obtained by sonication.

### Peer review

The authors report what is mainly a biochemical engineering study, although the carcinoma cell line utilised is gastrointestinal. The results are fairly limited but interesting, although clinical introduction of this kind of treatment is still many years away.

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## Sphincter of Oddi laxity: An important factor in hepatolithiasis

Ting-Bo Liang, Yu Liu, Xue-Li Bai, Jun Yu, Wei Chen

Ting-Bo Liang, Yu Liu, Xue-Li Bai, Jun Yu, Wei Chen, Department of Hepatobiliary and Pancreatic Surgery, Key Laboratory of Multi-Organ Transplantation of Ministry of Public Health, Key Laboratory of Organ Transplantation of Zhejiang Province, the First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China

**Author contributions:** Liang TB designed the study and performed the majority of the work; Liu Y collected all the patients' data and participated in the writing of the manuscript; Bai XL and Yu J answered for the follow-up of the patients; Chen W did the statistical treatment of the data.

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**Correspondence to:** Ting-Bo Liang, PhD, Department of Hepatobiliary and Pancreatic Surgery, Key Laboratory of Multi-Organ Transplantation of Ministry of Public Health, Key Laboratory of Organ Transplantation of Zhejiang Province, the First Affiliated Hospital, School of Medicine, Zhejiang University, 79 Qingchun Road, Hangzhou 310003, Zhejiang Province, China. [liangtingbo@zju.edu.cn](mailto:liangtingbo@zju.edu.cn)

Telephone: +86-571-87236688 Fax: +86-571-87236688

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dex was 0.070 in Group I and 0.010 in Group II ( $P < 0.001$ ). The mean frequency of biliary operation was 2.07 in Group I and 1.21 in Group II ( $P = 0.001$ ). Differences between the two groups are significant.

**CONCLUSION:** HL patients with SOL tend to have a higher risk of recurrence and a larger demand for reoperation than those without this condition.

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**Key words:** Sphincter of Oddi laxity; Hepatolithiasis; Recurrence index; Reoperation index; Choledochojejunostomy

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### Abstract

**AIM:** To evaluate the importance of sphincter of Oddi laxity (SOL) in hepatolithiasis (HL).

**METHODS:** Subjects included 98 patients diagnosed with HL between 2002 and 2007. Detailed histories were taken and the subjects were monitored until July 2008. HL patients were divided into two groups: Group I included 45 patients with SOL, and Group II included 53 patients without. Recurrence and reoperation indices of both groups were calculated and compared.

**RESULTS:** The recurrence index was 0.135 in Group I and 0.018 in Group II ( $P < 0.001$ ). The reoperation in-

### INTRODUCTION

Hepatolithiasis (HL) is prevalent in East Asia, especially in China<sup>[1-3]</sup>. While this condition results from multiple etiological factors, obstructive cholangitis is usually the main cause<sup>[2]</sup>. In our clinical practice, we have found few cases with obstruction of the common bile duct or sphincter of Oddi (SO); to the contrary, almost half of our clinical cases showed sphincter of Oddi laxity (SOL). We have also found that patients with SOL had a tendency for recurrence and always needed reoperation. The goal of this study was to determine the effect of SOL on recurrence and reoperation frequencies among patients with HL in order to recommend the most suitable therapy for this condition.

The SO is made up of the bile duct, pancreatic duct and ampulla sphincters. Regular contraction and relaxation maintain the normal pressure differences between the bile duct, pancreatic duct and the SO. The diameter of the duodenum papilla orifice is very small, no more than 2-3 mm even when the SO is completely relaxed with drugs. Normally, the SO can regulate the discharge of bile and pancreatic juice, and also prevent duodenum juice reflux. In patients with SOL, a larger diameter ( $\geq 10$  mm) of the biliary tract orifice facilitates the entry of bile juice into the duodenum without resistance, appearing as SO absence.

## MATERIALS AND METHODS

### Case selection

We considered 121 patients with HL who were admitted to the First Affiliated Hospital of the Medical College of Zhejiang University between April 2002 and March 2007. Seven cases were excluded due to histories of choledochojunostomy discovered during surgery, and ten cases were eliminated due to extensive complexity, resulting in a total of 104 selected cases. During the follow-up period of almost 6 years (until July 2008), six patients were lost. Thus, the study included 98 patients: 29 with simple intra-hepatolithiasis (IHL) and 69 with both IHL and choledocholithiasis.

Detailed histories were obtained from the patients, including age of first onset, frequency of recurrence, and previous biliary tract operation. The mean age of the patients was  $52.8 \pm 12.2$  years (range: 17-80 years), and mean age of the first onset was  $45.1 \pm 16.1$  years (range: 8-78 years) (Table 1). All patients were diagnosed by preoperative magnetic resonance cholangiopancreatography (MRCP) and B-ultrasonography (BUS), and diagnoses were confirmed in operation. The absence of residual stones was confirmed by routine choledochoscope (OL-YMPUS CHF P20, external diameter 4.9 mm) examination during operation. The following parameters were set for biliary tract operation: (1) simple cholecystectomy was removed from consideration; and (2) operation was defined as all procedures for clearing stones from the intrahepatic and extrahepatic bile ducts, including hepatectomy, endoscopic stone extraction, calculus removal from the T tube, and choledochojunostomy. Simple choledocholithotomy and T-tube drainage were performed on 35 patients, simple hepatectomy on 20 patients, choledocholithotomy and T-tube drainage with hepatectomy on 35 patients, simple choledochojunostomy on 5 patients, and choledochojunostomy with hepatectomy on 3 patients. BUS was routinely performed every 2 mo after operation, and abnormalities were confirmed with MRCP. Patients showing the symptoms related to HL were immediately examined with BUS and MRCP.

### SOL and patient groups

SOL, related to HL formation, may be primary or secondary. Patients without a history of choledochojunostomy or choledochoduodenostomy may be diagnosed

Table 1 Patient characteristics ( $n = 98$ )

Characteristics	
Male/female	35/63
Mean age (yr)	52.8
Mean age of first onset (yr)	45.1
Mean follow-up period (mo)	42.1
Follow-up prevalence	100%
Simple IHL/IHL with choledocholith	38/60
Recurrence rate of this operation	16.3%
Mean number of operations	1.60
Number of patients with 1 operation	67
Number of patients with 2 operations	19
Number of patients with 3 operations	7
Number of patients with 4 operations	1
Number of patients with 5 operations	1
Number of patients with 6 operations	2
Number of patients with 11 operations	1
Number of patients with simple choledocholithotomy and T-tube drainage	35
Number of patients with simple hepatectomy	20
Number of patients with choledocholithotomy, T-tube drainage and hepatectomy	35
Number of patients with simple choledochojunostomy	5
Number of patients with choledochojunostomy and hepatectomy	3
Recurrence index	0.072
Reoperation index	0.037

IHL: Intra-hepatolithiasis.

tomy or choledochoduodenostomy may be diagnosed with SOL according to either of the following criteria: (1) contrast media may be found in the common bile duct during duodenography; or (2) a Bake's dilator with a diameter of 10 mm is able to reach the duodenum *via* the SO without pre-dilation during surgery. Patients without a history of any sphincterotomy, including endoscopic sphincterotomy, can be defined as having primary SOL, while those with a history of sphincterotomy can be diagnosed with secondary SOL.

The patients were divided into two groups: HL patients with SOL (Group I) and those without (Group II). Group I included 45 patients (39 with primary SOL, 6 with secondary SOL) and Group II included 53 patients. The mean age of the patients, the mean age of the first onset, and operative procedures are presented in Table 2. Group I was divided into 2 subgroups according to the operative procedures: 6 patients who underwent choledochojunostomy (end-to-side anastomosis with common bile duct and jejunum) were placed in Group I A and 39 patients who did not undergo choledochojunostomy were placed in Group I B. The patients in Group I A had no operative histories before this hospitalization. Biliary visualization was undertaken with duodenography (hypaque meglumine 60%) in 19 (42.2%) patients of Group I and none in the patients of (0%) Group II.

### Recurrence and reoperation indices

Recurrence rate is a frequently used index in the study of lithiasis. As a traditional index, recurrence rate is al-

Table 2 Characteristics of patients in Groups I and II

	Group I	Group II
Number of patients	45	53
Male/female	18/27	17/36
Mean age (yr)	52.8	52.8
Mean age of first onset (yr)	41.9	47.8
Biliary visualization rate during duodenography	42.2%	0%
Mean follow-up period (mo)	38.4	41.9
Simple IHL/IHL with choledocholithiasis	15/30	14/39
Recurrence rate	22.2%	11.3%
Mean number of operations	2.07	1.21
Number of patients with 1 operation	22	45
Number of patients with 2 operations	12	7
Number of patients with 3 operations	7	0
Number of patients with 4 operations	1	0
Number of patients with 5 operations	0	1
Number of patients with 6 operations	2	0
Number of patients with 11 operations	1	0
Number of patients with simple choledocholithotomy and T-tube drainage	14	21
Number of patients with simple hepatectomy	7	13
Number of patients with choledocholithotomy, T-tube drainage and hepatectomy	18	17
Number of patients with simple choledochojejunostomy	4	1
Number of patients with choledochojejunostomy and hepatectomy	2	1

ways confined to consideration of a certain operation. Due to the frequent recurrence of HL, however, it is inappropriate to use only a single operation to evaluate the prognosis of this disease. To accurately describe and compare the whole history (from first onset to July 2008) of HL in each patient, we thus used the recurrence and reoperation indices concurrently. This allowed consideration of every recurrence or operation, and the intervals between two recurrences or two operations.

To determine the recurrence index, we recorded intervals (months) between each recurrence for a patient, calculated the reciprocal of each interval, and used the mean reciprocal as the recurrence index for that patient. If no recurrence occurred in the final operation, the corresponding reciprocal was designated as 0. A larger reciprocal value suggested a higher risk of recurrence. For example, the record of one patient showed that: 10 mo after the first operation, a recurrence occurred; the second recurrence occurred 5 mo after the second operation; and no recurrence occurred in the third operation. For this patient, the recurrence index is:  $(1/10 + 1/5 + 0)/3 = 0.1$ .

Like the recurrence index, there is a positive correlation between the reoperation index and the necessity of reoperation. To calculate the reoperation index, we recorded intervals (months) between every two closely succeeding operations for each patient, calculated the reciprocal of each interval, and used the mean reciprocal as the reoperation index for that patient. The reciprocal corresponding to the interval from the last operation to July 2008 was designated as 0. Thus, if the patient underwent only 1 operation, the reoperation index value was 0. For example, one patient's record shows: the second operation was performed 10 mo after the first operation;

Table 3 Comparison of Groups I and II

	Group I	Group II	P
Number of patients	45	53	-
Male/female	18/27	17/36	0.126
Mean age (yr)	52.8	52.8	0.771
Mean age of first onset (yr)	41.9	47.8	0.463
Mean follow-up period (mo)	39.9	43.9	0.145
Mean number of operations	2.07	1.21	< 0.001
Recurrence index	0.135	0.018	< 0.001
Reoperation index	0.07	0.01	< 0.001

the third and final operations were performed 5 mo after the second one. For this patient, the reoperation index is:  $(1/10 + 1/5 + 0)/3 = 0.1$ .

### Statistical analysis

All data are expressed as mean  $\pm$  SD. The *t* test was used to compare the differences of parameter mean values between groups. All *P* values were two-sided. A *P* value of < 0.05 indicated a statistically significant difference. All calculations were done using SPSS (version 11.5) software.

## RESULTS

### Patient data

The patients were monitored for a period ranging between 14 and 75 mo (median  $42.1 \pm 17.1$  mo). During this follow-up period, stones reappeared in the bile ducts of 16 of 98 patients (with a recurrence rate of 16.3%), including 2 cases of stones in the common bile duct, 4 cases in the intrahepatic bile duct, and 10 cases in both ducts. Seven of these patients received a reoperation, and the disease recurred again in 4 of them. The number of operations performed from the first onset to July 2008 are shown in Table 1; the mean number of operations was  $1.60 \pm 1.38$  (range: 1-11). The mean recurrence index was  $0.0717 \pm 0.193$  and the mean reoperation index was  $0.0373 \pm 0.127$  (Table 1).

### Comparison of Groups I and II

The patients were followed up for 16-73 mo (mean  $39.9 \pm 15.6$  mo) in Group I and for 14-75 mo (mean  $43.9 \pm 18.1$  mo) in Group II (*P* = 0.145). Recurrence was observed in 10 Group I patients (a recurrence rate of 22.2%) and 6 Group II patients (a recurrence rate of 11.3%). The mean number of operations from first onset to July 2008 was  $2.07 \pm 1.83$  (range: 1-11) in Group I, being significantly higher than that of Group II ( $1.21 \pm 0.63$ ; range: 1-5). The mean recurrence index was  $0.135 \pm 0.256$  for Group I, which was significantly higher than that of Group II ( $0.018 \pm 0.086$ ) (*P* < 0.001). The mean reoperation index was  $0.070 \pm 0.171$  for Group I, being also significantly higher than that of Group II ( $0.010 \pm 0.060$ ) (*P* < 0.001) (Tables 2 and 3).

### Comparison of Groups I A and I B

To evaluate the effect of choledochojejunostomy on the



Table 4 Comparison of Groups I A and I B

	Group I A	Group I B	P
Number of patients	6	39	-
Male/female	2/4	16/23	0.385
Mean age (yr)	58	52	0.845
Mean age of first onset (yr)	30.5	43.6	0.666
Mean follow-up period (mo)	42.5	39.5	0.905
Recurrence index	0	0.156	0.006
Reoperation index	0	0.081	0.034

SOL patients, we compared the recurrence and reoperation indices of Groups I A and I B. The follow-up period was between 18 and 61 mo (mean  $42.5 \pm 16.9$  mo) in Group I A, and between 18 and 73 mo (mean  $39.5 \pm 15.6$  mo) in Group I B ( $P = 0.905$ ). We found no recurrence among patients in Group I A (a recurrence rate of 0%), whereas recurrence occurred in 10 of 39 patients in Group I B (a recurrence rate of 25.6%). The mean recurrence indices were thus 0 for Group I A and  $0.156 \pm 0.270$  for Group I B. The mean reoperation indices were 0 for Group I A and  $0.081 \pm 0.182$  for Group I B. The differences in both the recurrence index and reoperation index values were statistically significant between groups (Table 4).

## DISCUSSION

HL is prevalent in the Asian countries of Japan, Korea and China<sup>[1-3]</sup>, and also occasionally occurs in Europe and America<sup>[4,5]</sup>. The disease is characterized by its intractable nature and frequent recurrence. Because of complications, potential carcinogenesis and even fatality<sup>[6-11]</sup>, most HL patients receive multiple operations. This study examined the role of SOL in the course of HL, and found that the frequencies of recurrence and reoperation were significantly higher in patients with SOL.

The obviously larger diameter of the papilla orifice in SOL patients indicates the injury to the SO muscle fibre and the absence of regular SO contraction. These conditions lead to duodenum juice reflux, similar to that seen in choledochoduodenostomy. During surgery on SOL patients, a Bake CBD dilator (CE-125-10-G, diameter = 10 mm) and choledochoscope (OLYMPUS CHF P20, external diameter = 4.9 mm) passed smoothly through the SO; even in some patients, a forefinger could enter the duodenum *via* the SO without any resistance, and food debris could be seen in the CBD. It results in *Escherichia coli* (*E. coli*) infection and decrease in biliary pH. *E. coli* can generate  $\beta$ -glucuronidase, which hydrolyzes direct bilirubin (water-soluble) into indirect bilirubin (water-insoluble) and may lead to the formation of stones in the biliary tract. Thus, HL tends to recur in SOL patients.

The specific causes of SOL, however, remain unknown. Congenital abnormality, mechanical injury by stones, chemical injury by bacteria, or iatrogenic injury are all possible etiological factors. Congenital maldevelopment of the smooth muscle and deficiency of some

neurotransmitter receptors can also contribute to SOL. Under inflammatory conditions where large amounts of inflammation mediators are generated over time<sup>[12]</sup>, the SO response to nitrergic neurotransmission is impaired, which may induce abnormal SO relaxation. Dilation of the common bile duct due to obstruction by stones may result in excessive dilation of the common bile duct sphincter and SOL development. In this study, absence of congenital abnormality in family histories and similarity among the mean age of the first onset ( $P > 0.05$ ) did not support congenital abnormality as a primary etiological factor of SOL.

It is not difficult to diagnose SOL. Duodenography is still considered to be a good method for describing the modality, despite its relatively low sensitivity (42.2%, Table 2). SO manometry is the best method of evaluating patients for sphincter dysfunction<sup>[13]</sup>, and it may be a feasible diagnostic method. The confidence of SO manometry, however, is suspected due to multiple factors (abdominal pressure, dynamic changes caused by the manometry, drug reactions). The invasive nature of manometry, the complexity of the operation, and the potential complication of pancreatitis restrict the use of SO manometry as a routine diagnostic method<sup>[14,15]</sup>. Due to the existence of intestinal juice reflux, simple calculus removal or hepatectomy are not sufficient for SOL patients, while choledochojejunostomy with an anti-reflux ansa intestinalis may be suitable for these patients<sup>[16]</sup>. The outcomes of six patients in Group I A showed the advantage of choledochojejunostomy, although the sample size is small. Further research on the effectiveness of this method should be conducted with a larger number of patients.

Endoscopic sphincterotomy may destroy the muscle fibres of the SO and cause iatrogenesis. The risks of cholangitis and cholangiocarcinoma have also been shown to markedly increase if the postoperative follow-up period is long enough<sup>[17]</sup>. This method should therefore be restricted to patients without SOL. Endoscopic balloon dilation is an effective alternative to endoscopic sphincterotomy<sup>[18-20]</sup>, which has been shown to result in complete recovery of sphincter function 21 d after operation<sup>[21]</sup>. An appropriate balloon size can preserve the SO and avoid undesirable effects due to an incompetent sphincter<sup>[22-26]</sup>.

SOL commonly arises in HL. HL patients with SOL tend to have a higher risk of recurrence and a larger demand for reoperation compared to those without SOL. Choledochojejunostomy with an anti-reflux ansa intestinalis may be the most promising therapy.

## COMMENTS

### Background

Hepatolithiasis (HL) is prevalent in East Asia, especially in China and obstructive cholangitis is usually considered the main cause. The authors found that almost half of the cases showed sphincter of Oddi laxity (SOL) in their clinical experience and the patients with SOL had a tendency for recurrence and always needed reoperation.

### Research frontiers

The obstructive cholangitis is usually considered as the main cause of HL. No report has put forward the conception of SOL up to date. The phenomenon and significance of SOL is still rarely known.

### Innovations and breakthroughs

The study has proposed the conception of SOL for the first time and considered SOL as a significant cause of occurrence or recurrence of HL. The authors of the study also put forward the recurrence and reoperation indices for the first time instead of recurrence rate, and suggested the choledochojejunostomy should be used as the surgical approach to the patients with SOL.

### Applications

By understanding SOL, this study may represent a new strategy in the treatment of HL patients with SOL.

### Terminology

SOL: an obviously large diameter of the papilla orifice makes the choledochoscope (OLYMPUS CHF P20, external diameter = 4.9 mm) pass smoothly through the SO. The recurrence and reoperation indices are used to accurately describe and compare the complete history of HL from the first onset to July 2008 in each patient, which allows consideration of each recurrence or operation, and the intervals between two recurrences or two operations.

### Peer review

The study revealed that SOL is an important factor associated with a higher risk of HL recurrence and reoperation frequency in the HL patients.

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## A study of the efficacy of bacterial biofilm cleanout for gastrointestinal endoscopes

Ying Fang, Zhe Shen, Lan Li, Yong Cao, Li-Ying Gu, Qing Gu, Xiao-Qi Zhong, Chao-Hui Yu, You-Ming Li

Ying Fang, Zhe Shen, Lan Li, Yong Cao, Li-Ying Gu, Qing Gu, Xiao-Qi Zhong, Chao-Hui Yu, You-Ming Li, Department of Gastroenterology, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China

Author contributions: Fang Y, Yu CH and Li YM designed the research; Fang Y, Gu LY, Gu Q, Zhong XQ and Cao Y performed the research; Fang Y, Shen Z and Li L analyzed the data and wrote the paper.

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Correspondence to: You-Ming Li, Professor, Department of Gastroenterology, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China. [zjulym@126.com](mailto:zjulym@126.com)

Telephone: +86-571-87236603 Fax: +86-571-87236611

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### Abstract

**AIM:** To compare the influence and clearance effect of enzymatic and non-enzymatic detergents against *Escherichia coli* (*E. coli*) biofilm on the inner surface of gastroscopes.

**METHODS:** Teflon tubes were incubated in a mixture of different detergents and *E. coli* culture ( $10^6$  CFU/mL) for 72 h at 15°C, and biofilms on the inner surface of the teflon tubes were analyzed by bacterial count and scanning electron microscopy. To evaluate the clearance effect of detergents, after biofilms were formed on the inner surface of Teflon tubes by 72 h lavage with *E. coli* culture, tubes were lavaged by enzymatic and non-enzymatic detergents at a speed of 250 mL/min, then biofilms on the inner surface were analyzed by bacterial count and scanning electron microscopy.

**RESULTS:** Non-enzymatic detergent had a better inhibition function on biofilm formation than enzymatic de-

tergent as it reduced bacterial burden by 2.4 log compared with the control samples ( $P = 0.00$ ). Inhibition function of enzymatic detergent was not significantly different to that of control samples and reduced bacterial burden by 0.2 log on average ( $P > 0.05$ ). After lavaging at 250 mL/min for 3 min, no living bacteria were left in the tubes. Scanning electron microscopy observation showed biofilms became very loose by the high shear force effect.

**CONCLUSION:** Non-enzymatic detergent has a better inhibition effect on biofilm formation at room temperature. High speed pre-lavage and detergents are very important in temporal formed biofilm elimination.

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**Key words:** Endoscopy; *Escherichia coli* biofilm; Scanning electron microscopy; Bacterial count

**Peer reviewer:** Antonello Trecca, MD, Digestive Endoscopy and Gastroenterology, Usi Group, Via Machiavelli, 22, 00185 Rome, Italy

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### INTRODUCTION

The concept of bacterial biofilm was proposed in 1936<sup>[1]</sup>. Bacteria adhere to wet surfaces easily, then form organized colonies of cells embedded in a self-excreted matrix, which is composed principally of polysaccharide, and the polysaccharide facilitates adhesion to the surface and to each other<sup>[1,2]</sup>. In clinical medicine, many environments provide optimal conditions for the formation of bacterial biofilm, such as contact lenses, central venous catheters,

urinary catheters and so on<sup>[3]</sup>. Similarly, the presence of biofilm on the surface of gastrointestinal endoscope channels has also been confirmed in recent studies<sup>[4-6]</sup>. Bacteria residing within biofilms are up to 1000 times more resistant to chemical inactivation than bacteria in suspension<sup>[7-9]</sup>. Biofilms are not only a reservoir of pathogenic bacteria that can detach, resume their planktonic state, and contaminate the patient, but also a source of endotoxins that may enter the circulation of the patient through ruptured mucosae and cause systemic disorders<sup>[10]</sup>. It was reported that if the endoscope channels were not cleaned prior to disinfection, the decontamination of endoscopes could become a failure<sup>[11,12]</sup>. Therefore, it is important to explore a reasonable cleaning agent of gastrointestinal endoscopes to achieve a satisfactory result of anti-biofilm under the present conditions.

In this controlled study, we compared the clearout effect of enzymatic and non-enzymatic detergents against *Escherichia coli* (*E. coli*) biofilm on the inner surface of gastroscopes.

## MATERIALS AND METHODS

### Preparation of bacterial suspension

A small amount of *E. coli* ATCC 25922 (Hangzhou Tianhe Biologics Corporation, Zhejiang, China) was taken from slant medium and inoculated into sterile Muller-Hinton (M-H) broth (pH 7.4) to obtain pure bacilli. The poured plate method was used to adjust the concentration of bacteria to 10<sup>6</sup> CFU/mL.

### Bacterial biofilm formation

A high temperature sterilized Teflon tube (digestive endoscope lumen material) was cut into 15 pieces with 0.5 cm × 0.5 cm per piece, which were randomly divided into 3 groups: Group 1: enzymatic detergent group; Group 2: non-enzymatic detergent group; Group 3: blank control group. To prepare a sterile biopsy bottle for each group, 5 mL of M-H broth and 100 µL of bacterial suspension with good preparation previously were added into each group. 13 mL of rapid multi-enzyme cleaner (3M Company, Sao Paulo, Minnesota, USA), 25 µL of a non-enzymatic detergent, intercept (Minntech Corporation, Minneapolis, Minnesota, USA) with a 1:200 dilution ratio (recommended maximum dilution ratio of the products) and 20 µL sterile distilled water were added into Group 1, Group 2 and Group 3, respectively, taking care not to overlap the various pieces of material. Then each group was put into a 15°C incubator to incubate. 5 mL bacilli fluid was aspirated by sterile syringe every 24 h, adding 5 mL M-H broth; it was then soaked in 0.1 mol/L phosphate buffered saline (PBS) for 15 min through two stages after 72 h. We discarded the PBS in order to remove bacteria loosely attached to the wall, then Teflon tubes were taken from each bottle with sterile tweezers to prepare for evaluation. One sample from each group was prepared for electron microscopic evaluation, and the other 4

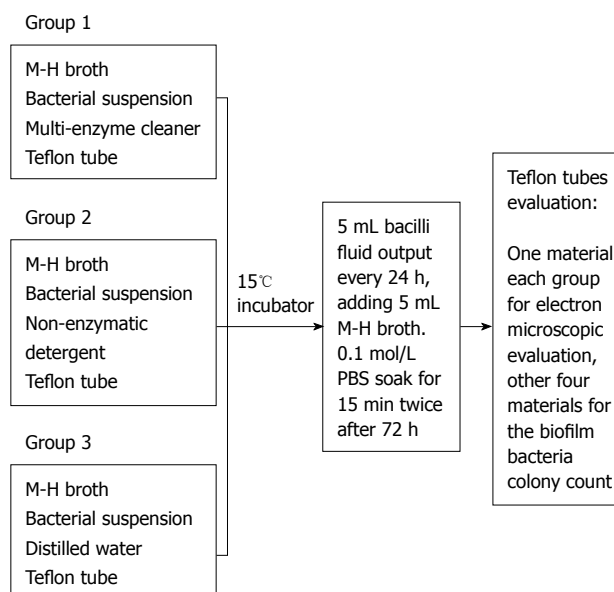


Figure 1 Bacterial biofilm formation assay.

samples were used for the biofilm bacteria colony count (Figure 1).

### Electron microscope evaluation of wall biofilm and bacterial colony counts

The teflon tubes material was transferred to a sterile Eppendorf (EP) tube, fixed with 1% osmium tetroxide for 1 h, then washed twice (each time for 15 min) in PBS buffer solutions. After washing, they were dehydrated for 15 min using alcohol and 100% acetone, replaced by amyl acetate eater, dried to CO<sub>2</sub> critical point, and sprayed gold with an ion sputtering instrument. Then they were scanned by electron microscope photographs (Stereoscan 260, Cambridge, UK). At the same time, another 12 Teflon tubes material in sterile EP tubes containing 1 mL of 0.1 mol/L PBS buffer solution were oscillated ultrasonically (38-47 kHz, 10 min) (Olympus Corporation, Tokyo, Japan), so that biofilm was stripped from the wall. We drew 100 µL PBS buffer solution from the above EP tube, then diluted the sample to 10000 times with 10 times serial dilution. 0.1 mL diluent was added into a sterile petri dish. M-H agar 20 mL cooled to 50°C was poured into the sterile petri dish, and the samples were mixed and cooled to 37°C. The petri dish was incubated for 24 h, and the growth of colonies and colony counts were examined.

### Production of biofilm-coated sample tubes

A high-temperature sterilized teflon tube with a length of 120 cm was placed in a sterilized beaker. Media contaminated with *E. coli* was run through the tube at a flow rate of 250 mL/min to mimic flow conditions (Minntech Co., Minneapolis, Minnesota, USA) during clinical endoscope procedures. The lavage lasted 4.5 h daily for 3 d, then 1/2 bacilli fluid was extracted and poured into the same amount M-H broth every 24 h.



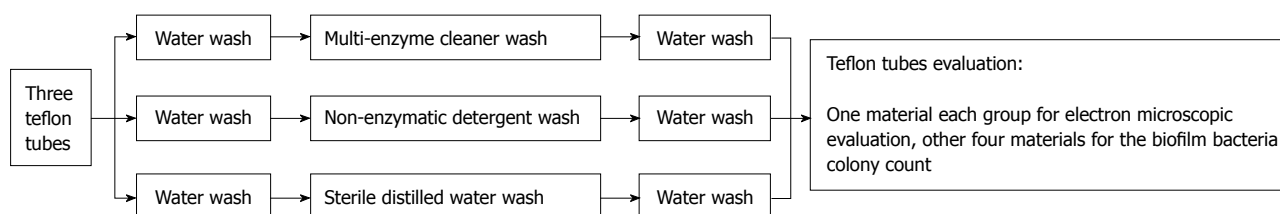


Figure 2 Cleaning steps of biofilm-coated sample tubes.

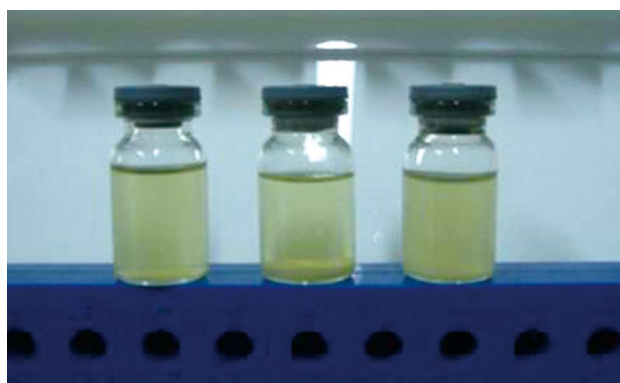


Figure 3 Mixture of detergent and bacteria cultured for 72 h. Left: The enzyme-containing washing, showing a homogeneous transparent liquid; Middle: A non-enzyme-containing detergent, showing in addition to a large amount of sediment at the bottom, the upper part was clean liquid; Right: The control group, showing bacteria bacilli and a small amount of sediment at the bottom.

### Cleaning of biofilm-coated sample tubes and biofilm evaluation

After lavage, the contaminated teflon tube was cut into 3 pieces. Next, the disconnected tubes were randomly divided into an enzyme wash group, a non-enzyme wash group, and a blank control group. According to Endoscope Disinfection Technical Operation Standards of China, after perfusion 3 sections of teflon tube were subjected to water wash (1 min), enzyme wash (3 min), and water wash again (1 min). In Group 1 enzyme wash was with multi-enzyme cleaner, in Group 2 non-enzymatic detergent was used instead of enzyme-containing detergent, and in Group 3 the enzyme wash step was replaced by sterile distilled water. After completing the above steps, tubing was cut with 5 cm spacing interception to produce 5 tubal walls of 0.5 cm × 0.5 cm, including 4 for the biofilm colony count, and 1 for scanning electron microscope observation of the concave wall biofilm (Figure 2).

### Statistical analysis

Statistical analysis was performed with SPSS 13.0 statistical package (SPSS Inc., Chicago, Illinois, USA). The mean value for different groups was compared using Student *t*-test.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Effects of detergent against bacterial biofilm formation on the wall

As shown in Figure 3, the bacterial suspension in the

Table 1 Wall residue biofilm colony count after bacterium and detergent suspension soaked

Detergents	Average actual colony count (CFU/ piece, $\pm$ SD)	Average standard colony count (lgCFU/ piece, $\pm$ SD)
Enzymatic detergent	$(1.15 \pm 0.86) \times 10^4$	$3.96 \pm 0.35^a$
Non-enzymatic detergent	$(6.85 \pm 2.90) \times 10$	$1.80 \pm 0.21^b$
Control	$(1.69 \pm 0.65) \times 10^4$	$4.19 \pm 0.22$

<sup>a</sup> $P = 0.00$  vs the control group, <sup>b</sup> $P = 0.25$  vs the control group.

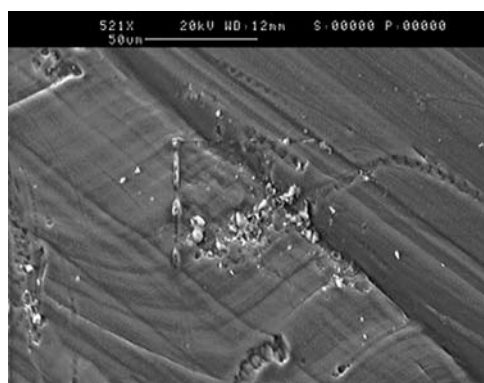


Figure 4 The concave wall structure after mixture of detergent and *Escherichia coli* cultured; surfaces are part of the biofilm formation.

enzymatic detergent group was limpid after culturing for 72 h, related primarily to the multi-enzyme ingredients to digest the organic components in detergent. The bacterial suspension in the non-enzymatic detergent group showed matrix components at the bottom, and the quantity was greater than the control group, which was associated with decomposed bacterial cell components and the organic components of the broth. After counting the number of bacteria in the biofilm, the non-enzymatic detergent had a better inhibition function on biofilm formation and reduced bacterial burden by 2.39 logs compared with water without any detergent ( $P < 0.05$ ). Enzymatic detergent reduced bacterial burden by 0.23 log compared with water without any detergent, and there was no significant difference between the two groups ( $P > 0.05$ ) (Table 1). On the surface of teflon tubes from any groups, the remaining biofilm was observed by electron microscopy (Figure 4). It was easier to find biofilm on a rough surface compared with a smooth non-porous surface, which showed that the compatibility of



**Figure 5** The concave wall structure after non-enzyme detergent (A), enzyme-containing detergent (B), and sterile distilled water (C) washing. A: No obvious biofilm residue; B: Massive biofilm structure residue; C: Relatively large biofilm attached to bacterial biomass, respectively.

detergent with the inner surface of endoscope channels was important for preventing the build-up of biofilm and removing a mature biofilm. It is generally accepted that smooth non-porous surfaces are the easiest to disinfect.

#### **Effects of detergent against bacterial biofilm removal on the wall**

After lavaging with bacterial suspension and reprocessing with different detergents, no living bacterial matter was left on the surface of teflon tubes. However, at higher magnification, scanning electron microscopy examination showed a few bacterial cells covering the internal surface of teflon tubes (Figure 5). The biofilm cells appeared as small microcolonies in the non-enzyme detergent group and blank group, but as a confluent membrane in the enzyme detergent group. The number of bacteria remaining was smaller in the non-enzyme detergent group than in the blank group.

## **DISCUSSION**

It has been reported that bacterial biofilm causes about 65% of bacterial infections in the clinic<sup>[13]</sup>. As research continues, bacterial biofilm is recognized as an ecological community composed of microbes and the irreversibly combined extracellular matrix, not simply a mixture of colonized bacteria and extracellular matrix. Biofilms have different phenotypes according to the difference in microbe growth and genetic transcription<sup>[14]</sup>. Biofilm induced biological material-related infections occurs widely in clinical departments, for instance, indwelling urinary catheter related infection, indwelling venous catheter related infection, artificial femoral head related infection. Antibiotics do not have a satisfactory effect on the infections due to the resistance of the biofilm<sup>[15]</sup>. Bacteria within biofilms are up to 1000 times more resistant to antimicrobials than the same bacteria in suspension. Endoscope related infection, as reported in articles<sup>[3,16]</sup>, also occurs in western countries with strict specifications of endoscope reprocessing procedures. Although the poor quality of disinfection of endoscopes is associated with inadequate compliance with reprocessing protocols, another explanation could be more related to the formation of biofilm on endoscope channels. We found that

an electron microscope scanning of an obsolete tube confirmed the existence of biofilm on the inner wall in a study.

In this experimental study, we adopted the endoscope lumen material (teflon) as the object of study. In the study on detergent effects on bacterial biofilm formation: biofilm colony count results showed that there was a significant difference in biofilm viable counts between non-enzyme detergent soaking teflon and the blank group ( $P < 0.05$ ), while there was no significant difference in biofilm viable counts between enzymatic detergent soaking teflon and the control group ( $P > 0.05$ ). This showed that non-enzyme detergent for biofilm formation had a better inhibitory effect than enzymatic detergent. The electron microscopy results showed that the biofilm was more easily deposited on the surface of non-flat areas, indicating detergent needs a better compatibility to the teflon tube in order to avoid producing a good place to form a biofilm because of the different affinity.

Endoscopes should be washed by water and enzymatic detergents before disinfection, otherwise the disinfectants have an inadequate effect because of the existence of blood, mucus and other organic materials<sup>[17]</sup>. Some data have revealed that a proper cleaning step could decrease the bacterial cells by 2-5 logs<sup>[18]</sup>. Our study confirmed the elimination of the number of bacteria within a biofilm by the shearing effect of high speed lavage. In the study for the effects of detergent against bacterial biofilm removal on the tube wall, lavage for 72 h to the teflon tube, then 1 min water wash, 3 min enzyme wash, and 1 min water wash again, produced a biofilm colony count of 0 in all samples of Teflon tube, but there were still differences in scanning electron microscopy; in the nearly 500-fold scan condition, a small amount of *E. coli* were found, showing small microcolonies for the non-enzyme detergent group and control group, but the number of bacteria remaining was fewer in the non-enzyme detergent group than in blank group. However, there was a confluent membrane of bacterial growth in the enzyme detergent group.

Many factors, as recognized and further researched, affect the formation or elimination of biofilms, including the characteristic of the fluid, the species of microbes,

surface of the tubes, velocity of flow, temperature and pH value<sup>[19]</sup>. The conservation of the endoscope should focus on avoiding minor injury to the inner wall of channels, which eases the formation of biofilm. It's also important to choose a green detergent with more cleansing power, but less corrosion to the tube, not only "with enzyme" or "without enzyme". According to the manual, the enzymatic detergent has maximum cleansing power at a temperature higher than room temperature. However, we observed the efficiency of detergent at a temperature of 15°C, which is a realistic temperature for use. It was reported that proteolytic enzymes in the detergent increased the risk of occupational asthma, which cannot be improved by avoidance of exposure with enzymes of detergent origin<sup>[20]</sup>.

During our short-term study, the elimination of bacterial biofilm on the inner wall of teflon tubes was studied and residual biofilm was detected by electron microscope scanning. Artificial contamination was performed using the *E. coli* bacteria only, whereas the actual contamination and the formation of biofilms is produced by miscellaneous bacteria in the clinic. Food residue, mucus, gastric acid and bile existing in the endoscope channels can decrease the shearing of the liquid and, therefore, should be completely removed to prevent the formation of biofilm. In this study, the current cleaning agents could not completely eliminate endoscopic biofilm, which should arouse our attention in the future. Therefore, a thorough cleaning of the endoscope to remove contaminants is one of many important and effective measures to prevent the formation of endoscopic biofilm. At the same time, how to effectively remove biofilm formed on the lumen is a continuing research goal. Further study on the formation and the removal of the biofilm is required and more effective detergents should be explored.

## COMMENTS

### Background

Many clinical environments provide optimal conditions for the formation of bacterial biofilm, including gastrointestinal endoscope channels. Biofilms are not only a reservoir of pathogenic bacteria, but also a source of endotoxins that may enter the circulation of the patient through ruptured mucosae and cause systemic disorders. However, if the endoscope channels are not cleaned prior to disinfection, the decontamination of endoscopes could become a failure because of biofilm formation. Therefore, it is important to explore a reasonable cleaning agent of the gastrointestinal endoscope to achieve a satisfactory result of anti-biofilm under the present conditions.

### Research frontiers

Endoscope related biofilm infections also occur in western countries with strict specifications of endoscope reprocessing procedures. Therefore, a thorough cleaning of the endoscope to remove contaminants is one of many important and effective measures to prevent the formation of endoscopic biofilm. To understand the effect of current detergents on biofilm, the authors produced a controlled study to compare the clearout effect of enzymatic and non-enzymatic detergents against *Escherichia coli* biofilm on the inner surface of gastroscopes.

### Innovations and breakthroughs

If endoscope channels are not cleaned prior to disinfection, the decontamination

of endoscopes could become a failure due to biofilms. Therefore, it is important to explore a reasonable cleaning agent of gastrointestinal endoscopes to achieve a satisfactory result of anti-biofilm under the present conditions. This study aims to draw attention to endoscopic workers to biofilms through the authors' common efforts to ensure the safety of clinical endoscopic work in the future.

### Applications

The study results suggest that the current cleaning agents cannot completely eliminate endoscopic biofilm, which should arouse our attention in the future. A thorough cleaning of the endoscope to remove contaminants is one of many important and effective measures to prevent the formation of endoscopic biofilm. How to effectively remove a biofilm that has already formed on the lumen is an ongoing research goal. Further study on the formation and the removal of biofilm is required and more effective detergents should be explored.

### Terminology

Bacterial biofilm: Bacterial biofilm is recognized as an ecological community composed of microbes irreversibly combined with the extracellular matrix, not simply a mixture of colonized bacteria and extracellular matrix.

### Peer review

The study is important as a means of exploring a reasonable cleaning agent of the gastrointestinal endoscope to achieve a satisfactory result of anti-biofilm under the present conditions. It deals with a very important topic on gastrointestinal reprocessing and it can add new information to this topic. It is also interesting and well designed.

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## Pathological differential diagnosis of solid-pseudopapillary neoplasm and endocrine tumors of the pancreas

Bao-An Liu, Zhuo-Ming Li, Zhan-San Su, Xiao-Ling She

Bao-An Liu, Zhuo-Ming Li, Department of Pathology, Xiangya Basic Medical School, Central-south University, Changsha 410013, Hunan Province, China

Zhan-San Su, Department of Pathology, Xiangya Third Hospital, Central-south University, Changsha 410013, Hunan Province, China

Xiao-Ling She, Department of Pathology, Xiangya Second Hospital, Central-south University, Changsha 410006, Hunan Province, China

**Author contributions:** Li ZM performed the majority of experiments and wrote the manuscript; Liu BA was involved in editing the manuscript and provided financial support; Su ZS reviewed all pathological slides; She XL participated in some experiments.

**Supported by** Department of Pathology, Xiangya Basic Medical School, Central-south University

**Correspondence to:** Bao-An Liu, Professor, Department of Pathology, Xiangya Basic Medical School, Central-south University, 110# Tongzipo Road, Changsha 410013, Hunan Province, China. [lba118@yahoo.com.cn](mailto:lba118@yahoo.com.cn)

Telephone: +86-731-82651410 Fax: +86-731-82651410

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papillary structure and discohesive appearance of the neoplastic cells were observed in all 10 (100%) cases. The results of immunostaining showed that nuclear expression of  $\beta$ -catenin and loss of E-cadherin in all the cases, was only seen in SPN. Molecular studies discovered that 9/10 (90%) cases harbored a point mutation of exon 3 in  $\beta$ -catenin gene. On the other hand, the mean age of PET patients was 43.1 years. Eight of 14 cases presented with symptoms caused by hypoglycemia, and the other 6 cases presented with symptoms similar to those of SPN. The mean size of the tumors was 2.9 cm, most of the tumors were solid, only 3/14 (21%) were a mixture of solid and cystic structures, and macroscopic hemorrhage and necrosis were much less common (3/14, 21%). Histologically, tumor cells were arranged in trabecular, acinar or solid patterns and demonstrated no pseudopapillary structure and discohesive appearance in all 14 (100%) cases. The results of immunostaining and mutation detection were completely different with SPN that membrane and cytoplasmic expression of  $\beta$ -catenin without loss of E-cadherin, as well as no mutation in  $\beta$ -catenin gene in all the cases.

### Abstract

**AIM:** To investigate differential points of solid-pseudopapillary neoplasm (SPN) of the pancreas and pancreatic endocrine tumor (PET).

**METHODS:** Ten cases of SPN and fourteen cases of PET were studied in this retrospective study. Clinical and pathologic features, immunostaining reactions and  $\beta$ -catenin gene mutations were analyzed.

**RESULTS:** The mean age of SPN patients was 25.6 years and these patients had no specific symptoms. The mean diameter of the tumors was 11.0 cm, 9/10 cases were cystic or a mixture of solid and cystic structures, and there was hemorrhage and necrosis on the cut surface in 8/10 (80%) cases. Characteristic pseudo-

**CONCLUSION:** Both macroscopic and microscopic features of SPN are quite characteristic. It is not difficult to distinguish it from PET. If necessary, immunostaining of  $\beta$ -catenin and E-cadherin is quite helpful to make the differential diagnosis.

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**Key words:** Solid-pseudopapillary neoplasm of the pancreas; Pancreatic endocrine tumor; Immunohistochemistry;  $\beta$ -catenin gene; Differential diagnosis

**Peer reviewer:** Aaron Vinik, MD, PhD, FCP, MACI, Professor of Medicine/Pathology/Neurobiology, Director of Research and Neuroendocrine Unit, Eastern Virginia Medical School, Strelitz Diabetes Center, 855 W Brambleton Avenue, Norfolk, VA 23510, United States

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## INTRODUCTION

Solid-pseudopapillary neoplasm (SPN) of the pancreas is a relatively rare and its histogenesis is still controversial. There are some similarities between SPN and pancreatic endocrine tumor (PET), especially the non-functioning ones, in clinical and pathological manifestations<sup>[1-3]</sup>. Both have few specific clinical symptoms and signs and lack exclusive features on ultrasonography, imaging examination and laboratory tests. Histopathologically, both may be very similar and the results of immunohistochemistry reported in the literature showed that expression profiles of the two tumors overlapped<sup>[4,5]</sup>, which sometimes results in difficulty in distinguishing the two entities. In recent years, studies have shown that the vast majority of SPN harbored a point mutation on exon 3 of  $\beta$ -catenin gene, which has not yet been discovered in other pancreas tumors. In this study we took the mutation of  $\beta$ -catenin gene as major diagnostic evidence and explored the major points of pathological differential diagnosis of SPN and non-functioning PET.

## MATERIALS AND METHODS

### Case selection

A total of 24 cases pathologically diagnosed as SPN or PET were retrieved from the files of Department of Pathology, Xiangya Hospital, Central South University, China, during the period from 1999 to 2008.

### Morphologic review

The clinical data, description of gross morphology, H&E sections and immunohistochemical staining of all the cases were reviewed and the pathological diagnoses were re-evaluated.

### Immunohistochemical study

Immunohistochemical stains were performed on formalin-fixed, paraffin-embedded 5  $\mu$ m sections from all patients. Eleven consecutive sections were prepared from each tissue block and stained for the following markers: pan cytokeratin (pan CK, DAKO), anti-trypsin (ACT, DAKO), anti-chymotrypsin (AACT, DAKO), vimentin (Vim, DAKO), synaptophysin (Syn, DAKO), chromogranin (CgA, Santa cruz), neuron-specific enolase (NSE, DAKO), insulin (Ins, Santa cruz), somatostatin (Som, Santa cruz), glucagon (Glu, Santa cruz), pancreatic polypeptide (PP, Santa cruz), E-cadherin (E-cad, Santa cruz),  $\beta$ -catenin (Santa cruz) and Ki-67 (DAKO). The sections were deparaffinized in xylene and rehydrated through

graded alcohols. Antigen retrieval was performed in 1 mmol/L of EDTA (pH 8.0) in a microwave oven at 98°C. Endogenous peroxidases were inactivated by immersing the sections in 0.3% hydrogen peroxide for 20 min. Staining was performed with the DAKO En Vision Kit (DAKO) and the sections were developed with 3,3'-diaminobenzidine tetrahydrochloride and counterstained with hematoxylin.

### DNA preparation, polymerase chain reaction (PCR) amplification

Tumor tissue was microdissected from formalin-fixed, paraffin-embedded blocks using a scalpel and placed into microcentrifuge tubes for DNA extraction. DNA extraction was performed with a FFPE DNA Isolation Kit from Omega Biotek following the manufacturers' protocol. Exon 3 of  $\beta$ -catenin was amplified by PCR. The primer sequences were as follows: sense: 5'-ATGGAACCAGACAGAAAAGC-3'; anti-sense: 5'-TTCCCACTCATACAGGACTT-3'. PCR cycling conditions were: 2 mmol/L MgCl<sub>2</sub> and 1 U Platinum-Taq polymerase (Takara), initial denaturation 5 min 94°C, 40 denaturation cycles 30 s 94°C, 30 s annealing 52-60°C, 30 s elongation 72°C, and final elongation 7 min 72°C. Using a PCR Purification Kit (Takara), purification of the PCR product was done essentially as recommended by the manufacturer.

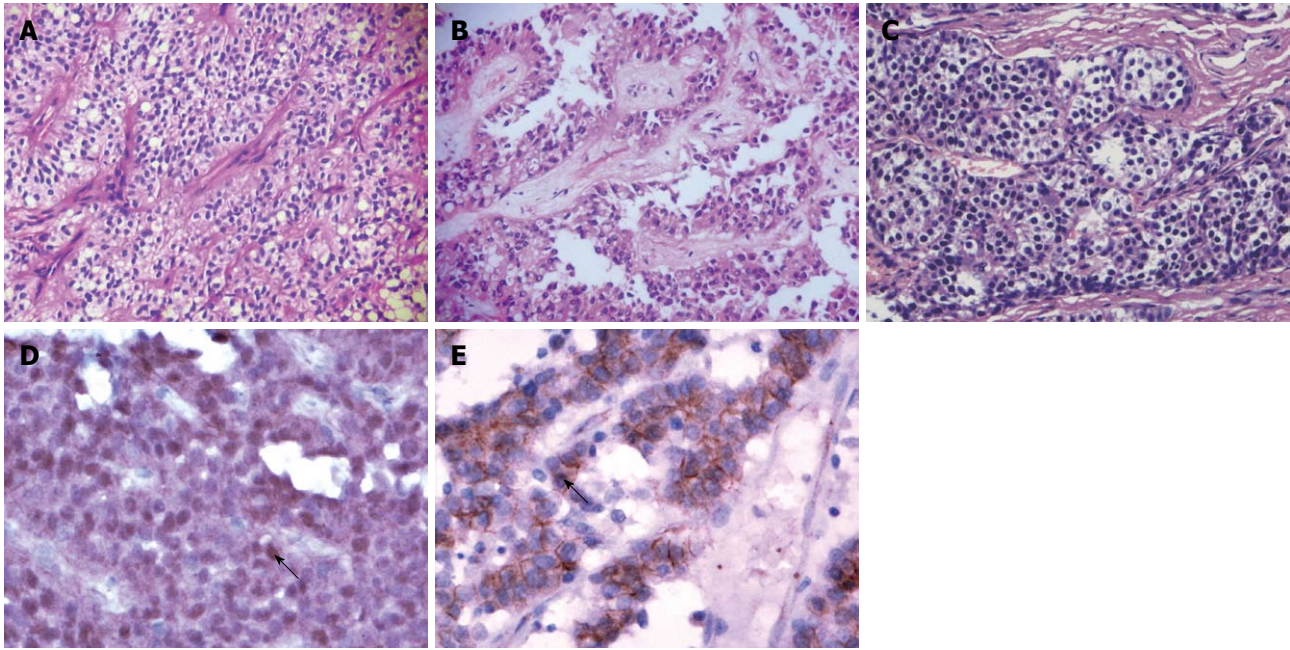
### DHPLC analysis, sequencing

DHPLC analysis was carried out on a WAVE DNA fragment analysis system (Transgenomic Inc.) equipped with a DNasep<sup>®</sup> Column. PCR products were denatured for 5 min at 95°C before being gradually reannealed by decreasing the sample temperature from 95 to 45°C over a period of 50 min to enable the formation of heteroduplexes. DHPLC analysis was carried out at a flow-rate of 0.9 mL/min and buffer B (0.1 mol/L TEAA, 25% acetonitrile), with a gradient increase of 2% per min for 4 min. Sequencing reactions were set up with 30 ng purified PCR fragment template and 10 pmol sequencing primer in 10  $\mu$ L total reaction volume following a dye terminator protocol. Sequencing primers were identical to amplification primers. Sequence alterations were verified by sequencing both DNA strands and by analyzing an independently generated PCR amplicon.

## RESULTS

Twenty-four cases, including 6 SPN and 18 PET, were re-evaluated according to clinical characteristics, gross morphology and microscopic features, results of immunohistochemistry and molecular findings. Six cases of SPN continued to have the original diagnosis, 14 cases of 18 PET were thought to be PET, the other 4 cases of PET were revised as SPN, and no other kind of tumor was found. Therefore there were 10 cases in the SPN group and 14 cases in the PET group.

There were 7 females and 3 males in the SPN group and 9 females and 5 males in the PET group. The mean



**Figure 1** Histopathological and immunohistochemical features of solid-pseudopapillary neoplasm (SPN) and pancreatic endocrine tumor (PET). A: SPN arranged in solid areas, patternless sheets of uniform epithelial cells with numerous small blood vessels (hematoxylin-eosin, original magnification  $\times 200$ ); B: SPN formed characteristic pseudopapillary changes due to the degenerative and discohesive nature of the tumor cells (hematoxylin-eosin, original magnification  $\times 200$ ); C: PET arranged in acinar-like pattern; the tumor cells are small and round with granular eosinophilic or clear cytoplasm (hematoxylin-eosin, original magnification  $\times 200$ ); D:  $\beta$ -catenin immunostaining of SPN: Nuclear translocation and accumulation of  $\beta$ -catenin protein (arrow) is seen in neoplastic epithelial cells (original magnification  $\times 200$ ); E:  $\beta$ -catenin immunostaining of PET: Membrane and cytoplasmic positive expression of  $\beta$ -catenin protein without nuclear stain (arrow) (original magnification  $\times 200$ ).

age was 25.6 years (range: 14–43 years) in the SPN group and 43.1 years (range: 16–52 years) in the PET group. Eight PET cases presented with symptoms of endocrine disorders, such as confusion, psychiatric disturbances, and even coma caused by hypoglycemia. All patients in the SPN group and 6 cases in the PET group had non-specific and similar clinical symptoms, including a dull aching pain in the abdomen (6 cases in the SPN group and 2 in the PET group); a painless abdominal mass in the epigastric region (3 in the SPN and 1 in the PET group); and non-specific abdominal symptoms for which they had been examined (1 case in the SPN group and 3 in the PET group).

Macroscopically all the SPN and PET cases were well-circumscribed single masses except for 1 PET case, which showed multiple masses. The mean diameters were 11.0 cm (range: 5–20 cm) in the SPN group and 2.9 cm (range: 1.5–5.7 cm) in the PET group. Cystic or cystic-solid areas with zones of hemorrhage and necrosis, or cystic spaces filled with necrotic debris were seen in 8/10 (80%) cases of SPN, and only 3/14 (21%) cases of PET. For both tumors there was no preferential localization within the pancreas.

On microscopy, SPN was composed of cells arranged in the form of solid sheets (Figure 1A), microcysts and pseudopapillary areas which showed characteristic pseudopapillae with the fibrovascular axis of the branch-shaped area surrounded by several layers of polygonal epithelioid cells (Figure 1B). The cells had moderate amounts of eosinophilic to vacuolated cytoplasm. The nuclei were ovoid and folded with indistinct

nucleoli and few mitoses. Regional cystic degeneration, hemorrhage, necrosis, aggregates of foamy histiocytes, cholesterol clefts were common. PET were usually arranged in three patterns: (1) trabecular or gyrus-like; (2) acinar- or duct-like (Figure 1C); and (3) solid or diffuse structure. The tumor cells were usually small and round with a granular eosinophilic or clear cytoplasm. Hemorrhage and necrosis were seen in only 3 cases, and pseudopapillary structures and cellular dyscohesive degeneration were not observed in all cases. The clinical and pathological features of SPN and PET are summarized in Table 1.

### Immunohistochemistry

The immunohistochemical results are summarized in Table 2. Nuclear translocation and accumulation of  $\beta$ -catenin protein was seen in neoplastic cells in all SPN cases (Figure 1D). The tumor cells of PET cases showed only normal membranous and cytoplasmic  $\beta$ -catenin labeling, but were negative for nuclear accumulation (Figure 1E). The other immunostains showed an overlap, even including synaptophysin and chromogranin A, between these two tumors. Ki-67 proliferative index was less than 1% in most of the tumors in both groups.

### DHPLC analysis

Nine of 10 SPN cases presented DHPLC heteroduplex bimodal or shoulder-type peaks on DHPLC mutation detection, which indicated mutations on exon 3 of  $\beta$ -catenin gene.

All 14 samples of PET showed a single peak on



Table 1 Clinical and pathological features of SPN and PET

Case No.	Sex/age (yr)	Tumor location	Size (cm)	Cystic or solid	H/N	PP areas
SPN group						
1	F/17	Body	9.5	Cystic	Present	Present
2	F/19	Tail	6.0	Cystic	Present	Present
3	F/14	Head	8.0	Cystic	Present	Present
4	M/20	Body & tail	10.0	Cystic & solid	Present	Present
5	F/24	Head	10.0	Cystic	Present	Present
6	F/33	Body & tail	5.0	Solid	Absent	Present
7	F/21	Body & tail	20.0	Cystic & solid	Present	Present
8	M/43	Body	10.5	Cystic & solid	Present	Present
9	F/27	Tail	12.0	Cystic & solid	Present	Present
10	F/38	Unclear	19.5	Cystic & solid	Present	Present
PET group						
11	F/16	Head	1.5	Solid	Absent	Absent
12	F/53	Body	2.0	Solid	Absent	Absent
13	M/44	Tail	1.5	Solid	Absent	Absent
14	F/40	Head	4.5	Solid	Absent	Absent
15	M/43	Head	3.0	Solid	Absent	Absent
16	F/49	Head	1.5	Solid	Absent	Absent
17	F/34	Head	2.0	Solid	Absent	Absent
18	F/46	Head	1.5	Solid	Absent	Absent
19	F/49	Tail	5.5	Solid	Absent	Absent
20	M/52	Head	3.0	Cystic & solid	Present	Absent
21	F/50	Tail	1.5	Solid	Absent	Absent
22	M/37	Tail	4.2	Cystic & solid	Present	Absent
23	F/45	Body	3.4	Solid	Absent	Absent
24	M/46	Tail	5.7	Cystic & solid	Present	Absent

H/N: Hemorrhage/necrosis; PP: Pseudopapillary; SPN: Solid-pseudopapillary neoplasm; PET: Pancreatic endocrine tumor.

Table 2 Immunohistochemical results of SPN and PET

Case No.	AACT	AAT	Vim	NSE	Syn	CgA	CK	EMA	Ins	Som	Glu	PP	E-cad	Beta	Ki-67 (%)
SPN group															
1	+	+	+	-	-	-	-	+	-	-	-	-	-	N/C	>1
2	+	+	+	+	+	-	+	+	-	-	-	-	-	N/C	>1
3	+	+	-	+	-	+	-	-	-	-	-	-	-	N/C	>1
4	+	+	-	+	+	-	-	-	-	-	-	-	-	N/C	>1
5	+	+	+	+	+	-	-	+	-	-	-	-	-	N/C	>1
6	-	+	+	+	+	-	+	-	-	-	-	-	-	N/C	>1
7	+	+	+	+	-	+	-	-	-	-	-	-	-	N/C	>1
8	+	-	+	-	+	-	-	-	-	-	-	-	-	N/C	>1
9	-	+	-	-	-	-	+	-	-	-	-	-	-	N/C	>1
10	+	-	-	-	+	+	+	-	-	-	-	-	-	N/C	2
PET group															
11	-	-	-	+	+	-	+	+	+	-	-	-	+	C/M	>1
12	-	-	-	+	+	+	-	+	+	+	-	-	+	C/M	>1
13	-	-	-	+	+	+	-	-	+	-	-	-	+	C/M	>1
14	-	-	+	+	+	+	+	+	-	-	-	-	+	C/M	>1
15	-	-	-	+	+	+	+	+	-	-	+	+	+	C/M	>1
16	-	-	-	+	+	+	+	-	-	-	-	-	+	C/M	>1
17	-	-	+	+	+	+	+	+	+	+	-	-	+	C/M	>1
18	-	-	-	+	+	+	+	+	+	-	-	-	+	C/M	>1
19	+	-	-	+	+	+	-	-	-	-	-	-	+	C/M	2
20	-	-	-	-	+	+	-	-	-	-	-	-	+	C/M	>1
21	+	-	-	+	+	+	+	+	+	-	-	-	+	C/M	>1
22	-	+	-	-	-	+	+	-	-	-	-	-	+	C/M	3
23	-	+	+	-	+	-	+	+	-	-	-	-	+	C/M	>1
24	+	-	+	-	-	-	-	+	-	-	-	-	+	C/M	4

N/C: Nuclear & cytoplasm; C/M: Cytoplasm & membrane; AACT:  $\alpha$ -1-Antichymotrypsin; AAT:  $\alpha$ -1-Antitrypsin; Vim: Vimentin; NSE: Neuron-specific enolase; Syn: Synaptophysin; CgA: Chromogranin; CK: Cytokeratin; Ins: Insulin; Som: Somatostatin; Glu: Glucagon; E-cad: E-cadherin.DHPLC mutation detection indicating no mutations of  $\beta$ -catenin gene exon 3 in PCR amplified fragments.**Sequencing**

Sequencing confirmed results of DHPLC mutation detec-



**Table 3** The details of  $\beta$ -catenin gene exon 3 mutation in SPN

Case No.	Codon	Base substitution	Amino acid conversion
1	33	TCT→TGT	Ser→Cys
2	37	TCT→TAT	Ser→Tyr
3	34	GGA→GTA	Gly→Val
4		Wild type	
5	32	GAC→TAC	Asp→Tyr
6	34	GGA→AGA	Gly→Arg
7	33	TCT→CCT	Ser→Pro
8	32	GAC→TAC	Asp→Tyr
9	32	GAC→GTC	Asp→Val
10	32	GAC→TAC	Asp→Tyr

tion that 9 cases of SPN had a point mutation of  $\beta$ -catenin gene exon 3. Mutations involving codons were as follows: 4 cases at codon 32, 2 at codon 33, 2 at codon 34 and 1 case at codon 37 (Table 3, Figure 2).

## DISCUSSION

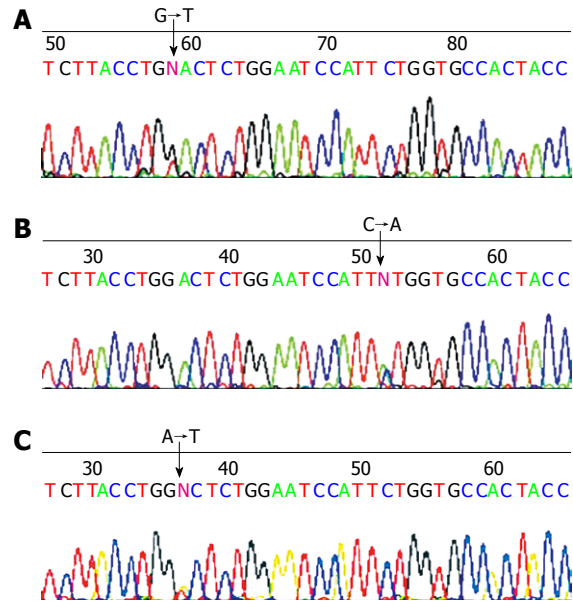
SPN of the pancreas is an uncommon tumor, constituting only about 1%-2% of exocrine pancreatic neoplasms<sup>[6]</sup>. SPN is clinically and histologically quite similar to the more common PETs, especially the non-functioning ones.

SPN predominantly occurs in adolescent girls and young women with a reported frequency of 87% to 90% (mean age of 22 to 25 years)<sup>[7]</sup>. Such a striking gender and age predilection of SPN is different from PET.

Functioning PET demonstrates specific clinical symptoms and signs which provides a suggestion for consideration of endocrine tumors. However, the clinical manifestations of SPN and non-functioning PET in our cases as mentioned in literature are not specific<sup>[8]</sup>. The patients in both groups present with unclear clinical features including abdominal discomfort, mild abdominal pain, poor appetite and nausea which are related to tumor compression on the adjacent organs, and a palpable mass in the abdomen<sup>[9,10]</sup>. Occasionally a mass may be found in the abdomen during complementary imaging studies such as ultrasound or CT scan<sup>[11,12]</sup>.

Both SPN and non-functioning PET have not been associated with specific clinical laboratory test findings including serum tumor markers<sup>[12,13]</sup>. The imaging features of both tumors present similarities in most cases, including ultrasonography, CT scanning, MRI and other imaging examinations. For both SPN and PET the best treatment is surgical resection. The routine imaging examinations are satisfactory for preoperative tumor location and understanding of the relationship between tumors and surrounding tissue. However, it is difficult for imaging studies to determine the nature of the lesions.

Both SPN and PET can occur in any part of the pancreas, though PET occurring in the pancreatic tail is more common<sup>[8]</sup>. SPN usually has much larger dimensions than PET. In contrast to the smaller mass (mean diameter 2.7 cm) of non-functioning PET, SPN is often



**Figure 2**  $\beta$ -catenin oncogene mutations in SPN. Representative DNA sequencing chromatograms demonstrate. A: A GAC→TAC mutation in codon 32 of case 5; B: A TCT→TAT mutation in codon 37 of case 2; C: A GAC→GTC mutation in codon 32 of case 9. Three samples of PET cases were randomly selected to be sequenced and the results confirmed that there was not any mutation on amplified PCR fragments of  $\beta$ -catenin gene exon 3.

much larger in size (mean of 10 cm at presentation in our study). Most of the cases in both groups are characterized with a tumor that is separated from the normal pancreas by a complete or incomplete fibrous capsule. On the cut surface, hemorrhagic and cystic areas are much more common in SPN than in PET.

Histologically, both SPN and PET could be arranged as solid areas and the size and shape of the tumor cells were relatively uniform with round or oval nuclei and vacuolated or eosinophilic large cytoplasm. It was difficult to distinguish these two kinds of tumor if only such histological pattern was used as the diagnostic basis. However, the unique morphological characteristics of SPN, large areas composing of pseudopapillary structures indicating evidence of cellular dyscohesive degeneration and cholesterol clefts, aggregation of foamy histiocytes, and characteristic microscopic features of SPN allow easy differential diagnosis from PET.

Immunohistochemical findings reported in early literature were of variation and absence of a specific profile. There was overlap of positive expression on immunostaining using such markers as  $\alpha$ 1-antitrypsin,  $\alpha$ 1-antichymotrypsin, NSE, Syn, progesterone receptor, carcinoembryonic antigen, pan CK, vimentin, CD10, CD56, and cyclin D1, so that immunohistochemistry was incapable of giving much helpful additional information for the differential diagnosis of SPN. However, more recently, the situation was changed by applications of E-cadherin and  $\beta$ -catenin which possess highly sensitivity and specificity. Literature reports<sup>[14-17]</sup> and our results showed that nuclear expression of  $\beta$ -catenin and loss of E-cadherin were seen in nearly all cases of SPN.

On the contrary, there was cytoplasmic and membrane expression of  $\beta$ -catenin and strong expression of E-cadherin in PET cases. The exclusive expression of  $\beta$ -catenin and E-cadherin in SPN can be applied to make definite differentiation from PET.

Most SPNs harbor mutations in the  $\beta$ -catenin gene and, as a result, most SPNs have an abnormal nuclear expression of  $\beta$ -catenin protein. We found 1 (case 4) in our cases without mutation in exon 3, however, it showed cystic and necrosis on macroscopy, pseudopapillary structure and discohesive appearance of the neoplastic cells on microscopy, cytoplasmic and nuclear staining for  $\beta$ -catenin, and loss of E-cadherin, which indicated alteration of  $\beta$ -catenin gene and suggested that there might exist a mutation on another exon of  $\beta$ -catenin gene.

In conclusion, SPN of the pancreas, compared with PET, is a cystic-solid or cystic tumor with a larger size mostly seen in young women, and has the morphological features of hemorrhage and necrosis on the cut surface and exclusive pseudopapillary structures on light microscopy caused by cellular dyscohesive degeneration. If one is aware of its clinical and histopathologic features, with sufficient sampling of the tumor, one usually does not confuse SPN with PET in most cases. If necessary, immunostaining of  $\beta$ -catenin and E-cadherin is quite helpful to make the differential diagnosis.

## COMMENTS

### Background

Solid-pseudopapillary neoplasm (SPN) is an uncommon pancreatic tumor with low malignant potential and unknown cell origin, constituting only about 1% of pancreatic neoplasms. Since it is rare and maybe morphologically similar to pancreatic endocrine tumor (PET), sometimes there is overlapped expression of conventional immunohistochemical markers in both tumors and a pathological misdiagnosis may take place.

### Research frontiers

In recent years, mutations of  $\beta$ -catenin, which are sufficient to induce pancreas tumorigenesis, have been found in SPN and the aberrant activation of the Wnt- $\beta$ -catenin pathway appears to be a constant feature in SPN. The mutations lead to the displacement of  $\beta$ -catenin and E-cadherin from their normal membrane location, and, as a result, SPNs have an abnormal pattern of labeling with antibodies to the  $\beta$ -catenin and E-cadherin protein which helps in differential diagnosis.

### Innovations and breakthroughs

In this study, mutations of the  $\beta$ -catenin gene were analyzed and immunostaining of  $\beta$ -catenin and E-cadherin protein were done in both SPN (10 cases) and PET (14 cases). Mutations of  $\beta$ -catenin and delocalization of  $\beta$ -catenin and E-cadherin were exclusively found in SPN.

### Applications

Characteristic morphological features and specific expressive patterns of  $\beta$ -catenin and E-cadherin make it to be easy to differentiate SPN from other pancreatic tumors.

### Terminology

$\beta$ -catenin (or Beta-catenin) gene is also known as CTNNB1 gene which encodes the protein  $\beta$ -catenin.  $\beta$ -catenin is a subunit of the cadherin complex and has been implicated as an integral component in the Wnt signaling pathway.

### Peer review

This is a nice retrospective analysis of the histological features of SPN of the pancreas compared with endocrine tumors and highlights some important differences.

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## Gastric duplication associated with pancreas divisum diagnosed by a multidisciplinary approach before surgery

Marta Di Pisa, Gabriele Curcio, Gianluca Marrone, Mariapina Milazzo, Marco Spada, Mario Traina

Marta Di Pisa, Gabriele Curcio, Mario Traina, Gastroenterology Service, IsMeTT - UPMC, via Tricomi 1, 90100 Palermo, Italy  
 Gianluca Marrone, Mariapina Milazzo, Department of Radiology, IsMeTT - UPMC, via Tricomi 1, 90100 Palermo, Italy  
 Marco Spada, Department of Surgery, IsMeTT - UPMC, via Tricomi 1, 90100 Palermo, Italy

Author contributions: All the authors contributed equally to this work; Di Pisa M wrote the paper.

Correspondence to: Dr. Marta Di Pisa, Gastroenterology Service, IsMeTT - UPMC, via Tricomi 1, 90100 Palermo, Italy. [mdipisa@ismett.edu](mailto:mdipisa@ismett.edu)

Telephone: +39-91-2192111 Fax: +39-91-2192200

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### INTRODUCTION

Gastric duplications are uncommon congenital anomalies<sup>[1]</sup>, which are rarely found in adults, are more common in females, and are typically diagnosed in children. They constitute 2%-7% of gastrointestinal duplications<sup>[2]</sup>. The clinical presentation is usually nonspecific, though gastric duplications can be associated with severe clinical presentations<sup>[2]</sup>. Traditionally, the majority of these duplications are cystic, non-communicating and located along the greater curvature or posterior wall; there are very few cases in the literature of gastric duplication completely communicating with the gastric lumen<sup>[3]</sup>.

We present a rare case of a communicating type gastric duplication associated with pancreas divisum in a young woman, diagnosed with a multidisciplinary approach, including computed tomography (CT), magnetic resonance imaging (MRI), endoscopy, histology and ultrasound endoscopy.

### CASE REPORT

A 48-year-old healthy female presented with epigastric pain, nausea and bloating. She was admitted to our hospital for further investigation.

Her medical history was unremarkable. Physical examination and laboratory data were normal. First, an upper gastrointestinal barium contrast study was done, which showed a double compartment stomach, with the accessory stomach starting at the esophagogastric junction and continuing along the entire greater curvature to the pylorus, where it joined the lumen of the primary stomach. Both gastric lumens were filled with barium, providing evidence of free communication of the duplication with the gastric cavity (Figure 1). The patency of the gastric duplication and primary stomach was confirmed by CT

### Abstract

We report a unique case of communicating gastric duplication associated with pancreas divisum, diagnosed with a multidisciplinary approach, including X-rays, computed tomography, magnetic resonance imaging, esophagogastroduodenoscopy, ultrasound endoscopy and histology. We believe that this approach constitutes a fuller diagnostic evaluation, resulting in better and safer surgery.

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**Key words:** Gastric duplication; Pancreas divisum; Computed tomography; Magnetic resonance imaging; Ultrasound endoscopy

**Peer reviewer:** Edward L Bradley III, MD, Professor of Surgery, Department of Clinical Science, Florida State University College of Medicine, 1600 Baywood Way, Sarasota, FL 34231, United States

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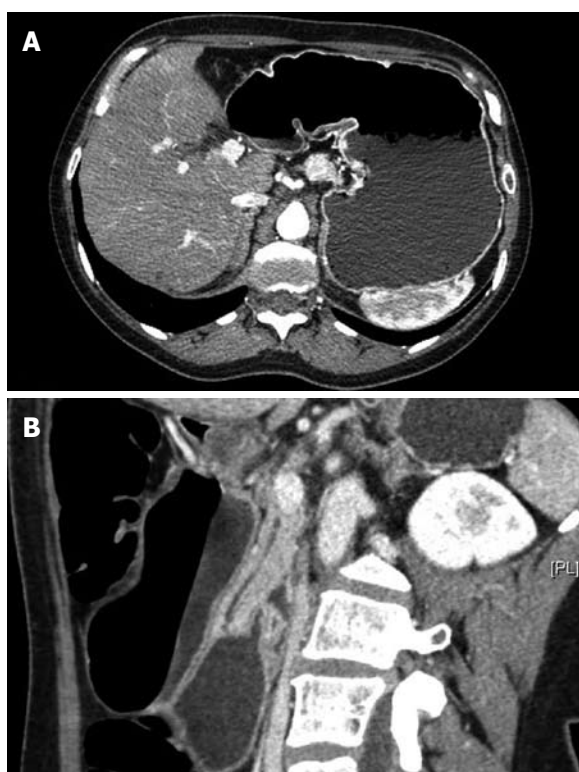




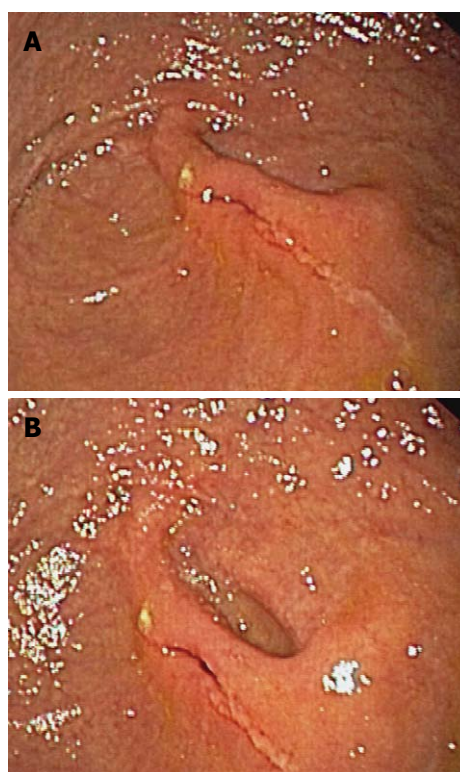
**Figure 1** Two free communicating cavities can be seen just beyond the esophagogastric junction.



**Figure 3** Magnetic resonance imaging showing the presence of an incomplete pancreas divisum.



**Figure 2** The communication of the 2 cavities visualized by computed tomography. A: Frontal view; B: Lateral view.



**Figure 4** The endoscopic view of the duplication. A: The first cavity with a similar gastric appearance continuing into the second one; B: The second proper gastric cavity continuing into the duodenum.

with gastrografin (Figure 2). Also, in order to rule out possible biliary and pancreatic complications, with which gastric duplications can be associated, and in view of potential surgery, MRI was performed. It showed an incomplete pancreas divisum, with no common bilio-pancreatic duct (Figure 3). To confirm the gastric nature of the wall of the double stomach, a gastroscopy with ultrasound examination was performed.

Under deep sedation, the patient was monitored continuously with electrocardiography, pulse oximetry and automatic recording of blood pressure and pulse. After introducing the scope into the esophagus, just behind the esophagogastric junction, a large cavity covered with normal mucosa was seen continuing into another cavity through a similar pylorus orifice. Beyond it, a new cavity,

continuing into the duodenum, was observed (Figure 4). During the same endoscopic examination, a 20 MHz miniprobe was inserted into the operative channel of the endoscope and it revealed, on the first cavity, the pathognomonic multilayered appearance of the wall suggestive of a digestive origin, including an echogenic inner mucosal layer and a hypoechoic muscular layer (Figure 5). Finally, multiple biopsies were taken to confirm the gastric origin of the first cavity, and the histology pattern showed the presence of gastric mucosa with moderate *Helicobacter pylori* (*H. pylori*)-related gastritis in both cavities.

Definitively attributing the patient's symptoms to this structure was difficult, but after 6 mo of omeprazole





**Figure 5** The typical gastric layer is visualized on endoscopic ultrasound.

therapy (associated with *H. pylori* eradication therapy), the patient was asymptomatic and was referred to a surgeon.

## DISCUSSION

Gastric duplications are uncommon congenital anomalies, rarely found in adults, and can involve any part of the gastrointestinal tract, from the mouth to the rectum<sup>[1]</sup>. They constitute about 4% of all alimentary tract duplications<sup>[1]</sup>. Gastric duplications share a common blood supply with the native bowel, though the stomach is the site least often affected, regardless of age. The etiology of duplication of the alimentary tract is likely multifactorial<sup>[2]</sup> (e.g. persistence of embryonic diverticula during development of the alimentary tract and recanalization and fusion of embryologic longitudinal folds). They can be cystic or tubular, are more common in females, and are, typically, diagnosed in children<sup>[2]</sup>. Gastric duplications can communicate with the gastric lumen or not. Communicating duplications usually do not require any intervention when both gastric lumens are patent, in contrast with non-communicating duplications, which require surgical treatment.

The clinical presentation is usually nonspecific; signs and symptoms of gastric duplications depend on the localization, size and whether or not there is communication with the gastric lumen. The most common symptoms are epigastric pain or discomfort, vomiting, and gastrointestinal bleeding<sup>[4]</sup>. In some cases, they can give rise to unusual complications, such as recurrent pancreatitis<sup>[5]</sup> or bile duct fistula<sup>[6]</sup>, infection, peptic ulcers developing in

the cyst, obstruction, and carcinoma arising in the cyst<sup>[7]</sup>. They can be associated with other congenital abnormalities: esophageal and duodenal diverticula, duplication cysts elsewhere in the digestive tract, annular and ectopic pancreas<sup>[8]</sup>, and spinal abnormalities<sup>[9]</sup>.

This is, to the best of our knowledge, the first report on a gastric duplication associated with a pancreas divisum. Alimentary tract duplications must be considered during evaluation of thoracic and abdominal masses, gastrointestinal hemorrhage of unclear etiology, intussusceptions and mechanical bowel obstructions.

This report presents a rare case of communicating gastric duplication associated with a pancreatic abnormality (pancreas divisum), diagnosed with a multidisciplinary approach, including not only imaging techniques (CT and MRI), but also ultrasound endoscopy and the histological pattern of the biopsy to confirm the gastric nature of both cavities, in order to refer the patient to surgery after a full evaluation.

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## Pancreatic pseudocyst filled with semisolid lipids mimicking solid mass on endoscopic ultrasound

Sang-Woo Cha, Sae Hee Kim, Hyang Ie Lee, Yun Jung Lee, Hyeon Woong Yang, Sung Hee Jung, Anna Kim, Min Koo Lee, Hyun Young Han, Dong Wook Kang

Sang-Woo Cha, Sae Hee Kim, Hyang Ie Lee, Yun Jung Lee, Hyeon Woong Yang, Sung Hee Jung, Anna Kim, Division of Gastroenterology, Department of Internal Medicine, Eulji University College of Medicine, Eulji University Hospital, Deajeon 302-779, South Korea

Min Koo Lee, Division of Gastroenterology, Department of Surgery, Eulji University College of Medicine, Eulji University Hospital, Deajeon 302-779, South Korea

Hyun Young Han, Division of Gastroenterology, Department of Radiology, Eulji University College of Medicine, Eulji University Hospital, Deajeon 302-779, South Korea

Dong Wook Kang, Division of Gastroenterology, Department of Pathology, Eulji University College of Medicine, Eulji University Hospital, Deajeon 302-779, South Korea

**Author contributions:** Cha SW interpreted the data and wrote the manuscript; all authors contributed to analyzing the patient's data and revising the article; Cha SW approved the final manuscript.

**Correspondence to:** Sang-Woo Cha, MD, Division of Gastroenterology, Department of Internal Medicine, Eulji University College of Medicine, Eulji University Hospital, Dunsan-dong, Seo-gu, Deajeon 302-779, South Korea. [swcha@eulji.ac.kr](mailto:swcha@eulji.ac.kr)  
 Telephone: +82-42-6113052 Fax: +82-42-6113853

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**Peer reviewers:** Dr. Ram Prakash Galwa, MBBS, MD, Department of Diagnostic Imaging, The Ottawa hospital, 751 Parkdale Avenue, Apartment 803, Ottawa, k1y1j7, Canada; Tarkan Karakan, Associate Professor, Gazi University, Department of Gastroenterology, Ankara, 06500, Turkey

Cha SW, Kim SH, Lee HI, Lee YJ, Yang HW, Jung SH, Kim A, Lee MK, Han HY, Kang DW. Pancreatic pseudocyst filled with semisolid lipids mimicking solid mass on endoscopic ultrasound. *World J Gastroenterol* 2010; 16(8): 1034-1038 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i8/1034.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i8.1034>

### INTRODUCTION

Routine use of cross-sectional imaging has resulted in an increased reporting of pancreatic cystic lesions. Some of these pancreatic cystic lesions may harbor microscopic malignancy or may degenerate into malignancy, therefore, it is important to differentiate between benign (serous), malignant/premalignant, and inflammatory (pseudocysts) cystic lesions<sup>[1]</sup>. Differentiation between pseudocysts and cystic neoplasms can be attempted by many modalities. At times, the differential diagnosis can be difficult. Although a prior history of pancreatitis cannot by itself justify the diagnosis of pancreatic pseudocysts, cystic lesions that occur without a history of pancreatitis suggest a diagnosis of cystic neoplasm<sup>[2]</sup>. Thus, the decision of whether to observe or operate on the pancreatic cystic lesion is difficult.

### CASE REPORT

A 51-year-old man was referred to our hospital for further evaluation of a probable gallbladder mass lesion and

### Abstract

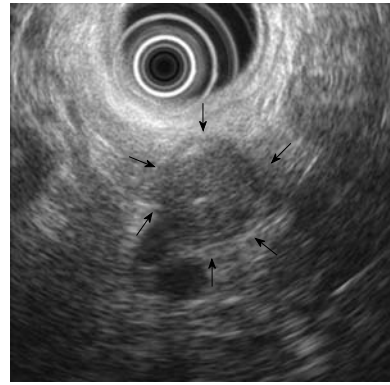
Pancreatic pseudocysts, which account for 70%-90% of pancreatic cystic lesions, characteristically are non-epithelially lined cystic cavities that are contiguous with the pancreas. Pancreatic pseudocysts can be caused by acute, chronic or traumatic pancreatitis and should be differentiated from other pancreatic diseases with cystic appearances, especially cystic neoplasms. We report a unique case of a pancreatic pseudocyst filled with semisolid lipids, which appeared by endoscopic ultrasound as a solid mass, and was therefore resected.

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**Figure 1** Image of the cystic lesion by contrast-enhanced abdominal computed tomography (arrow). There was a 17-mm well-marginated, round, low-density lesion in the body of the pancreas. The central contents of the lesion showed no enhancement in the post-contrast-enhanced image.

pancreatic cystic tumor. The patient initially visited another hospital because of fever and right upper quadrant abdominal pain. He had been treated for acute cholecystitis and showed clinical improvement. Abdominal computed tomography (CT) showed a probable gallbladder tumor and a pancreatic cystic tumor, therefore, he was referred to our hospital. At our initial evaluation, the patient was symptom free and the physical examination was normal. The patient had a history of alcohol consumption equivalent to 300 g/wk of ethanol. There was no history of pancreatitis. In laboratory analysis, white blood cell count was 5180/ $\mu$ L, hemoglobin 13.2 g/dL, and platelet count 218000/ $\mu$ L. Aspartate aminotransferase was 31 IU/L (normal 1-39 IU/L), alanine aminotransferase was 14 IU/L (normal 1-39 IU/L), total bilirubin 0.4 mg/dL (normal 0.1-1.2 mg/dL), amylase 23 IU/L (normal 20-104 IU/L), lipase 14 IU/L (normal 6-52 IU/L), carbohydrate antigen 19-9 11.3 U/mL (normal 0-37 U/mL), carcinoembryonic antigen 1.5 ng/mL (normal 0-5.0 ng/mL), total cholesterol 194 mg/dL (normal 1-240 mg/dL), and triglycerides 129 mg/dL (normal 1-250 mg/dL). A repeat abdominal CT revealed small stones in the gallbladder and mild dilatation of peripheral intrahepatic ducts without central intrahepatic and extrahepatic duct dilatation. Also, a 1.7-cm diameter, well-marginated, round, low-density lesion on the pancreatic body, compatible with a cystic lesion (Hounsfield units; pre-enhanced,  $\leq 20$  HU), was seen without post-contrast enhancement of the central portion. Rim enhancement equal to the adjacent pancreas parenchyma was seen. Pancreas parenchyma was seen with slightly atrophic changes (Figure 1). Endoscopic ultrasound (EUS) revealed a hyperechoic polypoid lesion of 14.1 mm on the fundal area of the gallbladder, with a posterior acoustic shadow. EUS also showed a 17 mm  $\times$  19 mm solid, round hypoechoic lesion on the pancreatic body. There were no other findings compatible with pancreatitis (Figure 2). EUS-guided fine needle aspiration (FNA) was not available at that time in our hospital. Endoscopic retrograde cholangiopancreatography (ERCP) was performed, which found irregular dilatations of the



**Figure 2** Image of the cystic lesion on EUS. It showed a 17-mm  $\times$  19-mm, well-circumscribed, round, homogeneous, hypoechoic solid lesion with central echogenicity (arrows).

peripheral intrahepatic ducts, without involvement of the extrahepatic and common bile ducts. Bile duct sweepings by balloon retrieval catheter showed bile sludge and *Clonorchis sinensis* (*C. sinensis*) adult organisms. Endoscopic pancreatography revealed mild displacement of the main pancreatic duct of the body due to a mass effect, but no cyst filling. After performing CT, EUS and ERCP, even though there was some disagreement about the pancreatic lesion, we thought that it was a solid neoplasm. Magnetic resonance imaging (MRI) was not performed to evaluate the pancreatic lesion further. At that time, we did not think about pseudocyst filled with semisolid lipid. The patient underwent laparoscopic cholecystectomy and body and tail pancreatectomy because of our concern that it was a solid pancreatic neoplasm. On pathological examination, the gallbladder showed xanthogranulomatous cholecystitis with *C. sinensis* infection. The pancreatic gross specimen revealed a well-encapsulated, unilocular cystic lesion filled with yellowish muddy material, which measured 17 mm in diameter (Figure 3). Microscopic findings of the resected cystic lesion were compatible with those for a pseudocyst. On hematoxylin-eosin (HE) staining, the lining of the cystic lesion consisted of fibrous and granulation tissue with no epithelial lining. Multiple extracellular fat vacuoles were shown in the cystic lesion. Findings were different from the lipocytes of a pancreatic lipoma. The surrounding pancreatic parenchyma showed mild inflammatory changes but there was no evidence of diffuse chronic pancreatitis. Sudan black B stain showed that the pseudocyst contents were lipid droplets (Figure 4A and B). No neoplasm was seen.

## DISCUSSION

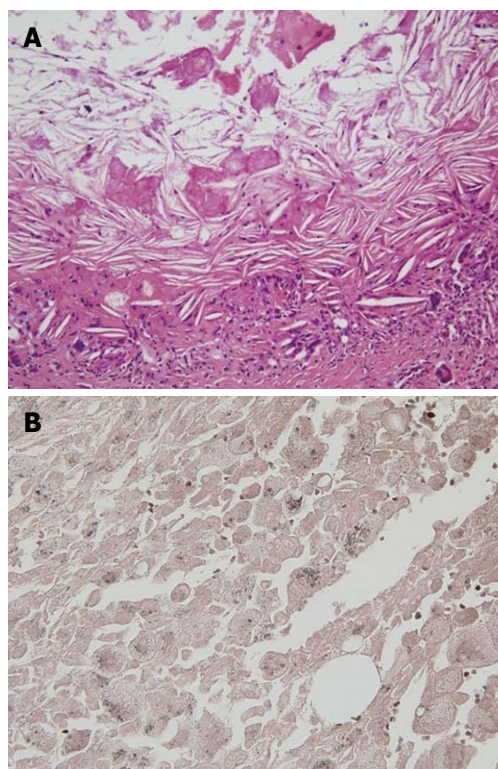
Cystic lesions of the pancreas are being detected more often as sensitive abdominal imaging tests are being used for multiple indications. Some of these lesions are either premalignant or malignant. Therefore, it is important to differentiate between benign (serous), malignant/premalignant, and inflammatory (pseudocysts) cystic lesions<sup>[1]</sup>. The decision with respect to observation or operation is further complicated by the fact that a significant percentage of the patients with cystic lesions have pseudocysts rather than cystic neoplasms. Pancreatic pseudocysts are non-neoplastic and have been reported to account for





**Figure 3 Gross finding of the pancreatic cystic lesion.** There was a 17-mm, unilocular, well-encapsulated cystic lesion filled with yellowish muddy material in the body of the pancreas.

70%-90% of pancreatic cystic diseases. The neoplastic pancreatic cystic diseases represent 10%-15% of all pancreatic cystic diseases<sup>[3]</sup>. However, more recent studies have reported that 40%-50% of patients with cysts have a history of pancreatitis. This suggests that pseudocysts only account for 40%-50% of pancreatic cystic lesions, and not 70%-90%<sup>[4,5]</sup>. They have explained that these dramatic differences were probably due to increased detection of small asymptomatic cysts in some studies, as well as differences in referral patterns<sup>[4]</sup>. Pseudocysts are cystic cavities that typically have or had connections with the pancreas, have no epithelial lining, and develop after acute or chronic pancreatitis, pancreatic trauma and surgery<sup>[6]</sup>. The diagnosis of a pancreatic pseudocyst depends on the clinical history and associated findings within the pancreas, such as gland atrophy, duct dilatation, calcification of the parenchyma, and calculi in the pancreatic duct. Although a prior history of pancreatitis cannot by itself justify the diagnosis of pancreatic pseudocysts, cystic lesions that occur without a history of pancreatitis suggest a diagnosis of cystic neoplasms<sup>[2]</sup>. Differentiation between pseudocysts and cystic neoplasms can be aided by CT, MRI, and EUS. The CT findings of a pseudocyst include a round or oval fluid collection with variably a thin or thick wall that shows evidence of contrast enhancement. Contrast-enhanced CT typically shows rim enhancement of pseudocysts<sup>[2]</sup>, which was present in our case. Most pseudocysts are unilocular and septations are infrequent<sup>[1]</sup>. The usefulness and accuracy of EUS in the differential diagnosis of pancreatic cystic diseases continues to evolve<sup>[7-14]</sup>. Koito *et al*<sup>[7]</sup> have reported the classification of the internal structures of solitary cystic lesions of the pancreas, and have compared them with pathological findings. They have concluded that the EUS findings were relatively accurate. However, Ahmad *et al*<sup>[8,9]</sup> have reported that endoscopic features alone (without FNA) cannot reliably differentiate between benign and malignant or neoplastic and non-neoplastic cystic lesions of the pancreas. Song *et al*<sup>[10]</sup> have reported that pseudocysts exhibit echogenic debris and parenchymal changes more often than do cystic tumors. In contrast, septae



**Figure 4 Microscopic finding of the pancreatic pseudocyst.** A: Microscopic finding of the pancreatic pseudocyst. HE staining showed multiple lipid droplets and cholesterol clefts; B: Sudan black B stain showed positive findings for lipid droplets, stained with a dark brown color.

and mural nodules are found more frequently in cystic tumors than in pseudocysts. In our case, EUS showed a well-circumscribed, hypoechoic, round lesion with central echogenicity. These findings are more compatible with a pancreatic solid mass than a cystic lesion. The lesion was not hyperechoic as in lipoma. We did not perform FNA. If we had done so, it would probably have shown lipids and debris that were suggestive of a pseudocyst. With endoscopic pancreatography, 70% of pseudocysts have communication with the pancreatic ductal system, while serous cystadenoma, mucinous cystadenoma, and mucinous cystadenocarcinoma usually do not. Our patient presented with no evidence of pancreatitis on medical history, laboratory findings or imaging evaluations. He had documented biliary calculus disease and a history of alcohol consumption equivalent to 300 g/wk of ethanol. As a result of suspicion of a solid pancreatic neoplasm, the pancreatic body and tail and spleen were resected. The pathology revealed a well-encapsulated, unilocular, pancreatic cystic mass that measured 17 mm in diameter, which was filled with yellowish muddy material. Sudan black B stain revealed that the mass was a pseudocyst with lipid contents (lipid droplets and cholesterol clefts). This is different from a pancreatic lipoma, which would have been composed of lobules of mature adipose cells that were sharply delineated from the surrounding pancreatic tissue by a thin fibrous capsule<sup>[13]</sup>. Lipomas are benign tumors of homogeneous adipose tissue that are



histopathologically identical to subcutaneous fat. CT findings of pancreatic lipoma include homogeneous distribution of dense adipose tissue, with no central or peripheral contrast enhancement<sup>[14]</sup>.

In our case, gallbladder stones and infection with *C. sinensis* could have been the causes of pancreatitis. However, our patient did not have clinical manifestations and a history of pancreatitis. The pathophysiological process that leads to development of this lesion is uncertain. As we known, pancreatic pseudocysts are formed by the walling off of areas of peripancreatic hemorrhagic fat necrosis with fibrous tissue. As such, they usually are composed of central necrotic-hemorrhagic material rich in pancreatic enzymes, surrounded by non-epithelial-lined fibrous walls of granulation tissue. Fat necrosis is the first step in many pseudocysts. Therefore, small amounts of necrotic fat are common in pseudocysts. To the best of our knowledge, this is the first reported case of pancreatic pseudocyst densely filled with lipids. Our patient was not obese (body mass index 19.1), and the CT scan showed small/normal amounts of peripancreatic and retroperitoneal fat. Necrosis of peripancreatic fat can progress to liquefaction, with subsequent organization and encapsulation. Our patient's lesion appeared to be more than 50% intrapancreatic. Another origin of fat material in the pancreas is focal fatty replacement of the pancreas. Pancreatic lipomatosis or fatty replacement of the pancreas is the most frequent pathological finding in the adult pancreas. It is associated with a variety of diseases, including obesity, diabetes mellitus, chronic pancreatitis, hereditary pancreatitis, obstruction of the pancreatic duct by calculus or tumor, and cystic fibrosis. It is typically distributed as multiple tiny nodules throughout the pancreas; however, some investigators have found uneven fatty replacement of the pancreas, which is seen as a focal fatty mass<sup>[15,16]</sup>. This appearance may simulate a pancreatic mass such as a cystic neoplasm or other pancreatic tumor. Our patient did not have a fatty pancreas by CT, nor in the resected specimen. It is possible that our patient had focal fatty pancreas, unrecognized pancreatitis in the past, and residual organizing fatty necrosis. Unenhanced CT has a diagnostic role in comparing adjacent retroperitoneal fat with a negative attenuation value and parenchymal fat. Moreover contrast-enhanced CT may not show a negative attenuation value of this focal fatty replacement because of normal adjacent pancreatic parenchymal contrast enhancement<sup>[17,18]</sup>. MRI may be helpful for confirming the presence of this type of focal fatty replacement of the pancreas. Chemical shift MRI has the advantage that the reduction in signal intensity of focal fatty replacement on opposed-phase images differentiates focal fatty replacement of the pancreas from true pancreatic tumors, which in general do not contain lipid<sup>[17-19]</sup>. Our patient's cystic lesion would likely have shown high fat content by MRI, if it had been performed. Another possibility of the origin of fat material in the pseudocyst is that this lesion is a thrombosed splenic artery aneurysm. The position in the pan-

creas is compatible with this possibility. The cholesterol-appearing spicules in the wall also are consistent with a vascular problem. When fat is stored in organs (e.g. liver or pancreas), the vast majority is in triglyceride, and not cholesterol. However, our patients did not have a splenic artery aneurysm or any vascular disorder by CT, nor in the resected specimen.

CT showed that our patient's lesion was cystic. EUS is thought to be more accurate, therefore, the presumed solid lesion was resected. We did not attempt to make a preoperative tissue diagnosis because we assumed that all solid masses needed resection. Even though the risk appears to be quite low, percutaneous FNA biopsy of the pancreas may disseminate tumor cells intraperitoneally or along the needle path in patients who are believed to be candidates for potentially curative resection. If we had performed MRI with fat content analysis, or EUS-guided FNA, we probably would have been able to identify the lipid content of the lesion. Resection more definitely evaluated for malignancy.

In summary, we reported a unique case of a pancreatic pseudocyst filled with semisolid lipids, which appeared by EUS as a solid mass, and was therefore resected. Additional preoperative testing may have identified the true nature of the lesion. A high lipid content of pseudocysts is rare.

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## Hepatic mucormycosis mimicking hilar cholangiocarcinoma: A case report and literature review

Ke-Wei Li, Tian-Fu Wen, Gan-Di Li

Ke-Wei Li, Tian-Fu Wen, Division of Liver Transplantation, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China

Gan-Di Li, Department of Pathology, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

**Author contributions:** Li KW and Wen TF contributed equally to this work; Li KW, Wen TF and Li GD performed research; Li KW and Wen TF wrote the paper.

**Correspondence to:** Tian-Fu Wen, PhD, Division of Liver Transplantation, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China. [vivian522513@163.com](mailto:vivian522513@163.com)

Telephone: +86-28-85513722 Fax: +86-28-85422411

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### Abstract

Mucormycosis is a rare but invasive opportunistic fungal infection associated with a high mortality rate, and normally occurs in immunocompromised patients. In this report, we describe an immunocompetent patient suffering from hepatic mucormycosis secondary to adrenal mucormycosis, which masquerades as hilar cholangiocarcinoma. After surgical procedure and treatment with amphotericin B and itraconazole, the patient recovered well and had a 2-year infection-free survival. To our knowledge, this special clinical manifestation of hepatic infection as well as adrenal mucormycosis has not been reported to date. Meanwhile, this is the first case of an immunocompetent patient with both adrenal and hepatic mucormycosis who has been treated successfully.

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**Key words:** Adrenal gland; Hilar cholangiocarcinoma; Liver; Mucormycosis

**Peer reviewers:** Salvatore Gruttadauria, MD, Assistant Professor, Abdominal Transplant Surgery, ISMETT, Via E.

Tricomi, 190127 Palermo, Italy; Giuseppe Orlando, MD, PhD, Department of Health Sciences, Wake Forest Institute for Regenerative Medicine, 391 Technology Way, Winston Salem, NC 27101, United States

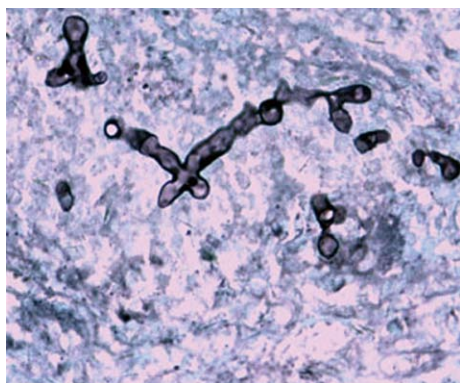
Li KW, Wen TF, Li GD. Hepatic mucormycosis mimicking hilar cholangiocarcinoma: A case report and literature review. *World J Gastroenterol* 2010; 16(8): 1039-1042 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i8/1039.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i8.1039>

### INTRODUCTION

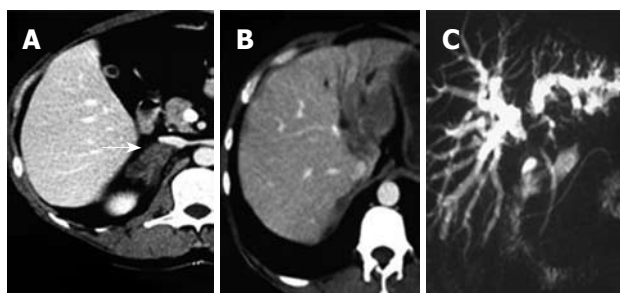
Mucormycosis caused by order mucorales<sup>[1,2]</sup>, a ubiquitous saprophytic mold found in soil and organic matter worldwide, is a rare but invasive opportunistic fungal infection. The disease in humans is mainly limited to people with risk factors such as neutropenia<sup>[3-6]</sup>, immune deficiencies<sup>[2-4,7,8]</sup>, malignant disease<sup>[2-8]</sup>, malnutrition<sup>[3,4]</sup>, diabetes<sup>[2-8]</sup>, trauma<sup>[2,6]</sup>, organ transplantation<sup>[2-5,7]</sup>, and iron overload<sup>[3-6]</sup>. The clinical infection due to mucorales includes rhinocerebral, pulmonary, cutaneous, gastrointestinal and disseminated diseases<sup>[3,5]</sup>. The first two are the most common diseases and all entities are associated with a high mortality rate<sup>[3]</sup>. To our knowledge, mucormycosis rarely involves liver, and hardly harms bile duct resulting in stenosis and cholestasis. This case report may present the first patient with adrenal mucormycosis in literature.

### CASE REPORT

A 36-year-old man was referred for evaluation of abdominal pain and icterus in April 2007. He was admitted to our center with a 3-mo history of abdominal pain, and 4-d icterus. He had nonsymptomatic adrenal mucormycosis characterized by mass location in the right adrenal gland area 5 mo ago. At surgical exploration in previous hospitalization, the mass was identified to be tenacious, confusing with right adrenal gland and there



**Figure 1** Adrenal mucormycosis, grocott methenamine sliver stain, × 100.



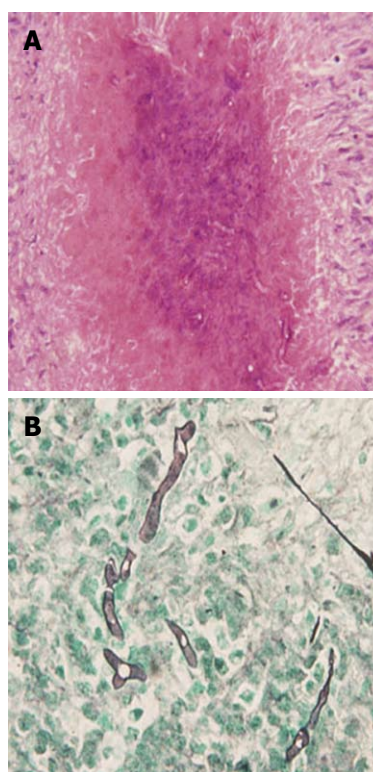
**Figure 2** Imaging changes. A: Arrow shows computed tomography (CT) features of adrenal mucormycosis; B: CT features of hepatic lesion. A well circumscribed hypodense lesion in hilar and left lateral lobe, surrounding the vessels without a mass effect, should suggest an angioinvasive organism. This lesion presents necrosis of liver tissue due to fungal thrombosis; C: Magnetic resonance cholangiopancreatography demonstrates an abrupt stenosis of the primary biliary confluence with symmetric upstream dilation of the intrahepatic bile ducts.

was adhesion between mass and adjacent tissues. The patient underwent grossly total resection of the mass combined with the right adrenal gland immediately and mucormycosis infection was demonstrated histopathologically (Figure 1 and Figure 2A). Thus he was treated with itraconazole, 100 mg *bid*, for 1 mo. After his discharge, he was not followed up.

On physical examination at this second admission, his vital signs were normal and his abdomen was healthy. On investigation, his white blood cell count was 15400/mm<sup>3</sup> with 86.2% neutrophils. Liver function test showed a bilirubin of 78.9 IU/L; direct bilirubin, 54.2 IU/L; alanine transaminase, 463 IU/L (normal range, < 55 IU/L); alkaline phosphatase, 180 IU/L (normal range, < 45 IU/L); albumin, 43 g/L (normal range, 35-55 g/L); and CA19-9, 108.6 U/mL (normal range, < 22 IU/L). Viral hepatitis screening (including tests for hepatitis A, B and C) revealed infection of hepatitis B. Chest X-ray was unremarkable. Abdominal computed tomography (CT) and magnetic resonance imaging (MRI) showed a lesion occupying the porta hepatis and partial left lateral lobe (Figure 2B); magnetic resonance cholangiopancreatography (MRCP) demonstrated an abrupt stenosis of the primary biliary confluence with symmetric upstream dilation of the intrahepatic bile ducts (Figure 2C). In light of initial evaluation, mucormycosis was suspected,



**Figure 3** Cut surface of the liver mass, a yellowish-white tissue. Vessels are present in mass. Arrow shows intrahepatic bile ducts dilatation, thickening and cholestasis.



**Figure 4** Pathological changes of liver tissue. Inflammatory focus containing irregularly shaped, broad, nonseptate hyphae with right angle branching typical of mucormycosis. A: Hematoxylin and eosin stain, × 40; B: Grocott methenamine sliver stain, × 100.

but hilar cholangiocarcinoma (HCCA) could not be excluded.

Therefore, an exploratory laparotomy was performed, revealed that the mass was yellowish-white and tenacious, with irregular but well-defined boundary, and no obvious necrotic center was detected. The sidewall of common hepatic duct became incrassate, forming a stenosis (nearly 3 mm of inside diameter) with dilatation and thickening of the intrahepatic bile ducts (Figure 3). It was unlike a malignant tumor grossly, hence partial resection of hepatic lesion for frozen section was achieved. Given the mucorales infection on the basis of intraoperative frozen section,



we made a liver debridement to the fullest extent possible and placed a T-tube stent for 3 mo. Histopathologically, the liver was necrotic, in which extensive fungal proliferations were examined. Hematoxylin and eosin (HE), and grocott methenamine silver (GMS) stains showed irregularly shaped broad nonseptate hyphae with the right angle branching typical of mucormycosis (Figure 4A and B). Intraoperative bile culture was performed but failed. Treatment with liposomal amphotericin B was initiated. Up to July 2007, he had received a cumulative 3 g of amphotericin B during the 3-mo hospitalization period. The patient was discharged in good general condition with an intraconazole prescription (100 mg *bid* for 4 mo). At this time, 2 years later, the patient remains alive and well. Abdominal MRI shows no evidence of the previous lesions.

## DISCUSSION

Mucormycosis is a rare fungal infection. It represents 12% of all documented filamentous mycosis observed in the participating centers during the same period (Pagano *et al.*<sup>[4]</sup>, 1993). Portals of entry for mucorales include sinuses, lungs, gastrointestinal tract and skin. In the present cohort, none of disseminated cases involves the sinuses. In contrast, they involve the lungs and/or the gastrointestinal tract<sup>[9]</sup>. Hepatic involvement is commonly presented with pulmonary or gastrointestinal infection and is considered a part of the disseminated disease<sup>[3,7]</sup>. Disseminated mucormycosis is the most severe form and is said to be uniformly fatal<sup>[8]</sup>. It is reported that the diagnosis is made ante mortem in only 12 of 185 patients<sup>[10]</sup>. The time lost before a correct diagnosis is made may be critical for survival.

Our case presented a typical hypodense mass lesion in CT findings. The hypodense hepatic lesion surrounding vessels without a mass effect (Figure 2B) suggest an angioinvasive organism causing fungal thrombosis and involvement of perivascular area subsequently. The CT findings are not pathognomonic but are valuable in narrowing the differential diagnosis<sup>[8]</sup>. Also MRI could reveal correspondences of abnormalities. MRCP demonstrated an abrupt stenosis of the primary biliary confluence with symmetric upstream dilation of the intrahepatic bile ducts, mimicking HCCA.

Differentiating mucormycosis from HCCA is important because of the differences in treatment. On imaging, obstruction at the hepatic duct confluence is generally due to HCCA. However, in up to 15% of patients, hilar obstruction could be caused by alternative diagnoses other than HCCA<sup>[11,12]</sup>. Involvement of second-order bile ducts is rare in patients with benign lesions, and was observed in only 26% of patients with HCCA-potentially helpful diagnostically in only a small proportion of cases. By contrast, vascular invasion and lobar atrophy are both common findings in patients with HCCA and significantly more common than in patients with benign lesions<sup>[11]</sup>. However, imaging ability to accurately distinguish HCCA from alternative diagnoses is limited. In addition, of the patients with benign lesions,

33% had elevated tumor markers (carcinoembryonic antigen and CA 19-9) (Jonathan Koea *et al.*<sup>[12]</sup>).

With regard to this case, culture and pathologic examinations of biopsy specimen provided the only definite diagnoses of mucormycosis. Mucorales appears in tissue as irregularly shaped, broad nonseptate hyphae with the right angle branching. The most characteristic features are perivascular and blood vessel invasions that result in arterial thrombosis and subsequent necrosis<sup>[3,9]</sup>. In our case, the sidewall of common hepatic duct became incrassate, forming a stenosis with upstream dilatation and thickening of the intrahepatic bile ducts, mimicking HCCA as the operation specimen and imaging represent. It suggested that the mucormycosis could likewise involve the bile ducts, leading to inflammation and incrassation of bile ducts. It has come to our knowledge that this characteristic has never been mentioned in the published series before.

Besides liver, there was also an adrenal gland involvement (primary infection), with no participation of the lung or other sites. As far as we know, involvement of the adrenal gland has not been described in the literature before, yet involvement of the brain, lung, kidney, stomach, colon, liver, spleen, thyroid gland, pancreas, and lymph nodes is known from autopsy<sup>[8]</sup>. However, the most initial portal of the infection remains unknown and deserves our attention.

The standard therapy for invasive mucormycosis is a combined medical-surgical approach<sup>[2,4,5,13]</sup>. High doses of amphotericin B should be used, rapidly reaching 1.0 mg/kg daily. Prolonged courses (> 6 wk) of amphotericin B are recommended, with total doses ranging between 1.5 and 3.0 g, according to the patient's underlying conditions<sup>[13]</sup>. Our patient underwent subtotal liver resection and received a cumulative 3 g of liposomal amphotericin B, and well responded to the treatment. Additionally, T-tube stent and intraconazole played an important part in the treatment.

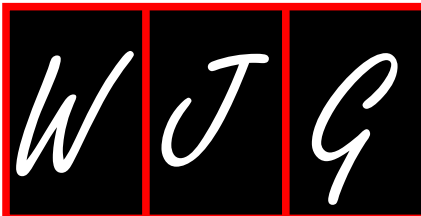
In conclusion, this case report illustrates a new form of clinical spectrum of mucormycosis. The successful treatment can be attributed to an early accurate diagnosis, anti-fungal therapy consisting of liposomal amphotericin B and intraconazole, as well as surgical debridement. As mentioned above, a therapeutic effect of T-tube stent cannot be excluded.

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**Tamara M Alempijevic, MD, PhD, Assistant Professor,** Clinic for Gastroenterology and Hepatology, Clinical Centre of Serbia, 2 Dr Koste Todorovica St., 11000 Belgrade, Serbia

**Paolo Aurello, MD, PhD,** Department of Surgery, University of Rome "La Sapienza", Faculty of Medicine 2, via di Grottarossa 1035, Rome 00189, Italy

**Shashi Bala, PhD, Post doctoral Associate,** Department of Medicine, LRB 270L, 364 Plantation street, UMass Medical School, Worcester, MA 01605, United States

**Roberto de Franchis, Professor** of Medicine (Gastroenterology), Department of Medical Sciences, University of Milan, Head, Gastroenterology 3 Unit, IRCCS Ospedale Maggiore Policlinico, Mangiagalli and Regina Elena Foundation, Via Pace 9, 20122 Milano, Italy

**Itaru Endo, MD, PhD, Professor and Chairman,** Department of Gastroenterological Surgery, Yokohama City University, Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama, 2360004, Japan

**Gianpiero Gravante, MD, BsC, MBBS,** Department of Hepatobiliary and Pancreatic Surgery, Leicester General Hospital, Flat 38, Room 8, Hospital Close, Leicester, LE5 4WU, United Kingdom

**Imran Hassan, MD, Assistant Professor,** Department of Surgery, SIU School of Medicine, 701 North Rutledge, PO Box 19638, Springfield, IL 62794, United States

**Toshiyuki Ishiwata, Associate Professor,** Department of Pathology, Integrative Oncological Pathology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan

**Milan Jirsa, Dr.,** Laboratory of Experimental Medicine - building Z1, Institute for Clinical and Experimental Medicine, Videnska 1958/9, Praha 4, 140 00, Czech

**Michael Kew, Professor,** Department of Medicine, Groote Schuur Hospital, Main Road, Observatory 7935, Cape Town, South Africa

**Limas Kupcinskas, Dr., Professor,** Gastroenterology of Kaunas University of Medicine, Mickeviciaus 9, Kaunas LT 44307, Lithuania

**Julio Mayol, MD, PhD,** Department of Digestive surgery, Hospital Clinico San Carlos, MARTIN-LAGOS S/n, Madrid, 28040, Spain

**Xiaofa Qin, MD, PhD,** Department of Surgery, UMDNJ-New Jersey Medical School, 185 South Orange Avenue, Newark, NJ 07103, United States

**Bernabe Matias Quesada, MD,** Department of Surgery, Hospital Cosme Argerich, Talcahuano 944 9<sup>a</sup>, Buenos Aires 1013, Argentina

**Georg Roth, MD,** Department of Anesthesiology and General Intensive Care, Medical University of Vienna, Waehringer Guertel 18-20, 1090 Vienna, Austria

**Mitsuo Shimada, Professor,** Department of Digestive and Pediatric Surgery, Tokushima University, Kuramoto 3-18-15, Tokushima 770-8503, Japan

**Subbaramiah Sridhar, MB, BS, MPH, FRCP (Edin), FRCP (Glasg), FRCP (Lond), FRSS (Lond), FRCPC (Medicine & gastroenterology), FACP, FACG, FASGE, AGAF,** Section of Gastroenterology, BBR 2544, Medical College of Georgia, 15th Street, Augusta, GA 30912, United States

**Swan N Thung, Professor,** Department of Pathology, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, United States

**Jian Wu, Associate Professor** of Medicine, Internal Medicine/Transplant Research Program, University of California, Davis Medical Center, 4635 2nd Ave. Suite 1001, Sacramento, CA 95817, United States



## Meetings

### Events Calendar 2010

January 25-26  
Tamilnadu, India  
International Conference on Medical  
Negligence and Litigation in Medical  
Practice

January 25-29  
Waikoloa, HI, United States  
Selected Topics in Internal Medicine

January 26-27  
Dubai, United Arab Emirates  
2nd Middle East Gastroenterology  
Conference

January 28-30  
Hong Kong, China  
The 1st International Congress on  
Abdominal Obesity

February 11-13  
Fort Lauderdale, FL, United States  
21th Annual International Colorectal  
Disease Symposium

February 26-28  
Carolina, United States  
First Symposium of GI Oncology at  
The Caribbean

March 04-06  
Bethesda, MD, United States  
8th International Symposium on  
Targeted Anticancer Therapies

March 05-07  
Peshawar, Pakistan  
26th Pakistan Society of  
Gastroenterology & Endoscopy  
Meeting

March 09-12  
Brussels, Belgium  
30th International Symposium on  
Intensive Care and Emergency  
Medicine

March 12-14  
Bhubaneswar, India  
18th Annual Meeting of Indian  
National Association for Study of  
the Liver

March 23-26  
Cairo, Egypt  
14th Pan Arab Conference on  
Diabetes PACD14

March 25-28  
Beijing, China  
The 20th Conference of the Asian

Pacific Association for the Study of  
the Liver

March 27-28  
San Diego, California, United States  
25th Annual New Treatments in  
Chronic Liver Disease

April 07-09  
Dubai, United Arab Emirates  
The 6th Emirates Gastroenterology  
and Hepatology Conference, EGHG  
2010

April 14-17  
Landover, Maryland, United States  
12th World Congress of Endoscopic  
Surgery

April 14-18  
Vienna, Austria  
The International Liver Congress™  
2010

April 28-May 01  
Dubrovnik, Croatia  
3rd Central European Congress  
of surgery and the 5th Croatian  
Congress of Surgery

May 01-05  
New Orleans, LA, United States  
Digestive Disease Week Annual  
Meeting

May 06-08  
Munich, Germany  
The Power of Programming:  
International Conference on  
Developmental Origins of Health  
and Disease

May 15-19  
Minneapolis, MN, United States  
American Society of Colon and  
Rectal Surgeons Annual Meeting

June 04-06  
Chicago, IL, United States  
American Society of Clinical  
Oncologists Annual Meeting

June 09-12  
Singapore, Singapore  
13th International Conference on  
Emergency Medicine

June 14  
Kosice, Slovakia  
Gastro-intestinal Models in  
the Research of Probiotics and  
Prebiotics-Scientific Symposium

June 16-19  
Hong Kong, China  
ILTS: International Liver  
Transplantation Society ILTS Annual  
International Congress

June 20-23  
Mannheim, Germany  
16th World Congress for  
Bronchoesophagology-WCBE

June 25-29  
Orlando, FL, United States  
70th ADA Diabetes Scientific  
Sessions

August 28-31  
Boston, Massachusetts, United States  
10th OESO World Congress on  
Diseases of the Oesophagus 2010

September 10-12  
Montreal, Canada  
International Liver Association's  
Fourth Annual Conference

September 11-12  
La Jolla, CA, United States  
New Advances in Inflammatory  
Bowel Disease

September 12-15  
Boston, MA, United States  
ICAAC: Interscience Conference  
on Antimicrobial Agents and  
Chemotherapy Annual Meeting

September 16-18  
Prague, Czech Republic  
Prague Hepatology Meeting 2010

September 23-26  
Prague, Czech Republic  
The 1st World Congress on  
Controversies in Gastroenterology &  
Liver Diseases

October 07-09  
Belgrade, Serbia  
The 7th Biannual International  
Symposium of Society of  
Coloproctology

October 15-20  
San Antonio, TX, United States  
ACG 2010: American College of  
Gastroenterology Annual Scientific  
Meeting

October 23-27  
Barcelona, Spain  
18th United European  
Gastroenterology Week

October 29-November 02  
Boston, Massachusetts, United States  
The Liver Meeting® 2010--AASLD's  
61st Annual Meeting

November 13-14  
San Francisco, CA, United States  
Case-Based Approach to the  
Management of Inflammatory Bowel  
Disease

December 02-04  
San Francisco, CA, United States  
The Medical Management of HIV/  
AIDS





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- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua 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

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- 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]

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 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*

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### 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.

### 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 *v* (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) =  $8.6 \pm 24.5$   $\mu$ 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 23 243 641.

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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.

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